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A Method for Determining the Growth Rate of Alkenone-producing Microalgae in Oceanic Waters

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Abstract

Extension of atmospheric CO_2 records to more ancient times through the development of a geological proxy for CO_2 is a major objective of paleoclimate studies. A promising CO_2 proxy has been carbon isotopic analysis of marine organic matter (Rau et al., 1992; Hayes et al., 1990). However, recent studies have demonstrated that microalgal growth rates and cell geometry in addition to CO_2 concentration affect carbon isotopic fractionation in marine microalgae. Results of modelling (Rau et al., 1996, 1997) and laboratory chemostat experiments (Laws et al., 1995, 1997; Bidigare et al., 1997; Popp et al., 1998) have begun to clarify these effects.

Isotopic analysis of alkenones provides a way to constrain the size and shape of the source organism because these compounds are only produced by a few select microalgae (Marlowe et al., 1990). Although laboratory and field studies suggest that isotopic analysis of alkenones shows great potential as a CO_2 proxy, the relationship between specific growth rate and carbon isotopic fractionation in natural samples is not well defined. This research establishes in the laboratory an alkenone ^{13}C-labeling method that can be used to evaluate the effect of growth rate on carbon isotopic fractionation in natural populations of the open ocean, alkenone-producing microalgae, *Emiliana huxleyi* and a major coastal variant, *Isochrysis galbana*. Our approach is analogous to the method for determining phytoplankton growth rates using ^{14}C-labeling of pigments (Goericke and Welschmeyer, 1992, 1993) but uses irmGCMS to determine the rate of ^{13}C incorporation into alkenones.

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Chapter 1: Introduction

Ice core studies have established linkages between climate change and atmospheric carbon dioxide (CO_2) on a number of time scales. Although ice core records of CO_2 and temperature variability extend instrumental data and allow greater understanding of paleoclimate records, ice cores are limited spatially to high latitude terrestrial environments and temporally to only about the last 0.7 my (Overpeck et al., 1997; Anderson et al., 2004). Extension of atmospheric CO_2 records to more ancient times through the development of a geological proxy for CO_2 is a major objective of paleoclimate studies. A promising CO_2 proxy has been carbon isotopic analysis of marine organic matter (Hayes et al., 1989, 1990; Rau et al., 1992; Popp et al., 1997).

However, recent studies have demonstrated that growth rate, temperature, nutrient availability, light availability and cell geometry also affect carbon isotopic fractionation by marine microalgae. These findings suggest that these environmental and physiological variables must be constrained before carbon isotopic analysis of marine organic matter can be used to understand changes in oceanic CO_2 levels (Rau et al., 1997; Schouten et al., 1998; Bidigare et al., 1999; Popp et al., 1999). Results of modeling (Rau et al., 1996, 1997) and laboratory chemostat experiments (Laws et al., 1995, 1997; Bidigare et al., 1997; Popp et al., 1998) have begun to clarify these physiological effects.

In field studies, cell geometry can be constrained only when the source of the phytoplankton carbon analyzed is known. Isotopic analyses of long-chain

ketones (alkenones) provide a way to constrain the size and shape of the source organism as well as the species because these compounds are only produced by a few select microalgae (Marlowe et al., 1990). Although laboratory and field studies suggest that isotopic analysis of alkenones show great potential as a CO_2 proxy because cell geometry can be estimated, the relationship between specific growth rate and carbon isotopic fractionation in natural samples is not well defined. Recent field investigations suggest that the growth rate of modern alkenone producers is correlated with phosphate concentration (Bidigare et al., 1997; Popp et al., 1999), consistent with the idea that an essential nutrient is often a factor controlling growth rate (Brown et al., 1981). However this relationship has not been demonstrated in the field because of the difficulty in determining the specific growth rates of individual algal species.

Redalje and Laws (1981) introduced a novel method for determining the growth rate and carbon content of natural phytoplankton by determining the specific activity of ^{14}C-labeled chlorophyll *a*. The assumption here is that the specific activity of the chlorophyll carbon and the total cellular carbon are equivalent after a given time period of isotope labeling. By combining the measured rate of carbon fixation with the calculated cell carbon concentrations, one can estimate the carbon-specific growth rate (Welschmeyer and Lorenzen, 1984; Laws, 1984). Goericke and Welschmeyer (1992, 1993) demonstrated that this technique could be adapted to many types of algae using various types of carotenoids. The purpose of this work is to show that the pigment ^{14}C-labeling

method can be adapted to other isotopes and that alkenone ^{13}C-labeling can be used to determine the growth rate of alkenone-synthesizing algae. ^{13}C-labeling was used as opposed to ^{14}C-labeling. ^{13}C-labeling was used to avoid the cost of cleanup of radioactive substances and also to circumvent the problems associated with isolating and analyzing ^{14}C-labeled alkenones. Stable carbon isotope analysis of alkenones is straightforward, sensitive and inexpensive (Bidigare et al., 1997)

The objective of this research is to establish in the laboratory the range of growth conditions where the alkenone ^{13}C-labeling method can yield reliable growth rates from alkenone-producing algae. Once established, this method can be used to evaluate the effect of growth rate on carbon isotopic fractionation in natural populations of alkenone-producing algae. We tested the method using continuous laboratory cultures (chemostats) of the open ocean, alkenone-producing microalgae, *Emiliana huxleyi* and a major coastal variant, *Isochryis galbana.* We compared the calculated growth rate obtained from the rate of ^{13}C labeling of alkenones to the true growth rate obtained from the dilution rate of nutrient media into the chemostat system.

The rationale for growing cultures in a chemostat is several fold. A chemostat is a system whose chemical characteristics do not change with time. Control over growth rate can be achieved since growth-rate is determined by the rate at which fresh medium is introduced into the growth chamber. At steady state, the culture is growing only as fast as the pumping rate of the medium,

allowing for accurate determination of growth-rate (Laws et al., 2001; Popp et al., 1998). Also, at steady-state, the isotopic composition of cells harvested from the growth chamber can be related to the growth chamber at the time of harvest (Laws et al., 2001).

The results of this study indicate that the ^{13}C-alkenone labeling method yields reliable estimates of growth rate for *Emiliana huxleyi* and *Isochryis galbana* over the range of 0.16 to 0.70 d^{-1}; providing evidence of the validity of the ^{13}C-alkenone labeling method. Results of this study can be applied in the field to obtain growth rates of natural alkenone-producing algae and aid in reconstructing ancient paleo-CO_2(aq) concentrations.

Chapter 2: Materials and Methods

Materials and Methods:

Axenic cultures of *Emiliania huxleyi* (CCMP 373 and 374) and *Isochrysis galbana* (CCMP 1323 and 1324) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and were grown on modified (100 μM nitrate) f/2 medium with silicate using 0.2 μm sterile filtered surface seawater from Kaneohe Bay, Oahu. Cultures were maintained in nitrate-limited chemostats at constant temperature (16° or 18°C), salinity (33‰) and light provided by a bank of daylight fluorescent lamps (~21.6 mol quanta m^{-2} d^{-1}). CO_2 concentration in solution was controlled by aerating the cultures with a mixture of gases from a tank of CO_2-free air with a tank of 2% CO_2. Mixing ratios were regulated by setting the flow rates of the gasses using mass flow controllers. Studies at a particular growth rate did not begin until the culture had been at steady state for at least four doubling times so that more than 93% of the biomass harvested had been grown with constant concentrations of phytoplankton biomass and constant concentrations and isotopic compositions of total dissolved inorganic carbon. Collection, processing of algae and water samples, chemical and isotopic analysis and calculations were identical to those of Laws et al. (1995, 1997) and Popp et al. (1998). Cultures were considered in steady state when the day-to-day variability in cell counts was less than ± 50,000 cells mL^{-1}, the variability in the concentration of total dissolved inorganic carbon was less than ± 10 μM and the variability in the carbon isotopic composition of total dissolved

inorganic carbon ($\delta^{13}C_{DIC}$) was within ± 0.1 ‰. Cell counts were determined using a Coulter counter.

Samples for total dissolved inorganic carbon (DIC) and $\delta^{13}C_{DIC}$ analysis were prepared using a system modified after Kroopnick (1985). Briefly, 9.67 mL of filtered seawater (preserved with 5 uL saturated mercuric chloride) and 1.2 mL of 25% 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 DIC was trapped using LN_2 on a multi-loop trap and transferred to a vacuum distillation line where the quantity of CO_2 was determined manometrically (MKS Baratron Model 122). Concentration of DIC based on the $P - V$ calculation yielded an accuracy and precision of less than ± 10 μM.

The concentration of CO_2(aq) was determined from the concentrations of DIC, phosphate and silicate as well as total alkalinity. The $\sum CO_2$ concentrations in mmol/kg as well as the alkalinity data in meq/kg are used to determine the partitioning of carbonate species to obtain values for CO_2(aq). Apparent dissociation constants used in this calculation were from Roy et al. (1993), Dickson (1990), and Millero (1995). The apparent constants were corrected for the effects of pressure (Millero, 1979). Nutrient analyses were performed using standard techniques on a Technicon Autoanalyzer II system. Total alkalinity was determined by the Gran method (Gran, 1952) using computer-controlled titration. Precision and accuracy as determined by analysis of a certified alkalinity seawater standard was determined to be less than 10 μeq kg^{-1}. Overall precision of our

calculation of [CO_2(aq)] was less than 10%. The isotopic composition of CO_2(aq) was determined from $\delta^{13}C_{DIC}$ using the equilibrium relationships of Mook et al. (1974) and Deines et al. (1974) and the calculated distribution of HCO_3^-, CO_3^{2-} and CO_2(aq). We use ε_p to specify the magnitude of isotopic fractionation associated with photosynthetic fixation of CO_2(aq) described by Popp et al. (1989) as modified by Freeman and Hayes (1992). The carbon isotopic composition of alkenones has been corrected by 4.2 ‰ to account for the isotope offset between alkenones and whole cell of *E.huxleyi* (Popp et al., 1998b).

Samples of phytoplankton for carbon isotopic analysis were filtered (Whatman GF/C precombusted at 400°C for 8 h) from 50 mL of seawater. The filtered material was wrapped in precombusted Al-foil and placed immediately in a freezer (-20°C) and stored until analysis. Samples were removed from the freezer and allowed to dry overnight in a 60°C oven. The isotopic composition of phytoplankton was determined using an on-line Carlo Erba CHN analyzer coupled to an isotope ratio mass spectrometer (Finnigan ConFlo II/Delta-Plus). Prior experience has indicated that the uncertainty using this technique is better than 1-2% for measurements of C and N isotopic weight percentage and approximately ±0.2 ‰ for isotopic C and N analysis. Isotopic compositions are reported in the standard δ-notation in permil relative to VPDB.

