A new method for estimating growth rates of alkenone-producing haptophytes

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Abstract

Laboratory culture experiments were performed to establish the range of growth conditions where ^{13}C labeling of di- and tri-unsaturated C_{37} methyl ketones yields reliable growth rates for alkenone-producing algae. Results document that even at low growth rates and short time intervals, ^{13}C labeling of the di-unsaturated C_{37} alkenone provides reasonable estimates of growth rate for *Isochrysis galbana*, *Isochrysis* sp., and three strains of *Emiliania huxleyi*. These findings suggest that although alkenone biosynthesis almost certainly involves a complex combination of intermediate pools, those pools must turn over at a rate sufficiently fast that the labeling of the di-unsaturated C_{37} alkenone is not greatly biased. However, bias was noted for the tri-unsaturated alkenone, suggesting that either growth rates in the field should be based on $K_{37:2}$ labeling or that long incubations should be used. Specific growth rates calculated from alkenone ^{13}C labeling experiments conducted in the subarctic Pacific decreased as a function of depth in the euphotic zone and were linearly correlated with photosynthetically active radiation below ~50 $\mu Ein\ m^{-2}\ s^{-1}$. These observations indicate that at depths greater than ~25 m, growth rates of the alkenone-producing algae were light-limited. Determination of the specific growth rates of the alkenone-producing algae was motivated by our present knowledge of the controls on stable carbon isotopic fractionation in marine microalgae. Development of our alkenone ^{13}C labeling technique for in situ growth rate determinations allows evaluation of the effect of growth rate on carbon isotopic fractionation in natural populations of *E. huxleyi* and *Gephyrocapsa oceanica* so that laboratory-based microalgal stable isotope fractionation hypotheses may be evaluated in the field.

Introduction

Specific rates of marine phytoplankton growth (μ) have until recently been difficult to measure. Two field methods used to determine phytoplankton-specific growth rate are the ^{14}C pigment labeling technique (Redalje and Laws 1981; Welschmeyer and Lorenzen 1984) and the dilution method (Landry and Hasset 1982). Redalje and Laws (1981) introduced the ^{14}C pigment labeling method for determining the growth rate and carbon content of natural phytoplankton by deter-

mining the specific activity of ^{14}C-labeled chlorophyll *a*. Their assumption was that the specific activities of chlorophyll carbon and total cellular carbon were equivalent after a given time period of isotope labeling. By combining the measured rate of carbon fixation with calculated cell carbon concentrations, one can estimate the carbon-specific growth rate (Welschmeyer and Lorenzen 1984; Laws 1984). Goericke and Welschmeyer (1992a,b, 1993a) further demonstrated that this technique could be adapted to various types of phytoplankton carotenoids for purposes of determining taxon-specific growth rates. An alternative field method for measuring the specific growth rate of phytoplankton is the dilution technique introduced by Landry and Hasset (1982). Determination of specific growth rate using this method is based on examining the rate of change of pigment concentration including carotenoids in an incubation experiment and can thus provide growth rate information with a similar algal-specificity to that of Goericke and Welschmeyer (1992a,b, 1993a). Recently, Pel et al. (2004) combined compound-specific isotope ratio mass spectrometry

and pyrolytic methylation-gas chromatography (Py-GC-IRMS) of fatty acids released from phytoplankton fractions collected using fluorescence-activated cell sorting to measure the δ^{13}C of natural cyanobacterial and algal populations in a lacustrine environment. In addition, they combined the use of flow cytometric cell sorting and Py-GC-IRMS with ^{13}C-labeling experiments to determine the standing stocks and population-specific growth rates of the predominant cyanobacterial and algal taxa in Lake Loosdrecht. Fluorescence-activated cell sorting is based on the pigment distribution in phytoplankton and thus provides a similar level of specificity as the pigment ^{14}C-labeling method and dilution techniques.

The purpose of our work is to show that these pigment-based methods can be adapted to stable isotope labeling of taxon-specific biomarkers. We investigated the use of ^{13}C labeling of alkenones. Alkenones are biosynthesized by select haptophytes as a set of C_{37}-C_{38} methyl and C_{38}-C_{39} ethyl ketones (Brassell 1993). We targeted analysis of the di- and tri-unsaturated C_{37} methyl ketone to increase the specificity of the growth rate measurement to only the common open-ocean alkenone-producing haptophytes.

Because typical methods for measuring algal growth rates rely on pigments possessed by many species, the growth rate of alkenone-producing algae can be measured only in the unique situation where these organisms dominate the phytoplankton community (e.g., growth rates were measured for the *E. huxleyi* bloom described in Holligan et al. [1993]). Because available techniques for determining taxon-specific growth rates are based on algal pigments, measured rates pertain to all algae that possess that pigment. Whereas many of the common oceanic haptophytes possess 19′-hexanoyloxyfucoxanthin (e.g., *Emiliania*, *Gephyrocapsa*, *Phaeocystis*, *Chrysochromulina*, *Corymbellus*, and *Imantonia*), only select haptophytes are known to produce alkenones in the open ocean (Conte et al. 1994; Jeffrey and Wright 1994; Thomsen et al. 1994; Volkman et al. 1995). As such, growth rates determined by, for example, the dilution and pigment labeling methods are not specific enough to determine the rate of growth of the alkenone-producing algae. To circumvent the nonspecificity of the pigment 19′-hexanoyloxyfucoxanthin among the oceanic haptophytes, we show here that the rate of ^{13}C incorporation into alkenones can be used to determine in situ growth rates specific to alkenone-producing haptophytes.

Determination of the specific growth rates for the alkenone-producing algae is motivated by our present knowledge of the controls on stable carbon isotopic fractionation in marine microalgae. Results of early studies of carbon isotopic compositions of marine particulate and sedimentary organic matter including biomarker compounds suggested that δ^{13}C variations were mainly due to changes in the concentrations of aqueous CO_2 (Arthur et al. 1985; Hayes et al. 1989; Popp et al. 1989; Rau et al. 1989) and to a lesser extent the physiology of marine phytoplankton (Sharkey and Berry 1985; Rau et al. 1992; Francois et al. 1993; Goericke et al. 1994; Thompson

and Calvert 1994). However, the fact that there is significant variability in correlations between δ^{13}C of marine suspended organic matter and $[CO_2(aq)]$ in the ocean (Goericke and Fry 1994; Rau 1994), implies that factors other than $[CO_2(aq)]$ play an important role in regulating isotopic fractionation in marine phytoplankton. Recent results of modeling (Goericke et al. 1994; Rau et al. 1996, 1997) and laboratory experiments (e.g., Laws et al. 1995, 1997; Bidigare et al. 1997, 1999; Popp et al. 1998a; Brukhardt et al. 1999; Riebesell et al. 2000) have begun to clarify these physiological effects.

Recent laboratory chemostat experiments demonstrated that microalgal growth rate and cell geometry in addition to $[CO_2(aq)]$ strongly affected carbon isotopic fractionation in marine phytoplankton (e.g., Laws et al. 1995; Popp et al. 1998a). In field studies, cell geometry can be quantitatively constrained only when the source of the phytoplankton carbon analyzed is known. Isotopic analyses of alkenones provide a way to constrain the size and shape of the source organism in the open ocean because although these compounds can be produced by four genera of haptophyte algae (*Emiliania*, *Gephyrocapsa*, *Isochrysis*, and *Chrysotila*), alkenone production in open-oceanic waters is predominantly by *E. huxleyi* and the closely related *G. oceanica* (Marlowe et al. 1984, 1990; Brassell 1993; Volkman et al. 1995), both of which are reasonably similar in size and shape.

Laboratory-based studies on carbon isotopic fractionation in *E. huxleyi* vary strongly in their results. Riebesell et al. (2000) examined carbon isotopic fractionation in a calcifying strain of *E. huxleyi* (B92/11) using dilute batch culture experiments over a wide range of CO_2 concentrations (1.1 to 53.5 μM L^{-1}) but a narrow range of growth rates (μ = 0.76 to 0.96 d^{-1}). They found a nonlinear relationship between carbon isotopic fractionation and the ratio μ/CO_2 and surmised a maximum fractionation of approximately 15‰. In contrast, Bidigare et al. (1997) used nitrate-limited continuous cultures (chemostat) to examine fractionation as a function of growth rate and $[CO_2(aq)]$ in a calcifying (B92/11) and non-calcifying (BT6) clone of *E. huxleyi*. They found a strong negative linear correlation between carbon isotopic fractionation and μ/CO_2 which yielded an estimated maximum fractionation of approximately 25‰ with no apparent differences between calcifying and noncalcifying strains. Although a variety of factors can affect carbon isotopic fractionation, the apparently conflicting results of these laboratory experiments argue that independent field evidence is needed to determine the effect of growth rate on carbon isotopic fractionation under natural conditions.

