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Controls on the molecular distribution and carbon isotopic composition of alkenones in certain haptophyte algae

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[1] **Abstract:** Although the biochemical functions and biosynthetic pathways of alkenones are still largely unknown, alkenone unsaturation ratios are now used extensively to infer ancient sea surface temperature, and their isotopic compositions have been used to reconstruct ancient atmospheric CO_2 levels. The inferred relationships between alkenone unsaturation ratios, isotopic compositions, and growth conditions are based on empirical laboratory and field studies and, in the case of isotope fractionation, on simple models of carbon acquisition and fixation. Significant uncertainty still exists concerning the physiological and ecological factors affecting cellular production of alkenones, unsaturation ratios, and isotopic composition. Phytoplankton culture conditions have been shown to affect alkenone unsaturation ($U^{K'}_{37}$), cellular alkenone content, intracellular isotopic compositions ($\Delta\delta$), and changes in fractionation (ε_P) as a function of the quotient of algal growth rate and aqueous carbon dioxide concentration (μ/CO_2). Such studies imply that plant physiology can affect the interpretation of environmental signals. The factor(s) controlling cellular alkenone concentrations and unsaturation ratios are reviewed, as well as the carbon isotopic composition of the alkenone-producing algae. A new technique is presented to determine growth rates of the alkenone-containing algae in natural settings that will facilitate testing laboratory-based hypotheses concerning the carbon isotopic fractionation and its relationship to growth rate/growth status of alkenone-producing algae in the field.

Keywords: *Emiliania huxleyi*; isotopic fractionation; alkenones; $U^{K'}_{37}$; coccolithophorid; algal cultures.

Index terms: Organic marine chemistry; geochemistry; stable isotopes; biogeochemical processes.

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Theme: Alkenones **Guest Editor:** John Hayes

1. Introduction

[2] *Emiliania huxleyi* is a haptophyte that occurs throughout the world's oceans, from polar regions of high productivity to the oligotrophic subtropical gyres [*Westbroek et al.*, 1993]. It is the dominant coccolithophorid in waters cooler than 20°C and warmer than 25°C but coexists with a diversity of related species at intermediate temperatures. In addition to its importance to the global carbon cycle, *E. huxleyi* produces biomarkers in the form of long-chain (C_{37}, C_{38}, and C_{39}) alkenones [see *Brassell*, 1993]. The closely related *Gephyrocapsa oceanica* also produces C_{37-39} alkenones and may be an important source of those compounds in certain oceanic regions [*Conte et al.*, 1994, 1995; *Volkman et al.*, 1995]. Alkenones are well preserved in marine sediments, and their molecular distributions and isotopic composition have been used to infer paleo-sea surface temperatures [*Brassell*, 1993] and pCO_2 values [*Jasper and Hayes*, 1990; *Jasper et al.*, 1994; *Pagani et al.*, 1999], respectively. *Grice et al.* [1998] have recently shown that the unsaturation ratio and carbon isotopic composition ($\delta^{13}C$) of long-chain alkenones in *Isochrysis galbana* were identical to those egested in fecal material and concluded that zooplankton herbivory does not invalidate the use of alkenone-based proxies for reconstructing sea surface temperature and pCO_2. While the noncalcifying haptophytes, *Crysotila* and *Isochrysis*, can also produce C_{37-39} alkenones, they are not considered a likely source of alkenones in open oceanic waters since their distributions are restricted to coastal waters [*Marlowe et al.*, 1990]. Because of *E. huxleyi*'s cosmopolitan distribution and contributions to the sedimentary record [*Westbroek et al.*, 1994], it is important to understand the variables that regulate alkenone metabolism in this haptophyte. Of particular importance are the factor(s) that control cellular alkenone concentrations, unsaturation ratios, and carbon isotopic compositions.

2. Culture Methods Used in the Study of Microalgae

[3] The goal of microalgal culture studies is to grow microalgae under a well-defined set of environmental conditions over which the experimentalist ideally has control. In this way the effects of environmental conditions on microalgal physiology and composition can be studied in a systematic way. The use of such culture work to study these relationships by no means implies that culture conditions simulate nature. Indeed, because the natural environment in which microalgae grow is constantly changing, no laboratory culture method can be said to exactly mimic conditions in nature. However, without some basic understanding of the way factors such as growth rate, temperature, and irradiance affect algal physiology and composition in a steady state or quasi-steady state system, it is impossible to interpret the implications of environmental indicators such as $U_{37}^{K'}$.

[4] Because seemingly subtle differences in culture conditions can sometimes cause large changes in algal composition, it is important that culture conditions be clearly defined and that experimentalists fully appreciate the strengths and weaknesses of various culture techniques. Studies of alkenone biosynthesis, unsaturation, and the relationship between carbon isotope fractionation and growth conditions have relied on two basic culture methods, batch and continuous culture. In batch culture, cells are inoculated into a growth medium containing a relatively high concentration of all essential nutrients [*Stein*, 1973]. After perhaps an initial lag phase the cells grow exponentially at a more or less constant rate until growth is slowed and ultimately stopped by some limiting factor. During the time that the cells are growing exponentially, factors such as temperature, salinity, irradiance, and cellular physiology determine the growth rate of the culture. While

experimentalists can measure and control factors such as temperature and irradiance, the relationship between these factors and growth rate is not under their control. Strictly speaking, the chemistry of the medium in a batch culture is not constant, since the uptake of essential nutrients and generation of waste products by the algae constantly changes the chemical environment in the growth medium. In dense batch cultures, significant changes in the concentration and isotopic composition of the inorganic carbon species occur. The isotopic signature of the harvested cells represents an integral average of the isotopic composition of the organic matter produced since the culture was inoculated. Assumptions must be made to interpret the relationship between isotope discrimination and concentrations of inorganic carbon species. In addition, the physical environment can also change due to absorption and scattering of light by the exponentially growing cells. If the concentration of inorganic carbon species changes significantly during the incubation, assumptions must be made about the relationship between isotope discrimination and the concentration of inorganic carbon species before the results can be interpreted.

[5] In dilute batch cultures, significant changes in parameters affecting the isotopic composition of the organic matter are avoided by harvesting the algae below a critical cell concentration. Preadaptation to the experimental conditions and 8–10 divisions of exponentially growing cells assure that their isotopic signature is not affected by organic matter produced prior to the experiment. If the culture is harvested at circa 30 μmol kg^{-1} particulate organic carbon (POC), the isotopic composition of the inorganic carbon source changes by less than 0.4‰, nutrient and inorganic carbon consumption can be considered negligible, and carbon speciation is largely unaffected because change in pH in seawater is typically less than 0.05 [*Burkhardt et al.*, 1999a, 1999b]. If light and nutrient supplies are kept at saturating levels, isotope discrimination can be directly related to the experimental CO_2 concentration, which remains the only parameter affecting the isotopic composition in such a system. Additional parameters such as a cyclical light:dark regime or different light intensities are easily incorporated in these experiments to address the question of the effect of colimitation by CO_2 and light supply on algal growth and isotope fractionation.

[6] From a technical point of view, the major advantage of dilute batch cultures lies in the opportunity to perform a large number of experiments over a short time period with highly reproducible results under quasi-steady-state conditions. Light attenuation in the culture vessels is minimized owing to the low cell density. Regardless of the factor controlling growth rate, the algae in all treatments experience similar cell concentrations until they are harvested. Discontinuity of cellular processes such as synchronized cell divisions or uncoupling of C and N assimilation in light:-dark cycles has no significant effect on the chemical and physical environment of dilute batch cultures. The disadvantages of dilute batch cultures are the requirements for large sample volumes and, especially when light:-dark cycles are applied, for precise timing of harvest. As in cyclostat or turbidostat systems, growth rate is not under direct control, which is an advantage of chemostat systems.

[7] In a nutrient-limited continuous culture system (*Herbert et al.* [1956] and Figure 1), fresh sterile medium is continuously pumped into the growth chamber, and an equal volume of the contents of the growth chamber is removed at the same time. The ratios of essential nutrients in the growth medium are adjusted so that one nutrient is limiting, and there is a definite excess of all other essential nutrients. The growth rate of the cells equals the pumping

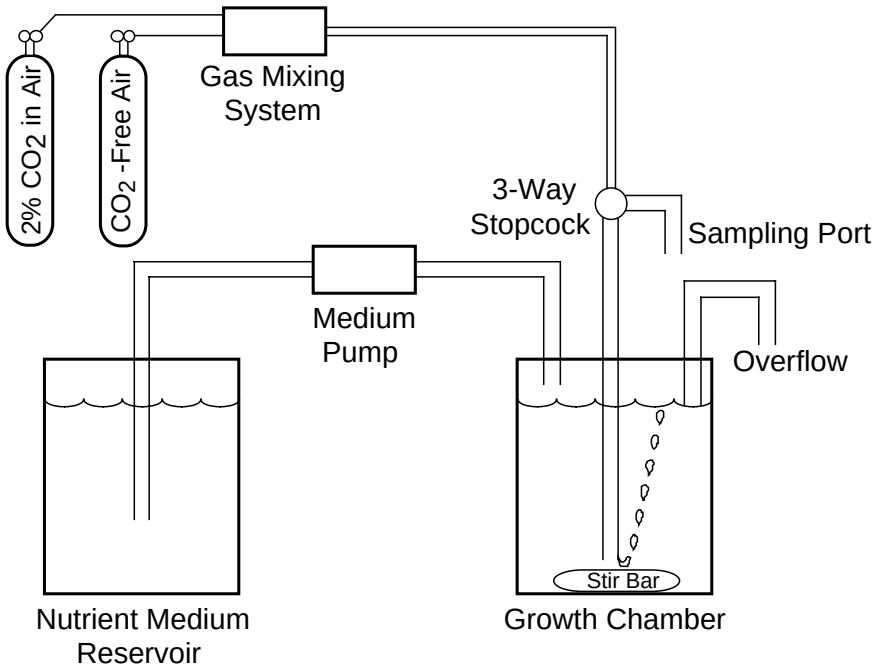


Figure 1. Schematic illustration of a continuous culture system. Phytoplankton growth rate is controlled by adjusting the rate of supply of a limiting nutrient to the growth chamber. The concentration of CO_2(aq) in air supplying the growth chamber is controlled by a gas mixing system.

rate of the medium divided by the volume of the growth chamber. The experimentalist directly controls the growth rate of the cells through control of the pumping rate. Steady state is assumed to exist when the pumping rate has been constant for at least four doubling times and cellular characteristics and the chemistry of the growth medium are constant to within experimental error. Under these steady state conditions the chemical and isotopic composition of cells harvested from the growth chamber can be directly related to the chemical and physical characteristics of the growth chamber at the time of harvest.

[8] Two variations on a standard, nutrient-limited continuous culture system are the cyclostat and turbidostat. In a cyclostat the irradiance on the growth chamber is cycled through a light and dark period to simulate light:dark (L:D) cycle effects in the real world [*Rhee et al.*, 1981]. Normally, the light source is turned on and off with a timer resulting in stepwise variations in the irradiance during each 24-hour cycle. Under these conditions the chemical and isotopic composition of the cells varies systematically on a 24-hour cycle, and there is no steady state in the usual sense of the word. However, when conditions are the same from one day to the next at the same time in the L:D cycle, the culture is said to be in a cyclic steady state. Under these conditions the average growth rate of the cells equals the pumping rate divided by the volume of the growth chamber. However, because cellular characteristics are systematically changing during the course of the L:D cycle, the growth rate of any particular biochemical will in general not equal the average growth rate between two points in time that are not separated by an integral multi-

ple of 24 hours. For example, if C_1 and C_2 are the concentrations of the cells at times T_1 and T_2, the growth rate μ of cells between T_1 and T_2 is given by

$$\mu = D + \frac{\ln(C_2/C_1)}{T_2 - T_1}, \quad (1)$$

where D is the dilution rate, defined to be the ratio of the pumping rate to the volume of the growth chamber. In the case of organic carbon we know that synthesis from inorganic carbon occurs only during the photoperiod and that respiration converts some organic carbon to inorganic carbon during the dark period. Let μ_i be the growth rate of organic carbon during the photoperiod and R the loss rate of organic carbon during the dark period. Let f be the photoperiod expressed as a fraction of 1 day. In a cyclic steady state it follows that

$$\mu = f\mu_i - (1-f)R \quad (2)$$

or $\mu_i = \mu/f + [(1 - f)/f]R$. If $f = 0.5$, for example, then $\mu_i = 2\mu + R$. In other words, if cells are growing on a 12:12 L:D cycle, one can expect that the growth rate of organic carbon during the photoperiod will be more than twice the 24-hour average growth rate (μ). This conclusion has important implications for carbon isotopic fractionation, because (1) carbon isotopic fractionation is determined by the growth rate at the time of synthesis and (2) dark respiration appears to have very little influence on carbon fractionation. For example, assume that carbon isotopic fractionation (ε_P) is observed when cells are growing at a rate of 1.0 d^{-1} on continuous light. Other factors being equal, this isotopic fractionation would be associated with cells growing at a 24-hour average rate of less than 0.5 d^{-1} in the field if the photoperiod in the field were 12 hours long.