Samples (200 mL) for alkenone analysis were filtered onto precombusted Whatman GF/F filters, immediately frozen in liquid N_2 and stored until extraction. Filters were extracted with methanol:chloroform (2:1) using

automated solvent extraction (Dionex ASE200, Macnaughton et al., 1997). Extracts were saponified using aqueous 0.5 N KOH/MeOH and the neutral lipid fraction recovered by extraction with hexane; alkenones were isolated by absorption chromatography on silica gel (Prahl et al., 1989).

Following derivatization of polar components (BSTFA), isotope-ratio-monitoring gas chromatography-mass spectrometry (irmGCMS, MAT252 with GC/C III; DB-1, 60 m) was used to determine δ values for alkenones (values of δ refer to $\delta^{13}C$ relative to the VPDB standard). The compound-specific isotopic results reported in this study were determined using techniques described by Hayes et al. (1990), Merritt and Hayes (1994), and Merritt et al. (1995). Isotopic analyses were performed in duplicate or triplicate when possible in order to calculate meaningful uncertainties. Accuracy of compound-specific isotopic analysis was assessed through analysis of internal standards in which the δ value was known. Precision of replicate irmGCMS analysis of compounds with natural abundance carbon isotope composition was found in most cases to be ±0.3‰ or better for individual peaks containing >5 nmoles of carbon with no indication of bias or inaccuracy in the results. Measured precision for compounds with modest ^{13}C-enrichment (δ-values ≈ 50-80 ‰) by irmGCMS was typically less than ±1.5‰.

After sampling for the natural abundance carbon isotopic composition of alkenone, cultures were allowed one week to regain steady-state, followed by the addition of an appropriate amount of ^{13}C-labeled bicarbonate to both the

chemostat and the nutrient reservoir in order to achieve an initial $\delta^{13}C_{DIC}$ of ~190 ‰. Cell counts, alkalinity, DIC, POC, and alkenone samples were taken at regular intervals (typically 6, 12, 24, 30, and 48 hours) to monitor the uptake of ^{13}C into the bulk phytoplankton and alkenones.

Calculations:

Cell count values were obtained directly from the Coulter counter with values expressed in cells mL^{-1}. Alkalinity values were obtained from the Dosimat readings. DIC values from the Baratron reading were in mM concentration. To account for the density of seawater, these values were divided by 1.025 with new units in mmol/kg. POC values (δ_p) were reported per mil from the EA reading. Alkenone values ($\delta_{alkenone}$) were reported in per mil units from the Thermo Finnigan Trace GC.

The true growth rate (μ_{true}) was obtained from the pump rate of the chemostat. The calculated growth rate (μ_{calc}) involved more complex equations that will be described in the discussion.

The CO_2(aq) was determined by partitioning of carbonate species using a computer program entitled Co2roy2 under Qbasic. The program used parameters such as depth, temperature, salinity, phosphate concentration, silicate concentration, alkalinity (meq kg^{-1}), and tCO_2 (mmol kg^{-1}) to determine the partitioning of the carbonate species and hence the CO_2(aq) concentration. With

this information, the total carbon isotopic fractionation (ε_p) can be calculated with eqn. 1.

$$\varepsilon_P = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{CO_2} + 1000}{\left(\frac{^{13}C}{^{12}C}\right)_{alkenone} + 1004.2} - 1 \right) 1000 \qquad (1)$$

The value of 1004.2 in calculating ε_p is needed to adjust for the isotopic difference between alkenones and total biomass (Popp et al., 1998). Besides these calculations, plots of the true growth rate versus the calculated growth rate were constructed, as well as plots of the $\mu/CO_2(aq)$ versus the total carbon isotopic fractionation.

Chapter 3: Results

Chemostats were used to compare the growth rate determined from the dilution rate of the medium with the growth rate calculated from the ^{13}C-labeling of alkenones. Equivalent rates would suggest that the alkenone ^{13}C-labeling provides reliable estimates of growth rate of alkenone-producing microalgae.

Three chemostats using two different strains of the microalga *Emiliania huxleyi* were completed. The first two were comprised of *E. huxleyi* CCMP373 at growth rates of 0.21 and 0.42 d^{-1}. The third was comprised of *E. huxleyi* CCMP374 with a growth rate of 0.16 d^{-1}. Five chemostats using two different strains of the alkenone-producing microalgae *Isochrysis galbana* were completed. The first three were comprised of *I. galbana*, CCMP1324, (strain TISO) grown at growth rates of 0.30, 0.50, and 0.70 d^{-1}. The last two were comprised of *I. galbana*, CCMP1323 grown at 0.28 and 0.53 d^{-1}.

Table 1 lists the nutrient concentrations, salinities, and temperature for each of the eight chemostats. The only variation comes from a 2 °C difference in temperature for two of the chemostats. Cell counts, alkalinity, total dissolved inorganic carbon concentrations ($\sum CO_2$), and the concentrations of CO_2(aq) for the chemostats are shown in Table 2.

Table 3 summarizes the isotopic data from the chemostats. Included in the table are isotopic data for the DIC, CO_2(aq), POC, and alkenones. The isotopic composition of the DIC was used to aid in determining the isotopic composition

of CO_2(aq), hence a similar pattern of enrichment was observed between the two. The POC isotopic data are presented as an average of two POC samples taken simultaneously, while the alkenone isotopic data is an average of the same sample analyzed two to four times to gather reasonable uncertainties of the calculations.

TABLE 1: Nutrient conditions as well as salinity and temperature measurements for all eight chemostat experiments

Species	Strain (CCMP)	μ_{true} (d^{-1})	PO_4 (µM)	SiO_2 (µM)	NO_2+NO_3 (µM)	Salinity (‰)	Temperature (°C)
E. huxleyi	373	0.21	33.75	100	0	33	18
E. huxleyi	373	0.42	33.75	100	0	33	18
E. huxleyi	374	0.16	33.75	100	0	33	16
I. galbana	1324	0.3	33.75	100	0	33	16
I. galbana	1324	0.5	33.75	100	0	33	16
I. galbana	1324	0.7	33.75	100	0	33	16
I. galbana	1323	0.28	33.75	100	0	33	16
I. galbana	1323	0.53	33.75	100	0	33	16

Since the nutrient medium did not change from experiment to experiment, neither do the nutrient concentrations in the chemostat. Nitrate was the limiting nutrient. Nitrate concentrations were below the limit of detection. The water for

the medium and chemostats came from Kaneohe Bay (salinity = 33 ‰) and the temperature of the chemostats was optimized for optimum growth.

TABLE 2: Cell counts in cells/ml, the alkalinity concentration in meq/kg, the total CO_2 concentration in mmol/kg as well as the CO_2(aq) concentration in μmol/kg for each chemostat during harvesting.

hour (labeled)	cell count (per ml)	alkalinity (meq/kg)	ΣCO_2 (mmol/kg)	CO_2(aq) (μmol/kg)
	***E. huxleyi* (CCMP373) μ = 0.21 d^{-1}**			
0	858800	2.44	2.13	12.97
6	822200	2.42	2.14	14.51
12	713600	2.44	2.14	13.18
18	632400	2.42	2.13	13.88
24	698800	2.43	2.14	13.67
48	767400	2.41	2.14	14.85
	***E. huxleyi* (CCMP373) μ = 0.42 d^{-1}**			
0	275400	2.33	2.13	15.85
6	269800	2.33	2.15	16.89
12	235800	2.33	2.14	16.38
18	208800	2.33	2.14	16.04
24	201200	2.32	2.15	17.56
48	216400	2.36	2.17	16.59
	***E. huxleyi* (CCMP374) μ = 0.16 d^{-1}**			
0	758000	2.35	2.18	22.49
6	803000	2.360	2.18	22.83
12	812000	2.370	2.19	22.77
24	890000	2.340	2.21	32.37
30	901000	2.330	2.23	39.26
48	712000	2.340	2.24	39.43
	***I. galbana* (CCMP1324) μ = 0.30 d^{-1}**			
0	754200	2.42	2.15	18.56
6	678000	2.36	2.14	17.95
12	618200	2.38	2.13	16.27
24	597600	2.36	2.13	17.08
30	584200	2.35	2.13	17.84
48	568800	2.37	2.12	16.06

Table 2: cont.

hour (labeled)		cell count (per ml)	alkalinity (meq/kg)	ΣCO_2 (mmol/kg)	CO_2(aq) (µmol/kg)
		***I. galbana* (CCMP1324) µ = 0.50 d^{-1}**			
0	A	1100000	2.39	2.17	18.87
6		996600	2.35	2.16	20.84
12	B	1019600	2.36	2.16	20.33
24		929000	2.34	2.15	21.07
30	L	919000	2.34	2.14	20.41
48		960600	2.34	2.19	27.40
	E				
		***I. galbana* (CCMP1324) µ = 0.70 d^{-1}**			
0		930000	2.38	2.11	14.18
6	2	1019000	2.38	2.13	15.17
12		978000	2.36	2.13	16.80
24	:	978200	2.4		14.44
30		961600	2.37	2.1	14.10
48		972800	2.37	2.15	18.69
	(	***I. galbana* (CCMP1323) µ = 0.28 d^{-1}**			
0	c		2.3	2.18	32.76
6			2.31	2.19	32.40
12	o		2.3	2.19	34.28
24			2.3	2.18	33.58
30	n		2.3	2.2	34.48
48			2.28	2.19	38.28
	t				
		***I. galbana* (CCMP1323) µ = 0.53 d^{-1}**			
	.				
0			2.35	2.18	23.64
6	)		2.3	2.19	31.86
12			2.31	2.19	33.09
24			2.29	2.18	35.03
30			2.29	2.19	38.06
48			2.28	2.19	38.22

Cell count data varied between chemostats due to varying growth rates and species. The total alkalinity remained surprisingly constant between chemostats with values between 2.28 and 2.44 meq/kg. Total dissolved inorganic carbon showed a similar trend with values between 2.10 and 2.20 mmol/kg. The CO_2(aq) concentrations were within range of normal seawater except for the last two chemostats that had values double the previous chemostat experiments. Cell count data for the last two chemostats are absent due to a malfunction of the Coulter counter. In these experiments, we tried to mimic open-ocean conditions, and for the most part, we were successful.

