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The effects of varying CO_2 concentration on lipid composition and carbon isotope fractionation in *Emiliania huxleyi*

Ulf Riebesell,[1] Andrew T. Revill,[2] Daniel G. Holdsworth,[2] and John K. Volkman[2,3,*]

[1]Alfred Wegener Institute for Polar and Marine Research, Postbox 120161, D-27515 Bremerhaven, Germany
[2]CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia
[3]Hanse-Wissenschaftskolleg, Postbox 1344, D-27749 Delmenhorst, Germany

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Abstract—We have measured the stable carbon isotopic composition of bulk organic matter (POC), alkenones, sterols, fatty acids, and phytol in the coccolithophorid *Emiliania huxleyi* grown in dilute batch cultures over a wide range of CO_2 concentrations (1.1–53.5 μmol L^{-1}). The carbon isotope fractionation of POC (ε_{POC}) varied by ca. 7‰ and was positively correlated with aqueous CO_2 concentration [CO_{2aq}]. While this result confirms general trends observed for the same alga grown in nitrogen-limited chemostat cultures, considerable differences were obtained in absolute values of ε_{POC} and in the slope of the relationship of ε_{POC} with growth rate and [CO_{2aq}]. Also, a significantly greater offset was obtained between the $\delta^{13}C$ of alkenones and bulk organic matter in this study compared with previous work (5.4, cf. 3.8‰). This suggests that the magnitude of the isotope offset may depend on growth conditions. Relative to POC, individual fatty acids were depleted in ^{13}C by 2.3‰ to 4.1‰, phytol was depleted in ^{13}C by 1.9‰, and the major sterol 24-methylcholesta-5,22E-dien-3β-ol was depleted in ^{13}C by 8.5‰. This large spread of $\delta^{13}C$ values for different lipid classes in the same alga indicates the need for caution in organic geochemical studies when assigning different sources to lipids that might have $\delta^{13}C$ values differing by just a few ‰. Increases in [CO_{2aq}] led to dramatic increases in the alkenone contents per cell and as a proportion of organic carbon, but there was no systematic effect on values of $U^{k'}_{37}$ used for reconstructions of paleo sea surface temperature. *Copyright © 2000 Elsevier Science Ltd*

1. INTRODUCTION

The carbon isotopic composition of particulate organic carbon ($\delta^{13}C_{POC}$) in the oceans varies significantly with latitude and reflects the effects of a variety of environmental variables. Early findings of a close correlation between seawater $\delta^{13}C_{POC}$ and the concentration of aqueous CO_2 [CO_{2aq}] (Rau et al., 1991a), raised the possibility of reconstructing paleo-CO_2 from the $\delta^{13}C$ values preserved in sedimentary organic matter (e.g., Rau et al., 1989; 1991b; Freeman and Hayes, 1992; Jasper et al., 1994). More recent compilations (e.g., Goericke and Fry, 1994), however, show more variability. Moreover, a direct link between the $\delta^{13}C$ value of organic matter in sediments and the CO_2 concentration in the surface layer is confounded by a multitude of additional factors including: species-specific differences in isotope fractionation among marine photosynthetic organisms (e.g., Popp et al., 1998b; Burkhardt et al., 1999a), effects of other environmental conditions such as irradiance, growth rate or the growth rate limiting resource on isotope fractionation in microalgae (e.g., Thompson and Calvert, 1995; Beardall et al., 1998; Laws et al., 1998; Popp et al., 1998b; Bidigare et al., 1999; Riebesell et al., 2000), physical isolation of primary producers, for example, in sea ice (Rau et al., 1991a), shifts in isotope composition of buried organic matter during degradation, and the input of terrestrial organic matter (with a different $\delta^{13}C$ value) into marine sediments (Jasper and Hayes, 1990).

Potentially clearer relationships between $\delta^{13}C$ values and palaeoenvironmental conditions can be obtained using specific biomarkers derived from particular marine taxa. It has now become possible using compound-specific isotope-ratio mass spectrometry to measure the $\delta^{13}C$ values of a great variety of individual compounds in the complex mixtures found in sediments and seawater. This technique is now widely used to determine the sources of organic matter in seawater and recent and ancient sediments (e.g., Freeman et al., 1990; Kohnen et al., 1992; Schoell et al., 1994; Canuel et al., 1997). Most studies have exploited relatively large isotope differences between C3 and C4 plants (e.g., Kuypers et al., 1999), or between plants, microalgae, and some bacteria to assign sources. However, the isotope differences between individual compounds in sediments can be quite small and within the ranges found in a particular source organism.

Degens et al. (1968) made an early attempt to address the issue of the difference between the $\delta^{13}C$ of POC and that of associated lipids. DeNiro and Epstein (1977) were the first to demonstrate that the low $\delta^{13}C$ values of lipids results from isotope fractionation during the oxidation of pyruvate to acetyl coenzyme *A*. Monson and Hayes (1982) were able to show that different biochemical pathways were likely to introduce a different level of isotope fractionation. Schouten et al. (1998) showed that the offset in $\delta^{13}C$ values for common lipids relative to the $\delta^{13}C$ value of POC varies significantly between microalgal species. This reflects, in part, the fact that the $\delta^{13}C$ of algal biomass is heavily influenced by variations in the relative amounts of the major biochemicals in the cell (i.e., proteins, carbohydrates, and lipids) since these have different $\delta^{13}C$ values. Further variation arises from isotopic fraction-

*Author to whom correspondence should be addressed (john.volkman@marine.csiro.au).

ations associated with different biosynthetic pathways and the site of reactions in the cell.

The primary aim of this study was to investigate possible variations in the $\delta^{13}C$ values of different lipid classes in *E. huxleyi* grown in nutrient-replete low-biomass batch cultures over a wide range of CO_2 concentrations. *E. huxleyi* is the predominant coccolithophorid in the modern ocean with a worldwide distribution (e.g., Westbroek et al., 1993). This species can form dense blooms in both coastal and oceanic waters (e.g., Brown and Yoder, 1994 and references therein), and is an important component of the oceanic carbon cycle (see review in Westbroek et al., 1993). *E. huxleyi* is one of several haptophyte microalgae that synthesizes a class of very long-chain (C_{37}–C_{39}) unsaturated ketones termed alkenones (Volkman et al., 1980; Marlowe et al., 1984) which are now widely used as proxies for sea surface temperature (e.g., Brassell et al., 1986; Müller et al., 1998). The isotope signature of these compounds has also been proposed as a paleobarometer of past CO_2 concentrations (Jasper and Hayes, 1990; Jasper et al., 1994; Bentaleb et al., 1996). Since there is still a need for a more fundamental understanding of the relationship between the isotopic fractionation of carbon in the lipids of microalgae and environmental variables such as $[CO_{2aq}]$, we also examined the relationship between $[CO_{2aq}]$ and $\delta^{13}C$ values for both the particulate organic carbon (POC) and the fatty acids, phytol, sterols, and alkenones.