[9] A turbidostat is operated much like a nutrient-limited continuous culture, but the ratios of essential nutrients in the sterile growth medium are adjusted so that nutrients are present in more-or-less optimal proportions, and the cell concentration in the growth chamber is maintained at a low enough level that there is an excess of all essential nutrients in the growth chamber. Under these conditions the growth rate of the culture is limited by the same factors that limit growth during the exponential phase of a batch culture, and the growth rate of the culture is not under the direct control of the experimentalist. The difference between a turbidostat and a batch culture is that fresh sterile medium is pumped into the turbidostat and an equal volume of the contents of the turbidostat removed so as to maintain a constant concentration of biomass in the growth chamber. Biomass is typically quantified by continuously monitoring the optical density (OD) of the culture. The medium pump turns on when the OD reading exceeds a preset value and turns off when the OD reading drops below that value. A variation on this experimental design is to dilute the growth chamber continuously at a fixed rate for a period of 1 day and to make appropriate adjustments to the dilution rate if the cell concentration increases or decreases. In this case, (1) is used to calculate the growth rate of the culture during a 24-hour period, and an adjustment is made to the dilution rate to try to exactly match the growth rate of the culture. After typically a few days of successively smaller adjustments the dilution rate matches the growth rate to within experimental error.

[10] One advantage of continuous culture systems over conventional batch cultures is that they provide constant or cyclic environmental conditions to which the chemical and isotopic composition of the algal cells can be related. In the case of nutrient-limited chemostats or cyclostats they also give the experimentalist direct control over the growth rate of the culture. To the extent that large amounts of material are needed for chemical or isotopic analysis, continuous culture systems also have

an advantage over batch cultures, because after coming to steady state continuous culture systems can be sampled day after day for weeks or even months. Furthermore, cells in continuous culture systems can be grown at high densities. The constancy of the chemical and physical environment in a continuous culture system does not require that the cultures be dilute. On the other hand, continuous culture methods by no means require that a culture be dense. Dilute cultures can be grown in a continuous mode if there is concern that high densities might produce artifacts (e.g., inhibition of growth caused by cell exudates) or confound measurements (e.g., the determination of the irradiance experienced by the cells is more easily done if the culture is optically thin).

[11] Extrapolation of laboratory data to the field must be done with caution because none of these techniques recreates natural environments, which can have changes in physical (e.g., temperature, mixing), chemical (e.g., major and trace nutrients), and biological (e.g., grazing pressures, species competition) processes. When possible, hypotheses developed using monospecific algal cultures should be tested in the field [cf. *Bidigare et al.*, 1997a, 1999b].

3. Physiological Factors Affecting $U_{37}^{K'}$

[12] Recent work has demonstrated that growth state may affect alkenone biosynthesis and polyunsaturation. *Conte et al.* [1998] and *Epstein et al.* [1998] documented an influence of cell physiology on concentrations and saturation levels of alkenones in cells grown in batch culture experiments. These authors found that the concentration of alkenones increased significantly when the cells reached late logarithmic and stationary phase growth. *Conte et al.* [1998] documented this trend in several strains of *Emiliania huxleyi* and one strain of *Gephyrocapsa oceanica*. The results of *Conte et al.* [1998] and *Epstein et al.* [1998] suggest that haptophytes under nutrient- or temperature-limited growth stress can increase total production of alkenones. *Epstein et al.* [1998] found a small increase in $U_{37}^{K'}$ when *E. huxleyi* cells reached stationary phase growth, whereas *Conte et al.* [1998] found that unsaturation ratios of C_{37} and C_{38} alkenones in some strains significantly decreased when cells entered late logarithmic stage growth. On the other hand, *Popp et al.* [1998b] found little change in alkenone concentration or unsaturation in two strains of *E. huxleyi* grown in nitrate-limited chemostat culture. *Popp et al.* [1998b] pointed out that the same strain of *E. huxleyi* grown in batch and chemostat culture also exhibit different $U_{37}^{K'}$-temperature relationships. They concluded that nutrient-limited growth rate effects should not produce serious errors in paleotemperatures calculated using $U_{37}^{K'}$. Recently, *Riebesell et al.* [2000a] determined that the concentration of CO_2(aq) had little effect on $U_{37}^{K'}$ values, although they found a weak positive correlation between alkenone cellular abundance and [CO_2(aq)]. These few studies indicate that environmental factors may influence cellular alkenone concentration and unsaturation. In order to determine the magnitude of these effects the growth status of cells in the field needs to be determined, especially in conjunction with alkenone flux to sediments, in order to fully understand the $U_{37}^{K'}$-temperature relationship. Growth status in this context means not only the growth rate as defined by (1) but also the factor(s) limiting growth and ancillary information such as temperature and photoperiod (e.g., equation (2)). In nutrient limited cases it may be impossible to determine what nutrient(s) is limiting, but in such cases an indication of whether the population is nutrient or light limited would facilitate the interpretation of data. The application of $U_{37}^{K'}$ to paleotemperature reconstructions will remain

empirical and vulnerable to potential misinterpretation until environmental effects on the $U^{K'}_{37}$-temperature relationship are understood.

4. Physiological Factors Affecting Biosynthesis

[13] The isotopic composition of organic biomarkers is often used to infer the isotopic composition of phytoplankton carbon associated with the particular biomarker. The use of biomarkers circumvents problems associated with separating phytoplankton carbon from other forms of carbon in field samples and in many cases constrains the species composition of the phytoplankton. The justification of using biomarkers in this way rests in part on the assumption that there is a more or less constant offset (Δ) between the $\delta^{13}C$ of the biomarker carbon and the associated phytoplankton carbon. Is there any reason to believe that this $\Delta\delta^{13}C$ is constant?

[14] A sufficient condition for a constant $\Delta\delta$ is that the bulk biochemical composition of phytoplankton carbon remains constant. To a good approximation, phytoplankton may be considered to consist of protein, carbohydrate, and lipid. There are consistent and systematic offsets in the $\delta^{13}C$ of these three classes of macromolecules, but if they are found in more or less constant relative proportions in phytoplankton cells, one would expect $\Delta\delta$ values to be constant as well.

[15] It is well known that the biochemical composition of phytoplankton can change dramatically in response to different growth conditions. One of the best theoretical analyses of this behavior is that of *Shuter* [1979]. In Shuter's model, phytoplankton carbon is allocated to one of four compartments: structural carbon, storage carbon, the dark reactions of photosynthesis, and the light reactions of photosynthesis. Shuter's model assumes that cells adjust their composition so as to achieve balanced growth and to maximize their rate of cell division under prevailing environmental conditions. Structural carbon is assumed to be a constant, and because of the requirement for the rate of cell division to be maximized, storage carbon in the form of carbohydrates and/or lipids is produced only when there is an excess of light energy, e.g., the rate of cell division is nutrient limited as opposed to light limited. To achieve balanced growth, the cells adjust the relative proportions of carbon in the light and dark reaction compartments.

[16] In accord with *Shuter*'s [1979] model, experimental studies have shown that when cells are growing at high relative growth rates (i.e., the growth rate is close to the nutrient-saturated value), ~50% of cellular carbon is allocated to proteins, 35% is allocated to carbohydrates, and ~15% is allocated to lipids. Algal protein contains carbon and nitrogen in a ratio of ~3.3 by weight [*Laws*, 1991], and ~85% of cellular nitrogen is allocated to protein [*DiTullio and Laws*, 1983]. The implication is that the C:N ratio in cells growing at a high relative growth rate is about (3.3)(0.85) / (0.50) = 5.6 by weight. This figure is virtually identical to the Redfield ratio of 5.7 by weight.

[17] There is now a general consensus that phytoplankton excrete some organic carbon [*Fogg*, 1983; *Raven*, 1993], and varying losses of exudates, if great enough, could lead to situations in which constant cellular compositions did not necessarily require constancy of isotopic fractionations. A likely mechanism is passive diffusion. *Raven*'s [1993] diffusion model indicates that a spherical cell with a diameter of 2 μm could lose 37.5% of its cell carbon per day by diffusion across the plasmalemma. If the cell were growing at one doubling per day, then 0.375 / (1 + 0.375) = 27% of the carbon it fixes would be released as dissolved organic carbon (DOC). This calcula-

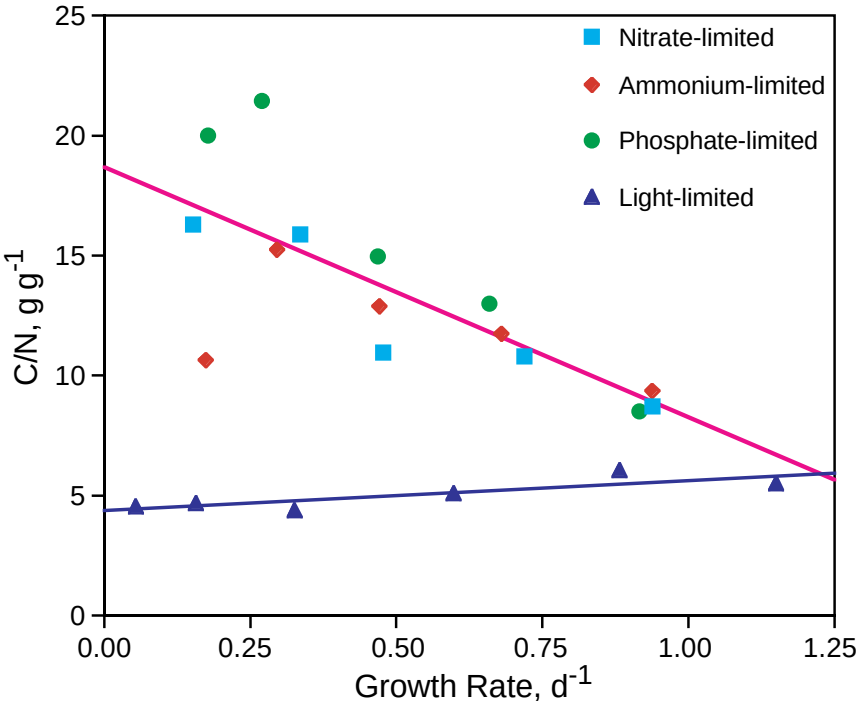


Figure 2. C:N ratios of the marine diatom *Thalassiosira weissflogii* grown under light- and nutrient-limited conditions. Data from *Laws and Bannister* [1980].

tion is relevant to phytoplankton communities dominated by picoplankton and is consistent with *Williams*'s [1981] model of planktonic food webs, which assumes that phytoplankton communities excrete 30% of the carbon they fix per day. The percentage loss is expected to scale as the surface-to-volume ratio and for cells with a diameter of 4 μm would therefore be roughly half that associated with 2-μm-diameter cells. *E. huxleyi*, with a diameter of ~5.2 μm [*Popp et al.*, 1998a], would be expected to excrete about 10% of the carbon it fixes at a growth rate of one doubling per day.