Table 3: Isotopic data describing the δ-^{13}C values of the DIC, the δ-^{13}C of the CO_2(aq) and the δ-^{13}C of the POC and alkenones.

hour (labeled)	^{13}C-DIC (‰)	^{13}C-CO_2(aq) (‰)	δ-^{13}C (POC) (‰)	δ-^{13}C (Alkenone) (‰)	μ_{calc} (d^{-1})
	***E. huxleyi* (CCMP373) $\mu = 0.21\ d^{-1}$**				
0	-5.42	-14.39	-34.5	-37.1	
6	137.75	127.48	-25.8	-32.5	0.2
12	123.88	113.73	-18.2	-27.9	0.2
18	110.28	100.25	-12.0	-24.6	0.2
24	94.60	84.72	-6.6	-22.1	0.2
48	64.57	54.96	6.0	-9.3	0.2
				Ave. μ_{calc}	**0.199**
	***E. huxleyi* (CCMP373) $\mu = 0.42\ d^{-1}$**				
0	-4.48	-13.47	-31.7	-36.8	
6	157.31	146.82	-13.2	-24.1	0.4
12	139.86	129.54	1.2	-10.4	0.406
18	130.33	120.11	11.2	-1.9	0.407
24	118.70	108.59	20.2	7.5	0.411
48	90.62	80.76	37.4	31.1	0.414
				Ave. μ_{calc}	**0.407**
	***E. huxleyi* (CCMP374) $\mu = 0.16\ d^{-1}$**				
0	-9.80	-18.92	-40.8	-45.0	
6	156.63	146.00	-32.1	-38.9	0.155
12	157.27	146.63	-25.2	-32.3	0.157
24	136.43	125.95	-14.5	-17.5	0.160
30	127.25	116.90	-11.7	-15.9	0.159
48	103.64	93.50	-2.6	-6.8	0.158
				Ave. μ_{calc}	**0.158**
	***I. galbana* (CCMP1324) $\mu = 0.30\ d^{-1}$**				
0	-10.41	-19.53	-40.4	-46.6	
6	136.23	125.76	-31.5	-41.6	0.273
12	LOST	Lost	-22.0	-34.0	0.289
24	109.11	98.89	-7.6	-17.3	0.310
30	97.67	87.55	-1.4	-15.3	0.289
48	76.84	66.92	12.5	-2.1	0.292
				Ave. μ_{calc}	**0.291**

TABLE 3: (cont.)

Hour (labeled)	^{13}C-DIC (‰)	^{13}C-CO_2(aq) (‰)	δ-^{13}C (POC) (‰)	δ-^{13}C (Alkenone) (‰)	μ_{calc} (d^{-1})
	***I. galbana* (CCMP1324) μ = 0.50 d^{-1}**				
0	-7.32	-16.47	-35.8	-43.2	
6	105.57	95.38	-17.9	-29.6	0.49
12	110.61	100.37	-5.5	-17.8	0.488
24	94.71	84.62	13.4	2.2	0.489
30	88.83	78.80	22.6	8.6	0.493
48	77.55	67.62	30.3	20.6	0.492
				Ave. μ_{calc}	**0.49**
	***I. galbana* (CCMP1324) μ = 0.70 d^{-1}**				
0	-5.83	-14.99		-44.0	
6	143.44	132.90		-19.7	0.684
12	138.84	128.34	12.5	-1.8	0.68
24	124.91	114.55	35.8	18.9	0.673
30	122.79	112.44	44.6	28.0	0.678
48	117.00	106.70	64.1	50.3	0.681
				Ave. μ_{calc}	**0.679**
	***I. galbana* (CCMP1323) μ = 0.28 d^{-1}**				
0	-9.85	-18.96	-45.4	-49.7	
6	153.01	142.40	-31.4	-46.1	0.276
12	144.68	134.15	-20.3	-39.4	0.253
24	124.29	113.93	-2.8	-23.9	0.262
30	115.18	104.93	3.6	-17.5	0.267
48	96.38	86.31	15.3	-1.3	0.271
				Ave. μ_{calc}	**0.266**
	***I. galbana* (CCMP1323) μ = 0.53 d^{-1}**				
0	-9.44	-18.57	-42.9	-47.4	
6	136.67	126.20	-21.2	-36.0	0.492
12	122.88	112.55	-4.7	-23.8	0.498
24	99.26	89.15	14.5	-4.1	0.508
30	91.01	80.99	19.4	1.4	0.514
48	78.31	68.40	27.4	15.7	0.516
				Ave. μ_{calc}	**0.506**

The ^{13}C-DIC and ^{13}C-CO_2(aq) data have similar trends. At t_o, no label has been added; giving unlabeled isotopic values. Label was then added and the uptake monitored in the DIC, POC and alkenone pools. The DIC isotopic value increases substantially from the addition of label. As the experiment proceeds, the label added to the chemostat equals that flushed out of the system. Decrease in label from the DIC is due to CO_2 bubbling from the 2% CO_2 gas cylinder, which had δ-^{13}C values much less than the spiked reservoir. In each case a steady decrease in ^{13}C-DIC and ^{13}C-CO_2(aq) was observed over time.

The ^{13}C-POC and ^{13}C-alkenone data show a reverse trend. Since the cells are growing at steady state, they take up $^{13}CO_2$. Their δ values then increase over time as label is assimilated. The amount of label taken up varies as a function of their growth rate. Cells growing rapidly take up label at a faster rate than the same strain maintained at a lower growth rate. Values given in bold in Table 3 show average calculated growth rates with the ^{13}C labeling method. Values remained very close to the growth rate obtained from the dilution rate of the chemostat, indicating the validity and usefulness of this approach.

Chapter 4: Discussion

The purpose of this study is to determine how reliable ^{13}C enrichment of alkenones tracks specific growth rate (μ) based on the turnover of cellular carbon (e.g., Goericke and Welschmeyer, 1992a,b; 1993a,b). The relevant theoretical analysis is as follows.

According to tracer kinetic theory, if A* is the atom % excess of the substrate pool (assumed to be constant), P* the atom % excess of the product pool, and (a) is the turnover rate of the product pool, then P* should follow the equation (Laws, 1984):

$$P^*(t) = A^*\left(1 - e^{-at}\right) \qquad (2)$$

If the product pool is stable, then in steady state growth (a) should equal μ, the growth rate of the algae. Under such conditions μ can be estimated by inverting equation 2 to obtain:

$$\mu = -\frac{1}{t}\ln(1 - P^*/A^*) \qquad (3)$$

The simplicity of this approach is complicated if there are intermediate pools between the external substrate pool and the final product. Such is logically the case when one is monitoring the photosynthetic incorporation of inorganic carbon into an end product such as an alkenone. If there is a single intermediate pool, then P* is described by the equation (Goericke and Welschmeyer, 1992a,b):

$$P^*(t) = A^*\left(1 - \frac{b}{b-\mu}\exp(-\mu t) - \frac{\mu}{\mu - b}\exp(-bt)\right) \quad (4)$$

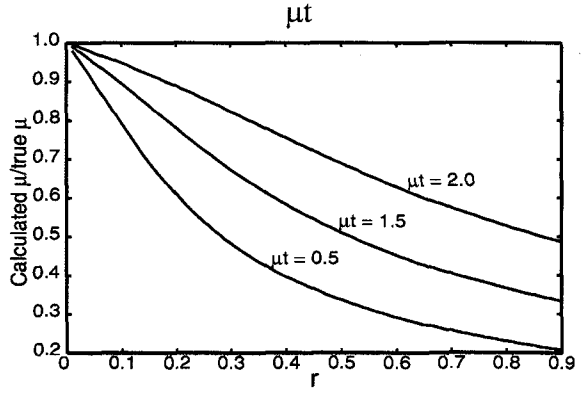
where b is the turnover rate of the intermediate pool. Rearranging equation 4 gives:

$$\ln(1 - P^*) = -\mu t + \ln\left(\frac{1}{1-r}\right) + \ln\left(1 - r\exp(\mu(r-1)t/r)\right) \quad (5)$$

where $r = \mu/b$.

The error incurred by calculating μ from equation 3 instead of equation 5 is shown in Figure 1. The error is a function of both μt and r and is small for large μt and small r.

Figure 1: Plot of r versus the ratio of calculated to true μ for assumed values of μt



A potential problem with the use of ^{13}C-labeling of alkenones to determine growth rates is the presence of intermediate pools that take up the ^{13}C-label at a rate slower than that of the alkenones. The problem is exasperated at

low growth rates. The existence of intermediate pools could slow down the labeling of alkenones and bias the growth rate estimates. The extent of bias will be negligible if r is sufficiently small and μt sufficiently large (Figure 1). Reliable estimates occur when the labeling of alkenones is equivalent to the labeling of the bulk phytoplankton. The purpose of laboratory studies was to explore the range of growth conditions where the alkenone-labeling method gives reliable estimates of growth rate and to identify under what conditions bias may be anticipated.

Growth rates for each time point are calculated using the following equation:

$$\mu_{calc} = \frac{\left[\left(\left(\frac{^{13}C}{^{12}C}\right)_{final} - \left(\frac{^{13}C}{^{12}C}\right)_{initial} e^{-t\mu_{true}}\right)\right]\mu_{true}}{\left(\left(\frac{^{13}C}{^{12}C}\right)_{CO_2-average}\right)\left(1-e^{-t\mu_{true}}\right)} \qquad (6)$$

where $^{13}C/^{12}C_{final}$ and $^{13}C/^{12}C_{initial}$ are the carbon isotope ratios of the POC or alkenone at time t and of the unlabeled POC or alkenone, respectively. The $^{13}C/^{12}C_{CO2(average)}$ is the average carbon isotope composition of CO_2(aq), t is time in days and μ_{true} is the dilution rate of the culture, which here is assumed to be the "true" growth rate of the phytoplankton. The dilution of the culture is taken into account due to washout of POC. The derivation of equation 6 is as follows. We

begin with an equation (eqn. 7) describing the addition and loss factors of ^{13}C-labeled bicarbonate in a chemostat.

$$\frac{dX}{dt} = -\mu X + \frac{\mu' AY}{DIC} \qquad (7)$$

where dX/dt represents the change in ^{13}C label, $-\mu X$ represents loss of label from washout of labeled POC with the remaining factor describing the uptake of label into the POC and hence the alkenones with μ' being the calculated growth rate, A representing the concentration of phytoplankton carbon and the Y term describes the ^{13}C in the DIC. Rearranging equation 7 yields equation 8.

$$\frac{dX}{dt} + \mu X = \frac{\mu' AY}{DIC} \qquad (8)$$

An integrating factor ($e^{\mu t}$) is applied to both sides to give equation 9.

$$(e^{\mu t} X)' = \frac{\mu' AY}{DIC} e^{\mu t} \qquad (9)$$

Integrating equation 9 gives equation 10.

$$e^{\mu T} X - X_{\circ} = \frac{\mu' A}{DIC} \int_0^T Y e^{\mu t} dt \qquad (10)$$

Solving for X yields equation 11.

$$X = X_{\circ} e^{-\mu T} + \frac{\mu' A}{DIC} e^{-\mu T} \int_0^T Y e^{\mu t} dt \qquad (11)$$

If Y and $e^{\mu t}$ are uncorrelated, equation 11 becomes,

$$X = X_{\circ} e^{-\mu T} + \frac{\mu' A}{\mu DIC} e^{-\mu T} (e^{uT} - 1) \frac{1}{T} \int_0^T Y dt \qquad (12)$$

Lastly we assume that Y_{ave} is the average ^{13}C value of the DIC between time points. The resulting equation is simplified and solved for μ' to give equation 13.

$$\mu' = \frac{\left(\frac{X}{A} - \frac{X_{\circ}}{A} e^{-\mu t}\right)\mu}{\frac{Y_{ave}}{DIC}(1 - e^{-\mu t})} \qquad (13)$$

Here μ' is the calculated growth rate and X/A is the $^{13}C/^{12}C$ ratio of the POC or alkenone at time t. The X_o/A term is the initial $^{13}C/^{12}C$ ratio of the POC or

alkenone, μ is the dilution rate of the chemostat and Y/DIC is the average $^{13}C/^{12}C$ ratio of the CO_2. Equation 13 is identical to equation 6.