The goal of our study was to develop a method to evaluate the effect of growth rate on carbon isotopic fractionation in natural populations of the alkenone-producing algae so that laboratory-based microalgal fractionation hypotheses may be field-tested. Our key objective was 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. In this article, we show that reli-

able specific growth rates can be determined using ^{13}C labeling of C_{37} di-unsaturated alkenones. We tested this method on two clones of the open ocean alkenone-producing algae *E. huxleyi* and two strains of the major coastal alkenone-producer, *Isochrysis*, using continuous laboratory cultures (chemostats) run at several dilution rates. In these experiments, ^{13}C-labeled bicarbonate was added once steady state was achieved, and samples were collected during time series ranging from 6 to 48 h. These primary experiments were supplemented by batch culture experiments using *E. huxleyi*. Results indicated that even at low growth rates and short time intervals, ^{13}C labeling of C_{37} di-unsaturated alkenones yielded reliable estimates of growth rate for *E. huxleyi* and *I. galbana* over the μ range of 0.16 to 0.70 d^{-1}, providing evidence that the ^{13}C-alkenone labeling method is valid. We also show results of in situ field incubations in the subarctic Pacific using the alkenone ^{13}C labeling technique. In these field experiments, growth rates decreased as a function of depth and light limitation on growth rates was clearly apparent.

Materials and procedures

Laboratory experiments—Axenic cultures of *Emiliania huxleyi* (CCMP 373 and 374), *Isochrysis galbana* (CCMP 1323), and *Isochrysis* sp. (CCMP 1324) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, ME, USA) and grown in 2.3 L nitrate-limited continuous culture on modified (100 μM nitrate) f/2 medium with silicate using 0.2 μm sterile filtered surface seawater from Kaneohe Bay, Oahu, HI. Cultures of *E. huxleyi* strain CCMP 373 were grown at 18 °C, whereas all other experiments were run at 16 °C. All experiments were performed under continuous saturating (~250 μEin m^{-2} s^{-1}) irradiance provided by a bank of daylight fluorescent lamps. CO_2 concentration in solution was controlled by aerating the cultures with a mixture of gases from a tank of CO_2-free air and a tank of 2% CO_2 in air. The mixing ratio of these gases was regulated 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 harvested biomass had been grown with constant phytoplankton biomass and constant concentration and isotopic composition of total dissolved inorganic carbon (DIC). Cultures were considered in steady state when day-to-day cell counts varied less than about ±5%, the concentration of DIC varied less than ±10 μM and the natural abundance carbon isotopic composition of total dissolved inorganic carbon ($\delta^{13}C_{DIC}$) varied within ±0.1‰. After sampling for the natural abundance carbon isotopic composition of alkenones, cultures were allowed approximately 1 week to regain steady state, at which time ^{13}C-labeled bicarbonate was added to both the chemostat and the nutrient reservoir. We used a modest enrichment of ^{13}C in the DIC pool (~190‰), which provided a sufficiently unambiguous isotopic signal in the alkenones to determine ^{13}C uptake even at low growth rates (~0.1 d^{-1}) and

short time intervals (6 h). These label additions required no more than a 0.3% increase in the ambient DIC concentration. Cell counts, alkalinity, DIC, POC, and alkenone samples were taken at regular intervals (typically 6, 12, 24, 30, and 48 h) to monitor the ^{13}C uptake into bulk organic matter and alkenones. Carbonate system parameters were not measured for the initial time point after the ^{13}C label was added. The initial isotopic composition of ^{13}C-labeled DIC was calculated using isotope mass balance knowing DIC concentration of the initial time point, the $\delta^{13}C_{DIC}$ of the prelabeled sample, and the isotopic composition and volume of the stock ^{13}C-trace solution added to the prelabeled sample.

Cultures of *E. huxleyi* (CCMP 1742) obtained from the CCMP were grown in batch cultures in 4 L polycarbonate bottles under conditions of nutrient and light saturation (~315 μEin m^{-2} s^{-1}) at constant, controlled temperature (15.3 °C and 19.5 °C). Each dilute batch culture consisted of 3.8 L sterilized, 0.2-μm filtered Kaneohe Bay seawater with added nutrients (f/2 media). Cultures were inoculated with ~300 cells mL^{-1}. Growth rates, determined by daily change in cell density, varied from 0.32 to 0.47 d^{-1}. Initial samples were taken when cultures reached a cell density of ~30,000 cells mL^{-1}. Cell growth at this density has been found to have a minimal effect on seawater inorganic carbon chemistry (Laws et al. 2001). Once cell density reached this level, ^{13}C-labeled bicarbonate was added. Cell counts, alkalinity, DIC, POC, and alkenone samples were taken at regular intervals (0 and 12 h or 0, 12, and 24 h) to monitor the ^{13}C uptake into the bulk phytoplankton and alkenones.

Procedures for collection and processing of algal and water samples and chemical and isotopic analysis and calculations were identical to those of Laws et al. (1995, 1997) and Popp et al. (1998a). Cell density for all experiments was determined using a Coulter Z1 dual threshold particle counter using a 50-μm aperture tube and a 3- to 8-μm threshold to optimize detection of these approximately 5-μm-diameter cells.

Field experiments—We field-tested the alkenone ^{13}C labeling method during R/V *Kilo Moana* cruise KM-0311 (22 June to 4 August 2003). Results presented here are from KM-0311 Station 2 located in the subarctic Pacific (42 °N, 152 °W). Sample collection followed the protocols and methods described in Prahl et al. (2005). We present here only a brief description and refer the reader to that publication for further details. Suspended particles for natural abundance $\delta^{13}C$ analysis of alkenones were collected from the depth of incubations and were pressure filtered (~10 psi) sequentially through a single precombusted glass-fiber filter (148 mm or 90 mm diameter; Whatman GF/F or Millipore APFF, each 0.7 μm nominal pore size) from water (~80 L) taken from 10 L PVC sample bottles on dedicated CTD casts. Water samples from the depth of incubations were also taken from PVC sample bottles on dedicated CTD casts for determination of total alkalinity, nutrient and total dissolved inorganic carbon (DIC) concentration, and the natural abundance $\delta^{13}C_{DIC}$.

Water samples (~50-100 L) for incubation experiments were transferred from PVC bottles on the CTD rosette to acid-cleaned 25-L polycarbonate bottles, ^{13}C-labeled bicarbonate was added soon thereafter, and the bottles were sealed. The polycarbonate bottles were then attached to an in situ array which was deployed during predawn hours and collected at sunrise the following day. The free-floating array design was used to replicate as closely as possible the natural light and temperature conditions at the depth of collection. Upon retrieval of the in situ array, $\delta^{13}C_{DIC}$ samples were taken from each carboy to confirm the level of isotopic enrichment. Remaining water from all carboys at each depth was then filtered and stored frozen (–20 °C) until needed for analysis.

Sample analysis—Samples from culture experiments for analysis of DIC concentration and $\delta^{13}C_{DIC}$ were prepared using a flow-injection system modified after Kroopnick (1985). Briefly, our system used a sample loop containing 9.67 mL filtered (0.22 µm, Millipore Millex-GP) seawater (preserved with 5 µL saturated mercuric chloride) and an acid loop with 1.2 mL 25% H_3PO_4. The sample and acid were injected into a 13-mL column fitted at the base with a fritted glass disk and sparged with N_2 for 6 min. The sparged CO_2 from acidification of DIC was trapped using liquid N_2 on a multiloop trap and transferred to a vacuum distillation line where the quantity of CO_2 was determined manometrically (MKS Baratron Model 122). DIC concentration based on the $P – V$ calculation yielded an accuracy and precision better than ±10 µmol kg^{-1}. $\delta^{13}C_{DIC}$ from KM-0311 were analyzed using a GasBench (Thermo-Finnigin) coupled to a Delta-Plus XP mass spectrometer (B. N. Popp, T. M. Rust and R. J. Wallsgrove, unpublished method). Overall precision as determined by replicate analysis of samples and standards for these methods was ±0.1‰ for natural abundance and ±1.5‰ for labeled samples. DIC concentration in field samples was determined coulometrically at the NOAA Pacific Marine Environmental Laboratory using a Single-Operator Multi-Metabolic analyzer (SOMMA) system similar to that described by Johnson et al. (1993).

The concentration of $[CO_2(aq)]$ was determined using the program developed by Lewis and Wallace (1998). This program (available at http://cdiac.ornl.gov/oceans/co2rprt.html) calculates the concentration of carbonate species from temperature, salinity, total alkalinity, and the concentrations of DIC, phosphate, and silicate. Apparent dissociation constants used were from Roy et al. (1993) and Dickson (1990a,b). 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 on filtered seawater (0.22 µm; Millipore Millex-GP) was determined by the Gran method (Gran 1952) using computer-controlled titration. Precision and accuracy as determined by multiple analysis of a certified seawater alkalinity standard were determined to be better than ±10 µeq kg^{-1}. Overall precision of our calculation of $[CO_2(aq)]$ was better 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 $[CO_2(aq)]$. The carbon isotopic composition of alkenones was corrected by 4.2‰ before calculating growth rate to account for the isotopic offset between alkenones and whole-cell carbon based on previous analyses of *E. huxleyi* (Popp et al. 1998b). We used the measured difference between the $\delta^{13}C$ of $[CO_2(aq)]$ and cells to correct for isotopic discrimination rather than using an assumed value (e.g., Welschmeyer and Lorenzen 1984).