2. EXPERIMENT

2.1. Algal Cultures

Stock cultures of coccolith-bearing *Emiliania huxleyi* (Lohmann) Hay and Mohler clone PML B92/11 (generously supplied by J. Green, Plymouth Marine Laboratory) were acclimatized to experimental conditions (see below) for 7 days prior to the start of the experiments. Preadapted cultures were incubated in dilute batch cultures at 16°C in sterile filtered (0.2 μm) *f*/2-enriched natural seawater (Guillard and Ryther, 1962) without silicate. The cultures were nutrient saturated under all conditions. The incident photon flux density was 150 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 16:8 hour light:dark regime. In two experimental runs with six (series 1) and four (series 2) sets of triplicate batch cultures, cells were grown over a range of concentrations of aqueous CO_2. To obtain the desired $[CO_{2aq}]$, the inorganic carbonate system was manipulated in two ways:

(1) The speciation of the dissolved inorganic carbon (DIC) was adjusted by addition of 0.5 N NaOH or 0.5 N HCl. This changed the alkalinity and pH of the medium in addition to $[CO_{2aq}]$, but kept total DIC approximately constant (series 1, $[CO_{2aq}]$ range: 1.1–14.6 μmol L^{-1}).
(2) Total DIC was increased by addition of $NaHCO_3$. This changed alkalinity and DIC, but kept pH approximately constant (series 2, $[CO_{2aq}]$ range: 15.0–53.5 μmol L^{-1}).

At each CO_2 level, triplicate 2.3 L batch cultures and one additional control bottle were inoculated from acclimatized stock cultures to provide an initial cell density of 100 cells mL^{-1}. Bottles were closed without headspace and incubated for 7–12 days, depending on algal growth rate, in Rumed 1200 light thermostats. Cells were kept in suspension by inversion two to three times per day. Cell counts were obtained daily from 20 mL subsamples from the control bottle using a Coulter Multisizer particle counter.

Average daily growth rates for the duration of the experiment were estimated according to

$$\mu = \ln (N_f/N_0)/t, \tag{1}$$

where N_0 and N_f are the cell densities at the beginning and end of the experiment and t is the duration of incubation in days. The cultures were harvested at a cell density of ca. 8×10^4 cells mL^{-1}.

The growth rate (μ) was determined as an average over both light and dark periods. To account for differences in the light:dark regime between this and previous studies (e.g., Bidigare et al., 1997), instantaneous growth rate (i.e., the rate of growth during the light period), was used in a direct comparison (see below). Instantaneous growth rate (μ_i) was calculated according to

$$\mu_i = \frac{(L + D)\mu}{L - Dr}, \tag{2}$$

where L and D are the duration of light and dark periods, respectively, and the factor r accounts for dark respiration. The value of r was assumed to equal 0.15, following the work of Laws and Bannister (1980).

2.2. Bulk Measurements

Samples for isotope analyses of particulate organic carbon (POC; 80–350 mL, in triplicate), and lipids (ca. 1.7 L, one for each CO_2 concentration) were filtered onto precombusted (12 h, 500°C) Whatman GF/C glass fiber filters. POC filters were stored frozen in acid-washed and acetone-rinsed glass dishes until analysis. Filters for lipid extractions were freeze dried over liquid N_2 and wrapped in aluminium foil until analysis. $\delta^{13}C_{POC}$ was determined using an ANCA–SL 20–20 Europa Scientific mass spectrometer. Inorganic C was removed by fuming filters with concentrated HCl overnight.

The filtrates obtained at the beginning and the end of the experiment were used for analysis of dissolved inorganic carbon (DIC), total alkalinity and $\delta^{13}C_{DIC}$. The samples for DIC and $\delta^{13}C_{DIC}$ determination were preserved with $HgCl_2$ (0.01% final concentration) and refrigerater stored in glass bottles without headspace until analysis.

DIC was measured coulometrically with a UIC Inc. coulometer at 20°C (Johnson et al., 1993). $\delta^{13}C_{DIC}$ was analyzed on a Finnigan MAT Delta-S mass spectrometer after phosphoric acid extraction (Mackensen et al., 1996). Total alkalinity was measured potentiometrically using a computer-controlled Gran titration technique (Gran, 1952) with a Metrohm 713 pH-meter and 665 Metrohm Dosimat at 20°C. pH was measured using a WTW 3000 Microprocessor pH-meter and a combined AgCl/KCl pH electrode calibrated with NBS buffers. $[CO_{2aq}]$ was calculated from DIC, total alkalinity, temperature and salinity (S = 32) and the concentrations of silicate and phosphate using dissociation constants of Goyet and Poisson (1989).

The isotope fractionation of POC (ε_{POC}) was calculated according to

$$\varepsilon_{POC} = \left(\left[\frac{\delta^{13}C_{CO_2} + 1000}{\delta^{13}C_{POC} + 1000} \right] - 1 \right) \times 1000, \tag{3}$$

where $\delta^{13}C_{POC}$ is the isotope composition of the total particulate organic matter collected at the end of the experiment. $\delta^{13}C_{CO_{2aq}}$ is the isotopic composition of the CO_2 source, calculated following Mook (1986) according to

$$\delta^{13}C_{HCO3} = \delta^{13}C_{DIC} - (\varepsilon_1[CO_2] + \varepsilon_2[CO_3^{2-}]/[DIC]), \tag{4}$$

$$\delta^{13}C_{CO_{2aq}} = \delta^{13}C_{HCO3} - \varepsilon_1[CO_2], \tag{5}$$

where $\delta^{13}C_{HCO3}$ and $\delta^{13}C_{DIC}$ are the isotopic compositions of HCO_3^- and DIC, respectively, $\varepsilon_1 = -9866/T + 24.12‰$ and $\varepsilon_2 = -867/T + 2.52‰$, with T being the absolute temperature in Kelvin.