Results of analysis indicate that even at low growth rate (0.16 d^{-1}) and a short duration of labeling (6 hrs), calculated growth rates agree with those determined from the dilution rate of the chemostat (0.158 d^{-1} for μ_{calc} versus 0.16 d^{-1} for the dilution rate of the chemostat at the 6 hr time point, see Figure 2). This indicates that the rate of labeling of alkenones matches closely the labeling rate of the bulk phytoplankton. In other words, values of μ_{calc}/μ_{true} are always above 0.9. These results suggest that b was large compared to μ in all cases (resulting in low values of r) and that the intermediate pool was turning over on time scales less than 6 hours (eqn 5). Errors incurred by calculating μ from equation 3 will be small, even with a large range of μt. Figure 2 shows how closely μ_{calc} matches the dilution rate during harvesting of the chemostats.

Figure 2: Calculated growth rates from ^{13}C-labeling of alkenones at each time point for all eight chemostat experiments

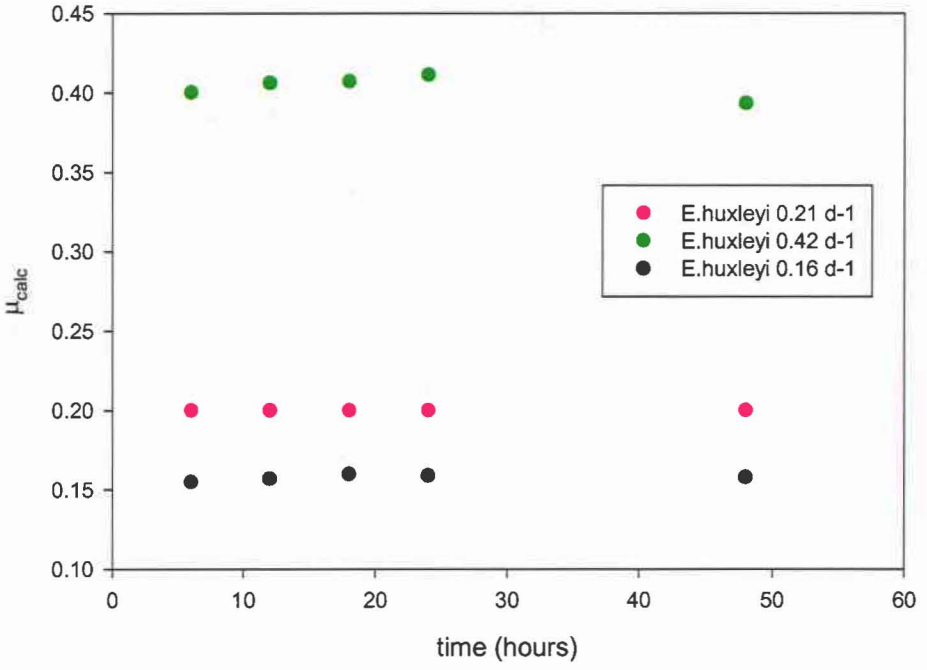
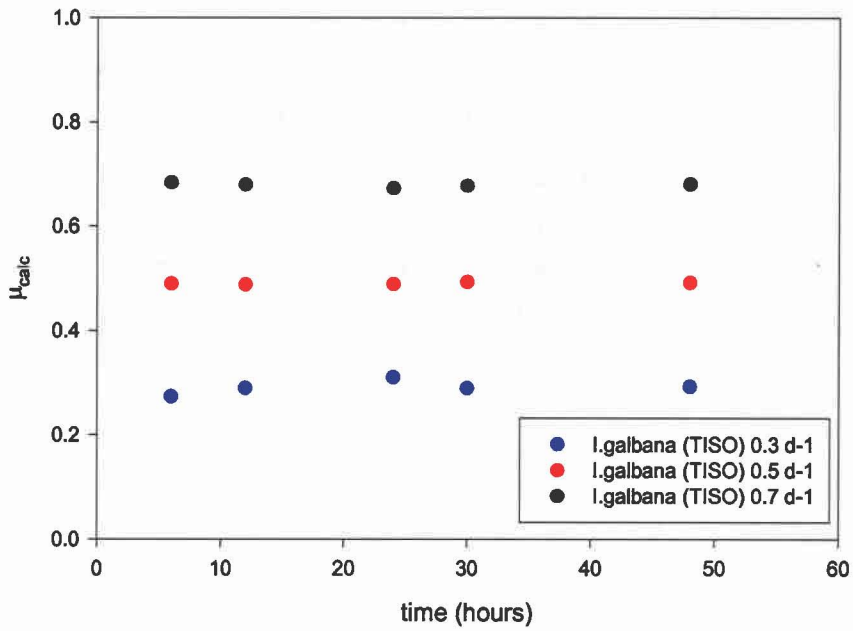
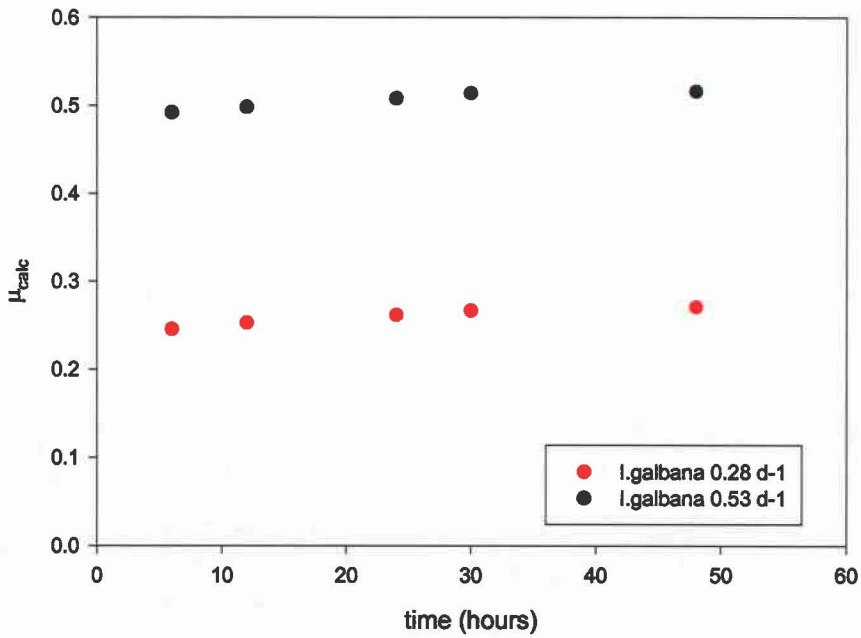


Figure 2: (cont.)

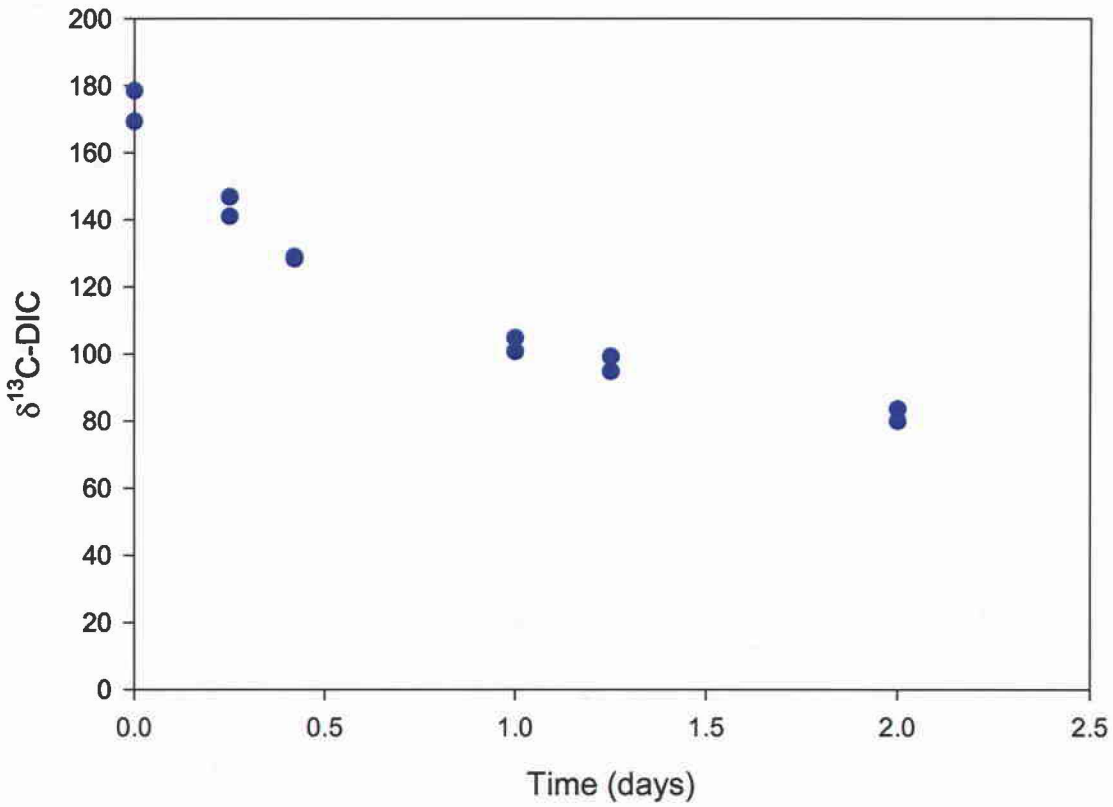




These findings suggest that this method gives reliable estimates of growth rate with CO_2(aq) concentrations ranging from 14 μmol/kg up to above 30 μmol/kg. Table 2 shows general chemical characteristics at the time of harvesting for each chemostat. Results from the cell count, alkalinity, and DIC analysis reveal that for each chemostat, these values do not change appreciably, indicating steady-state growth. Steady-state growth is needed for reliable estimates of growth rate from isotopic labeling. For six of the eight chemostats, the concentrations of CO_2(aq) are typical of open-ocean waters. The two chemostats comprised of *I. galbana* (CCMP1323) have CO_2(aq) values double the previous experiments, yet calculated growth rates in Table 3 still agree with those obtained from the dilution rate of the chemostat.