Samples of phytoplankton cells for carbon isotopic analysis of total organic carbon (TOC) were filtered (Whatman GF/C precombusted at 400 °C for 8 h) from 50 mL (chemostat cultures) or 250 mL (batch cultures) seawater. The filtered material was wrapped in precombusted Al-foil and immediately frozen (–20 °C). When needed for analysis, samples were removed from the freezer, defrosted, decalcified with concentrated sulfurous acid (Verardo et al. 1990), and dried overnight in a 60 °C oven. The isotopic composition of TOC was then determined using an on-line Carlo Erba CN analyzer coupled to an isotope ratio mass spectrometer (Finnigan ConFlo II/Delta-Plus). Prior experience has indicated that the uncertainty of this technique is better than ±1-2% for measurements of C and N weight percentage and better than ±0.2‰ for C isotopic analysis. Carbon isotopic compositions ($\delta^{13}C$) are reported in the standard δ-notation in permil relative to V-PDB.

Samples (200 mL from chemostat cultures or 400-700 mL from batch cultures) for alkenone analysis were filtered (Whatman GF/C precombusted at 400 °C for 8 h), immediately frozen in liquid N_2, and stored until extraction. Filters were extracted with methanol:chloroform (2:1) using automated solvent extraction (Dionex ASE200) (e.g., Sachs and Lehman 1999). Extracts were saponified using aqueous 0.5 N KOH/MeOH, and the neutral lipid fraction was recovered by partitioning into 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 the $\delta^{13}C$ of alkenones. Reported compound-specific isotopic results were obtained 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 of known δ value. Precision of replicate irmGCMS analysis of compounds with natural abundance carbon isotope composition was found in most cases to be ±0.3‰ (±0.00033 ^{13}C atom

%, where atom % = $\left(\dfrac{^{13}C}{^{12}C + ^{13}C} \right) 100$: the $^{13}C/^{12}C$ ratio of the

sample can be calculated directly from the measured δ value knowing the $^{13}C/^{12}C$ ratio of V-PDB = 0.011237) or better for indi-

vidual peaks containing > 5 nM of carbon with no indication of bias or inaccuracy in the results. Measured precision for compounds with modest ^{13}C enrichment (δ values ≈ 50 to 80‰) by irmGCMS was typically better than ±1.5‰ (±0.0016 ^{13}C atom %).

Assessment

Theoretical framework—The purpose of this study was to determine if the rate of ^{13}C enrichment in alkenones tracks specific growth rate (μ). Analysis based on the turnover of cellular carbon (e.g., Goericke and Welschmeyer 1992a,b, 1993a,b) provides the relevant theoretical background, 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):

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

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 1 to obtain

$$\mu = -\frac{1}{t}\ln(1 - P*/A*) \qquad (2)$$

The simplicity of this approach is complicated if there are intermediate pools between the external substrate pool and the final product. Such could be the case when one is monitoring the photosynthetic incorporation of inorganic carbon into an end product such as alkenones, which are now known to serve some type of energy storage function in the algal cell (Epstein et al. 1998; Prahl et al. 2003; Eltgroth et al. 2005). If there is a single intermediate pool, then $P*$ is described by the equation (Goericke and Welschmeyer 1992a,b)

$$P*(t) = A*\left(1 - \frac{b}{b-\mu}e^{-\mu t}\frac{\mu}{\mu-b}e^{-bt}\right) \qquad (3)$$

where b is the turnover rate of the intermediate pool. Rearrangement of equation 3 gives

$$\ln(1 - P*) = -\mu t + \ln\left(\frac{1}{1-r}\right) + \ln\left(1 - re^{\frac{\mu(r-1)t}{r}}\right) \qquad (4)$$

where $r = \mu/b$.

The error incurred by calculating μ from equation 2 instead of equation 4 is a function of both μ*t* and *r* and is small for large μ*t* and small *r*. If μ calculated from the rate of alkenone labeling agrees with that obtained for our culture by other means (e.g., culture dilution rate, ^{13}C labeling rate of TOC in cells, change in cell density), then it suggests that *b* is quite large compared to μ. This would be the case if the intermediate pool was turning over on a time scale of, for example, 1 h. Although the intermediate pool is undoubtedly a complex combination of intermediate pools, it is for practical purposes unnecessary to model anything more complicated. The point is that the existence of intermediate

pools would slow down labeling of the final product (alkenones) and lead to an underestimate of growth rate. The extent of this bias would be negligible if *r* were sufficiently small and μ*t* sufficiently large. The purpose of our work was to explore the range of growth conditions where the alkenone-labeling technique gives reliable estimates of growth rate and to identify under what conditions significant bias is anticipated. Goericke and Welschmeyer (1992a,b, 1993a) used this theoretical framework to evaluate the labeling kinetics of algal pigments. We adapted the same approach here to evaluate the labeling kinetics of alkenones and their precursor compounds.

Experimental background—The rationale for growing phytoplankton in continuous culture (chemostat) is severalfold. 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 constant-volume growth chamber. At steady state, the culture is growing at the rate the limiting nutrient in the medium is supplied, allowing for accurate control of growth rate (Laws et al. 2001). Also, at steady state, the isotopic composition of cells harvested from the growth chamber can be related to the chemistry of the growth chamber medium at the time of harvest (Laws et al. 2001). However, when performing ^{13}C-labeling experiments with a continuous culture, pumping medium into the growth chamber produces loss of isotopically labeled materials, and the $CO_2(g)$ in the aeration gases equilibrates with total dissolved inorganic carbon in the culture medium. Both processes must be considered.

The rate of change of ^{13}C in cells or alkenones in a chemostat is the difference between the rate of uptake of label from the inorganic carbon pool and the loss of labeled cells or alkenones via the overflow, i.e.,

$$\frac{dX}{dt} = -\mu X + \frac{\mu' AY}{DIC} \qquad (5)$$

where *X* is ^{13}C in cells or alkenones, μ is the dilution (or growth) rate of the culture, *A* is the organic carbon in cells or alkenones, *Y* is ^{13}C in dissolved inorganic carbon, DIC is the concentration of dissolved inorganic carbon, and μ′ is the growth rate of the cells at time *t*. There is no a priori reason that μ must equal μ′. If μ and μ′ are unequal, then $A = A_o e^{(\mu'-\mu)t}$ and equation 5 becomes

$$\frac{dX}{dt} + \mu X = \frac{Y}{DIC}\mu' A_o e^{(\mu'-\mu)t} \qquad (6)$$

The fraction of ^{13}C in organic carbon at time zero is X_o/A_o, and the fraction of ^{13}C in organic carbon at time *T* is $\frac{X}{A_o e^{(\mu'-\mu)t}}$. Integrating gives

$$Xe^{\mu T} - X_o = A_o\mu' \int_0^T \frac{Y}{DIC} A_o e^{\mu't} dt \qquad (7)$$

Assuming that *Y*/DIC is independent of time (which turns out to be a good assumption), equation 7 becomes

$$Xe^{\mu T} - X_o = A_o \frac{Y}{DIC}\left(e^{\mu' T} - 1\right) \qquad (8)$$

where Y/DIC is understood to be the average value of the fraction of ^{13}C in DIC during the incubation. Multiplying by $e^{\mu T}$ gives

$$X - X_o e^{-\mu T} = A_o \frac{Y}{DIC} e^{-\mu T}\left(e^{\mu' T} - 1\right) \qquad (9)$$

Dividing both sides by $A_o^{(\mu' - \mu)t}$ gives

$$F - F_o e^{-\mu' T} = \frac{Y}{DIC}\left(1 - e^{-\mu' T}\right) \qquad (10)$$

where F is the fraction of ^{13}C in the organic matter. Equation 10 shows that there is no explicit dependence on the pumping rate μ. Substituting F_{DIC} for Y/DIC and solving for μ' yields

$$\mu' = \frac{1}{T}\ln\left(\frac{F_{DIC} - F}{F_{DIC} - F_o}\right) \qquad (11)$$

Equation 11 is identical to equation 2 and has the obvious property that $F = F_o$ when $T = 0$, and F equals F_{DIC} in the limit as $T \rightarrow \infty$. However, the latter is not strictly true because of isotopic discrimination associated with photosynthesis (Welschmeyer and Lorenzen 1984). Therefore, F_{DIC} was corrected using the natural abundance isotopic fractionation observed in our prelabeled sample.

Derivation of equation 11 assumed that Y/DIC was independent of time. Results indicate a slow exponential decline in values of $\delta^{13}C_{DIC}$ and thus the $\delta^{13}C$ of $CO_2(aq)$ as a function of time in all chemostat experiments (Table 1). This change was driven by equilibration of the $CO_2(g)$ in the aeration gases with $CO_2(aq)$ in the culture medium. Figure 1 shows the change in the ^{13}C atom % of DIC in a continuous culture with no phytoplankton. Since the change in the value of this property was very small in this experiment and all others, we used the average ^{13}C atom % of $CO_2(aq)$ between sampling points in equation 11. We used isotopic enrichment in $CO_2(aq)$ as opposed to total dissolved inorganic carbon since $CO_2(aq)$ is the primary inorganic carbon substrate used by *E. huxleyi* (Rost et al. 2003). Changes in temperature and the distribution of carbonate species in seawater affect carbon isotopic fractionation between total dissolved inorganic carbon (mainly bicarbonate) and $CO_2(aq)$. Therefore, using only the $\delta^{13}C$ of DIC to calculate growth rate may not represent the isotopic composition of the inorganic carbon phase being fixed by the algae. Although this effect is small at large addition of ^{13}C-labeled bicarbonate, the use of ^{13}C atom % DIC with small ^{13}C additions can cause variation up to 10% in calculated growth rates between oceanic sites simply owing to differences in temperature and the distribution of carbonate species between sites and their affect on the equilibrium isotope differences in the ^{13}C activity of bicarbonate and $CO_2(aq)$. Although the isotope chemistry of inorganic carbon changed in our experiments, this phenomenon did not compromise the results of our chemostat experiments because we calculated the ^{13}C atom % of $CO_2(aq)$ and used those values in our growth rate calculations.