2.3. Lipid Extraction

Freeze-dried filter samples were shredded and placed in a 15 mL glass extraction tube to which solvent was added (chloroform:methanol:water; 1:2:0.8; 5 mL). The contents were stirred, sonicated for 10 minutes, centrifuged and the supernatant transferred to a separating funnel. The extraction was then repeated a further three times and the extracts combined. Chloroform:water (1:1) was then added to the separating funnel until the final solvent ratio was 1:1:0.9 chloroform:methanol:water. This was shaken gently and left to stand overnight to allow phase separation. The chloroform layer was reduced under vacuum to ca. 2 mL and transferred to a glass tube for saponification. 5%

Table 1. Algal growth conditions and results from batch culture incubations with parallel measurements of cellular lipid contents and isotopic compositions.

Series	$[CO_{2aq}]$ (μmol L^{-1})	TA (μEq L^{-1})	DIC (μmol L^{-1})	pH	μ (d^{-1})	μ_i (d^{-1})	μ_i/CO_2 (L μmol^{-1} d^{-1})	$\delta^{13}C_{DIC}$ (‰)	$\delta^{13}C_{CO_2}$ (‰)	$\delta^{13}C_{POC}$ (‰)	ε_{POC} (‰)	C $cell^{-1}$ (pg $cell^{-1}$)	N $cell^{-1}$ (pg $cell^{-1}$)	C:N (molar)
1	1.1	3373	2125	9.12	0.52	0.85	0.77	−0.06	−9.97	−16.9	7.04	5.3	0.8	7.5
1	2.7	2955	2113	8.83	0.81	1.32	0.49	−0.01	−9.92	−17.3	7.53	6.0	1.0	7.4
1	4.0	2787	2109	8.69	0.92	1.49	0.37	0.13	−9.78	−17.7	8.05	9.2	1.3	8.2
1	5.3	2599	2047	8.58	0.97	1.57	0.30	0.59	−9.32	−17.7	8.49	10.2	1.4	8.4
1	7.6	2505	2060	8.44	0.96	1.56	0.21	0.42	−9.49	−19.1	9.81	9.0	1.3	8.2
1	14.5	2352	2079	8.19	0.90	1.46	0.10	0.40	−9.51	−21.2	11.9	10.4	1.3	9.4
1	14.6	2354	2083	8.19	0.90	1.46	0.10	0.36	−9.55	−21.7	12.4	9.4	1.3	8.6
2	15.0	2309	2047	8.17	0.82	1.32	0.09	0.61	−9.30	−21.4	12.3	11.4	1.7	8.0
2	16.3	2779	2458	8.21	0.78	1.26	0.08	−0.13	−10.0	−22.0	12.3	10.6	1.7	7.5
2	36.4	4742	4351	8.12	0.78	1.27	0.03	−2.08	−12.0	−24.4	12.7	10.0	1.6	7.5
2	53.5	9739	8884	8.25	0.76	1.23	0.02	−3.30	−13.2	−26.6	13.8	11.7	1.8	7.6

potassium hydroxide in methanol:water (80:20; 3 mL) was added to the total extract and the mixture heated at 80°C for 2 hours. The mixture was then extracted with hexane:chloroform (4:1 v/v) to obtain a neutral fraction. The mixture was then acidified (hydrochloric acid) and re-extracted with hexane:chloroform (4:1 v/v) to obtain the fatty-acid fraction. The fatty-acid fraction was reduced to dryness and methylated by adding methanol:HCl:chloroform (10:1:1 v/v/v; 3 mL) and heating (80°C, 2 hours). Internal standards were added to each fraction: a C_{23} fatty-acid methyl ester (50 μl of 250 ng/μl) to the fatty acids and n-C_{22} alkane (40 μl of 280 ng/μl) to the neutral fraction.

2.4. Gas Chromatography

Each extract was analyzed by gas chromatography on a 50 m, nonpolar methyl silicone fused–silica capillary column (HP-1, 0.32 mm i.d., 0.17 μm film thickness) using a Varian 3410 gas chromatograph equipped with a SPI injector programmed from 45°C at injection to 310°C at 200°C/minute and held there for 40 minutes. Hydrogen was the carrier gas. The GC oven temperature program was 45°C for 1 minute followed by a ramp to 140°C for the fatty acids or 160°C for the neutrals at 30°C/min and then at 3°C/min to 310°C. The oven was then maintained isothermally for 12 minutes.

2.5. Gas Chromatography–Mass Spectrometry

In order to confirm identifications and check peak purity, selected samples were analyzed by gas chromatography–mass spectrometry using a Fisons Instruments MD800 quadrupole MS and Masslab software. The MD800 was operated in full scan mode (650 to 50 Dalton). GC conditions were as above except that helium was used as the carrier gas. Typical operating conditions were a source temperature of 250°C, an ionization energy of 70 eV and an interface temperature of 310°C.

2.6. Gas Chromatography–Isotope Ratio Mass Spectrometry

Gas chromatography–isotope-ratio mass spectrometry (GC–IRMS) was carried out using a Finnigan MAT Delta S isotope ratio mass spectrometer linked to a Hewlett Packard 5890 series II GC *via* a cupric oxide/nickel/platinum combustion furnace (940°C) and a copper reduction furnace (640°C). Values of $\delta^{13}C$ were referred to an external CO_2 standard introduced at intervals through the GC run and checked using a standard mix of deuterated n-alkanes and n-C_{40}. The latter was initially co-injected with the neutral fractions (alkenones), but due to co-elution problems it was necessary to run the calibration standard mix after every 1–2 analyses. Accuracy and reproducibility between analyses were also monitored using the $\delta^{13}C$ value of the internal n-C_{22} alkane standard for neutrals and 23:0 fatty-acid methyl ester for fatty acids. Note that the C_{18} polyunsaturated fatty acids were not separated to baseline and thus a composite value was calculated for these compounds.

The $\delta^{13}C$ values for fatty-acid methyl esters were corrected for the addition of the extra carbon atom in the methyl ester derived from methanol in the derivatizing solution. We used a correction factor determined by analyzing a standard free fatty acid (n-C_{23}) as the free acid and after methylation. Previous experience in our laboratories has shown that the methylation procedure goes to completion with only traces of free fatty acids so no kinetic isotope effect would be expected. The method is analogous to the use of BF_3/methanol which has been shown to be a suitable methylation procedure for isotope studies (Abrajano et al., 1994). In a similar way, the $\delta^{13}C$ values of sterols and phytol were corrected for the carbon atoms in the TMSi group using correction factors determined from measurements of free and TMSi–ether cholesterol.