[18] Figure 2 is one example of the dependence of phytoplankton C:N ratios on growth rate for light- and nutrient-limited cultures of the marine diatom *Thalassiosira weissflogii*. Figure 2 illustrates two important points about microalgal composition. First, the C:N ratios extrapolate to a value close to the Redfield ratio (5.7 g g^{-1}) at maximum growth rate. Second, the dependence of C:N ratio on growth rate is very different under light- and nutrient-limited conditions. Under nutrient-limited conditions, there is a negative correlation between C:N ratios and growth rate. Under light-limited conditions the C:N ratio is positively correlated with growth rate. The very different relationships between C:N ratios and growth rate under light- and nutrient-limited conditions have important implications for studies of $\Delta\delta$ as a function of growth rate. $\Delta\delta$ would be expected to systematically change as a function of growth rate under both nutrient-limited and light-limited conditions, but the correlation between $\Delta\delta$ and growth rate would differ in the two cases. When growth rates are low and light-limited, the percentage of protein is high and the percentage of carbohydrates is low. When growth rates are low and nutrient-limited, the

percentage of protein is low and the percentage of carbohydrates is high.

[19] The fact that cycling of elements in the ocean is often observed to follow Redfield stoichiometry is consistent with the assumption that algal biochemical composition is rather constant and follows the 50:35:15 protein:carbohydrate:lipid pattern. Extreme conditions such as temperature stress or nutrient starvation can cause algal composition to deviate significantly from this pattern [*Laws*, 1991], but for obvious reasons such extreme growth conditions are not associated with high rates of production. Even in the oligotrophic subtropical gyres, studies of phytoplankton growth rates have shown that relative growth rates are high [*Laws et al.*, 1987]. The implication is that the great majority of phytoplankton biomass produced in the ocean is probably synthesized under conditions where the elemental composition of the phytoplankton is not far from Redfield stoichiometry, and protein, carbohydrate, and lipid carbon are being produced in roughly a 50:35:15 ratio. The implication is that while a given algal species can be forced to grow slowly and exhibit compositional characteristics very different from Redfield stoichiometry in the laboratory, competition in the field tends to select for the species that can grow most rapidly under the prevailing environmental conditions. The species that win this competition are growing at high relative growth rates and hence display compositional characteristics consistent with Redfield stoichiometry (Figure 2). This conclusion supports the assumption of reasonably constant $\Delta\delta$ values. Direct laboratory evidence for the constancy of $\Delta\delta$ values is presented in Table 1.

5. Biosynthesis of Alkenones

[20] Unfortunately, the physiological functions of alkenone biomarkers remain an enigma for biogeochemists and paleoceanographers. Despite the high levels of alkenones found in *E. huxleyi* (up to 15 pg cell^{-1}; see *Epstein et al.* [1998] and discussion therein), the cellular locations, biochemical functions, and biosynthetic pathways of these long-chain hydrocarbons remain elusive [*Conte et al.*, 1994; *Schouton et al.*, 1998]. Since the degree of alkenone unsaturation varies as a function of growth temperature [*Prahl and Wakeham*, 1987], it is tempting to speculate they have a role in regulating membrane fluidity. *Volkman et al.* [1981] examined the fatty acid composition of *E. huxleyi* and found that this alga contains a di-unsaturated C_{36} alkenoic acid (1.5% relative to total fatty acids). These authors suggest that this 36:2 fatty acid is biosynthetically related to the long-chain alkenes and ketones found in *E. huxleyi*. Furthermore, *Volkman et al.* [1981] note the lack of a homologous series of fatty acid precursors in *E. huxleyi* and suggest that 36:2 fatty acid synthesis involves short-chain coupling reactions rather than sequential chain elongation.

[21] Intracellular variations in the $\delta^{13}C$ of lipid biomarkers can provide insights into the metabolic pathways responsible for their biosynthesis. Conversely, knowledge of lipid synthesis routes for a given organism may be useful for interpreting intracellular variations in the $\delta^{13}C$ of their lipid biomarkers. Until recently, it was assumed that fatty acids and polyisoprenoids were synthesized from a common precursor, acetyl-CoA, via the classical fatty acid synthetase and acetate/mevalonate (MVA) pathways, respectively [*Lehninger*, 1975]. While C_{16-18} fatty acids are synthesized from acetyl-CoA in the plastid, synthesis of the isoprenic C_5 monomer, isopentenyl diphosphate (IPP), is less straightforward. It has recently been documented, through the use of elegant ^{13}C- and ^{2}H-labeling experiments, that there are two distinct pathways used by plants for IPP synthesis [*Schwender et al.*, 1996; *Disch et al.*,

Table 1. Mean Δδ Values Reported for Various Lipid Biomarkers of *Isochrysis galbana* and *Emiliania huxleyi* Grown in Continuous and Batch Culture[a]

Culture Conditions and Organism	Biomarker	Δδ (*n*)[b]
	Continuous Culture	
Isochrysis galbana	Phytol	−2.8 (1)[c]
	C_{37+38} alkenones	−3.1 ± 0.2 (2)[c]
	24-methylcholesta-5,22E-dien-3β-ol	−7.2 (1)[c]
	16:0 fatty acid (palmitic acid)	−7.7 (1)[c]
	18:n fatty acid	−5.6 (1)[c]
Emiliania huxleyi	Phytol	−4.5 ± 0.4 (6)[d]
	$C_{37:2}$ alkenone	−4.3 ± 0.4 (10)[e]
	$C_{37:3}$ alkenone	−4.3 ± 0.5 (10)[e]
	24-methylcholesta-5,22E-dien-3β-ol	−7.3 ± 0.6 (6)[d]
	14:0 fatty acid (myristic acid)	−6.2 ± 0.5 (6)[d]
	16:0 fatty acid (palmitic acid)	−6.5 ± 0.9 (6)[d]
	18:1 fatty acid (oleic acid)	−6.6 ± 0.8 (6)[d]
	Batch Culture	
Emiliania huxleyi	Phytol	−2.0 ± 0.3 (10)[f]
	$C_{37:2}$ alkenone	−3.8 (1)[g]
	$C_{37:2}$ alkenone	−5.4 ± 0.3 (10)[f]
	$C_{37:3}$ alkenone	−5.2 ± 0.6 (10)[f]
	$C_{38:2}$ alkenone	−5.3 ± 0.5 (10)[f]
	$C_{38:3}$ alkenone	−4.8 ± 0.9 (10)[f]
	24-methylcholesta-5,22E-dien-3β-ol	−8.3 ± 0.9 (10)[f]
	14:0 fatty acid (myristic acid)	−2.3 ± 0.8 (10)[f]
	16:0 fatty acid (palmitic acid)	−2.5 ± 0.9 (10)[f]
	Σ18:n fatty acids	−4.0 ± 0.4 (10)[f]

[a]Mean Δδ values are $\delta^{13}C_{biomarker} - \delta^{13}C_p$, ‰.
[b]Uncertainties are expressed as the standard deviation ($n \geq 3$) or the range ($n = 2$).
[c]*Schouten et al.* [1998].
[d]*Bidigare et al.* [1997b]; $\delta^{13}C_{POC}$ values ranged from −27.6 to −46.6‰.
[e]*Popp et al.* [1998a]; $\delta^{13}C_{POC}$ values ranged from −27.6 to −46.6‰.
[f]*Riebesell et al.* [2000a]; $\delta^{13}C_{POC}$ values ranged from −16.89 to −26.63‰.
[g]*Jasper and Hayes* [1990].

1998; *Lichtenthaler*, 1999]. For the synthesis of cytoplasmic sterols (and ubiquinone-10) by higher plants, red algae (*Cyanidium caldarium*), and heterokont microalgae (*Ochromonas danica*), IPP is formed via the classical MVA pathway (Figure 3). In contrast, these organisms synthesize plastidic isoprenoids (phytol, carotenoids, and plastoquinone-9) from IPP generated via the novel 1-deoxy-D-xyolose-5 phosphate (DOXP) pathway (Figure 3). In this

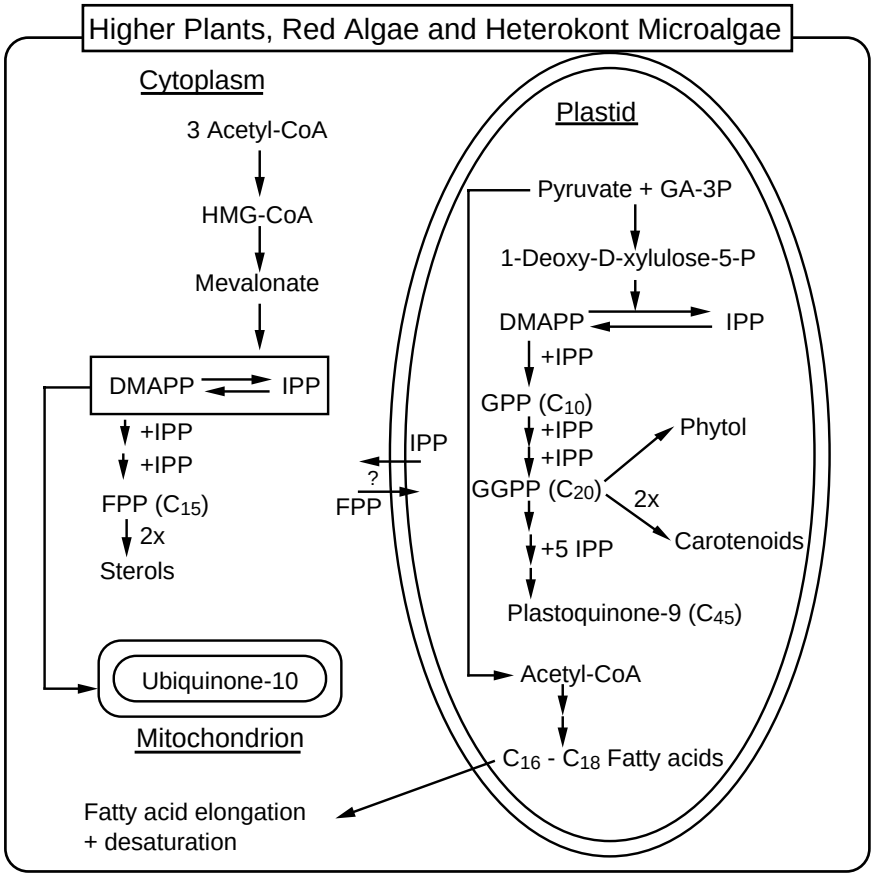


Figure 3. Pathways of lipid biosynthesis in higher plants, red algae, and heterokont microalgae [after *Lichtenthaler*, 1999]. In this scheme, acetyl-CoA and pyruvate/glyceraldehyde-3-phosphate serve as precursors for the synthesis of cytoplasmic (sterols and ubiquinone-10) and plastidic (phytol, carotenoids, and plastoquinone-9) isoprenoids, respectively. Fatty acids (C_{16-18}) are synthesized in the plastid from acetyl-CoA and transported to the cytoplasm for subsequent elongation and desaturation. Abbreviations used: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

nonmevalonate pathway, IPP is formed from the intermediate DOXP. The latter is generated via DOXP synthase, an enzyme that catalyzes the condensation of pyruvate and glyceraldehyde-3-phosphate. In the case of green algae, both cytoplasmic and plastidic isoprenoids are synthesized from IPP produced via the DOXP pathway (Figure 4). It should be noted that the pathway(s) used for IPP formation by nonheterokont, chlorophyll *c*-containing microalgae (e.g., *Emiliania huxleyi*, *Isochrysis galbana*, and *Gephyrocapsa oceanica*) have not been established. *Sessions et al.* [1999] provide evidence that the hydrogen isotopic characteristics of the MVA and DOXP pathways are different and suggest that δD biomarker analysis may be a useful tool for investigating the occurrence of the DOXP pathway.