Laboratory experiments—Results of laboratory culture experiments indicate that there is little bias in growth rates calculated from the ^{13}C labeling of the $C_{37:2}$ alkenone ($K_{37:2}$, di-unsaturated C_{37} ketone) (Figure 2, Tables 1 and 2). The slope of a least-squares regression of growth rates calculated from the $K_{37:2}$ ^{13}C-labeling rate onto the dilution rate of the chemostat cultures or growth rates determined from the cell density change in batch culture experiments (Figure 2B) is not significantly ($P < 0.05$) different from unity. Average growth rate calculated from the regression equation in Figure 2B is within 0.07 d^{-1} of the dilution rate of the chemostat cultures or growth rates determined from the cell-density change in batch culture experiments. Furthermore, $K_{37:2}$-calculated growth rates are, on average, within < 0.05 d^{-1} of growth rates calculated from the rate of ^{13}C labeling of whole cells (Tables 1 and 2). Even at low dilution rate (0.16 d^{-1}) and short duration (6 h) of labeling, the growth rate calculated from the ^{13}C labeling of $K_{37:2}$ is essentially identical to the dilution rate of the chemostat. Therefore, results of the alkenone ^{13}C-labeling experiments indicate that μ_{calc}/μ_{true} was always greater than 0.9, where μ_{calc} is the calculated $K_{37:2}$ growth rate and μ_{true} is the culture growth rate. These results suggest that b was large compared to μ in all cases (resulting in low values of r) and that the $K_{37:2}$ alkenone intermediate pool was turning over on time scales less than approximately 6 h (equation 4). Therefore, errors incurred by calculating μ for alkenone-producing algae from equation 2 as opposed to equation 4 should be small, even over a large range of μt.

Growth rates calculated from the ^{13}C labeling of $K_{37:2}$ in cultures vary as a function of time. In part, removal of the 400 mL required for sampling the chemostat at each sampling interval may have perturbed cell growth. During a 6-h sampling interval, there was insufficient time for the chemostat to refill at pumping rates < 0.7 d^{-1}. However, even at a dilution rate of 0.7 d^{-1}, it took > 5 h to replenish the volume of material removed for sampling. Therefore, the first 6-h sampling interval was the only time when dilution rate was equal to the overflow rate. Conservatively, this sampling interval represents the time when cells were most likely to have remained at steady-state growth. Difference between the growth rates calculated from the ^{13}C labeling of $K_{37:2}$ and dilution rates for the four experiments averaged < 0.09 d^{-1} (Table 1). On the other hand, the average difference between growth rates calculated from the ^{13}C labeling of $K_{37:2}$ and dilution rate or the growth rate determined by changes in cell counts at the 12-h sampling interval was only 0.06 d^{-1}. Results of 6- and 12-h sampling are consistent with the conclusion that the $K_{37:2}$-alkenone intermediate pool was turning over on time scales less than approximately 6 h.

When both compounds were measured in the same experiment, the rate of ^{13}C labeling of the di-unsaturated C_{37} ketone ($K_{37:2}$) always exceeded that of the tri-unsaturated C_{37} ketone ($K_{37:3}$) (Tables 1 and 2). Consequently, growth rates calculated from the ^{13}C-labeling rate for $K_{37:3}$ significantly underestimate

Table 1. Summary of measured and calculated variables in chemostat culture experiments.

μ, d⁻¹	t, d	ΣCO₂, μmol kg⁻¹	Σalkalinity, μeq kg⁻¹	δ_{DIC}, ‰	[CO₂(aq)], μmol kg⁻¹	δ_{CO2}, ‰	δ_{cells}, ‰	$\delta_{37:2}$, ‰	$\delta_{37:3}$, ‰	μ'_{cells}, d⁻¹ [a]	$\mu'_{37:2}$, d⁻¹ [a]	$\mu'_{37:3}$, d⁻¹ [a]
					E. huxleyi (CCMP 373)							
0.21	Presample	2.12	2.44	−5.4	13.0	−14.4	−34.5		−32.9			
0.21	0.00	2.14	2.44	157.6	14.5	147.2						
0.21	0.25	2.14	2.42	137.8	13.2	127.5	−25.8		−28.3	0.238		0.129
0.21	0.50	2.14	2.44	123.9	13.9	113.7	−18.1		−23.7	0.253		0.152
0.21	0.75	2.13	2.42	110.3	13.7	100.3	−12.0		−20.4	0.241		0.127
0.21	1.00	2.14	2.43	94.6	14.9	84.7	−6.6		−17.9	0.207		0.116
0.21	2.00	2.14	2.41	64.6	13.0	55.0	6.0		−5.1	0.157		0.228
0.42	Presample	2.09	2.33	−4.7	16.9	−13.7	−31.7		−36.8			
0.42	0.00	2.08	2.33	174.3	15.8	163.7						
0.42	0.25	2.09	2.33	157.3	16.9	146.8	−13.2		−24.1	0.446		0.313
0.42	0.50	2.09	2.33	139.9	16.4	129.5	1.2		−10.4	0.460		0.414
0.42	0.75	2.09	2.33	130.3	16.0	120.1	11.2		−1.9	0.401		0.315
0.42	1.00	2.10	2.32	118.7	17.6	108.6	20.1		7.5	0.445		0.424
0.42	2.00	2.12	2.36	90.6	16.6	80.8	37.4			0.370		
					E. huxleyi (CCMP 374)							
0.16	Presample	2.16	2.35	−10.3	21.9	−19.4	−40.8	−45.1	−45.0			
0.16	0.00	2.18	2.36	169.4	22.5	158.6						
0.16	0.25	2.18	2.36	156.6	22.8	146.0	−32.1	−37.9	−40.6	0.210	0.148	0.101
0.16	0.50	2.19	2.37	145.7	22.8	135.1	−25.2	−32.3	−36.9	0.188	0.177	0.094
0.16	1.00	2.21	2.34	136.4	32.4	126.0	−14.5	−17.5	−29.1	0.167	0.230	0.110
0.16	1.25	2.23	2.33	127.3	39.3	116.9	−11.7	−15.9	−27.3	0.100	0.057	0.056
0.16	2.00	2.24	2.34	103.6	39.4	93.5	−2.6	−6.8	−20.0	0.135	0.135	0.085
					I. galbana (CCMP 1323)							
0.28	Presample	2.16	2.30	−10.6	28.9	−19.7	−45.4		−49.7			
0.28	0.00	2.18	2.30	176.2	32.8	165.4						
0.28	0.25	2.19	2.31	153.0	32.4	142.4	−31.4	−44.3	−46.1	0.339		0.079
0.28	0.50	2.19	2.30	144.7	34.3	134.2	−20.3	−28.6	−39.4	0.324		0.174
0.28	1.00	2.18	2.30	124.3	33.6	113.9	−2.8		−23.9	0.323		0.231
0.28	1.25	2.19	2.30	115.2	34.5	104.9	3.6		−17.5	0.312		0.248
0.28	2.00	2.19	2.28	96.4	38.3	86.3	15.3		−1.3	0.264		0.261
0.53	Presample	2.18	2.35	−9.5	24.5	−18.7	−42.9		−47.4			
0.53	0.00	2.18	2.35	171.4	23.6	160.6						
0.53	0.25	2.18	2.30	136.7	31.9	126.2	−21.2	−31.3	−36.0	0.580		0.264
0.53	0.50	2.19	2.31	122.9	33.1	112.6	−4.7	−7.0	−23.8	0.618		0.386
0.53	1.00	2.18	2.29	99.3	35.0	89.2	14.5		−4.1	0.547		0.408
0.53	1.25	2.19	2.29	91.0	38.1	81.0	19.4	15.9	1.4	0.458		0.361
0.53	2.00	2.19	2.28	78.3	38.2	68.4	27.4	26.7	15.7	0.413		0.448
					Isochrysis sp. (CCMP 1324)							
0.30	Presample	2.14	2.42	−10.8	13.7	−19.9	−40.4	−46.6	−47.4			
0.30	0.00	2.15	2.36	156.5	18.6	145.9						
0.30	0.25	2.14	2.36	136.2	18.0	125.8	−31.5	−41.6	−44.9	0.237	0.130	0.060
0.30	0.50	2.13	2.38	120.6	16.3	110.3	−22.0	−34.0	−41.0	0.308	0.234	0.109
0.30	1.00	2.13	2.36	109.1	17.1	98.9	−7.6	−19.2	−33.5	0.295	0.281	0.123
0.30	1.25	2.13	2.35	97.7	17.8	87.6	−1.8	−15.3	−29.0	0.303	0.184	0.172
0.30	2.00	2.12	2.37	76.8	16.1	66.9	12.5	−2.1	−16.7	0.368	0.293	0.194
0.50	Presample	2.13	2.39	−6.7	15.0	−15.8	−35.8	−43.2	−42.1			
0.50	0.00	2.18	2.39	178.9	18.9	168.1						
0.50	0.25	2.16	2.35	135.3	20.8	124.8	−17.9	−29.6	−34.8	0.471	0.345	0.161