Optimum GC separation was achieved using on-column injection (duck-bill) onto a J&W DB-1 capillary column (60 m × 0.32 mm i.d.; 0.25 μm film). The oven was initially held at 40°C for 1 minute and then programmed to 160°C at 30°C/min, then to 220°C at 10°C/min and finally to 325°C at 3°C/min and held isothermally for 25 minutes.

The isotopic fractionation (ε_{lipid}) of selected lipids relative to that of CO_2 was calculated in an analogous fashion to (ε_{POC}) according to

$$\varepsilon_{lipid} = \left(\left[\frac{\delta^{13}C_{CO_2} + 1000}{\delta^{13}C_{lipid} + 1000} \right] - 1 \right) \times 1000. \quad (6)$$

The isotopic composition of the various lipids relative to that of POC and the C_{16} fatty acid were calculated as a simple difference (following Jasper et al., 1994; Schouten et al., 1998). That is

$$\Delta\delta^{13}C_{POC\text{-}lipid} = \delta^{13}C_{POC} - \delta^{13}C_{lipid}, \quad (7)$$

$$\Delta\delta^{13}C_{16:0\text{-}lipid} = \delta^{13}C_{16:0} - \delta^{13}C_{lipid}. \quad (8)$$

This gives similar values to those for $\varepsilon_{biomarker}$ (as used by Hayes, 1993; Summons et al., 1994; Popp et al., 1998a) calculated for lipids relative to POC. $\Delta\delta^{13}C_{POC\text{-}lipid}$ and $\varepsilon_{biomarker}$ values differ at most by a few tenths of a per mil when δ values are reasonably close (i.e., <10‰) as in our study.

3. RESULTS

3.1. General Observations

Aqueous CO_2 concentrations covered a wide range of values from 1.1 to 53.5 μmol L^{-1} (Table 1) and thus extended well beyond the range found in natural waters (8–25 μmol L^{-1}: Tans et al., 1990; Rau et al., 1992). During our experiments, the dissolved inorganic carbon (DIC) concentrations and total alkalinities (TA) decreased only slightly (by ~5% and ~2.5%, respectively), due to organic carbon and calcite production by the microalgae. Changes in pH $[CO_{2aq}]$, and in $\delta^{13}C_{DIC}$ during the course of incubation thus remained small (i.e., ≤0.15 units, ≤20%, and ≤0.4‰, respectively). Based on the observed changes in DIC and total alkalinity we calculated the ratios of carbonate precipitation (ΔTA) and organic matter production

Table 2. Percentage composition and contents of individual fatty acids (fg/cell) in *E. huxleyi* as a function of CO_2 concentration (μmol L^{-1}).

$[CO_{2aq}]$	1.1	2.7	4.0	5.3	7.6	14.5	15.0	16.3	36.4	53.5
Fatty acid					Percentage composition*					
14:0	2.0	9.2	9.9	8.2	12.4	10.1	10.0	15.8	12.4	16.6
16:0	6.4	5.8	6.2	6.3	5.4	6.6	13.4	7.9	7.5	8.8
18:0	1.1	1.4	1.4	4.3	0.9	1.2	2.6	0.6	0.8	0.8
18:1	8.5	9.4	12.3	10.2	11.4	12.9	23.8	21.1	18.3	20.1
18:2	1.9	3.8	3.8	4.0	5.7	6.3	6.3	5.6	6.2	6.7
18:3	8.3	7.4	7.4	6.9	6.3	6.8	4.8	5.7	5.4	5.1
18:4	11.3	9.9	8.8	8.3	8.5	8.1	5.4	7.5	7.5	6.9
18:5	21.8	18.3	17.3	18.3	16.9	17.3	9.5	9.9	11.8	11.0
22:6	38.6	34.9	32.9	33.4	32.6	30.7	24.2	25.9	30.1	24.0
Sum%	100	100	100	100	100	100	100	100	100	100
Fatty acid					Content fg/cell					
14:0	10.8	68.7	86.9	77.2	125.3	110.3	56.1	113.7	84.9	178.2
16:0	34.9	42.9	54.5	59.4	54.4	72.4	75.1	57.2	51.3	94.2
18:0	6.1	10.2	12.5	40.4	9.2	13.5	14.6	4.4	5.2	8.2
18:1	46.0	69.9	108	95.8	115	141	133	152	125	216
18:2	10.4	28.2	33.4	37.6	57.7	68.6	35.4	40.4	42.1	71.7
18:3	45.0	54.8	64.9	64.9	63.3	74.5	27.0	40.7	36.7	54.8
18:4	61.1	73.6	76.8	77.9	85.8	88.5	30.0	54.1	51.3	74.5
18:5	118	136	152	172	171	190	53.5	71.5	81	118
22:6	209	259	289	314	329	336	136	186	206	258
Sum (fg/cell)	542	743	878	938	1011	1094	561	720	683	1074

* Minor components (such as 15:0, 16:1, and 20:5) are not included since they collectively represented less than 5% of the total fatty acids. Analytical precision for replicate analyses is about ±5%, but cell counts are less reliable (±10%) and so estimates of amounts per cell are believed accurate within ±15%.

(ΔDIC–ΔTA). These calculations neglect the small increase in alkalinity due to uptake of nitrate by phytoplankton growth (Brewer and Goldman, 1976). Ratios of calcite to organic matter production ranged from 0.89 to 2.25 (mean: 1.41 ± 0.50) which are representative of high-calcifying cells of *E. huxleyi* (van Bleijswijk et al., 1994). As might be expected, higher ratios were obtained in incubations in which additional $NaHCO_3$ was added to the growth medium leading to DIC concentrations 2–3 times natural seawater concentrations.

3.2. Growth Rates and C:N Ratios

Growth rates (μ) ranged from 0.52 to 0.97 d^{-1} (Table 1); the slowest growth occurred at the lowest CO_2 concentration. Although somewhat lower growth rates were obtained during the second series of experiments, μ remained within a relatively narrow range of 0.76 to 0.97 d^{-1} for $[CO_{2aq}] > 2$ μmol L^{-1}. The C:N atomic ratio varied between 7.4 and 9.4 with a mean value of 8.0 ± 0.6. Although C:N ratios did not vary consistently with increasing concentrations of CO_2, there appears to be an overall increase in C:N with increasing $[CO_{2aq}]$ in the first series of experiments. In contrast, lower C:N ratios were obtained at the high CO_2 concentrations of DIC-enriched medium in the second series. The total carbon and nitrogen contents per cell increased towards higher $[CO_{2aq}]$ by factors of 2.2 and 2.3, respectively, over the $[CO_{2aq}]$ range tested. Increasing carbon content per cell is usually associated with an increase in cell size (e.g., Montagnes et al., 1994), but we were not able to measure this directly.