[22] An exception to the IPP schemes depicted in Figures 3 and 4 is found in the euglenophyte

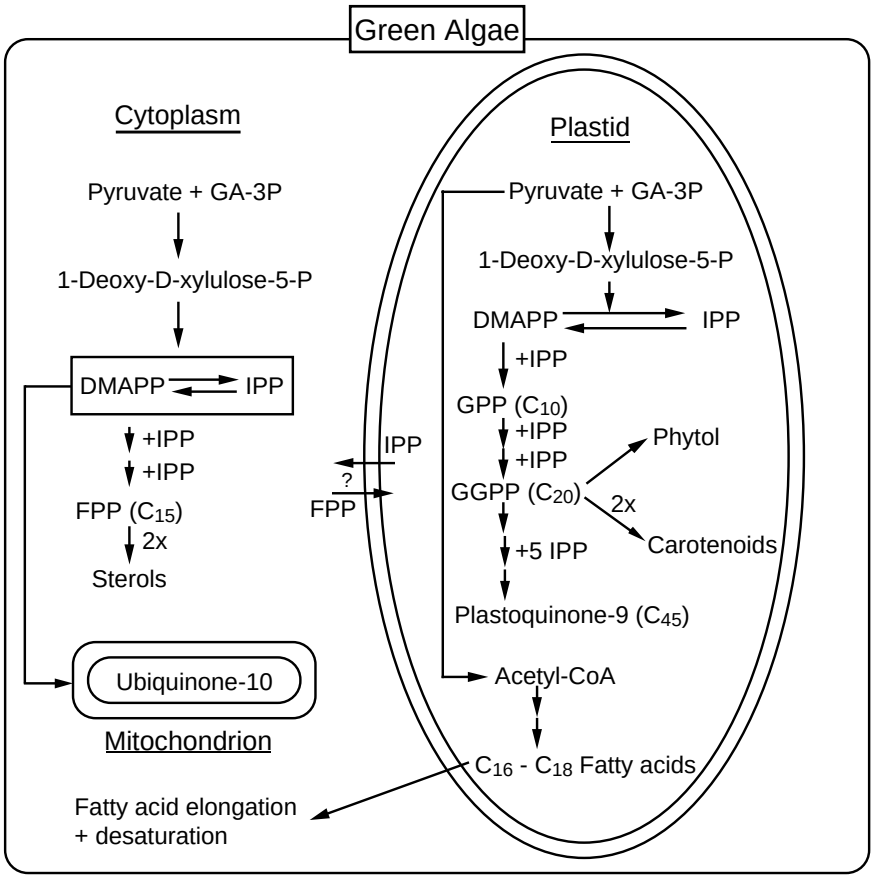


Figure 4. Pathways of lipid biosynthesis in green algae [after *Lichtenthaler*, 1999]. In this scheme, pyruvate/glyceraldehyde-3-phosphate serves as precursors for the synthesis of both cytoplasmic (sterols and ubiquinone-10) and plastidic (phytol, carotenoids, and plastoquinone-9) isoprenoids. Fatty acids (C_{16-18}) are synthesized in the plastid from acetyl-CoA and transported to the cytoplasm for subsequent elongation and desaturation.

Euglena gracilis. This chlorophyll *b*-containing alga uses the MVA pathway to generate IPP for the synthesis of all cellular isoprenoids (Figure 5). In this organism all lipids (including fatty acids) are derived from acetyl-CoA, albeit in different compartments. Acetyl-CoA is produced via the decarboxylation of pyruvate, a reaction that is catalyzed by the enzyme pyruvate dehydrogenase. As the result of kinetic isotopic fractionation, pyruvate dehydrogenase yields a product, acetyl-CoA, whose carboxyl carbon is depleted in ^{13}C [*DeNiro and Epstein*, 1977]. *Hayes* [1993] extended this line of reasoning and hypothesized that acetogenic lipids (e.g., fatty acids) in MVA pathway-containing organisms should be more depleted in ^{13}C than polyisoprenoids (e.g., phytol, sterols, and carotenoids) since they possess a higher content of carboxyl carbon.

[23] Since growth rate and [CO_2(aq)] variations lead to differences in $\delta^{13}C$ of marine phytoplankton [*Laws et al.*, 1995, 1997], compound-specific isotopic values are typically expressed relative to that of the source organism (i.e., $\Delta\delta \equiv \delta^{13}C_{\text{biomarker}} - \delta^{13}C_{\text{p}}$). The latter facilitates

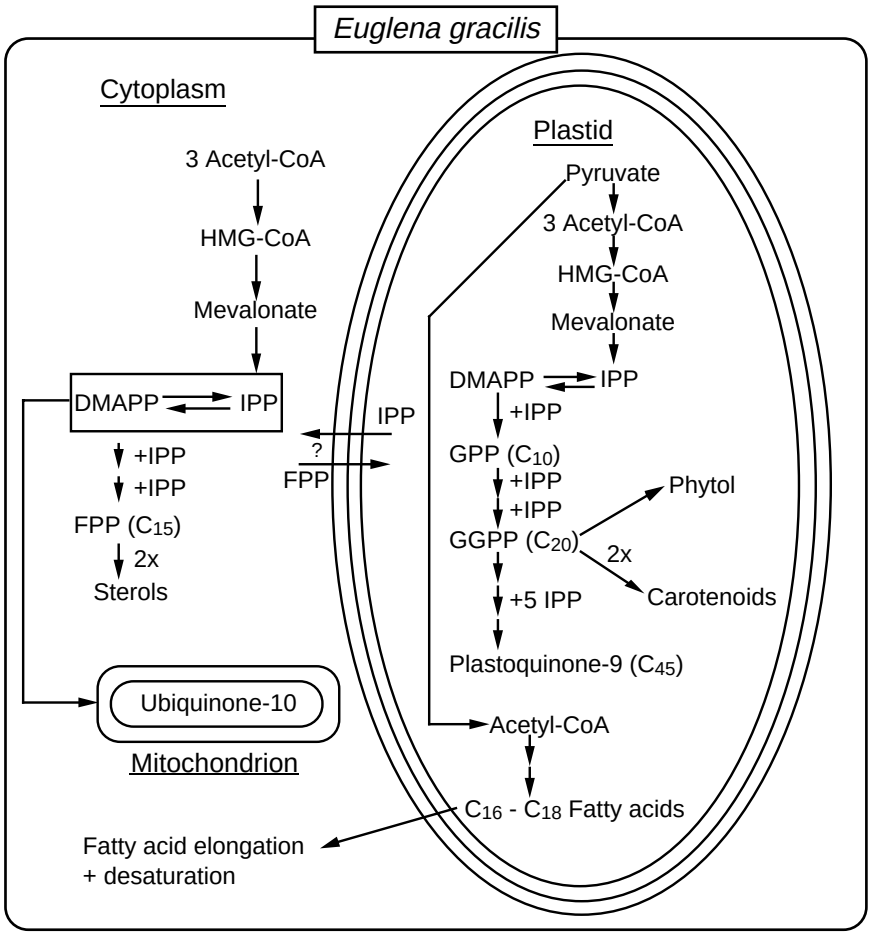


Figure 5. Pathways of lipid biosynthesis in *Euglena gracilis* [after *Lichtenthaler*, 1999]. In this scheme, acetyl-CoA serves as a precursor for the synthesis of both cytoplasmic (sterols and ubiquinone-10) and plastidic (phytol, carotenoids, and plastoquinone-9) isoprenoids. Fatty acids (C_{16-18}) are synthesized from acetyl-CoA in the plastid and transported to the cytoplasm for subsequent elongation and desaturation.

comparison of isotopic signatures of lipid biomarkers between phytoplankton species grown under different culture conditions. It should be noted that *Schouten et al.* [1998] recommended that $\Delta\delta$ values be calculated relative to $\delta^{13}C_{16:0}$. Since the 16:0 fatty acid occurs in a wide range of marine organisms, this approach is not suitable for reconstructing $\delta^{13}C$ values for natural assemblages of phytoplankton. In addition, any compound versus compound comparison adds an unwelcome degree of freedom (i.e., the abundance of the "base compound") that is avoided in compound versus biomass comparisons.

5.1. Continuous Culture

[24] Values of $\Delta\delta$ determined for the alkenone-producing haptophytes, *E. huxleyi* and *I. galbana*, are summarized in Table 1. Isotopic analyses of phytol, alkenone, sterol, and fatty acid biomarkers were performed by isotope ratio monitoring Gas Chromatography/Mass Spectrometry (irmGC/MS) [*Hayes et al.*,

1990]. A ~5‰ variation in $\Delta\delta$ was observed, with values ranging from −2.8 to −7.7‰. On average, phytol, alkenones, fatty acids, and sterols are depleted in ^{13}C (relative to the source organism) by 3.6, 3.9, 6.5, and 7.2‰, respectively. The mean $\Delta\delta$ value determined for phytol, ~4‰, is consistent with measurements performed for natural phytoplankton communities [*Bidigare et al.*, 1999b] and the sedimentary record [*Hayes et al.*, 1990]. The plastidic isoprenoid phytol is enriched in ^{13}C by 3.6‰ relative to that observed for cytoplasmic sterol 24-methylcholesta-5,22E-dien-3β-ol. While large variations in $\Delta\delta$ were observed between lipid classes, values for an individual compound displayed only minor variations ($\leq$1.7‰) between organisms. Smaller uncertainties in $\Delta\delta$ (<0.9‰) were observed for individual biomarkers determined for a given organism. The large (and sometimes anomalous) intercompound variations in $\Delta\delta$ could arise from (1) the use of different IPP pathways for acyclic and cyclic isoprenoid biosynthesis [*Schwender et al.*, 1996; *Disch et al.*, 1998; *Lichtenthaler*, 1999], (2) the use of different acetate pools for biomarker biosynthesis (compartmentalization [cf. *Flesch and Rohmer*, 1988]), and/or (3) biosynthetic fractionation of biomarkers occurring downstream from acetate [cf. *Summons et al.*, 1994]. Knowledge of the pathway(s) and fractionations associated with IPP biosynthesis in haptophyte algae is required before interpreting the $\Delta\delta$ variations observed by *Bidigare et al.* [1997b] and *Schouton et al.* [1998].

5.2. Batch Culture

[25] Values of $\Delta\delta$ determined for *E. huxleyi* grown in batch culture are summarized in Table 1. A ~6‰ variation in $\Delta\delta$ was observed, with values ranging from −2.0 to −8.3‰. On average, phytol, alkenones, fatty acids, and sterols are depleted in ^{13}C (relative to the source organism) by 2.0, 4.9, 2.9, and 8.3‰, respectively. Interestingly, there was better agreement in $\Delta\delta$ values determined for two different species (*E. huxleyi* and *I. galbana*) grown in nitrate limited chemostat culture than for one species (*E. huxleyi*) grown under nitrate-limited (chemostat) and nutrient-saturated (dilute batch cultures) conditions. In addition to the mechanisms described above, interexperimental variations in $\Delta\delta$ could arise from (1) differences in the culture conditions used for phytoplankton cultivation (nutrient-limited versus nutrient-saturated growth) and (2) differences in the biochemical composition of the cultures investigated [cf. *Shuter*, 1979]. The data presented in Table 1 document that phytoplankton culture conditions have a profound effect on the magnitude of $\Delta\delta$ for individual algal biomarkers. However, caution should be exercised in applying results from either culture method to the field.