Continued

TABLE 1—*Continued*

0.50	0.50	2.16	2.36	110.6	20.3	100.4	−5.5	−17.8	−26.9	0.480	0.425	0.242
0.50	1.00	2.15	2.34	94.7	21.1	84.6	13.3	2.2	−11.8	0.558	0.534	0.320
0.50	1.25	2.14	2.34	88.8	20.4	78.8	20.0	8.6	−6.1	0.606	0.499	0.333
0.50	2.00	2.19	2.34	77.6	27.4	67.6	30.3	20.6	7.6	0.389	0.481	0.346
0.70	Presample	2.15	2.38	−6.3	17.3	−15.5	[b]-39.8	−44.0	−44.3			
0.70	0.00	2.11	2.38	160.3	14.2	149.7						
0.70	0.25	2.13	2.38	143.4	15.2	132.9		−19.7	−28.8		0.679	0.397
0.70	0.50	2.13	2.36	138.8	16.8	128.3	12.5	−1.8	−16.5	0.834	0.597	0.391
0.70	1.00	2.13	2.40	124.9	14.4	114.6	35.8	18.9	−1.1	0.654	0.499	0.287
0.70	1.25	2.10	2.37	122.8	14.1	112.4	44.6	28.0	5.5	0.736	0.603	0.319
0.70	2.00	2.15	2.37	117.0	18.7	106.7	64.1	50.3	24.5	0.903	0.746	0.376

[a]μ' calculated using equation 11.

[b]Assumed based on $\delta_{37:2}$

the dilution rate of the chemostat cultures and growth rates determined from the cell density change in batch culture experiments (Figure 2C). Because growth rates based on the ^{13}C-labeling rate of $K_{37:2}$ best match the culture growth rates, we recommend that growth rates be based on $K_{37:2}$ (see also below).

The concentration ratio of $K_{37:2}/(K_{37:2} + K_{37:3})$ in natural alkenone-producing populations (the so-called $U^{K'}_{37}$ index) changes linearly as a function of temperature (e.g., Muller et al. 1998). The fact that the concentration ratio for these compounds changes with temperature, however, implies nothing about growth rates calculated from their rate of formation because growth rate is defined as dX/dt divided by X. Changes in $K_{37:2}$ and $K_{37:3}$ concentrations affect the analytical pool of alkenones in a sample. The relationship of Muller et al. (1998; $U^{K'}_{37} = 0.033T + 0.044$) indicates that the $K_{37:2}$ alkenone is dominant above approximately 14 °C. Therefore, sufficient quantities of $K_{37:2}$ alkenone should exist for analysis in many environments where these algae are common.

Alkenone labeling kinetics—To investigate alkenone-labeling kinetics, we examined the relative ^{13}C incorporation rates into $K_{37:2}$ and $K_{37:3}$ over the range of growth rates and sampling intervals by plotting the relative specific activity ($P^* \equiv$ [13]C atom % excess $K_{37}/$[13]C atom % excess CO_2) as a function of $\mu \cdot t$ or growth rate–normalized time (Figure 3A, B). It is well known that the quantitative response of $U^{K'}_{37}$ to growth temperature can differ significantly between species and even between strains of the same species of alkenone-producing haptophytes (Conte et al. 1998). Despite the high growth temperatures, the major alkenone produced by algae in our experiments was $K_{37:3}$ except in experiments using *E. huxleyi* strain

**Table 2**. Summary of measured and calculated variables in batch culture experiments.

| μ, d⁻¹	t, d	ΣCO_2, μmol kg⁻¹	Σalkalinity, μeq kg⁻¹	δ_{DIC}, ‰	$[CO_2(aq)]$, μmol kg⁻¹	δ_{CO2}, ‰	δ_{cells}, ‰	$\delta_{37:2}$, ‰	$\delta_{37:3}$, ‰	μ_{cells}, d⁻¹ [a]	$\mu_{37:2}$, d⁻¹ [a]	$\mu_{37:3}$, d⁻¹ [a]
				E. huxleyi (CCMP 1742), temperature = 15.3 °C								
0.32 (± 0.03)	0.00	1.77	2.29	0.4	4.1	−8.8	−34.3	−36.1	−34.1			
0.32 (± 0.03)	0.50	1.77	2.29	209.4	4.1	198.3	15.2	−0.7	−5.6		0.382	0.305
0.32 (± 0.03)	1.00	1.77	2.29	209.4	4.1	198.3		29.9	20.7	0.275	0.392	0.317
0.32 (± 0.03)	0.00	1.73	2.28	0.5	3.7	−8.6	−27.8	−36.2	−33.4			
0.32 (± 0.03)	0.50	1.73	2.28	239.6	3.7	228.2	17.0	2.5	−1.5	0.422	0.352	0.289
0.32 (± 0.03)	1.00	1.73	2.28	239.6	3.7	228.2	51.4	38.4	27.3	0.409	0.372	0.296
				E. huxleyi (CCMP 1742), temperature = 19.5 °C								
0.47 (± 0.06)	0.00	1.83	2.29	0.3	6.2	−8.5	−30.2	−33.1	−33.8			
0.47 (± 0.06)	0.50	1.83	2.29	125.9	6.2	116.0	4.5	−3.7	−11.2	0.658	0.549	0.406
0.47 (± 0.06)	1.00	1.83	2.29	125.9	6.2	116.0	25.3	22.4	9.2	0.595	0.604	0.430
0.47 (± 0.06)	2.00	1.83	2.29	125.9	6.2	116.0	56.8	52.4	40.3	0.607	0.599	0.460
0.47 (± 0.13)	0.00	1.84	2.30	0.4	6.3	−8.4	−29.3	−18.1	−20.6			
0.47 (± 0.13)	0.50	1.84	2.30	126.0	6.3	116.1	−3.9	−0.7	−8.7	0.459	0.350	0.277
0.47 (± 0.13)	1.00	1.84	2.30	126.0	6.3	116.1	20.7	21.6	10.2	0.517	0.456	0.325
0.47 (± 0.13)	2.00	1.84	2.30	126.0	6.3	116.1	48.6	47.8	36.1	0.496	0.468	0.358

[a]μ calculated using equation 2.

1742. Alkenone unsaturation in this strain ($U^{K'}_{37} = 0.034T + 0.039$: Prahl et al. 1988) is similar to that found in the natural alkenone-producing populations representing the open ocean (e.g., Muller et al. 1998).