Isotopic data show the pattern of enrichment in the CO_2(aq), POC, and alkenones, whose values are used to calculate growth (Table 3). The $\delta^{13}C_{DIC}$ values show a pattern of enrichment after the addition of ^{13}C-label followed by a general decrease in enrichment as the phytoplankton take up the label. A decrease in enrichment results from CO_2 bubbling from the 2% CO_2 gas cylinder that had a much lower isotopic value than the ^{13}C-labeled chemostat and nutrient reservoir. Results from an abiotic ^{13}C-labeled chemostat experiment with a 2% CO_2 gas cylinder show identical trends of enrichment, suggesting that the decrease in enrichment was due to CO_2 bubbling (Figure 3).

Figure 3: Results from an abiotic ^{13}C-labeled chemostat experiment (in duplicate) showing the effect of CO_2 bubbling on the isotopic composition of DIC over time.



The POC and alkenone enrichment increases over time as more and more label is incorporated into the phytoplankton. Phytoplankton exhibiting high growth rates take up the label faster than slower growing phytoplankton of the

same species. The calculated growth rates from the ^{13}C-labeling of alkenones agree surprisingly well with those obtained from the dilution rate of the chemostat with values of μ_{calc}/μ_{true} above 0.9. The highest deviations tend to occur at low-growth rate with short intervals (6 hrs). The average calculated growth rates are, however, very close to the true growth rates in all cases as shown in Table 4 and Figure 4. The points in Figure 4 remain close to the 1:1 values, indicating close agreement between the calculated and true growth rates.

Table 4: The relationship between the growth rate obtained from the dilution rate of the chemostat (μ_{true}) to that calculated from the ^{13}C-labeling of alkenones (μ_{calc})

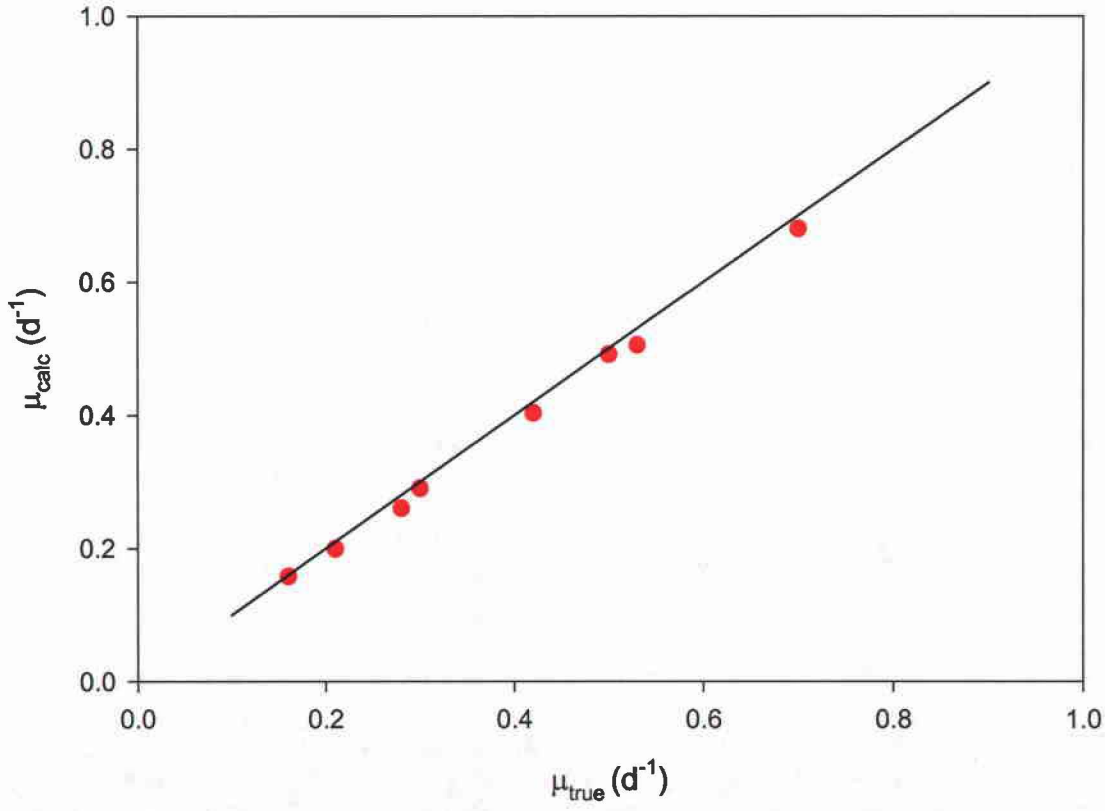
Species	Strain (CCMP)	μ_{true}[a]	μ_{calc}[b]	Isotope signature[c]
E. huxleyi	374	0.16	0.16	37:2
E. huxleyi	373	0.21	0.20	37:2
E. huxleyi	373	0.42	0.40	37:2
I. galbana	1324	0.30	0.29	37:2
I. galbana	1324	0.50	0.49	37:2
I. galbana	1324	0.70	0.68	37:2
I. galbana	1323	0.28	0.26	37:3
I. galbana	1323	0.53	0.51	37:3

[a]Growth rate derived from the dilution rate of the chemostat

[b]Average calculated growth rate from ^{13}C-labeling of alkenones

[c]Specifies between the di (37:2) or tri (37:3)-unsaturated alkenone in growth rate calculations

Figure 4: The relationship between the growth rate obtained from the dilution rate of the chemostat to the calculated growth obtained from the ^{13}C-labeling of alkenones



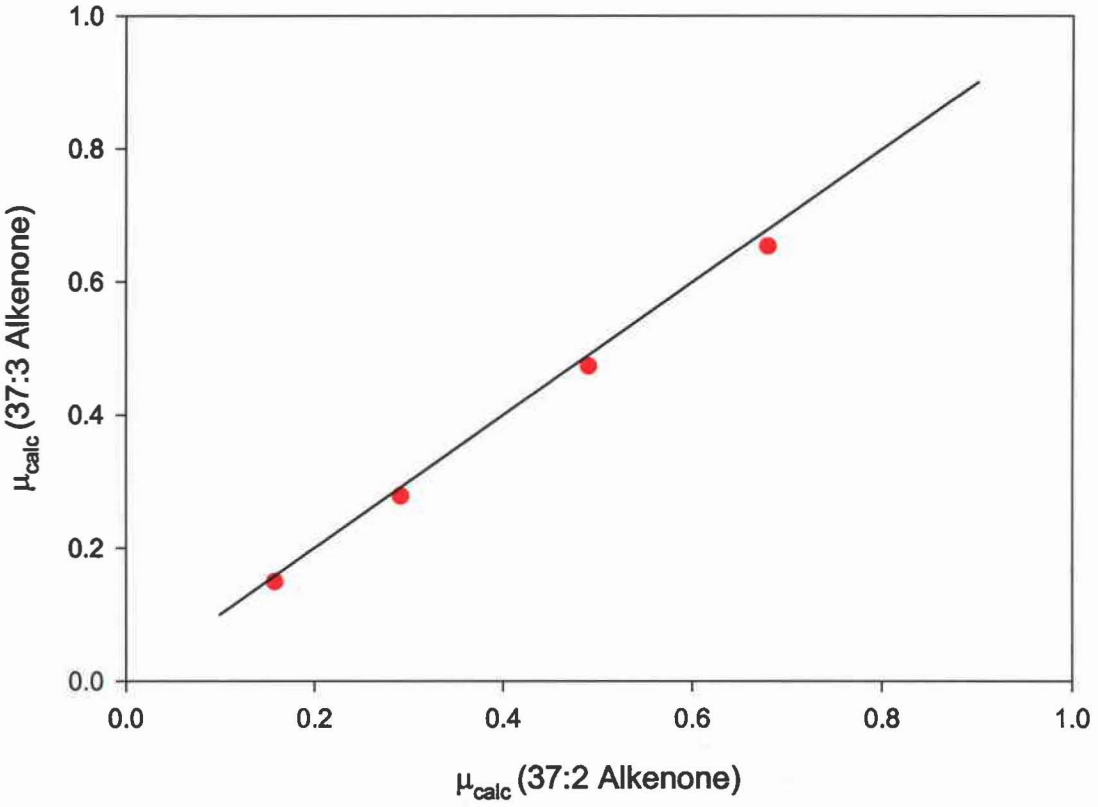
In Table 4 both the 37:2 and the 37:3 alkenone are used to calculate growth rates of alkenone-producing microalgae. To study the bias incurred from using the 37:2 alkenone versus the 37:3 alkenone in the growth rate calculation, four of the chemostat experiments yielded isotopic data from both the 37:2 and 37:3 alkenone. In each case the calculated growth rates from the 37:3 alkenone were slightly lower than the growth obtained from the 37:2 alkenone. The error incurred from using isotopic data from the 37:3 alkenone will be minimal (< 5%) since the difference in calculated growth rates between the di and tri-unsaturated is so small (Table 5 and Figure 5).

Table 5: The relationship between the calculated growth rates from the 37:2 and 37:3 alkenones for four chemostat experiments

Species	Strain (CCMP)	Growth rate (d^{-1})[a]	37:2[b]	37:3[c]
E. huxleyi	374	0.16	0.158	0.149
I. galbana	1324	0.3	0.291	0.278
I. galbana	1324	0.5	0.49	0.473
I. galbana	1324	0.7	0.679	0.653

[a]Growth rate determined from the dilution rate of the chemostat
[b]Average growth rate (d^{-1}) determined from the labeling of the di-unsaturated Alkenone
[c]Average growth rate (d^{-1}) determined from the labeling of the tri-unsaturated Alkenone

Figure 5: The relationship between the calculated growth rates from the 37:2 and 37:3 alkenone for four of the eight chemostats



Potential concerns with this method include active bicarbonate uptake by the phytoplankton, variations in the alkenone content per cell, nutrient limitation, and the ability to apply this method to batch culture.