6. Controls on the Carbon Isotopic Composition of Phytoplankton

[26] As in many other fields, theoretical understanding of the relationship between carbon fractionation by marine phytoplankton and growth conditions has closely followed the publication of relevant experimental results. *Francois et al.* [1993] showed that ε_P, approximately the difference between the $\delta^{13}C$ of the aqueous CO_2 and phytoplankton carbon, should closely follow the equation

$$\varepsilon_P = \varepsilon_1 + f(\varepsilon_2 - \varepsilon_{-1}), \quad (3)$$

where ε_1, ε_{-1}, and ε_2 are the isotopic discriminations associated with whatever process brings inorganic carbon through the plasmalemma into the cell, diffusion back into the surrounding water, and enzymatic carboxylation to produce phytoplankton, respectively, and f is the fraction of the inorganic carbon taken up by the cell that diffuses back into the water. Assuming that the rate of diffusion of CO_2 back into the water is proportional to the

intracellular concentration of CO_2 (C_i), (3) can be rearranged to give

$$\varepsilon_P = \varepsilon_1 + \frac{\varepsilon_2 - \varepsilon_{-1}}{1 + \frac{\mu C}{PC_i}}, \quad (4)$$

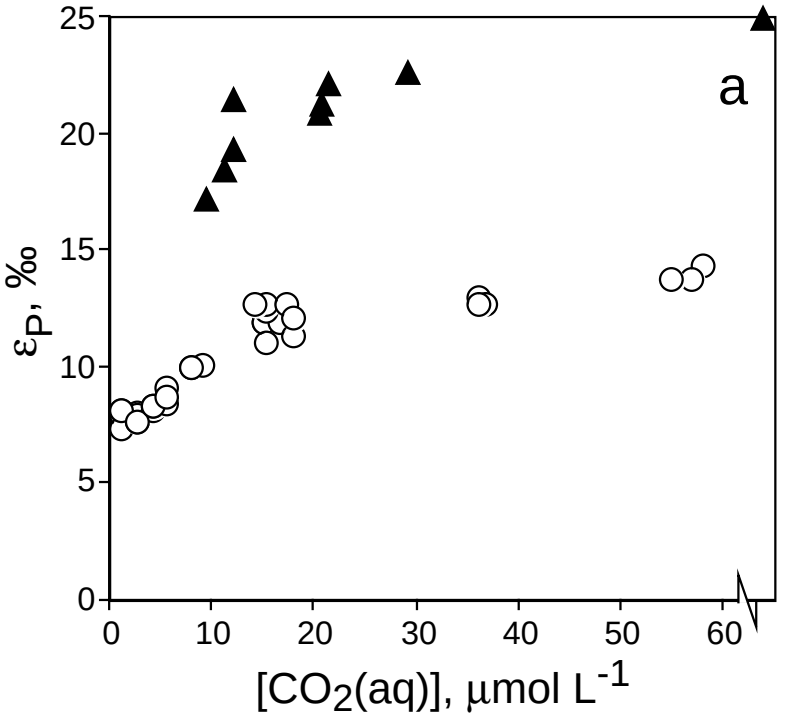
where μ is the growth rate of the cell, C is the organic carbon content of the cell, and P is the permeability of the plasmalemma to CO_2. Testing this equation requires knowledge of C_i, which is virtually never known. However, if one is willing to assume that inorganic carbon enters the cell by passive diffusion of CO_2, (4) can be rearranged to give

$$\varepsilon_P = \varepsilon_1 + (\varepsilon_2 - \varepsilon_{-1})\left(1 - \frac{\mu C}{PC_e}\right) \quad (5)$$

where C_e is the concentration of CO_2 in the external medium [*Farquhar et al.*, 1982; *Francois et al.*, 1993; *Laws et al.*, 1995; *Rau et al.*, 1996]. Experimental data consistent with (4) were reported for the marine diatom *Phaeodactylum tricornutum* by *Laws et al.* [1995]. One expects that P will be proportional to the surface area of the cell, and theoretical models incorporating that assumption were developed by *Rau et al.* [1996] and *Popp et al.* [1998a]. *Popp et al.* [1998a] showed that experimental data from several species with very different surface:volume ratios were consistent with the surface area hypothesis. Results by *Burkhardt et al.* [1999a] also indicate that some of the variability in isotope fractionation could be accounted for by differences in cellular carbon content and cell surface area between species. Nevertheless, *Burkhardt et al.* [1999a] found large residual variability in ε_P for any given combination of growth rate, cellular C content, cell surface area, and CO_2 concentration. Some of the variability was attributed to species-specific differences in mechanisms of inorganic carbon acquisition or in the properties of RUBISCO. However, large differences in isotopic fractionation were also found within single species, indicating that other unknown factors contributed to the isotopic composition of algal cells.

[27] Although the primary role of growth rate and CO_2 concentration in controlling ε_p was demonstrated by experimental results obtained in chemostat cultures [*Laws et al.*, 1995, 1997; *Bidigare et al.*, 1997a; *Popp et al.*, 1998a], no clear relationships were obtained in studies using batch culture incubations [*Thompson and Calvert*, 1994, 1995; *Hinga et al.*, 1994; *Johnston*, 1996; *Burkhardt et al.*, 1999a, 1999b]. Part of the discrepancy may be due to poorly constrained experimental conditions in some batch culture incubations or species-specific differences in isotope fractionation. Nevertheless, the principal disagreement in some of these results remains a matter of concern. For instance, at high [CO_2(aq)] and low growth rate, nitrate-limited chemostat and light-limited turbidostat incubations have consistently yielded ε_p values of ~25‰, i.e., close to isotopic fractionation of the carboxylating enzyme RUBISCO [*Laws et al.*, 1995, 1997; *Bidigare et al.*, 1997a; *Popp et al.*, 1998a; *Rosenthal et al.*, 1999]. At similar [CO_2(aq)] and growth rates, in batch culture incubations in which growth rates were either controlled by photon flux density or were nutrient saturated, isotope fractionation was found to be much lower [*Hinga et al.*, 1994; *Johnston*, 1996; *Korb et al.*, 1996; *Burkhardt et al.*, 1999a, 1999b; *Riebesell et al.*, 2000a, 2000b]. In addition, results from *E. huxleyi* grown in chemostat [*Bidigare et al.*, 1997a] and turbidostat [*Rosenthal et al.*, 1999] experiments show a strong dependence of ε_P on μ/CO_2, whereas similar results from dilute batch culture show only a small dependence (see below and Figure 6) [*Riebesell et al.*, 2000b].

[28] On the basis of studies with metabolic inhibitors and estimates of intracellular CO_2 concentrations, algal physiologists have shown that marine phytoplankton possess a CO_2 concentrating mechanism (CCM) and that inorganic carbon enters the cell in part via active transport [*Kerby and Raven*, 1985; *Patel and*


a
εP, ‰
[CO2(aq)], µmol L-1
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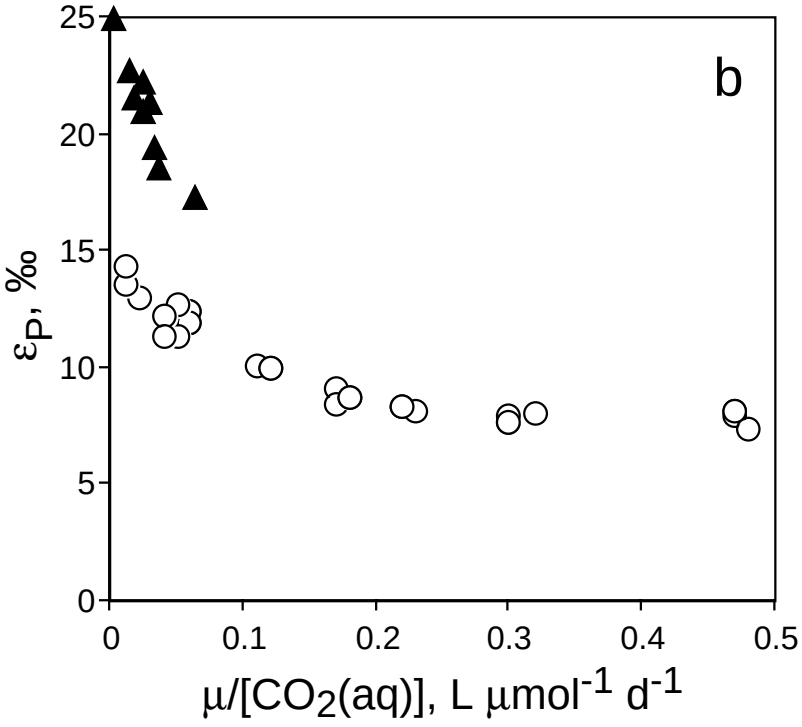
b
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0
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0.5

Merrett, 1986; *Burns and Beardall*, 1987; *Dixon and Merrett*, 1988; *Nimer et al.*, 1996]. The first isotopic evidence for active uptake was reported by *Laws et al.* [1997]. In their extended studies with *P. tricornutum*, *Laws et al.* [1997] showed that (5) gave an inadequate description of the relationship between ε_P and μ/C_e when the range of μ/C_e was extended to high values. *Laws et al.* [1997] postulated that CO_2 entered the cell both by passive diffusion and by active transport and that via active transport the cell adjusted its internal CO_2 concentration so as to minimize the energetic costs of transporting CO_2 from the external medium to the site of carboxylation. On the basis of this energy minimization model, they concluded that the relationship between ε_P and μ/C_e would be nonlinear. A least squares fit of their *P. tricornutum* data yielded an equation of the form

$$\frac{\mu}{C_e} = \frac{k_{-1}}{C(1+b)}\frac{\varepsilon_2' - \varepsilon_P}{\varepsilon_P - \varepsilon_1'} \quad (6)$$

where $\varepsilon_2' = \varepsilon_2 - \varepsilon_{-1} + \varepsilon_1$ and $\varepsilon_1' = \varepsilon_1 + ((\beta)/\ (1 + \beta))\ (\varepsilon_2 - \varepsilon_{-1})$, and β is a constant. Subsequently, *Keller and Morel* [1999] developed a model that allows for active transport of either CO_2 or bicarbonate. Their equation for ε_P takes the form

$$\varepsilon_P = \varepsilon_2 + \frac{1}{1 + \frac{C_e P}{\gamma \mu C}} \times \left(\delta^{13}_{CO_2} + \varepsilon_1 - \varepsilon_{-1} - \delta^{13}_{source} - \frac{\varepsilon_2 - \varepsilon_{-1}}{\gamma}\right) \quad (7)$$

where γ is the ratio of active transport to carbon fixation and δ^{13}_{source} is the $\delta^{13}C$ of the inorganic carbon that is actively transported into the cell. It is straightforward to show that (6) and (7) are virtually identical if the source is CO_2 and $\beta = \gamma - 1$. An examination of (7) makes it clear that isotope fractionation alone cannot be used to distinguish between active uptake of CO_2 and active uptake of bicarbonate [see *Keller and Morel*, 1999]. For example, if CO_2 is the form of inorganic carbon being actively transported, then $\delta^{13}_{CO_2} - \delta^{13}_{source} = 0‰$. However, if bicarbonate is the form of inorganic carbon being actively transported, then $\delta^{13}_{CO_2} - \delta^{13}_{source} = -8‰$. This change will have no effect on the calculated value of ε_p if γ increases by an appropriate amount and P changes in direct proportion to γ. For example, *Keller and Morel* [1999] are able to obtain a good fit to *Laws et al.*'s [1997] *P. tricornutum* data by assuming active transport of CO_2 with $\gamma = 1.2$ or active transport of bicarbonate with $\gamma = 2.2$. In the former case the implication is that the cells are actively transporting CO_2 at a rate 20% higher than the photosynthetic rate in order to make up for diffusional losses of CO_2. In the latter case the cells are actively transporting bicarbonate at a rate 120% higher than the photosynthetic rate in order to make up for diffusional losses. Clearly, the bicarbonate active transport scenario implies a much less efficient use of actively transported carbon, but from a strictly mathematical standpoint there is no reason to prefer the one scenario to the other.