3.3. Lipid Distributions

Total lipid extracts of *E. huxleyi* contained a relatively simple distribution of compounds including phytol, a suite of fatty acids, a single major sterol (24-methylcholesta-5,22E-dien-3β-ol), the long-chain alkenones and the 36:2 methyl and ethyl alkenoates (analyzed as the methyl esters after saponification and transmethylation). The fatty acids consisted mainly of 14:0, 16:0, 18:4($n-3$)[1], 18:5($n-3$), and 22:6($n-3$) which collectively comprised over 80% of the total fatty acids (Table 2). Minor components included 16:1($n-7$), 18:2($n-6$), 18:3($n-3$), and traces of 20:5($n-3$). The relative proportion of 16:0 was low compared with most other microalgae (e.g., Volkman et al., 1989), but very similar distributions of fatty acids have been reported for other strains of *E. huxleyi* (e.g., Conte et al., 1995). The alkenones consisted of the 37:3, 37:2, 38:3, and 38:2 methyl ketones, and the 38:3, 38:2, 39:3, and 39:2 ethyl ketones (the latter two compounds being present in very low abundance; Table 3). Similar distributions have been reported in other strains of *E. huxleyi* (e.g., Volkman et al., 1980; Conte et al., 1995). The long-chain alkenes that occur in a few strains of *E. huxleyi* (e.g., Volkman et al., 1980; Rieley et al., 1998) were not observed here.

3.4. Effects of CO_2 Concentration on Lipid Distributions

Although the same fatty acids were found to predominate in all samples, the percentage composition and cellular contents varied with CO_2 concentration (Table 2). The distribution at the lowest CO_2 concentration contained the highest percentage of polyunsaturated fatty acids with 22:6($n-3$) comprising over 38% of the total. In contrast, the distribution at the highest CO_2

[1] Fatty acids are denoted by $x:y(n-z)$ where x is the number of carbon atoms, y is the number of double bonds, and z is the position of the double bond from the terminal methyl group.

Table 3. Contents of organic carbon (pg/cell), phytol, sterol, and alkenones (each fg/cell and as percentage of C_{org}), and $U^{k'}_{37}$ values in *E. huxleyi* as a function of CO_2 concentration (μmol L^{-1}).

$[CO_{2aq}]$	1.1	2.7	4.0	5.3	7.6	14.5	15.0	16.3	36.3	53.5
pg C_{org}/cell	5.3	6.0	9.2	10.2	9.0	9.4	9.4	11.4	10.0	11.7
Phytol	40	35	37	26	30	35	36	34	33	43
Sterol	56	63	45	39	35	49	75	50	70	86
Alkenones										
$C_{37:3}$ methyl	54	113	214	207	237	239	550	434	400	676
$C_{37:2}$ methyl	23	48	96	88	122	139	287	203	158	317
$C_{38:3}$ ethyl	26	45	63	61	60	62	121	102	100	162
$C_{38:3}$ methyl	2.3	7.2	18	16	17	21	68	51	42	72
$C_{38:2}$ ethyl	21	40	47	59	70	81	156	119	99	186
$C_{38:2}$ methyl	0.6	2.5	9.1	12	14	18	21	11	10	14
$C_{39:3}$ ethyl	1.0	2.8	4.4	4.3	4.2	4.7	9.6	10	6.7	13
$C_{39:2}$ ethyl	0.2	1.6	3.2	3.2	3.6	5.5	9.5	9.0	4.4	10
Sum alkenones	128	260	454	451	528	570	1222	939	820	1450
Phytol/C_{org} %	0.75	0.58	0.40	0.36	0.33	0.36	0.38	0.29	0.33	0.36
Sterol/C_{org} %	1.1	1.0	0.49	0.38	0.39	0.52	0.79	0.44	0.69	0.73
37:3/C_{org} %	1.0	1.9	2.3	2.0	2.6	2.5	5.9	3.8	4.0	5.8
$U^{k'}_{37}$	0.30	0.30	0.31	0.30	0.34	0.37	0.34	0.32	0.28	0.32

concentration showed much reduced percentages of the 18:5($n-3$) and 22:6($n-3$) polyunsaturated fatty acids and the highest content of 14:0 fatty acid. There are few obvious trends in the fatty-acid percentage compositions if the data are viewed as a single data set, but consistent changes are observed when the two experiments are viewed separately.

In the first series where the total DIC was kept constant, there was a strong decline in the percentage composition of the more highly unsaturated fatty acids 18:5($n-3$) and 22:6($n-3$), a modest decline in 18:3 and 18:4, little change in 18:0, a small increase in 16:0 and a significant increase in the proportion of the 14:0, 18:1, and 18:2 fatty acids with increasing CO_2 from 1.1 to 14.6 μmol L^{-1} (Table 2). In absolute terms, the content of fatty acids per cell approximately doubles over this CO_2 range and even the abundances of 18:5 and 22:6 fatty acids showed a modest increase (Table 2).

Similar trends can be seen in the second series (Table 2), although there was more scatter and comparisons are limited by the smaller number of data points. However, there was a major difference between the two experiments with cells at the start of the second series having much lower contents of polyunsaturated fatty acids than would be expected based on data from the first series. This led to a distinct difference in the proportions of fatty acids between the two sets of experiments, with the percentage of 18:1($n-9$) being about 2 times as abundant in the second set of experiments. The reasons for this change are not known, but it should be noted that the second series of experiments was conducted several weeks after the first. Thus, the observed differences in fatty-acid abundances may reflect changes in the physiological conditions of the stock cultures. Despite this difference in absolute abundances, the isotope fractionations show a single trend across the complete data set (Fig. 1).