The relative rates of K_{37} labeling increase exponentially and approach values close to 1 for large t (Figure 3A, B). The relative rates are independent of the culturing method used, but dependent on the degree of unsaturation in K_{37}. The labeling pattern shown in Figures 3A and B is typical of continuous incorporation of a tracer and is consistent with kinetics expected for $P^* = 1 - e^{-\mu t}$ (Shlyk 1970; Welschmeyer and Lorenzen 1984). Goericke and Welschmeyer (1992a,b, 1993a) proposed several models describing the effects of potential biosynthetic pathways of pigments and their precursor compounds on the rates of pigment labeling. They showed that the kinetics of pigment labeling could be examined graphically using relative pigment labeling transformed by $R^* \equiv \ln(1 - P^*)$. Relative $K_{37:2}$-alkenone labeling transformed by $\ln(1 - P^*)$ is linearly correlated with growth rate–normalized time (i.e., $\mu \cdot t$) (Figure 3C). The slope and intercept of this relationship are not significantly different ($P < 0.05$) from -1 and 0, respectively. This labeling pattern is consistent with kinetics of compound labeling described by $P^* = 1 - e^{-\mu t}$ (Goericke and Welschmeyer 1992a,b, 1993a) where the slope of the line equals μ. By viewing time-normalized growth rate, measured μ values should be equal to the rate of growth as if the algae had grown at 1 d^{-1} (Goericke and Welschmeyer 1992a,b). Growth-
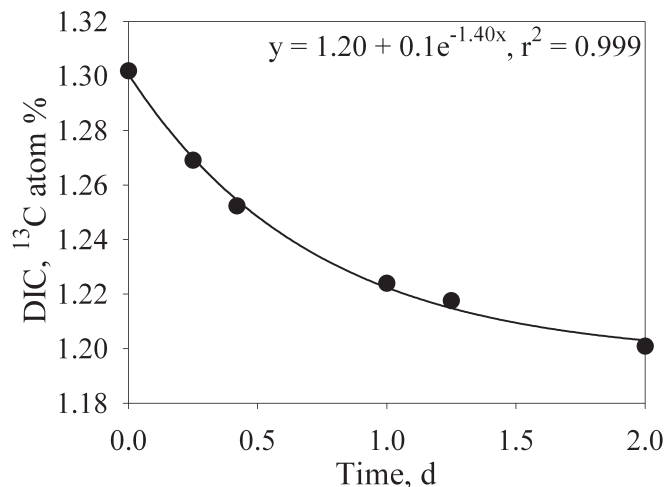
Fig. 1. Change in the ^{13}C atom % of total dissolved inorganic carbon (DIC) as a function of time in a chemostat lacking actively growing cells. At a constant dilution rate of 1 d^{-1} and constant aeration using the mixture of gases (2% CO_2 in air and CO_2-free air) and gas flow rates typical of our experiments, ^{13}C-labeled bicarbonate was added to sterile seawater in the nutrient medium and culture chamber. Because no phytoplankton were introduced to the culture chamber, the change in the ^{13}C atom % of DIC can be attributed only to equilibration of the $CO_2(g)$ in the aeration gases with DIC in the culture media. The line through the data are the best-fit regression using an exponential decay of the form shown.
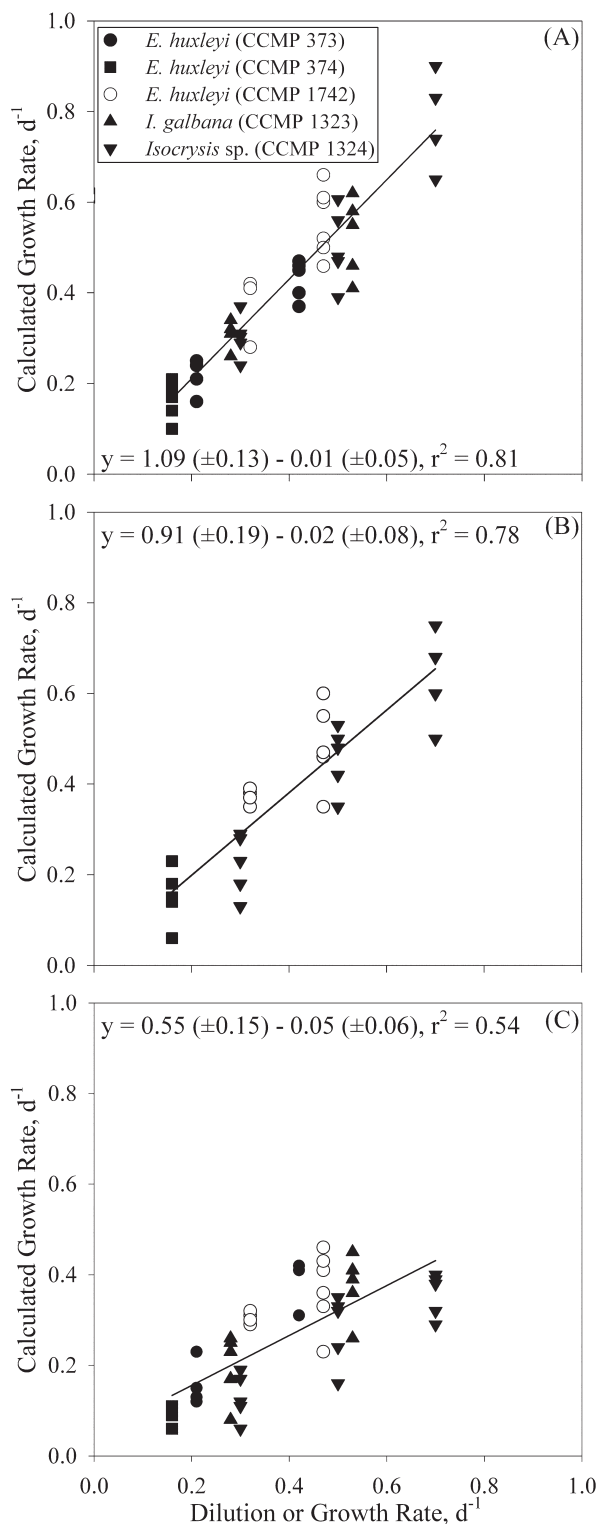
Fig. 2. Growth rates calculated from the ^{13}C-labeling rate of total cellular carbon (A), $K_{37:2}$ (B), and $K_{37:3}$ (C) plotted as a function of the chemostat culture dilution rate or the growth rate determined from the cell density change in batch culture experiments. The solid lines and equations shown on each plot represent results from least-squares regression analysis.
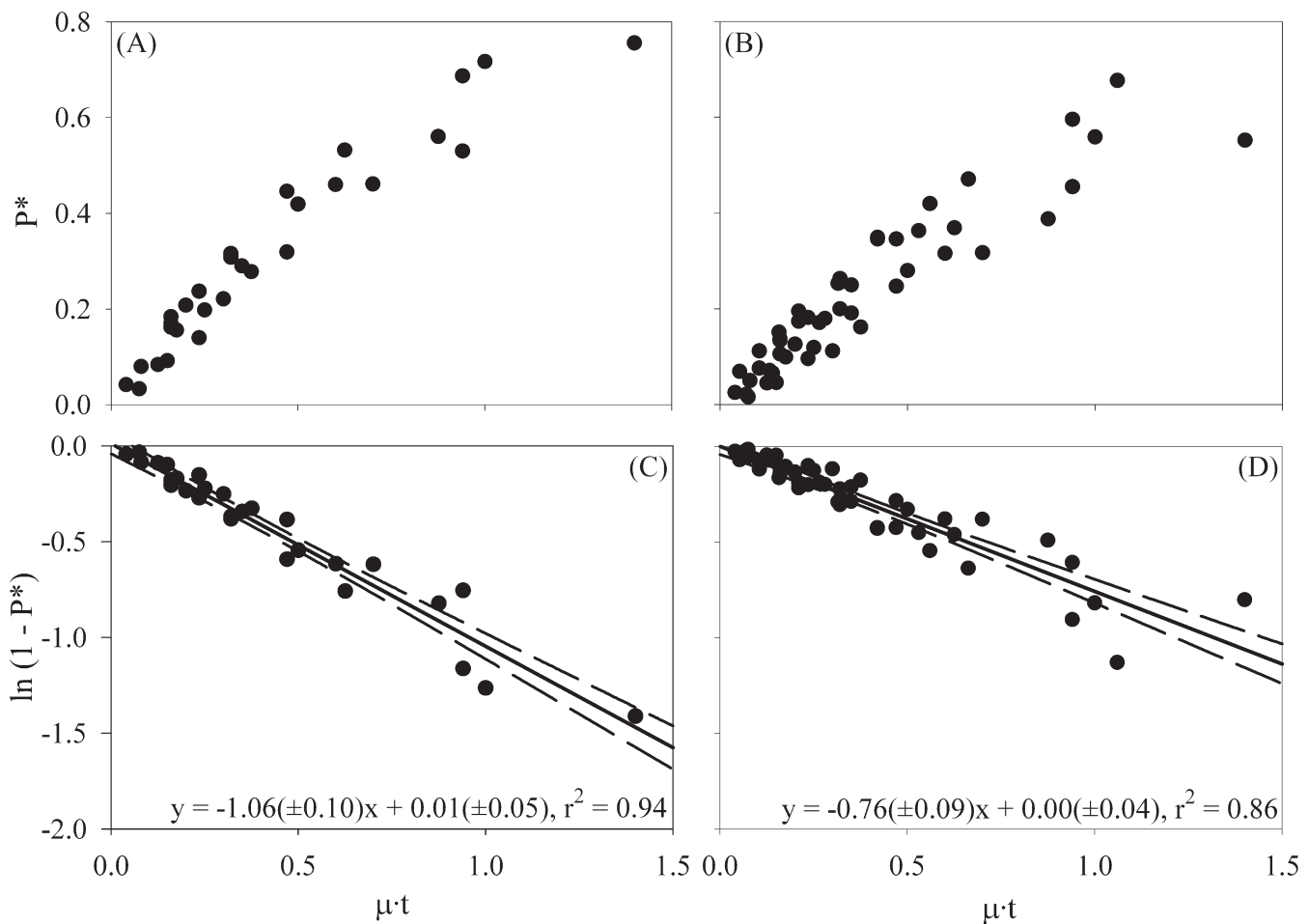
Fig. 3. The time course of ^{13}C-labeling for $K_{37:2}$ (A and C) and $K_{37:3}$ (B and D) observed in all experiments (batch and chemostat cultures) plotted as a function of growth rate normalized time ($\mu \cdot t$). (A) and (B) P^*, defined as the K_{37} ^{13}C atom % excess divided by CO_2(aq) ^{13}C atom % excess, plotted as a function of $\mu \cdot t$. (C) and (D) P^* transformed by $\ln(1 - P^*)$ plotted as a function of $\mu \cdot t$. The solid lines in C and D are least-squares regressions, and the dashed lines show the 95% confidence interval of that regression.

rate–normalized labeling kinetics of $K_{37:2}$ are not significantly different from 1 (Figure 3b), suggesting that at the 95% level of confidence, the kinetics of compound labeling can be adequately described by the simplest model $P^* = 1 - e^{-\mu t}$. Although $K_{37:2}$ biosynthesis must involve a complex combination of intermediate pools, those pools must turn over at a rate sufficiently fast that $K_{37:2}$ labeling is not biased. The implication of these results is that $K_{37:2}$ labeling should allow growth rate determinations using equation 2 (or 11) for experiments involving reasonably short incubation times.