7. Carbon Isotopic Fractionation in *Emiliania huxleyi*

7.1. Laboratory Studies

[29] Laboratory-based studies of carbon isotopic fractionation in *E. huxleyi* vary in their approach and conclusions. *Hinga et al.* [1994] used closed system batch cultures grown on a

Figure 6. Carbon isotope fractionation (ε_p) of *Emiliania huxleyi* in relation to (A) [CO_2(aq)] and (B) μ/[CO_2(aq)] determined in chemostat incubations (solid triangles, clones BT6 and B92/11) by *Bidigare et al.* [1997a] and in dilute batch culture incubations (open circles, clone B92/11) by *Riebesell et al.* [2000b]. CO_2 concentrations of *Bidigare et al.* [1997a] were converted from μmol kg^{-1} to μmol L^{-1}. The data point in the right upper corner of Figure 6a corresponds to [CO_2(aq)] = 274.1 μmol kg^{-1}.

12:12 L:D cycle to examine the effects of $[CO_2(aq)]$ and growth rate on ε_P in a noncalcifying strain of *E. huxleyi* (clone BT6). They found a strong negative trend between ε_P and μ_i/CO_2. Their results indicate a maximum fractionation of ~12.5‰ at a photoperiod growth rate (μ_i, see equation (2)) of 0.6 d^{-1} and $[CO_2(aq)]$ exceeding 100 μmol kg^{-1}. Maximum fractionation (ε_f) will be achieved when $f \rightarrow 1$ in (3) and should equal the flux-weighted average of isotope effects associated with all carbon-fixing reactions active in the cell. *Raven and Johnston* [1991] and *Goericke et al.* [1994] calculated maximum fractionations in eukaryotic microalgae of 25–28‰.

[30] *Thompson and Calvert* [1995] grew a calcifying and noncalcifying strain of *E. huxleyi* in closed batch culture experiments under different light intensities. They modeled carbon isotopic fractionation as a Rayleigh distillation process and suggested that cells were utilizing bicarbonate as a major source of inorganic carbon for growth. A maximum fractionation of 24.6‰ in these experiments was achieved at an irradiance of 4.8 photons m^{-2} d^{-1}, conditions which yielded highest growth rates [*Thompson and Calvert*, 1995]. Reevaluation of Thompson and Calvert's results by *Laws et al.* [1998] indicated that an error was made in the use of the Rayleigh distillation model and that the relationship between the $\delta^{13}C$ and the concentration of dissolved inorganic carbon (DIC) at the time of harvest could be equally well described by a model assuming bicarbonate or CO_2 uptake. Evidence for direct bicarbonate utilization by *E. huxleyi* and *G. oceanica* is equivocal (see *Nimer et al.* [1997] and *Sikes et al.* [1980] and below). However, several authors have suggested that coccolithophores may supplement the supply of $CO_2(aq)$ to the cell through calcification *Paasche*, 1964; *Nimer and Merrett*, 1992; *Brownlee et al.*, 1994; *Nimer et al.*, 1997]. Recent work by *Buitenhuis et al.* [1999] is consistent with this hypothesis. *Buitenhuis et al.* [1999] measured rates of photosynthesis and calcification in *E. huxleyi* (strain Ch 24–90) as a function of different concentrations of chemical species of inorganic carbon. *Buitenhuis et al.* [1999] found that at constant $[CO_2(aq)]$, rates of photosynthesis increased with $[HCO_3^-]$ and suggested that the CO_2 used for photosynthesis in *E. huxleyi* is derived externally from seawater ($CO_2(aq)$) and internally from carbonate precipitation. These experiments may address the effect of calcification on isotopic fractionation since *Buitenhuis et al.* [1999] indicated that isotopic analyses of particulate inorganic and organic carbon were conducted and will be published in a companion paper.

[31] Available evidence, however, indicates that the effect of calcification on fractionation is not straightforward in *E. huxleyi*. *Laws et al.* [1998] found evidence suggesting that in the experiments conducted by *Thompson and Calvert* [1995] at low growth rates, $CO_2(aq)$ supplied via calcification dominated the inorganic carbon pool, whereas at high growth rates, *E. huxleyi* used $CO_2(aq)$ directly. At the two lowest growth rates, coccolith carbon was enriched in ^{13}C by 35‰ relative to organic carbon and 16–17‰ relative to external bicarbonate. These results suggested that the coccoliths were being formed from an internal pool of inorganic carbon that had been enriched in ^{13}C as a result of photosynthesis and that calcification and carbon fixation were both working on an internal pool that was chemically and isotopically homogeneous [*Laws et al.*, 1998]. At high growth rates, coccolith carbon was depleted in ^{13}C by 10‰ relative to external bicarbonate carbon. These results suggested that the inorganic carbon pool used for calcification might have been impacted by isotopically light CO_2 derived from respiration. The implication of these findings is that the substrate pool used for calcification is influenced by photosynthesis and respiration [*Laws et al.*, 1998].

Recently, *Riebesell et al.* [2000c] demonstrated using laboratory cultures that calcite production was reduced at elevated CO_2(aq) concentrations in *E. huxleyi* and *G. oceanica*. Similar results were obtained in mixed algal cultures in field studies. If CO_2(aq)-dependent changes in rates of calcification affect inorganic carbon acquisition, they may also affect the carbon isotopic composition of the alkenone-producing algae directly or indirectly through the production of coccoliths.

[32] *Bidigare et al.* [1997a] used nitrate-limited continuous cultures with constant irradiance to examine fractionation as a function of growth rate and [CO_2(aq)] in a calcifying (B92/11) and noncalcifying (BT6) clone of *E. huxleyi*. A strong negative linear correlation was found between ε_P and μ/CO_2, which resulted in an estimated ε_f of ~25‰. *Bidigare et al.* [1997a] found little difference in ε_P between calcifying and noncalcifying strains of *E. huxleyi* over a range of [CO_2(aq)] and growth rates in this limited data set. Fractionation in the calcifying strain B92/11 was recently examined using dilute batch culture experiments using a 16 hours light: 8 hours dark regime over a wide range of CO_2 concentrations (1.1–53.5 μmol L^{-1}) but a fairly narrow range of photoperiod growth rates (0.76 – 0.96 d^{-1}) [*Riebesell et al.*, 2000b]. These authors found large differences in fractionation between chemostat and dilute batch culture incubations (Figure 6). Estimates for ε_P are up to 8‰ higher and the slope of the ε_P versus μ_i/[CO_2(aq)] relationship is considerably steeper in nitrate-limited chemostats [*Bidigare et al.*, 1997a] compared to the nitrate-saturated batch cultures [*Riebesell et al.*, 2000b]. Also, owing to the wider range of μ_i/[CO_2(aq)] values covered in batch culture experiments, it becomes evident that the relationship between ε_P and μ_i/[CO_2(aq)] is nonlinear. The apparent difference in ε_P responses between nitrate-limited and nitrate-saturated cultures of *E. huxleyi* suggests a principal difference in carbon acquisition mechanisms for different growth-rate-limiting resources in this species. Similar differences in ε_P were found for the diatom *Phaeodactylum tricornutum* grown in nitrate-saturated dilute batch cultures and in a nitrate-limited chemostat [*Riebesell et al.*, 2000a]. When grown under nitrogen deficiency (nitrate-limited chemostat), ε_P of *P. tricornutum* decreases with increasing growth rate. In contrast, under nitrogen-saturated conditions, ε_P values are considerably lower at comparable μ and [CO_2(aq)] and are largely insensitive to light-dependent changes in growth rate (data not shown). In both experimental approaches, ε_P shows a relatively small CO_2 sensitivity in the range of CO_2 concentrations naturally occurring in the ocean (8–25 μmol kg^{-1}). *Riebesell et al.* [2000a] suggested that the inconsistency in ε_P responses between nitrate-limited chemostat cultures and nitrate-saturated dilute batch cultures might reflect differences in the regulation of carbon uptake relative to carbon fixation.

[33] Recently, *Rosenthal et al.* [1999] presented results of continuous culture light-limited (turbidostat) experiments. A linear fit of the turbidostat results indicated that the $\varepsilon_P - \mu/CO_2$ relationship was not statistically different from that found in chemostat experiments [*Rosenthal et al.*, 1999]. Thus it appears that nitrate and light-limited growth in continuous cultures yield nearly identical $\varepsilon_P - \mu/CO_2$ relationships and ε_f values. At this time it is not known why results from batch culture experiments differ from chemostat and turbidostat results or the extent to which variability in ε_P noted in culture studies represents that found in the field.

[34] *Nimer et al.* [1997] recently examined carbonic anhydrase (CA) activity and bicarbonate utilization in *E. huxleyi* (strain Bigelow 88E) and *G. oceanica* (strain PCC NZ90) as a function of [CO_2(aq)]. They found extracellular CA activity only in *E. huxleyi* under conditions

of very low CO_2(aq) concentrations (<2 umol kg^{-1}). It is reasonable to assume that if these strains are indicative of species growing in nature, they would not be expected to express CA activity in the ocean since CO_2(aq) levels this low are rarely encountered. Carbonic anhydrase can catalyze the dehydration of bicarbonate and thus affect the supply of CO_2 to a cell. *Nimer et al.* [1997] suggested that *G. oceanica* was able to utilize bicarbonate as an inorganic carbon source directly through an anion-exchange type mechanism. In contrast to the results of *Nimer et al.* [1997], *Sikes et al.* [1980] used short-term ^{14}C tracer experiments to show that CO_2(aq) was the substrate for photosynthesis and bicarbonate was the form of carbon supplied to the calcification site in *E. huxleyi.* It seems reasonable to ask which, if any, culture methods faithfully recreate natural growth conditions for *E. huxleyi*. Before addressing the issue of the applicability of culture conditions, we present results of field studies of alkenone isotopic variations.

7.2. Field Studies of the Alkenone-Containing Algae

[35] *Bidigare et al.* [1997a] showed that carbon isotopic fractionation of alkenone-producing algae in natural marine environments varied systematically with the concentration of soluble reactive phosphate. They argued that when PO_4 concentrations were greater than ~0.1 μM, PO_4 provided a monitor of change in growth rates of the alkenone-containing algae. They reasoned that this correlation was due to trace-metal limitations of algal growth rather than a direct control by PO_4 levels. An additional trace element, selenium, could be included in the list of growth-limiting factors since *Danbara and Shiraiwa* [1999] have shown that *E. huxleyi* (and *G. oceanica*) has a selenium requirement. The fact that cobalt [*Saito*, 2000], zinc, and selenium can exhibit phosphate-like distributions in the ocean adds further support to the "trace metal-growth rate" hypothesis advanced by *Bidigare et al.* [1997a]. The *Bidigare et al.* [1997a] relationship between fractionation and PO_4 was derived almost exclusively from upwelling environments in the Pacific Ocean. We examine details of that relationship, including new (Table 2) and recently published [*Popp et al.*, 1999; *Eek et al.*, 1999] data from low and high [PO_4] environments.