Phytol contents all fell within the narrow range from 26 to 43 fg/cell and there was no obvious trend with varying CO_2 concentration (Table 3). When normalized to organic carbon, phytol contents were approximately 2 times as high in the experiments where $[CO_2]$ was lowest, but they then declined to

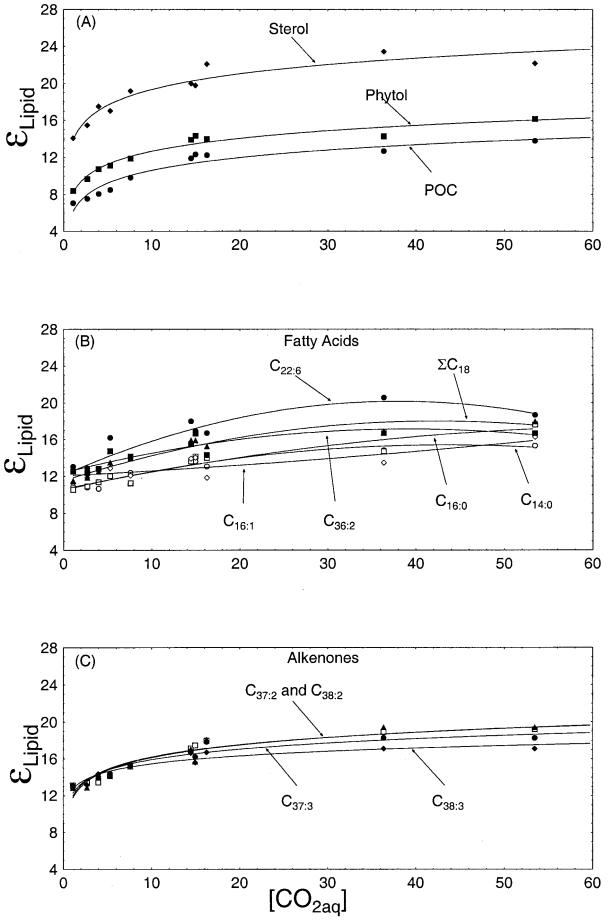

Fig. 1. Isotopic fractionations of POC (ε_{POC}) and individual lipids (ε_{lipid}) versus CO_2 concentration. **A**: (●) POC, (■) phytol, (◆) sterol; **B**: (○) $C_{14:0}$, (□) $C_{16:0}$, (◇) $C_{16:1}$, (△) ΣC_{18}, (●) $C_{22:6}$, (■) $C_{36:2}$; **C**: (□) $C_{37:2}$, (▲) $C_{38:2}$, (●) $C_{37:3}$, (◆) $C_{38:3}$. Note that the curves for $C_{37:2}$ and $C_{38:2}$ alkenones are superimposed. The isotope fractionation was calculated according to $\varepsilon_{lipid} = [(\delta^{13}C_{CO_2} + 1000)/(\delta^{13}C_{lipid} + 1000)] - 1) \times 1000$.

be within the range 0.33 ± 0.05 (Table 3). This is in marked contrast to the changes observed in the fatty-acid contents (Tables 2 and 3). The contents of the only major sterol, 24-methylcholesta-5,22E-dien-3β-ol, were also within a narrow range, with all but the value at the highest CO_2 concentration being in the range 54 ± 20 fg/cell. When normalized to organic carbon, the sterol contents behaved similarly to those of phytol.

In contrast, the alkenone contents per cell increased by more than a factor of 10 over the CO_2 range studied, i.e., significantly larger than the factor of 2 increase in cellular carbon contents (Table 3). Alkenone contents per cell increased with increasing [CO_{2aq}] in the first experiment, although when expressed as a percentage of the organic carbon content the variation was less marked. However, in the second series of experiments, where additional bicarbonate was added and [CO_{2aq}] values were much higher, the alkenone contents per cell (and even when normalized to organic carbon) were significantly greater (Table 3). As with the fatty acids, there was a distinct difference in contents of alkenones between the two series of experiments, but in this case the alkenone contents were elevated in the second series as if in (over)compensation for the reduced fatty-acid content (compare Tables 2 and 3). *E. huxleyi* does not synthesize high contents of storage lipids such as triacyglycerols and so these data are consistent with the possibility that one of the functions of alkenones is to store excess photosynthate as storage lipids. In our experiments, alkenone contents were enhanced at high [CO_{2aq}], but enhanced contents have also been observed when cells enter the stationary phase (Epstein et al., 1998).

Despite these variations in absolute contents per cell, the relative proportions of individual alkenones were affected very little by changes in CO_2 concentration resulting in little systematic variation in the $U^{k'}_{37}$ ratio (Table 3). Although there has been speculation that some environmental variables, in addition to temperature, might influence $U^{k'}_{37}$ (Epstein et al., 1998), our results show for the first time that changes in CO_2 concentration have little effect.

3.5. Effects of CO_2 Concentration on Carbon Isotopes

The $\delta^{13}C$ of the DIC ranged from −3.3‰ to 0.6‰ (Table 1), largely due to addition of $NaHCO_3$ with a slightly more negative isotopic signal than that of the seawater DIC in the high CO_2 experiments. This corresponds to calculated $\delta^{13}C$ values for CO_2 between −13.2‰ and −9.3‰. The $\delta^{13}C_{POC}$ value decreased with increasing [CO_{2aq}] by about 10‰ over the [CO_{2aq}] range from 1.1 to 53.5 μmol L^{-1}. Accounting for the change in isotopic composition of the source DIC, this yields isotope fractionation values for POC (ε_{POC}), between 7‰ and ca. 13.8‰ over the [CO_{2aq}] range tested (Table 1; Fig. 1).

The observed changes in $\delta^{13}C_{POC}$ as a function of CO_2 concentration (Table 1) closely corresponded to CO_2-related changes in the isotope compositions of the various lipids, particularly that of the sterol and phytol (Fig. 1; Table 4). As [CO_{2aq}] increased from 1.1 to 53.5 μmol L^{-1}, the $\delta^{13}C$ values became progressively lighter and the isotope fractionation of almost all lipids followed a similar nonlinear trend. Thus, the ε_{lipid} value for phytol changed by 7.7‰, for the 14:0 fatty acid by 4.5‰, for 24-methylcholesta-5,22E-dien-3β-ol (isotopically the lightest of all the lipids examined) by 8.1‰, and for the 37:3 alkenone by 5.3‰ (Fig. 1). Even within the [CO_{2aq}] range representative for the modern ocean (8–25 μmol L^{-1}), the ε_{lipid} values of the various lipids changed by 4–5.5 ‰.

Within the fatty acids, different responses were observed (Fig. 1). The behavior of the 14:0, C_{18} PUFA and 22:6 fatty acids mirrored that of POC, but the isotopic response of the 16:0 and 16:1 fatty acids showed different trends (Fig. 1). The ε_{lipid} values of the 37:2 and 38:2 alkenones were closely similar, but diverged from the 37:3 and 38:3 homologues at higher CO_2 concentrations, with differences of up to 2‰ in the second series of experiments. Note that in our experiments the isotope values of the C_{38} methyl and ethyl alkenones were measured together since they were not sufficiently separated on the capillary GC column for reliable values to be obtained separately. Over the [CO_{2aq}] range found in modern seawater the difference in isotope fractionation between the various alkenones was not statistically different.