Active bicarbonate uptake can affect the $\delta^{13}C$ of the cell. Recently, Cassar et al. (2003) have shown that bicarbonate uptake by Southern

Ocean phytoplankton is significant, and that under most environmental conditions, the carbon concentrating mechanism (CCM) is constitutive. Recent experiments by Rost et al. (2003), however, suggest that *E. huxleyi* has a poorly developed CO_2 concentration mechanism that has low carbonic anhydrase activity and can only supply a steady but low concentration of bicarbonate to the cell. In addition, since the ^{13}C-label is distributed among all the species in the DIC, an active uptake of bicarbonate in principle should not affect values of μ_{calc}. The close agreement between μ_{calc} and the growth rate obtained from the dilution rate of the media in this study supports this notion.

It is possible that the alkenone content per cell can bias growth rate calculations. Recently, Prahl et al. (2003) have shown that when *E. huxleyi* are grown under nutrient limitation in batch culture, the intracellular alkenone concentrations increase. In this case, the alkenones should be labeled at a faster rate than the bulk phytoplankton, biasing growth rate calculations to a higher value. When the cells are placed in the dark, cellular alkenone concentrations decrease, potentially biasing growth rate calculations to lower values, until the light cycle resumes. These observations suggest that alkenones may serve the role as storage molecules as opposed to serving only as membrane lipids (Prahl et al., 2003). Experiments on the role of changing alkenone content per cell and their affect on the rate of alkenone ^{13}C labeling are needed in order to apply this

method to field conditions in which cells are subject to light/dark cycles and may be under nutrient limitation.

Results show that in batch culture ^{13}C-labeling of the POC and alkenones closely agree with the absolute value, suggesting that this method can be applied to batch culture conditions. Unpublished batch data are shown in Table 6. These data show calculated growth rates of a batch culture of *E. huxleyi* (B91/11) from ^{13}C-labeling of POC and alkenones. Absolute growth rate was computed from changes in POC concentration.

Table 6: *E. huxleyi* calculated growth rates in batch culture from isotopic labeling of POC and alkenones

	Method	Growth rate (calculated)
change in POC	1	0.23
Isotopic labeling (POC)	2	0.21
Isotopic labeling (alkenone)	3	0.25

Isotopic fractionation as a function of μ/CO_2 is used to provide estimates of growth of natural populations of alkenone-producing algae in the field. In calculating ε_p, the unlabeled ^{13}C-CO_2 isotopic value and the isotopic value of the ^{13}C-unlabeled alkenone are described by Freeman and Hayes (1992). Since growth rate and CO_2 variability lead to differences in the $\delta^{13}C$ of phytoplankton,

biomarker isotopic values are expressed relative to the source organism (Laws et al., 2001) with a stable (Popp et al., 1998) and accepted difference of 4.2 ‰ (Jasper et al., 1994; Popp et al., 1998; Riebesell et al., 2000) for alkenones. Shown in Figure 5 is a plot of ε_p versus μ/CO_2 for three separate studies. The slope of this study is similar to that of Bidigare et al. (1997), with a negative relationship between ε_p versus μ/CO_2. This study yielded a maximum fractionation of 26 to 28‰, relative to 24 to 25‰ observed in the study by Bidigare et al, (1997). The difference results from using data from *I. galbana* in conjunction with *E. huxleyi* in this study as opposed to only *E. huxleyi* (clone BT6 and B92/11) in the other two studies. *I. galbana* has its own size and cell geometry among other characteristics, which contributes to its slightly higher ε_p values at a given value of μ/CO_2. The slope and intercept of this study differs from that of Riebesell et al. (2000). Batch culture and a light/dark cycle were used in their experiment as opposed to a continuous light-chemostat culture in this study. Much of the difference can be explained by the use of a light/dark cycle and potential differences in the growth-limiting resource (Riebesell et al., 2000).

Table 7: The relationship between the total isotopic fractionation (unlabeled-ε_p) to the ratio of growth rate over CO_2 concentration

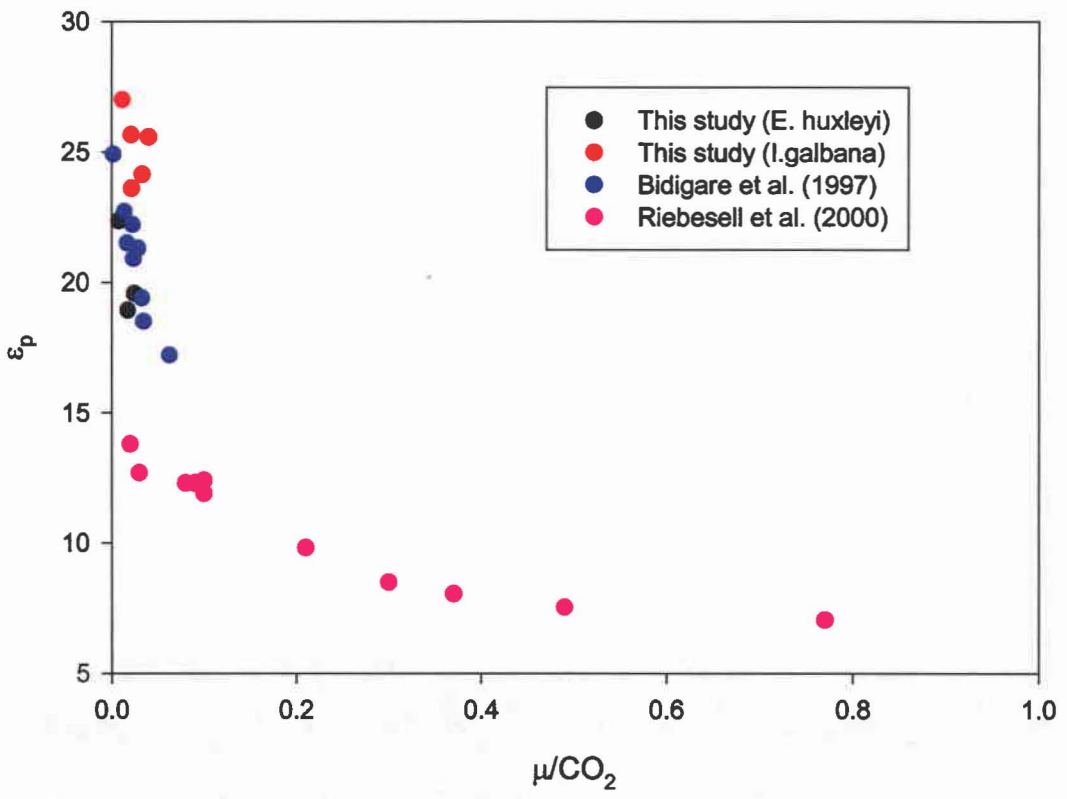
Species	Strain (CCMP)	μ_{true}[a]	μ/CO_2[b]	ε_p[c]
E.huxleyi	374	0.16	0.0079	22.36
E.huxleyi	373	0.21	0.017	18.93
E.huxleyi	373	0.42	0.025	19.57
I.galbana	1324	0.3	0.022	23.59
I.galbana	1324	0.5	0.033	24.13
I.galbana	1324	0.7	0.040	25.56
I.galbana	1323	0.28	0.012	26.99
I.galbana	1323	0.53	0.022	25.65

[a]Growth rate derived from the dilution rate of the chemostat

[b]Growth rate divided by CO_2(aq)

[c]Total carbon isotopic fractionation (unlabeled)

Figure 6: The relationship between total isotopic fractionation and growth rate over the CO_2 concentration in unlabeled samples for three studies



This study also contributes to data relating the natural abundance fractionation of biomarkers (alkenones) to the bulk phytoplankton (POC). Current estimates suggest a difference of 4.20 ‰ between the isotopic compositions of the alkenones and the bulk cells. This study gave a weighted mean of the 37:2 alkenone to be 5.55 ‰ with higher values obtained from the 37: alkenone. When taking into account only the chemostats containing *E. huxleyi*, the weighed mean (37:2 alkenone) becomes 2.63‰, lower than excepted values. Disregarding the *E.huxleyi* 0.21 d^{-1} data (Table 8), the *E. huxleyi* weighed mean increases to 4.72‰, slightly higher than excepted values. Interestingly, the average of the difference between the isotopic composition of the alkenones versus the bulk phytoplankton is 4.17‰ (37:2 alkenone), very close to the excepted value of 4.20 ‰. In any case, such small differences from the accepted value will have negligible effects in calculating ε_p from the ^{13}C-labeling of alkenones due to the fact that 4.2 is added to 1000 in calculating ε_p.

Table 8: Weighted means and averages determined by the fractionation between the bulk phytoplankton and alkenones

Species	Strain (CCMP)	Growth rate (d^{-1})[a]	$\delta^{13}C_{POC}$[b]	$\delta^{13}C_{37:2}$[b]	$\varepsilon_{cell-37:2}$[c] (‰)	$\delta^{13}C_{37:3}$[b]	$\varepsilon_{cell-37:3}$[c] (‰)
E.huxleyi	373	0.16	-40.84	-45.13 ± 0.33	4.49	-44.95 ± 0.30	4.3
E.huxleyi	373	0.21	-34.6	-37.1 ± 0.034	2.6		
E.huxleyi	374	0.42	-31.7	-36.79 ± 0.52	5.28		
I.galbana	1324	0.3	-40.38	-46.64 ± 0.02	6.57	-47.36 ± 0.02	7.33
I.galbana	1324	0.5	-35.77	-43.20 ± 0.49	7.77	-42.10 ± 0.09	6.61
I.galbana	1324	0.7		-44.04 ± 0.39		-44.35 ± 0.63	
I.galbana	1323	0.28	-45.4			-49.74 ± 0.09	4.57
I.galbana	1323	0.53	-42.87			-47.40 ± 0.04	4.76
				Grand mean[d]	5.55 ± 0.017	Grand mean[d]	6.72 ± 0.017
				Average[e]	5.34 ± 0.017	Average[e]	5.51 ± 0.017

[a]Growth rate determined from the dilution rate of the chemostat

[b]$\delta^{13}C$ of the bulk cell (POC), the di-unsaturated alkenone (37:2), and the tri-unsaturated alkenone (37:3)

[c]Fractionation between whole cells and alkenones determine by $\varepsilon = 1000\{[(\delta^{13}C_{POC} + 1000)/(\delta^{13}C_{Alkenone} + 1000)] - 1\}$

[d]Weighted mean = $\sum(\varepsilon_i/\sigma_i^2)/\sum(1/\sigma_i^2)$; Standard Deviation = $[\sum(1/\sigma_i^2)]^{-0.5}$

[e]Average ε values

Alkenones are produced in the ocean by a few select species, notably *E. huxleyi* and *I. galbana*. *E. huxleyi* is the most widespread coccolithophore species, ranging from the subartic to the subantartic (Marlowe et al., 1990). Alkenones are known to date back into pre-Pleistocene sediments (Marlowe et al., 1990). Alkenones-producing microalgae (*E. huxleyi)* form large blooms, visible in satellite images. Alkenones began to gather attention when it was discovered that the unsaturation ratio of alkenones reflects the organism's growth temperature (Goldman and Carpenter, 1974; Brown et al., 1993; Conte et al., 1994, 1998; Sikes et al., 1997; Müller et al., 1998; Epstein et al., 1998; Prahl et al., 2003). These compounds are also well preserved in the sediments (Brassell, 1993). Studies like this one allow researchers to understand one of the most widespread phytoplankton species on the planet. A method for determining growth rates in the field from ^{13}C-labeling of alkenones provides a way to more fully study these organisms, even during non-bloom conditions.