[36] Results of isotopic analyses of alkenones from additional oceanic regions (Figure 7a and Table 2) are in general agreement with those of *Bidigare et al.* [1997a]. The convention used here was introduced by *Rau et al.* [1992] and *Jasper et al.* [1994] and modified by *Bidigare et al.*, 1997a] and relates fractionation to the inverse of [CO_2(aq)], that is,

$$\varepsilon_P = \varepsilon_f - \frac{b}{CO_2}, \tag{8}$$

where b should be proportional to growth rate of the alkenone-containing algae [see also *Rau et al.*, 1996, 1997; *Bidigare et al.*, 1997a; *Popp et al.*, 1998a]. The fact that b is related to phosphate concentration by an equation of the form $b = 79 + 120[PO_4]$ (Figure 7a) implies that growth rates remain finite even as [PO_4] approaches zero. This observation probably reflects a combination of two factors. First, phytoplankton are able to cleave phosphate groups from dissolved organic phosphorus compounds [*Ammerman and Azam*, 1985; *Karl and Tien*, 1997; *Benitez-Nelson*, 2000]. Hence phosphate concentrations approaching zero do not imply the absence of microalgal growth; that is, there is another source of phosphorus. As has been pointed out by *Bidigare et al.* [1997a], *E. huxleyi* is known to have low phosphorous requirements for growth [*Paasche and Brubak*, 1994] and can outcompete other phytoplankton when growing under elevated N:P ratios [*Riegman et al.*, 1992]. Second, the correlation between b and phosphate most likely reflects limitation by a nutrient other

Table 2. Sample Location, Pertinent Environmental Information, and Calculated Fractionation for Samples Collected During the U.S. Joint Global Ocean Flux Study Arabian Sea and Southern Ocean Expeditions

Sample	Location	Date	*d*, m	PO_4, μM	CO_2(aq), μmol kg^{-1}	ε_P,[a] ‰	*b*,[b] ‰ μmol kg^{-1}
			Arabian Sea				
1	19.88°N, 65.89°E	March 17, 1995	5	0.41	10.6	12.72	130
2	19.17°N, 67.17°E	March 18, 1995	5	0.43	10.3	11.74	137
3	10.00°N, 64.89°E	March 23, 1995	5	0.20	10.2	14.55	106
4	14.46°N, 64.86°E	March 26, 1995	5	0.25	10.2	13.07	121
5	16.00°N, 61.98°E	March 30, 1995	5	0.32	10.4	14.88	106
6	17.21°N, 59.77°E	April 2, 1995	5	0.35	10.6	14.33	113
7	18.09°N, 58.00°E	April 6, 1995	5	0.34	11.0	14.32	118
8	23.22°N, 61.03°E	Nov. 2, 1995	5	0.50	10.5	13.69	119
9	19.17°N, 67.10°E	Nov. 6, 1995	5	0.33	10.5	15.82	97
10	10.90°N, 64.95°E	Nov. 10, 1995	5	0.30	10.6	16.46	91
11	14.47°N, 64.96°E	Nov. 13, 1995	5	0.27	10.5	16.06	94
12	17.07°N, 59.83°E	Nov. 16, 1995	5	0.55	11.5	13.90	127
13	18.51°N, 57.29°E	Nov. 18. 1995	5	0.39	10.8	14.84	110
14	18.27°N, 60.32°E	Nov. 23, 1995	5	0.58	11.6	13.46	134
			Southern Ocean				
15	60.28°S, 170.05°W	Nov. 22, 1996	5	1.48	22.7	16.39	195
16	60.30°S, 170.10°W	Nov. 22, 1996	50	1.49	22.6	14.86	229
17	56.90°S, 170.17°W	Nov. 24, 1996	5	1.38	17.7	12.94	213
18	56.98°S, 170.17°W	Nov. 25, 1996	50	1.39	17.8	12.91	216
19	53.03°S, 174.73°W	Nov. 25, 1996	5	0.73	17.1	15.07	170

[a]Values of ε_P are based on $\delta_{37:2}$ and $\delta_{CO_{2(aq)}}$ assuming a depletion of ^{13}C relative to carbon biomass of 4‰.
[b]Here, $b = (25 - \varepsilon_P)CO_2$; see text.

than phosphate, probably a trace metal (see discussion above). Rearranging the *b* correlation equation (Figure 7a) gives

$$\varepsilon_P = 25 - \frac{79 + 120[PO_4]}{CO_2}. \quad (9)$$

According to (9), in the limit of zero phosphate concentration ε_P approaches $25 - 79/CO_2$. At a typical CO_2 concentration of 10 μmol kg^{-1} this limiting value becomes 17‰. The implication of this analysis is that alkenone-producing phytoplankton are virtually always growing at some finite rate even as the concentration of soluble reactive phosphate approaches zero. Isotopic fractionations approaching 25‰ are therefore unlikely to be observed in the field unless alkenone-producing algae are growing slowly in waters with CO_2 concentrations greatly exceeding 10 μmol kg^{-1}.

[37] The accuracy and precision at which maximum fractionation ε_f can be determined in *E. huxleyi* has implications for calculation of [CO_2(aq)] from isotopic analyses of alkenones [see *Bidigare et al.*, 1997a; *Pagani et al.*, 1999]. Results of chemostat and turbidostat as well as some batch culture experiments [e.g., *Thompson and Calvert*, 1995] suggest ε_f values of 25‰. Although maximum isotopic fractionation in dilute batch culture experiments rarely exceeded ~17‰ (e.g., Figure 6), U. Riebesell (unpublished data, 2000) observed isotopic fractionation approaching 25‰ in diatoms (*S. costatum* and *P. tricornutum*) grown in dilute batch culture experiments at concentrations of CO_2(aq) exceeding 150 μmol kg^{-1}. Although similar experiments have not been performed, it is reasonable to assume that *E. huxleyi* would respond similarly.

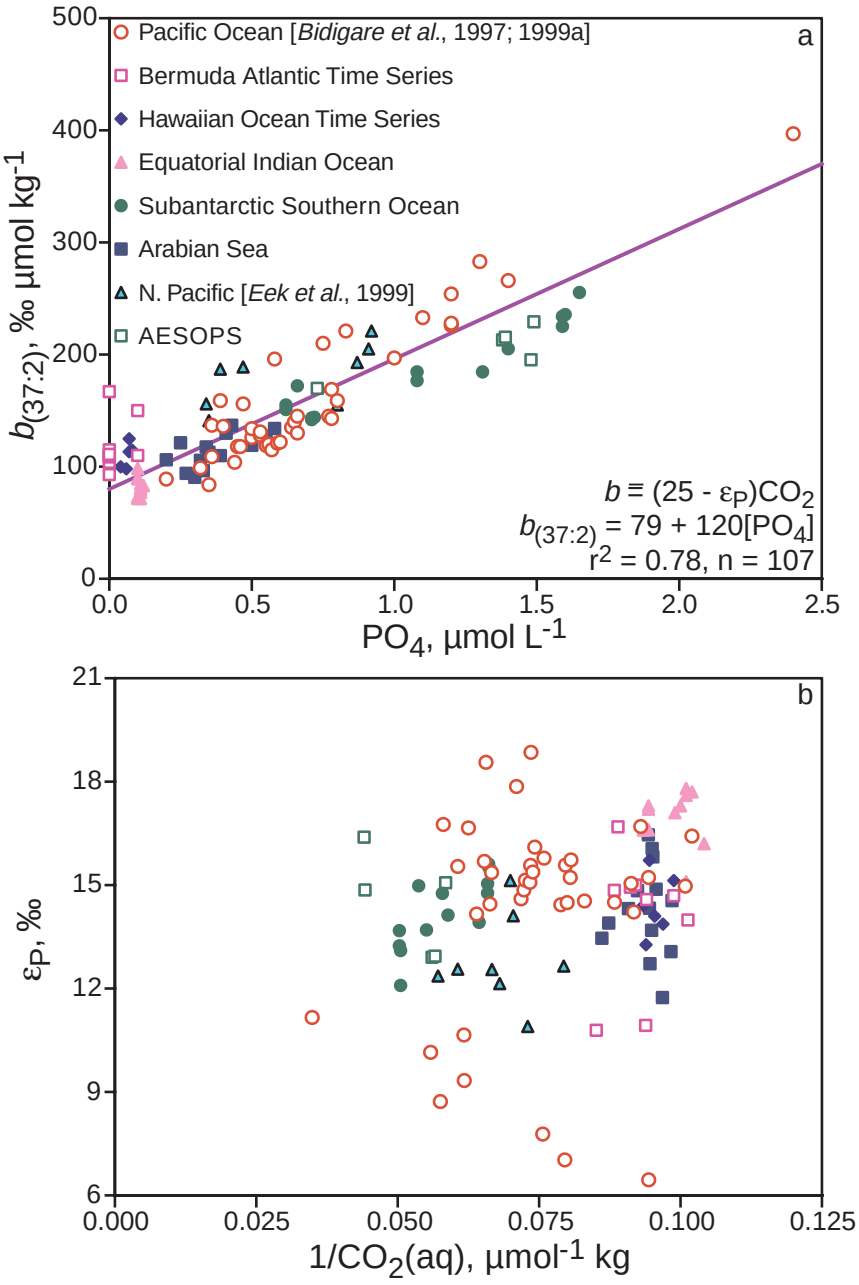

Figure 7. Relationships between (a) *b* and phosphate concentration and (b) [CO_2(aq)] and phosphate concentration. Data for the Pacific Ocean and the Bermuda Atlantic time series study (BATS) samples are from *Bidigare et al.* [1997a, 1999a]. Data from Hawaii Ocean time series (HOT), the equatorial Indian Ocean, and the subantarctic Southern Ocean are from *Popp et al.* [1999]. Data from the North Pacific are from *Eek et al.* [1999]. The line and equation in Figure 7a represent a geometric mean regression analysis (reduced major axis) and include all data.

[38] The $\delta^{13}C$ values of alkenones from the Santa Monica Basin have been corrected after recently discovering an error in their reported value [*Bidigare et al.*, 1999a] that affected the values of ε_P, μ, and b. The recalculated values of b fall above those observed for most other Pacific Ocean localities. The results of *Eek et al.* [1999] from Line P (between Vancouver Island and Station Papa [*Whitney and Freeland*, 1999]) are consistent with the recalculated Santa Monica Basin data. *Popp et al.* [1999] showed that values of b from the Hawaiian Ocean Time-series site and equatorial Indian Ocean (low-PO_4 environments) overlap with those from the Bermuda Atlantic Time Series site. They suggested that these results imply that the micronutrient controls on growth discussed by *Bidigare et al.* [1997a] apply to all results regardless of the concentrations of PO_4. The *Popp et al.* [1999] results of b from the subantarctic Southern Ocean and the new results from the Joint Global Ocean Flux Study (JGOFS) Arabian Sea and Southern Ocean (AESOPS) studies are consistent with the results of *Bidigare et al.* [1997a] from the Pacific Ocean.

[39] If the concentration of $CO_2(aq)$ were the major factor controlling the carbon isotopic fractionation in the alkenone-producing algae, a negative correlation between ε_P and the reciprocal of $[CO_2(aq)]$ would be expected [e.g., *Rau et al.*, 1992]. Results shown in Figure 7b indicate no correlation between ε_P and $1/CO_2(aq)$, implying that physiological factors are also important in controlling ε_P. As pointed out by *Bidigare et al.* [1997a], the relationship in Figure 7a could result simply from a correlation between concentrations of dissolved CO_2 and phosphate. *Bidigare et al.* [1997a] investigated a possible pseudocorrelation between b and $[PO_4]$ by examining correlations between concentrations of $CO_2(aq)$ and phosphate. They concluded that the weak correlation between $[CO_2(aq)]$ and $[PO_4]$ was not the primary cause of the highly significant dependence of b on $[PO_4]$ for samples from the Pacific Ocean [see also *Popp et al.*, 1999]. However, the correlation between $CO_2(aq)$ and $[PO_4]$ ($r^2 = 0.8$) for this larger data set is as good as that between b and $[PO_4]$. To determine the effect of $[PO_4]$ on fractionation in this larger set of data, *Popp et al.* [1999] examined the effect of $[PO_4]$ on ε_P at relatively constant $[CO_2(aq)]$. At $CO_2(aq)$ concentrations between 12 and 16.5 μmol kg^{-1}, ε_P and $[PO_4]$ are well correlated ($r^2 = 0.64$, $n = 33$, $p < 0.0005$), whereas the coefficient of determination for the dependence of ε_P on $[CO_2(aq)]$ is only 0.02. *Popp et al.* [1999] suggested that the fact that ε_P is significantly correlated with $[PO_4]$ at essentially invariant $[CO_2(aq)]$ indicates that the correlation between b and $[PO_4]$ is not driven by correlations between $[CO_2(aq)]$ and $PO_4]$ in surface waters.

[40] *Popp et al.* [1999] also examined details of the effect of $[PO_4]$ on ε_P in samples from the subantarctic Southern Ocean. They found that variations in ε_P were more closely linked to changes in the concentration of PO_4 than $CO_2(aq)$. Concentrations of PO_4 and $CO_2(aq)$ north of the Polar Frontal Zone at $\sim$51°S are low and relatively constant, whereas south of $\sim$51°S, concentrations of both dissolved species increase as a function of latitude. In samples collected south of 51°S, *Popp et al.* [1999] found that a decrease in ε_P was correlated with an increase in $[PO_4]$, implying an increase in the rate of growth of the alkenone-producing algae that affected carbon isotopic fractionation.