The mean isotopic differences between POC and the various lipids ($\Delta\delta^{13}C_{POC\text{-}lipid}$) spanned a range from 1.9 ± 0.5 ‰ for phytol to 8.5 ± 1.1 ‰ for the sterol (Table 5). No consistent trend in $\Delta\delta^{13}C_{POC\text{-}lipid}$ values was observed with changing [CO_{2aq}]. Mean values between 4.9 ± 1.0 ‰ and 5.4 ± 0.5 ‰ were obtained for isotopic offsets between POC and the various alkenones, without statistically significant differences between them.

4. DISCUSSION

4.1. C:N Ratios and Microalgal Growth Rates

Culturing microalgae invariably involves some differences between the conditions in the natural environment and those simulated in the culture vessel. In our experiments we have chosen to use low-biomass nutrient-enriched batch cultures in an attempt to minimize possible effects of inorganic carbon and nutrient utilization and biomass accumulation on growth conditions. The C:N ratios with a mean value of 8.0 ± 0.6 and range of 7.4–9.4 are close to the Redfield ratio and within ranges expected for natural populations. For comparison, Biddanda and Benner (1997) reported a range of 7.6–10.6 in batch cultures of *E. huxleyi*, with values greater than 9.0 occurring in stationary phase cultures, while van Bleijswijk et al. (1994) reported a range between 5.6 and 7.4. The apparent increase in C:N with increasing [CO_{2aq}] in the first experimental series (Table 1) is consistent with observations by Burkhardt and Riebesell (1997) and Burkhardt et al. (1999c) who demonstrated CO_2-related changes in C:N and C:P ratios in six species of diatoms and a dinoflagellate at [CO_{2aq}] < 10 μmol kg^{-1}.

The growth rates obtained in this study, which ranged between 0.52 to 0.97 d^{-1}, are within the range of 0.2–1.2 d^{-1} reported for field populations (e.g., Egge and Heimdal, 1994; Bidigare et al., 1997). Although a maximum value of 0.35 d^{-1} was obtained by Thompson and Calvert (1995) in their *E. huxleyi* culture experiments, higher maximum specific growth rates up to 1.9 d^{-1} were found in culture experiments by Brand and Guillard (1981). In our first series of experiments, growth was reduced at the lowest concentration of CO_2 which is comparable with CO_2-dependent responses in growth rates by other marine phytoplankton, such as diatoms (Riebesell et al., 1993; Burkhardt et al., 1999c). It is presently not clear what

Table 4. Results of compound specific isotope analysis of *E. huxleyi* lipids ($\delta^{13}C_{lipid}$, ‰).*

		Fatty acids							Alkenones			
$[CO_{2aq}]$	Phytol	14:0	16:0	16:1 ($n-7$)	ΣC_{18}	22:6	36:2	Sterol	37:3	37:2	38:3	38:2
1.1	−18.2 (0.4)	−20.6 (0.2)	−20.3 (0.4)	−21.0	−21.2 (0.4)	−22.7 (0.3)	−22.2 (0.4)	−23.7 (0.7)	−22.6 (0.4)	−22.8 (0.6)	−22.8 (0.7)	−22.5 (0.6)
2.7	−19.4 (0.6)	−20.5 (0.2)	−20.6 (0.3)	−21.5	−21.5 (0.3)	−22.5 (0.4)	−22.0 (0.8)	−25.0 (0.1)	−23.0 (0.3)	−23.0 (0.4)	−22.8 (0.4)	−22.5 (0.2)
4.0	−20.3 (0.8)	−20.2 (0.4)	−20.9 (0.4)	−22.3	−22.0 (0.3)	−22.2 (0.1)	−22.3 (0.8)	−26.8 (0.8)	−23.3 (0.3)	−22.9 (0.6)	−23.8 (0.5)	−23.8 (0.5)
5.3	−20.2 (0.3)	−21.1 (0.1)	−21.1 (0.4)	−21.9	−22.5 (0.4)	−25.1 (0.9)	−23.7 (0.4)	−25.9 (0.15)	−23.4 (0.2)	−23.2 (0.3)	−23.3 (0.1)	−23.1 (0.3)
7.6	−21.1 (0.3)	−21.6 (0.3)	−20.5 (0.6)	−21.3	−23.1 (0.1)	−23.2 (0.1)	−23.3 (0.0)	−28.1 (0.4)	−24.5 (0.2)	−24.3 (0.5)	−24.3 (0.0)	−24.5 (0.8)
14.5	−23.1 (0.2)	−23.0 (0.1)	−22.8 (0.1)	−23.1	−25.0 (0.2)	−27.0 (0.4)	−24.6 (0.5)	−28.9 (0.6)	−25.8 (0.4)	−26.2 (0.2)	−25.7 (0.8)	−26.1 (0.8)
14.6	−22.6 (0.4)	nd	nd	nd	nd	nd	nd	−29.2 (0.1)	−25.6 (0.4)	−25.9 (0.6)	−26.0 (0.6)	−26.1 (0.5)
15.0	−23.3 (0.6)	−22.6 (0.1)	−23.1 (0.4)	−23.0	−24.8 (0.2)	−25.8 (0.3)	−25.5 (0.2)	−28.5 (0.1)	−25.1 (0.1)	−26.3 (0.9)	−24.5 (0.3)	−24.6 (0.5)
16.3	−23.7 (0.9)	−22.8 (0.2)	−23.7 (0.1)	−21.6	−24.9 (0.1)	−26.3 (0.3)	−24.0 (0.6)	−31.4 (0.2)	−27.4 (0.3)	−27.5 (0.4)	−26.3 (0.9)	−27.6 (1.2)
36.4	−25.9 (1.1)	−26.4 (0.1)	−26.3 (0.2)	−25.1	−28.4 (0.1)	−31.9 (0.5)	−28.2 (0.2)	−34.6 (1.2)	−29.7 (0.7)	−30.3 (0.1)	−28.6 (0.7)	−30.8 (0.7)
53.5	−28.9 (0.1)	−28.1 (0.1)	−30.3 (0.1)	−29.0	−30.6 (0.2)	−31.3 (0.3)	−29.4 (0.3)	−34.6 (0.1)	−30.9 (0.1)	−31.8 (0.1)	−29.8 (0.4)	−32.0 (0.6)

* Values are $x(y)$ where x = mean value and y = either standard deviation if three analyses were performed or the range if two concurrent analyses were obtained. nd = not determined. ΣC_{18} includes all polyunsaturated C_{18} fatty acids since these were not adequately separated on the capillary column used for isotope analysis. CO_{2aq} concentrations are μmol L^{-1}.

caused the somewhat lower growth rates in the second series of experiments conducted in DIC-enriched medium.