Relative $K_{37:3}$-alkenone labeling transformed by $\ln(1 - P^*)$ is linearly correlated with growth rate–normalized time (Figure 3D). The intercept of this relationship is not significantly different ($P < 0.05$) from 0; however, the slope is significantly less ($P < 0.05$) than –1. Although the linear fit of these data with an intercept through the origin indicates that the kinetics of $K_{37:3}$ labeling follow the form $P^* = 1 - e^{-\mu t}$ (Goericke and Welschmeyer 1992a,b, 1993a), the fact that the slope is not 1

suggests that labeling of a precursor pool significantly slows labeling of $K_{37:3}$. This interpretation is supported by the fact that the discrepancy between growth rates calculated based on the labeling of $K_{37:2}$ and $K_{37:3}$ diminishes in most experiments as the incubation proceeds (Tables 1 and 2). The implications of these findings are that growth rates based on $K_{37:3}$ labeling would be lower than cellular growth rates during short incubation times, and that $K_{37:2}$ is a biosynthetic precursor to $K_{37:3}$. The latter is consistent with the biosynthetic pathway of polyenoic fatty acids in higher plants, whereby 18:2 and 18:3ω3 are formed by the sequential desaturation of 18:1 (Somerville and Browse 1996).

Field experiments—Specific growth rates calculated from ^{13}C labeling of alkenones using equation 2, corrected for respiration and the length of the photoperiod in the subarctic Pacific, decreased as a function of depth in the euphotic zone (Figure 4A). We corrected for day length and respiration using the approximation of Bidigare et al. (1997):
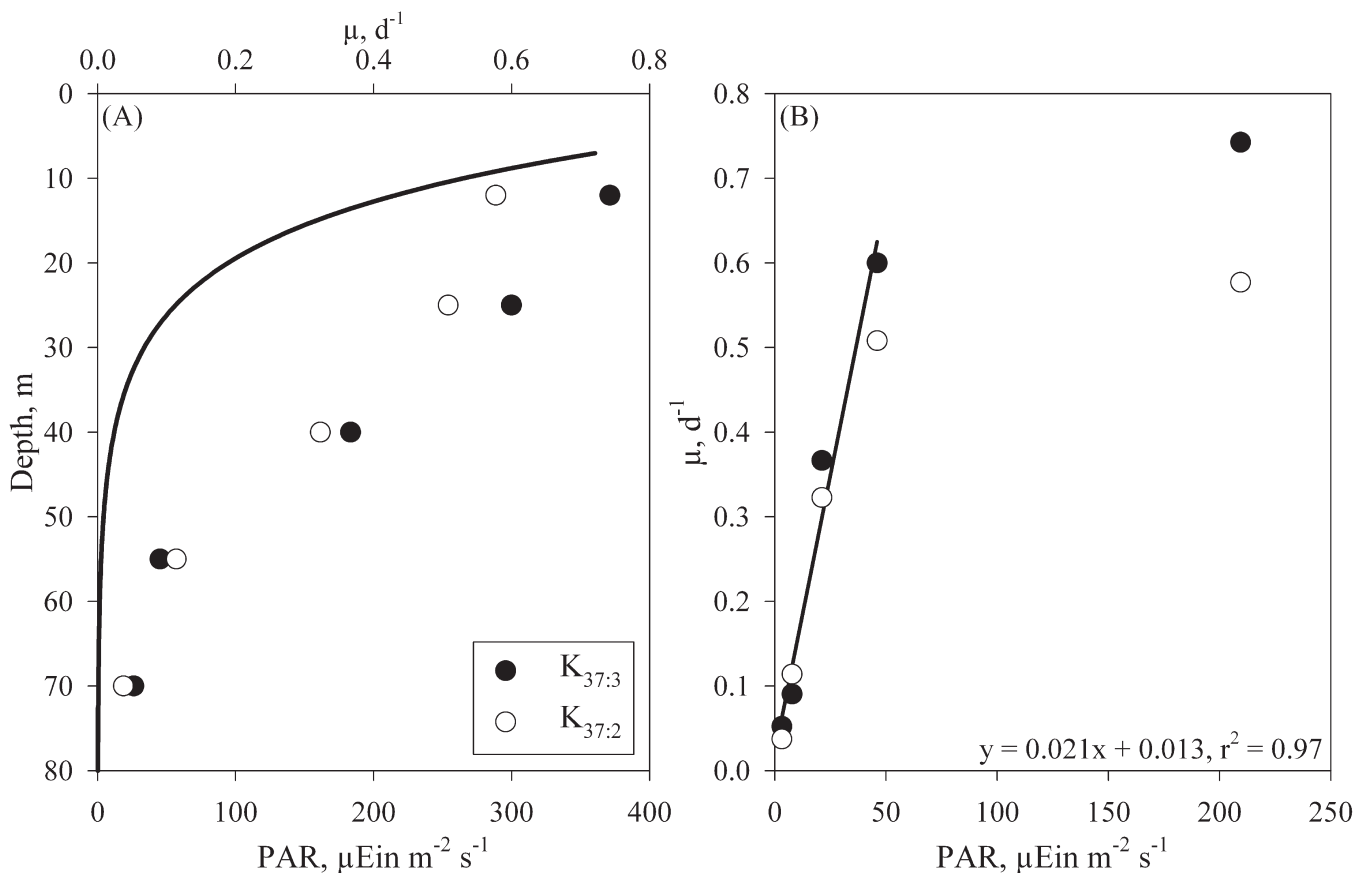
Fig. 4. (A) Plot of in situ growth rates of alkenone-producing algae (● based on $K_{37:3}$ and ○ based on $K_{37:2}$) and the measured noontime photosynthetic active radiation (PAR) as a function of euphotic zone depth. (B) Plot of in situ growth rates of the alkenone-producing algae as a function of PAR in the subarctic Pacific (42 °N, 152 °W). The line in B is a least-squares regression through growth rate data based on $K_{37:3}$ with PAR less than ~50 µEin m^{-2} s^{-1}. PAR was measured in situ using a Biospherical Instruments QSP-200L on the CTD package.

$$\mu = [\mu_{24:0}/(24/t_p)]0.8 \qquad (12)$$

where µ refers to the 24-h average growth rate, $\mu_{24:0}$ is growth rate in continuous light, t_p is the day length, and the factor 0.8 adjusts the growth rate for dark respiration (see Laws et al. 1995). These measured photoperiod growth rates are within the range of those determined for *E. huxleyi* during a mono-specific bloom (Holligan et al. 1993) and for the 19′-hexanoy-loxyfucoxanthin-containing haptophytes (0.1-0.8 d^{-1}, Muggli and Harrison 1996; see also Table 4 in Bidigare et al. [1997]). However, the maximum growth rate measured in subarctic Pacific alkenone-producing algae (0.7 d^{-1}) (Figure 4) is lower than the maximum growth rates for the alkenone-producing coccolithophorids *E. huxleyi* and *G. oceanica* determined by Brand and Guillard (1981) in laboratory cultures (1.7-1.9 d^{-1} when grown on 14:10 light:dark cycle). On the other hand, our field measurement agrees with the maximum growth rates (approximately 0.6 d^{-1}) determined in laboratory cultures of several strains of *E. huxleyi* isolated from a nearby

location (50 °N, 145 °W) in the subarctic Pacific (Muggli and Harrison 1996).

Photoperiod growth rates for alkenone-producing algae in the subarctic Pacific appear to obey Michaelis-Menten kinetics when plotted as a function of in situ measured noontime photosynthetic active radiation (PAR) at the depth of incubation. First-order kinetics are indicated by the linear correlation between growth rate and PAR below approximately 50 µEin m^{-2} s^{-1} (Figure 4B); thus at depths greater than approximately 25 m, growth of the alkenone-producing algae was limited by light. Saturating light intensities reported for *E. huxleyi* range from 72 to > 600 µEin m^{-2} s^{-1} for photosynthesis (Paasche 1964: 107 µEin m^{-2} s^{-1}; Nielsen 1995: 72–114 µEin m^{-2} s^{-1}; Nielsen 1997: 117–299 µEin m^{-2} s^{-1}; Balch et al. 1992: 351–1000 µEin m^{-2} s^{-1}; Muggli and Harrison 1996: 60 µEin m^{-2} s^{-1}; Zondervan et al. 2002: ~80 µEin m^{-2} s^{-1}). Results from the subarctic Pacific are consistent with the lower limit of saturating irradiances for *E. huxleyi* in laboratory culture studies (i.e., ~ 50-100 µEin m^{-2} s^{-1}) and suggest that these experiments are indicative of natural populations.

Results show that growth rates calculated from the rate of labeling of $K_{37:3}$ exceed those calculated using $K_{37:2}$ (Figure 4), indicating that $K_{37:3}$ labeled more rapidly than $K_{37:2}$. This pattern is opposite that observed in our laboratory experiments and indicates that $K_{37:3}$ turnover in the field was somehow more rapid than that of $K_{37:2}$. Because our kinetic analysis of the relative rates of alkenone labeling in laboratory experiments indicates that precursor labeling affects $K_{37:3}$ more than $K_{37:2}$, we suggest that the higher turnover of $K_{37:3}$ relative to $K_{37:2}$ in the field experiments resulted from preferential consumption of $K_{37:3}$. Prahl et al. (2003) demonstrated preferential consumption of $K_{37:3}$ relative to $K_{37:2}$ when cells are kept in complete darkness for several days in laboratory experiments using *E. huxleyi* strain 1742. Although the depth of the shallowest incubation was above the apparent lower limit of saturating irradiances for *E. huxleyi*, we suggest that the physiological state of these algae must have somehow influenced the rate of $K_{37:3}$-alkenone turnover and ^{13}C labeling. It should be noted that the relative difference between growth rates calculated using $K_{37:2}$ and $K_{37:3}$ are reasonably constant (i.e., the percent difference in calculated growth rates falls within 1 standard deviation). The absolute difference appears larger at shallower depths because the growth rates are higher.