When the value of ε_p (carbon isotopic fractionation) is determined from the isotopic composition of the alkenones, as well as the Cd/Ca measurements of planktonic foraminifera to constrain growth rate, paleo-[CO_2(aq)] can be determined in the field using the relationships developed in the laboratory (Popp et al., 1998, 1999). The Cd/Ca measurements can be used to estimate ancient

phosphate levels, which in turn, can be used to infer microalgal growth rates (Bidigare et al., 1997). This gives great potential for the use of alkenones as a CO_2 proxy because cell geometry and growth rate of alkenone-producing algae can be estimated.

Our approach is analogous to the method for determining phytoplankton growth rates using ^{14}C-labeling of pigments (Goericke and Welschmeyer, 1984, 1992, 1993) but uses irmGCMS to determine the rate of ^{13}C incorporation into alkenones.

Besides providing a method for understanding these alkenone-producing microalgae, this study also aids in understanding controls on isotopic fractionation. The role of growth rate on isotopic fractionation and uptake is one of the major objectives in this study.

Results agree with the growth rate obtained from the dilution rate of the chemostat, suggesting that the ^{13}C-labeling of alkenones gives reliable estimates of growth rate. It also suggests that this method can be applied to field conditions. The development and evaluation of the alkenone ^{13}C-labeling method is needed to allow the paleoceanographic community to use this proxy to better understand mechanisms of climate change. Results from these experiments may help to reveal the pathways of formation and biochemical functions of alkenones. This technique may also lend itself to the development of methods to estimate growth rates of other algae and bacteria such as the Archaea *Crenarchaeota*, which produces the biomarker crenarchaeol (Damste et al., 2002) as well as the

Archaea *Methanococcus burtonii*, a known producer of the *sn*-2-hydroxyarchaeol biomarker (Hinrichs et al., 1999). In the future, this method will be used to test laboratory based ^{13}C-isotopic fractionation hypotheses in the field by comparing growth rates determined using the ^{13}C alkenone labeling method in conjunction with field incubations with those calculated using the natural abundance carbon isotopic composition of alkenone and laboratory-derived fractionation relationships.

Chapter 5: Conclusions

Results from this study show that ^{13}C-labeling of alkenones gives reliable estimates of growth over a wide range of growth rates (0.16 d^{-1} to 0.70 d^{-1}) with differing alkenone-producing species (*E. huxleyi* CCMP373 and 374, *I. galbana* CCMP1323 and 1324). Reliable estimates of growth were obtained even on short intervals (6 hrs) and low growth rate (0.16 d^{-1}). A doubling of CO_2(aq) concentration from around 15 μmol/kg to over 30 μmol/kg appeared to have little to no effect on calculated growth rates, which suggests that the method can be applied to the range of CO_2(aq) concentrations found in the open ocean.

In determining growth rates of natural open-ocean alkenone-producers (namely *E. huxleyi*), equation 3 can be used to calculate growth rates even using short incubation times (e.g. 6-12 hrs) due to the fact that the labeling of alkenones matches that of the bulk phytoplankton on time scales of six hours or less.

Results from this study show that reliable estimates of growth can be obtained by both the di and tri-unsaturated alkenone. Although the tri-unsaturated alkenone consistently gave slightly lower growth rate estimates versus the di-unsaturated alkenone, they were still acceptable and within a few percent of the calculated growth rate from the di-unsaturated alkenone.

This study also expands on the small database on the difference between the natural abundance carbon isotopic composition of alkenones versus whole cell. While the weighted mean of the isotopic difference between the two was higher than literature values, the average of the *E. huxleyi* experiments was within

a few percent of the accepted value (4.17 ‰ versus the accepted value of 4.20 ‰).

Since the growth rate estimates from the ^{13}C-labeling of alkenones was so close to the true growth rate derived from the dilution rate of the chemostat, this gives merit to the application of the method to determine growth rate of other aquatic organisms that express unique biomarkers.

Appendix: Growth Rate Calculation Data

Table 1 E.huxleyi 0.21d^{-1} CCMP 373
Growth Rate Calculations
0.21 per day

time, d	u, d-1	$e^{-\mu t}$	13C/12C initial POC	13C/12C final POC	13C/12C DIC	GROWTH RATE		
0.25	0.21	0.948854	0.010849	0.010946	0.012670	0.21		
0.25	0.21	0.948854	0.010849	0.010949	0.012670	0.21		Alkenone u
0.25	**0.21**	**0.948854**	**0.010821**	**0.010872**	**0.012670**	**0.20**		**0.20**
0.5	0.21	0.900325	0.010849	0.011029	0.012618	0.21		0.20
0.5	0.21	0.900325	0.010849	0.011037	0.012618	0.21		0.20
0.5	**0.21**	**0.900325**	**0.010821**	**0.010924**	**0.012618**	**0.20**		0.20
0.75	0.21	0.854277	0.010849	0.011101	0.012464	0.21		0.20
0.75	0.21	0.854277	0.010849	0.011104	0.012464	0.21		
0.75	**0.21**	**0.854277**	**0.010821**	**0.010961**	**0.012464**	**0.20**	ave	0.199
1	0.21	0.810584	0.010849	0.011160	0.012305	0.21	stdev	0.003
1	0.21	0.810584	0.010849	0.011165	0.012305	0.21		
1	**0.21**	**0.810584**	**0.010821**	**0.010988**	**0.012305**	**0.20**		
2	0.21	0.657047	0.010849	0.011298	0.012078	0.21	Average u	stdev
2	0.21	0.657047	0.010849	0.011310	0.012078	0.21	0.21	0.007
2	**0.21**	**0.657047**	**0.010821**	**0.011132**	**0.012078**	**0.20**		

Table 2 E.huxleyi 0.42 d^{-1} CCMP 373
Growth Rate Calculations
0.42 per day

time, d	u, d-1	e^{-ut}	13C/12C initial POC	13C/12C final POC	13C/12C DIC	GROWTH RATE		
0.25	0.42	0.900325	0.010881	0.011089	0.012887	0.42		
0.25	0.42	0.900325	0.010881	0.011089	0.012887	0.42		Alkenone u
0.25	**0.42**	**0.900325**	**0.010824**	**0.010967**	**0.012887**	**0.40**		**0.400**
0.5	0.42	0.810584	0.010881	0.011241	0.012822	0.42		0.406
0.5	0.42	0.810584	0.010881	0.011260	0.012822	0.42		0.407
0.5	**0.42**	**0.810584**	**0.010824**	**0.011120**	**0.012822**	**0.41**		**0.411**
0.75	0.42	0.729789	0.010881	0.011337	0.012658	0.42		0.414
0.75	0.42	0.729789	0.010881	0.011389	0.012658	0.42		
0.75	**0.42**	**0.729789**	**0.010824**	**0.011216**	**0.012658**	**0.41**	average	0.408
1	0.42	0.657047	0.010881	0.011461	0.012544	0.42	stdev	0.005
1	0.42	0.657047	0.010881	0.011466	0.012544	0.42		
1	**0.42**	**0.657047**	**0.010824**	**0.011321**	**0.012544**	**0.41**		
2	0.42	0.431711	0.010881	0.011627	0.012353	0.41	Average u	stdev
2	0.42	0.431711	0.010881	0.011688	0.012353	0.42	0.42	0.007
2	**0.42**	**0.431711**	**0.010824**	**0.011587**	**0.012353**	**0.41**		

Table 3 E.huxleyi 0.16d^{-1}, CCMP 374

Growth Rate Calculations

0.16 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.16	0.960789	0.010778	0.010876	0.012878	0.16		
0.25	0.16	0.960789	0.010778	0.010876	0.012878	0.16		alkenone u
0.25	**0.16**	**0.960789**	**0.010730**	**0.010800**	**0.012878**	**0.16**		0.155
0.5	0.16	0.923116	0.010778	0.010954	0.012880	0.16		**0.157**
0.5	0.16	0.923116	0.010778	0.010953	0.012880	0.16		0.160
0.5	**0.16**	**0.923116**	**0.010730**	**0.010874**	**0.012880**	**0.16**		0.159
1	0.16	0.852144	0.010778	0.011076	0.012807	0.16		**0.158**
1	0.16	0.852144	0.010778	0.011073	0.012807	0.16		
1	**0.16**	**0.852144**	**0.010730**	**0.011040**	**0.012807**	**0.16**	ave	0.158
1.25	0.16	0.818731	0.010778	0.011107	0.012619	0.16	stdev	0.002
1.25	0.16	0.818731	0.010778	0.011106	0.012619	0.16		
1.25	**0.16**	**0.818731**	**0.010730**	**0.011058**	**0.012619**	**0.16**		
2	0.16	0.726149	0.010778	0.011203	0.012463	0.16	Average u	stdev
2	0.16	0.726149	0.010778	0.011212	0.012463	0.16	0.16	0.003
2	**0.16**	**0.726149**	**0.010730**	**0.011161**	**0.012463**	**0.16**		