[41] Few studies exist that evaluate effects of active transport of inorganic carbon on isotopic fractionation in marine phytoplankton growing under natural conditions. Recently, *Tortell et al.* [2000] demonstrated that rapidly growing phytoplankton in diatom-dominated nutrient-replete coastal environments could use a carbon-concentrating mechanism to supplement diffusion of $CO_2(aq)$ into their cells. They

suggested that a lack of correlation between isotopic fractionation and cellular growth rates and CO_2(aq) concentration resulted from active assimilation of inorganic carbon. From a comparison of field and laboratory data of isotopic fractionation, growth rates, and CO_2(aq) concentrations, *Tortell et al.* [2000] predicted that active assimilation would produce a nonlinearity in ε_P when μ/CO_2 exceeded 0.2. Thus *Tortell et al.* [2000] suggested that nonlinear behavior would be expected to occur in phytoplankton growing at $\mu > 1.1$ d^{-1} in air-equilibrated seawater with a CO_2(aq) concentration of $\sim$10 μmol kg^{-1}. The minimum CO_2(aq) concentration for the data shown in Figure 7 is 9.6 μmol kg^{-1} (equatorial Indian Ocean [see *Popp et al.*, 1999]). If the predicted relationship of *Tortell et al.* [2000] can be extended to *E. huxleyi*, it suggests that ε_P should be a linear function of μ/CO_2 in the contemporary ocean since growth rates of the haptophytes rarely exceed $\sim$0.8 d^{-1} in the field [see *Bidigare et al.*, 1997a, Table 3] or $\sim$0.95 d^{-1} in the laboratory [*Riebesell et al.*, 2000b]. If, however, assimilation of inorganic carbon is linked with coccolith formation, active inorganic carbon uptake could contribute significantly to cellular carbon acquisition. Increased rates of calcification have been found in *E. huxleyi* grown under phosphate limitation [*Paasche and Brubak*, 1994] and under low CO_2(aq) concentrations [*Riebesell et al.*, 2000c]. Although haptophyte growth rates are likely to be low in warm oligotrophic waters [*Latasa et al.*, 1997; *Gieskes and Kraay*, 1989], enhanced supply of inorganic carbon through calcification could contribute to cellular carbon acquisition under the low [PO_4] and low [CO_2(aq)] typical of these regions.

[42] *Bidigare et al.* [1997a] suggested that if the isotopic compositions of alkenones are used to estimate ancient [CO_2(aq)], then variations in growth rate must be constrained. If the concentration of PO_4 in surface waters can be estimated, then the correlation between b and PO_4 (Figure 7a) may be used to estimate growth rates of the alkenone-producing algae. Laboratory culture studies indicate that the Cd/Ca ratio in calcareous tests of certain planktonic foraminifera is sensitive to ambient PO_4 levels [*Mashiotta et al.*, 1993]. However, recent field data suggest that this Cd/Ca $-$ PO_4 relationship is masked by a temperature dependency of Cd uptake in these organisms [*Rickaby and Elderfield*, 1999]. *Pagani et al.* [1999] minimized possible changes in growth rates of alkenone-producing algae by examining isotopic records of alkenones in low-productivity, open ocean settings, where variations in surface water phosphate may be minimal. Schrag and colleagues (D. Schrag, personal communication, 1999) have preliminary field data that suggest that growth rate controls the Sr uptake in coccoliths and thus implies that the Sr/Ca ratio in coccoliths may be used to infer growth rate variations in alkenone-producing algae. Since there are two potential approaches to constrain growth rates in the alkenone-producing algae and because use of alkenones can limit uncertainty in cell size and shape, isotopic analyses of alkenones remains a potential isotopic tool for reconstruction of ancient atmospheric CO_2 levels.

[43] Building on their previous work, *Pagani et al.* [2000] recently reconstructed surface water [CO_2(aq)] using alkenone $\delta^{13}C$ values extracted from surface sediments across a latitudinal transect in the central Pacific Ocean (175°E from 45°N to 15°S). The depth of haptophyte production was estimated from $U^{K'}_{37}$ temperatures. Preindustrial [CO_2(aq)] was reconstructed at each site using the alkenone $\delta^{13}C$ values, the probable ranges of [PO_4] and the $\delta^{13}C$ of CO_2(aq) at the depth of production, and the b $-$ PO_4 relationship of *Popp et al.* [1999]. *Pagani et al.* [2000] found that sedimentary alkenone-based [CO_2aq] values were lower than actual water column CO_2(aq) mea-

surements. However, when measured water column [CO_2(aq)] were adjusted to account for a 20% increase in anthropogenic carbon dioxide, the alkenone-based [CO_2(aq)] reconstruction accurately captured water column CO_2(aq) concentrations to within ± 0.5 μmol kg^{-1} for the majority of the sample sites [*Pagani et al.*, 2000]. These authors also provided evidence suggesting that the growth rates of alkenone-containing haptophytes are not light-limited in the upper central Pacific Ocean.

7.3. Relationship Between Laboratory Results and Field Data

[44] Results of laboratory experiments and field studies imply that isotopic fractionation in *E. huxleyi* changes as a function of cellular growth rate and ambient CO_2 concentration. Although the overarching consistency in the response of *b* to changing [PO_4] (Figure 7) and the astonishing ability of this method to reconstruct preindustrial CO_2(aq) concentrations by *Pagani et al.* [2000] support the interpretation that growth rate affects fractionation, these correlations do not prove that growth rates are changing in the alkenone-producing algae. Conclusive experiments are required to link the growth rate of natural populations of alkenone-producing algae with PO_4 (or micronutrient) concentrations and to resolve the differences in fractionation behavior found in laboratory studies (i.e., batch versus chemostat cultures). However, field tests of hypotheses based on observations of laboratory cultures have not been possible because it has been too difficult to assess growth rates of specific phytoplankton in natural assemblages. Two field methods commonly used to determine phytoplankton growth rate are the dilution method [*Landry and Hasset*, 1982] and the ^{14}C pigment labeling technique [*Redalje and Laws*, 1981; *Welschmeyer and Lorenzen*, 1984]. Since the dilution and ^{14}C-labeling techniques are based on the rate of change and specific radioactivity of pigments, respectively, these rates pertain to algae that possess that pigment. For example, these methods could not be used to test fractionation hypotheses in the alkenone-containing algae because growth rates determined by these methods rely on the use of the carotenoid 19′-hexanoyloxyfucoxanthin. While many of the common oceanic haptophytes possess 19′-hexanoyloxyfucoxanthin (e.g., *Emiliania*, *Gephyrocapsa*, *Phaeocystis*, *Chrysochromulina*, *Corymbellus*, and *Imantonia*), only *E. huxleyi* and *G. oceanica* are known to produce alkenones in the open ocean [*Conte et al.*, 1994; *Jeffrey and Wright*, 1994; *Thomsen et al.*, 1994]. To circumvent this problem, we recently developed in the laboratory a technique to measure the growth rate of the alkenone-containing algae in the field. The technique parallels the ^{14}C-labeling method but uses irmGCMS to determine the rate of uptake of ^{13}C by alkenones and thus yields the growth rate of only the alkenone-containing algae. Results of a batch culture time series (18, 24, 40, and 48 hours) experiment indicate that growth rate determined by ^{13}C incorporation into alkenones did not differ from growth rates determined by cellular ^{13}C uptake or by change in particulate organic carbon (POC) (Table 3). These results show that growth rates inferred from ^{13}C incorporation into alkenones and rates of carbon fixation are similar (see *Goericke and Welschmeyer* [1992a, 1992b, 1993a, 1993b] for a discussion of the kinetics of pigment ^{14}C labeling and its effect on determination of growth rates). These findings suggest that growth rates of natural populations of alkenone-synthesizing algae may be determined using ^{13}C alkenone labeling experiments using shipboard or in situ incubations. Such experiments would for the first time allow laboratory-based isotopic fractionation hypotheses regarding growth rates to be tested in the field.

Table 3. Results of Batch Culture Time Series Labeling Experiment With *Emiliania huxleyi* Strain B92/11[a]

Method	Growth Rate	Error	n
ΔPOC	0.23	0.05[b]	4
Isotopic labeling, POC	0.21	0.01	3
Isotopic labeling, alkenone	0.25	0.02	4

[a]Absolute growth rate was computed from time-dependent changes in particulate organic carbon (POC) concentration. Growth rate was also estimated from ^{13}C enrichment using a modification of equation (3) of *Welschmeyer and Lorenzen* [1984] to take into account stable isotopic values.

[b]Standard error from slope of regression ln POC versus time; slope of relationship yields μ.

8. Concluding Remarks and Issues to Be Addressed

[45] Although cellular locations, biochemical functions, and biosynthetic pathways of alkenones remain elusive, the consistent $U^{K'}_{37}$-temperature relationship for the world's oceans [*Müller et al.*, 1998] suggests that alkenone unsaturation can be reliably used to infer paleo-sea surface temperature. In addition, despite the distressingly poor correlation between ε_P and CO_2 in the contemporary ocean (Figure 7b), the consistency in the response of b to changing $[PO_4]$ in the same samples (Figure 7) and the coherent downcore variation in the isotopic composition of alkenones from low-productivity, open ocean settings show it to have potential for tracking changes in ancient pCO_2 [*Pagani et al.*, 1999]. This review indicates that much progress has been made at understanding how physiology of the alkenone-producing algae affects cellular abundance and unsaturation of alkenones, intracellular carbon isotope effects, and carbon isotopic fractionation associated with photosynthesis. However, significant uncertainty still exists concerning physiological and ecological factors affecting production and isotopic composition of alkenones. Phytoplankton culture conditions can have a profound effect on $\Delta\delta$, $U^{K'}_{37}$, alkenone cellular content, and changes in ε_P as a function of μ/CO_2. On the other hand, b (where $b \equiv (\varepsilon_f - \varepsilon_P)CO_2$) is systematically related to PO_4 concentration in the world's oceans, suggesting that physiological and environmental variables contribute only variance to the observed correlation between b and PO_4, rather than influencing the nature of the correlation. This premise, however, cannot easily be evaluated because few studies exist that allow laboratory-based relationships to be rigorously tested in the field. The following lists several significant questions that remain to be addressed concerning how alkenone biosynthesis and the physiology of alkenone-producing algae can affect cellular alkenone concentrations, unsaturation ratios, and carbon isotopic compositions.

1. What are the subcellular locations and functions of alkenones in *E. huxleyi* and *G. oceanica*? Do interorganelle variations in the $\delta^{13}C$ of alkenones exist?
2. What controls the cellular concentration of alkenones? Do changes in the ratio of protein:carbohydrate:lipid adequately explain variations in $\Delta\delta$?
3. What are the pathways of formation of polyisoprenoids and alkenones in *E. huxleyi*? Can these be determined using ^{13}C-tracer or other studies?
4. What is the growth status of alkenone-producing algae in the field, and how does growth status affect the cellular alkenone abundance and $U^{K'}_{37}$?
5. How does carbon isotopic fractionation ε_P in *E. huxleyi*/*G. oceanica* change in response to changes in $[CO_2(aq)]$ and

growth rate μ during bloom and nonbloom conditions? What are the effects of irradiance and light limitation on ε_P? Does light-limited growth occur frequently in the field? Do results from either batch or continuous culture adequately describe this response? Can these results be used to "calibrate" this paleo-CO_2 indicator?

6. What is the maximum fractionation ε_f in *E. huxleyi*/*G. oceanica*? What is the variability in ε_f in nature? (Note: $b \equiv (\varepsilon_f - \varepsilon_P)CO_2$, where ε_f is assumed constant.)
7. Does *E. huxleyi* possess an inorganic carbon concentrating mechanism? Under what conditions does it affect the carbon isotopic composition of *E. huxleyi*?

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