Results of laboratory experiments suggest that growth rate should be based on the rate of ^{13}C labeling of $K_{37:2}$. In addition, we have performed similar in situ alkenone ^{13}C-labeling experiments in the subtropical North Pacific (Station ALOHA, Popp et al. 2006) and the Gulf of California (Guaymas Basin, B. N. Popp, F. G. Prahl, and R. J. Wallsgrove, unpublished data). In these field experiments the rate of $K_{37:2}$ ^{13}C labeling always exceeded that of $K_{37:3}$ in experiments where both compounds were measured. However, higher rates of $K_{37:3}$ labeling relative to $K_{37:2}$ are not without precedent. Shin et al. (2000) found higher turnover of $K_{37:3}$ relative to $K_{37:2}$ at one of the four stations they examined in the Bering Sea (see their Figure 3, p. 123). We note, however, that growth rates determined in the subarctic Pacific using the rate of labeling of $K_{37:3}$ are within 20% of those determined using the rate of labeling of $K_{37:2}$ (Figure 4A) and that light limitation of the growth of alkenone-producing algae may be determined using the rate of labeling of either $K_{37:2}$ or $K_{37:3}$ (Figure 4B).

Discussion

Results obtained for phytoplankton grown under controlled laboratory conditions indicate that carbon isotopic fractionation depends primarily on $[CO_2(aq)]$, growth rate (μ), and the ratio of cellular surface area to carbon content. Although data from field studies support this hypothesis (e.g., Laws et al. 1995; Bidigare et al. 1997; 1999b; Popp et al. 1999a,b), there have been no rigorous field tests of the laboratory-derived relationships because of difficulty determining carbon isotopic fractionation and growth rate of the same algae. The goal of our work was to develop a method to measure the growth rate of a single marine phytoplankton species or a group of taxonomically closely related algae so that effects of microalgal growth rate on carbon isotopic fractionation of marine organisms may be evaluated in natural settings.

Our laboratory results suggest that growth rates of alkenone-producing algae in the ocean may be determined using in situ incubation combined with ^{13}C labeling of the C_{37} di-unsaturated alkenone. Importantly, our field evidence clearly indicates that light limitation on the growth of alkenone-producing algae may be determined using this method. Most laboratory culture experiments have grown *E. huxleyi* under saturating light irradiance with either excess nutrients (Riebesell et al. 2000) or nutrient limitation (Bidigare et al., 1997). Consequently, ^{13}C labeling of alkenone can be used to compare carbon isotopic fractionation as a function of growth rate and $[CO_2(aq)]$ from cells that are not light-limited in the field with relationships developed in laboratory culture experiments.

Carbon isotopic fractionation in marine microalgae is known to vary also as a function of cell geometry (Popp et al. 1998a; Brukhardt et al. 1999). As far as we are aware, no other organic compound has the taxonomic specificity to a marine alga or group of marine algae that allows the size and shape of phytoplankton to be constrained. For example, whereas the sterol dinosterol is thought to derive exclusively from dinoflagellates, it can also be present in certain diatoms (Volkman et al. 1993) and the geometry of dinoflagellates can vary widely. The use of alkenones to determine growth rate using the ^{13}C-labeling technique circumvents this difficulty. Measurements of the natural abundance δ^{13}C of alkenones and of $[CO_2(aq)]$ allows calculation of carbon isotopic fractionation associated with photosynthesis by the alkenone-producing haptophytes. Together with ^{13}C-labeling experiments, which yield reasonably accurate growth rates, the relationship of carbon isotopic fractionation to μ/CO_2, as postulated by Bidigare et al. (1997) based on laboratory cultures, could be field-tested. Consequently, growth rate estimated from laboratory culture experiments can be compared with in situ growth rates determined using the ^{13}C-labeling technique to evaluate the effect of μ on carbon isotopic fractionation, thus providing one test of laboratory-derived fractionation hypotheses in the ocean.

Stable isotope labeling of taxonomically distinctive individual compounds may also allow development of methods to estimate growth rates of other algae and bacteria that produce unique biochemicals that are amenable to analysis using irmGCMS. For example, the Archaea *Crenarchaeota* produces the unique biomarker crenarchaeol (Sinninghe Damsté et al. 2002) and the Archaea *Methanococcus burtonii* is the only known producer of *sn*-2-hydroxyarchaeol (Hinrichs et al. 1999, 2000).

Comments and recommendations

Laboratory and field data presented here suggest that growth rates of the alkenone-producing algae may be determined using the ^{13}C-labeling rates of alkenones. Implicit in

the relationship is the assumption that under all oceanographic conditions the alkenone cell quota is reasonably constant. Available evidence indicates that alkenone cellular concentrations are indeed reasonably stable over a range of exponential growth conditions, implying that alkenone carbon is synthesized at rates similar to cellular carbon. Alkenone concentrations of ~1 pg cell^{-1} have been found in laboratory cultures of *E. huxleyi* (Prahl et al. 1988; Conte et al. 1998; Popp et al. 1998b), and available information suggests that similar concentrations are found in natural populations (see slope of line in Figure 1 of Conte and Eglinton [1993]). Two exceptions appear when cells are allowed to reach late logarithmic or stationary-phase growth in batch cultures (Conte et al. 1998; Epstein et al. 1998; 2001; Prahl et al. 2003) and when cells are held in complete darkness for several days (Epstein et al. 2001; Prahl et al 2003). For example, Conte et al. (1998) found that alkenone concentration increased when cells reached late logarithmic and stationary-phase growth in several strains of *E. huxleyi* and one strain of *G. oceanica*. Prahl et al. (2003) showed that *E. huxleyi* cells in stationary phase (i.e., nutrient starved) continued to produce alkenones, with cells reaching total alkenone concentrations 3 times higher than when dividing exponentially. Under these conditions of physiological stress, the concentration of $K_{37:2}$ increased relative to that of $K_{37:3}$. In contrast, Prahl et al. (2003) showed that cells kept in culture for 5 days of continuous darkness resulted in a 75% decrease in the total cellular alkenone concentrations with preferential loss of $K_{37:3}$. These observations are consistent with the role of alkenones as cellular energy stores (Epstein et al. 2001; Pond and Harris 1996; Prahl et al. 2003) and could affect growth rate measurements using alkenone-specific ^{13}C labeling. Under conditions of extreme nutrient limitation, alkenone-producing algae could continue to biosynthesize alkenones without actually dividing. Production of alkenones as energy storage products could result in uptake of ^{13}C label and would thus overestimate growth rate determinations. Cells consuming alkenone storage products in darkness may also bias upward growth rates determined using alkenone ^{13}C labeling by increasing the turnover of these cellular biomarkers. Currently, it is not known at what light level alkenone consumption occurs, so the extent of bias in natural populations is unknown. The fact that growth rates based on $K_{37:3}$ were higher than rates determined from $K_{37:2}$ in the subarctic Pacific (Figure 4) implies that the light level where alkenone consumption occurs may be above saturating irradiance. Future laboratory studies should investigate ^{13}C labeling of alkenone in stationary growth phase cells as well as ^{13}C labeling as a measure of growth rate during light-limited growth and as a function of irradiance.

Results of our laboratory experiments indicate that the ^{13}C labeling rates of $K_{37:2}$ exceed those of $K_{37:3}$ and that calculated growth rates based on $K_{37:2}$ match culture growth rates reasonably well. Consequently, we recommend that growth rates of alkenone-producing algae be based on the ^{13}C-labeling rate of

the $K_{37:2}$ alkenone whenever possible. Because the relative $K_{37:2}$ to $K_{37:3}$ concentrations vary as function of temperature, isotopic analysis of $K_{37:2}$ may not always be practical. Under these conditions, we suggest either large volume incubation to increase the analytical pool of the $K_{37:2}$ alkenone or, when practical, longer incubations. Growth rates based on the ^{13}C-labeling rate of $K_{37:2}$ and $K_{37:3}$ converge with time (Tables 1 and 2). Future experiments should determine the length of incubation time necessary for reliable growth rates based on the ^{13}C-labeling rate of $K_{37:3}$. If larger-volume and longer-duration incubations are impractical, it should be noted that laboratory experimental results indicate that for 24-h incubation, growth rates based on $K_{37:3}$ were up to ~55% lower than rates based on $K_{37:2}$ in culture experiments.

With the exception of the method described here, there is currently no recognized alternative way to measure the growth rate of alkenone-producing algae in the field except in rare monospecific blooms. Nonetheless, growth rates determined using the ^{13}C-labeling method should be compared with more traditional methods to confirm the alkenone ^{13}C-labeling method under natural conditions. We suggest that growth rate measurements be performed using methods based on analysis of 19′-hexanoyloxyfucoxanthin and compared with those determined using ^{13}C labeling of alkenones during a bloom of alkenone-producing algae.

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