Table 4 E.huxleyi 0.16d^{-1}, CCMP 374

Growth Rate Calculations

0.16 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.16	0.960789	0.010778	0.010876	0.012878	0.16		alkenone u
0.25	0.16	0.960789	0.010778	0.010876	0.012878	0.16		0.149
0.25	**0.16**	**0.960789**	**0.010732**	**0.010781**	**0.012878**	**0.15**		**0.148**
0.5	0.16	0.923116	0.010778	0.010954	0.012880	0.16		0.149
0.5	0.16	0.923116	0.010778	0.010953	0.012880	0.16		0.150
0.5	**0.16**	**0.923116**	**0.010732**	**0.010822**	**0.012880**	**0.15**		**0.151**
1	0.16	0.852144	0.010778	0.011076	0.012807	0.16		
1	0.16	0.852144	0.010778	0.011073	0.012807	0.16	ave	0.149
1	**0.16**	**0.852144**	**0.010732**	**0.010910**	**0.012807**	**0.15**	stdev	0.001
1.25	0.16	0.818731	0.010778	0.011107	0.012619	0.16		
1.25	0.16	0.818731	0.010778	0.011106	0.012619	0.16		
1.25	**0.16**	**0.818731**	**0.010732**	**0.010931**	**0.012619**	**0.15**		
2	0.16	0.726149	0.010778	0.011203	0.012463	0.16	Average u	stdev
2	0.16	0.726149	0.010778	0.011212	0.012463	0.16	0.16	0.006
2	**0.16**	**0.726149**	**0.010732**	**0.011012**	**0.012463**	**0.15**		

Table 5 TISO $0.3d^{-1}$, CCMP 1324

Growth Rate Calculations

0.3 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.3	0.927743	0.010783	0.010884	0.012650	0.29		
0.25	0.3	0.927743	0.010783	0.010883	0.012650	0.29		Alkenone u
0.25	**0.3**	**0.927743**	**0.010713**	**0.010770**	**0.012650**	**0.27**		**0.273**
0.5	0.3	0.860708	0.010783	0.010992	0.012179	0.30		0.289
0.5	0.3	0.860708	0.010783	0.010988	0.012179	0.30		0.310
0.5	**0.3**	**0.860708**	**0.010713**	**0.010855**	**0.012179**	**0.29**		**0.289**
1	0.3	0.740818	0.010783	0.011141	0.011608	0.31		0.292
1	0.3	0.740818	0.010783	0.011163	0.011608	0.32		
1	**0.3**	**0.740818**	**0.010713**	**0.011043**	**0.011608**	**0.31**	ave	0.291
1.25	0.3	0.687289	0.010783	0.011217	0.012306	0.30	stdev	0.013
1.25	0.3	0.687289	0.010783	0.011228	0.012306	0.30		
1.25	**0.3**	**0.687289**	**0.010713**	**0.011065**	**0.012306**	**0.29**		
2	0.3	0.548812	0.010783	0.011369	0.012144	0.30	Average u	stdev
2	0.3	0.548812	0.010783	0.011385	0.012144	0.30	0.30	0.012
2	**0.3**	**0.548812**	**0.010713**	**0.011214**	**0.012144**	**0.29**		

Table 6 TISO $0.3d^{-1}$, CCMP 1324

Growth Rate Calculations

0.3 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.3	0.927743	0.010783	0.010884	0.012650	0.29		
0.25	0.3	0.927743	0.010783	0.010883	0.012650	0.29		Alkenone u
0.25	**0.3**	**0.927743**	**0.010705**	**0.010733**	**0.012650**	**0.26**		**0.263**
0.5	0.3	0.860708	0.010783	0.010992	0.012179	0.30		0.276
0.5	0.3	0.860708	0.010783	0.010988	0.012179	0.30		0.292
0.5	**0.3**	**0.860708**	**0.010705**	**0.010777**	**0.012179**	**0.28**		**0.277**
1	0.3	0.740818	0.010783	0.011141	0.011608	0.31		0.283
1	0.3	0.740818	0.010783	0.011163	0.011608	0.32		
1	**0.3**	**0.740818**	**0.010705**	**0.010861**	**0.011608**	**0.29**	ave	0.278
1.25	0.3	0.687289	0.010783	0.011217	0.012306	0.30	stdev	0.011
1.25	0.3	0.687289	0.010783	0.011228	0.012306	0.30		
1.25	**0.3**	**0.687289**	**0.010705**	**0.010911**	**0.012306**	**0.28**		
2	0.3	0.548812	0.010783	0.011369	0.012144	0.30	Average u	stdev
2	0.3	0.548812	0.010783	0.011385	0.012144	0.30	0.29	0.015
2	**0.3**	**0.548812**	**0.010705**	**0.011049**	**0.012144**	**0.28**		

Table 7 TISO $0.5d^{-1}$, CCMP 1324

Growth Rate Calculations

0.50 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.5	0.882497	0.010835	0.011037	0.012309	0.51		
0.25	0.5	0.882497	0.010835	0.011035	0.012309	0.51		Alkeone u
0.25	**0.5**	**0.882497**	**0.010752**	**0.010905**	**0.012309**	**0.49**		**0.490**
0.5	0.5	0.778801	0.010835	0.011175	0.012328	0.50		0.488
0.5	0.5	0.778801	0.010835	0.011177	0.012328	0.50		0.489
0.5	**0.5**	**0.778801**	**0.010752**	**0.011037**	**0.012328**	**0.49**		**0.493**
1	0.5	0.606531	0.010835	0.011384	0.012306	0.50		0.492
1	0.5	0.606531	0.010835	0.011390	0.012306	0.50		
1	**0.5**	**0.606531**	**0.010752**	**0.011262**	**0.012306**	**0.49**	ave	0.490
1.25	0.5	0.535261	0.010835	0.011462	0.012166	0.50	stdev	0.002
1.25	0.5	0.535261	0.010835	0.011519	0.012166	0.51		
1.25	**0.5**	**0.535261**	**0.010752**	**0.011334**	**0.012166**	**0.49**		
2	0.5	0.367879	0.010835	0.011573	0.012081	0.50	Average u	stdev
2	0.5	0.367879	0.010835	0.011582	0.012081	0.50	0.50	0.007
2	**0.5**	**0.367879**	**0.010752**	**0.011469**	**0.012081**	**0.49**		

Table 8 TISO $0.7d^{-1}$, CCMP 1324

Growth Rate Calculations

0.70 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.7	0.839457	0.011237	0.011237	0.012731	0.62		
0.25	0.7	0.839457	0.011237	0.011237	0.012731	0.62		alkenone u
0.25	**0.7**	**0.839457**	**0.010742**	**0.011016**	**0.012731**	**0.68**		0.684
0.5	0.7	0.704688	0.011237	0.011377	0.012714	0.64		**0.680**
0.5	0.7	0.704688	0.011237	0.011377	0.012714	0.64		0.673
0.5	**0.7**	**0.704688**	**0.010742**	**0.011217**	**0.012714**	**0.68**		0.678
1	0.7	0.496585	0.011237	0.011636	0.012628	0.67		**0.681**
1	0.7	0.496585	0.011237	0.011643	0.012628	0.67		
1	**0.7**	**0.496585**	**0.010742**	**0.011450**	**0.012628**	**0.67**	ave	0.679
1.25	0.7	0.416862	0.011237	0.011739	0.012517	0.68	stdev	0.004
1.25	0.7	0.416862	0.011237	0.011739	0.012517	0.68		
1.25	**0.7**	**0.416862**	**0.010742**	**0.011552**	**0.012517**	**0.68**		
2	0.7	0.246597	0.011237	0.011958	0.012479	0.68	Average u	stdev
2	0.7	0.246597	0.011237	0.011956	0.012479	0.68	0.66	0.023
2	**0.7**	**0.246597**	**0.010742**	**0.011802**	**0.012479**	**0.68**		

Table 9 Isochrysis galbana 0.28d^{-1}, CCMP 1323

Growth Rate Calculations

0.28 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.28	0.932394	0.010727	0.010882	0.012837	0.28		
0.25	0.28	0.932394	0.010727	0.010887	0.012837	0.29		alkenone u
0.25	**0.28**	**0.932394**	**0.010678**	**0.010719**	**0.012837**	**0.25**		0.276
0.5	0.28	0.869358	0.010727	0.011009	0.012806	0.28		**0.253**
0.5	0.28	0.869358	0.010727	0.011009	0.012806	0.28		0.262
0.5	**0.28**	**0.869358**	**0.010678**	**0.010794**	**0.012806**	**0.25**		0.267
1	0.28	0.755784	0.010727	0.011206	0.012669	0.28		**0.271**
1	0.28	0.755784	0.010727	0.011205	0.012669	0.28		
1	**0.28**	**0.755784**	**0.010678**	**0.010969**	**0.012669**	**0.26**	ave	0.266
1.25	0.28	0.704688	0.010727	0.011278	0.012484	0.28	stdev	0.009
1.25	0.28	0.704688	0.010727	0.011277	0.012484	0.28		
1.25	**0.28**	**0.704688**	**0.010678**	**0.011041**	**0.012484**	**0.27**		
2	0.28	0.571209	0.010727	0.011407	0.012347	0.28	Average u	stdev
2	0.28	0.571209	0.010727	0.011411	0.012347	0.28	0.27	0.013
2	**0.28**	**0.571209**	**0.010678**	**0.011222**	**0.012347**	**0.27**		

Table 10 I.galbana 0.53d^{-1}, CCMP 1323

Growth Rate Calculations

0.53 per day			13C/12C	13C/12C	13C/12C	GROWTH		
time, d	u, d-1	e^{-ut}	initial POC	final POC	DIC	RATE		
0.25	0.53	0.875903	0.010755	0.010999	0.012655	0.53		
0.25	0.53	0.875903	0.010755	0.010999	0.012655	0.53		alkenone u
0.25	**0.53**	**0.875903**	**0.010705**	**0.010833**	**0.012655**	**0.49**		0.492
0.5	0.53	0.767206	0.010755	0.011186	0.012604	0.53		**0.498**
0.5	0.53	0.767206	0.010755	0.011184	0.012604	0.53		0.508
0.5	**0.53**	**0.767206**	**0.010705**	**0.010970**	**0.012604**	**0.50**		0.514
1	0.53	0.588605	0.010755	0.011401	0.012414	0.53		**0.516**
1	0.53	0.588605	0.010755	0.011400	0.012414	0.53		
1	**0.53**	**0.588605**	**0.010705**	**0.011192**	**0.012414**	**0.51**	ave	0.506
1.25	0.53	0.515561	0.010755	0.011459	0.012208	0.53	stdev	0.010
1.25	0.53	0.515561	0.010755	0.011451	0.012208	0.53		
1.25	**0.53**	**0.515561**	**0.010705**	**0.011253**	**0.012208**	**0.51**		
2	0.53	0.346456	0.010755	0.011546	0.012100	0.52	Average u	stdev
2	0.53	0.346456	0.010755	0.011545	0.012100	0.52	0.52	0.013
2	**0.53**	**0.346456**	**0.010705**	**0.011414**	**0.012100**	**0.52**		

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