4.2. Effects of CO_2 Concentrations on Cellular Contents of Fatty Acids, Sterols, and Phytol

The lipid content and composition of microalgae can be significantly affected by changes in environmental conditions: critical factors include nutrient status, light intensity and temperature (e.g., Shifrin and Chisholm, 1981; Sukenik et al., 1989; Dunstan et al., 1993 and references therein). Much less is known about the effects of varying the CO_2 concentration. Our data suggest that in *E. huxleyi* the CO_2 concentration can have a significant effect on the abundances of some lipids such as fatty acids and alkenones and a relatively minor effect on others such as sterols and phytol (Tables 2 and 3).

Presumably this reflects the different functions of the lipids within the cell and their different modes of biosynthesis. Thus, one might not expect phytol contents to vary greatly in experiments where the light regime is kept constant since phytol is primarily associated with chlorophyll *a*. The similar amounts of sterol per cell is noteworthy and is probably a reflection of the role of sterols in membrane composition and function. Fatty acids have multiple roles within the cell and their abundance appears to be particularly sensitive to environmental conditions.

Only a few studies have specifically examined the effects of varying CO_2 concentration on microalgal lipid biochemistry. Gordillo et al. (1998a) studied the lipid class composition of the green alga *Dunaliella viridis* under four different photon flux densities and in darkness, and under two different conditions of CO_2 supply (atmospheric and 1%) with and without nitrogen depletion. Nitrogen limitation led to greater changes in lipid class composition under 1% CO_2 than under atmospheric CO_2 levels. The main reserve lipid, triacylglycerols, increasing from 1% to 22% of total lipids under 1% CO_2 during nitrogen limitation. In the cyanobacterium *Spirulina platensis*, reduced protein and pigment contents and increased carbohydrate contents occurred in culture grown under elevated CO_2 concentrations (Gordillo et al., 1998b). Summons et al. (1996) have shown that synthesis of dimethyl alkanes in a cyanobacterium is affected by changing CO_2 concentrations. Pronina et al. (1998) demonstrated in a green microalga a high correlation between the induction of a CO_2-concentrating mechanism and an acceleration of fatty-acid desaturation. Interestingly there was also some increase in polyunsaturated fatty-acid biosynthesis when CO_2 concentration was reduced to low levels, as seen in our experiments.

4.3. Effects of CO_2 Concentration on Alkenone Distributions and $U^{k'}_{37}$

Alkenones are well preserved in marine sediments and have been found in sediments from recent to mid-Cretaceous (Farrimond et al., 1986). Alkenones are now widely used to reconstruct paleo-sea surface temperatures (see review by Brassell, 1993), through calibrations of the $U^{k'}_{37}$ index. Early concern that this calibration might vary greatly because of differences between haptophyte species has now been allayed by the work of Müller et al. (1998) who showed that a single calibration can be applied to most of the world's oceans. It is still an open question whether other environmental effects might influence this ratio (e.g., Epstein et al., 1998), and whether sedimentary diagenetic processes might affect the ratio in some situations (e.g., Gong and Hollander, 1999).

In Table 3 we present data for $U^{k'}_{37}$ over the wide range of CO_2 concentrations examined in our study. It is apparent that although there are small variations in the value of $U^{k'}_{37}$ in the different experiments there is no systematic trend with $[CO_{2aq}]$ despite large changes in the content of alkenones per cell. This implies that changing CO_2 concentrations were probably not a significant influence on the changes in $U^{k'}_{37}$ values recorded in the geological record.

Popp et al. (1998a) examined the effect of growth rate on $U^{k'}_{37}$ values for a noncalcifying strain and a calcifying strain of *E. huxleyi* grown in chemostat cultures at constant temperature. The calcifying strain was the same as that used in our experiments. The change in $U^{k'}_{37}$ with growth rate was small for both strains, suggesting that nutrient-limited growth rate effects do not produce significant errors in paleotemperature determinations using either U^{k}_{37} or $U^{k'}_{37}$ (Popp et al., 1998a). Interestingly, the $U^{k'}_{37}$ values for the two strains differed significantly from each other and from values obtained in batch cultures (e.g., Prahl and Wakeham, 1987). Our values of $U^{k'}_{37}$ of 0.32 ± 0.04 for strain B92/11 are also lower than the $U^{k'}_{37}$ value of 0.57 expected for the alga grown at a temperature of 16°C. It appears that this strain synthesizes more 37:3 alkenone than most other strains at this temperature. Similarly low values of $U^{k'}_{37}$ (0.27–0.33) were obtained by Popp et al. (1998a) for a noncalcifying strain of *E. huxleyi* grown at slightly higher temperature of 18°C in a chemostat culture. However, their culture of strain B92/11 gave higher values than we found, although these are still less than would be predicted based on natural seawater calibrations (e.g., Müller et al., 1998). Conte et al. (1995) studied replicate cultures of the same strain of *E. huxleyi* and found significant variability in their biomarker profiles, including alkenone distributions, even though the culture temperature varied by only ±0.3°C. These data indicate that alkenone synthesis is not only influenced by temperature but by other environmental and/or physiological variables.

4.4. Effects of CO_2 Concentration on Overall Isotope Fractionation

Over the range of CO_2 concentrations tested (1.1 to 53.5 μmol L^{-1}), ε_{POC} changes by ca. 7‰ and is positively correlated with $[CO_{2aq}]$ (Fig. 2a). Isotope fractionation is influenced by a number of factors including growth rate, light intensity and duration, and cell size and geometry (e.g., Laws et al., 1995; 1997; Popp et al., 1998b; Burkhardt et al., 1999a; 1999b). In our experiments variations in growth rate were relatively small (for $[CO_{2aq}] > 2$ μmol L^{-1}), but carbon content per cell and hence probably also cell size varied over the CO_2 range studied (Table 1) which, in concert with differences in CO_2 concentrations (Fig. 2a), likely explains the observed changes in ε_{POC}.

Isotope fractionation by the same strain of *E. huxleyi* (PML B92/11) in relation to growth rate and $[CO_{2aq}]$ was previously investigated by Bidigare et al. (1997). Both data sets yield an inverse relationship of ε_{POC} when plotted against growth rate divided by CO_2 concentration ($\mu_i/[CO_{2aq}]$; Fig. 2b). However,

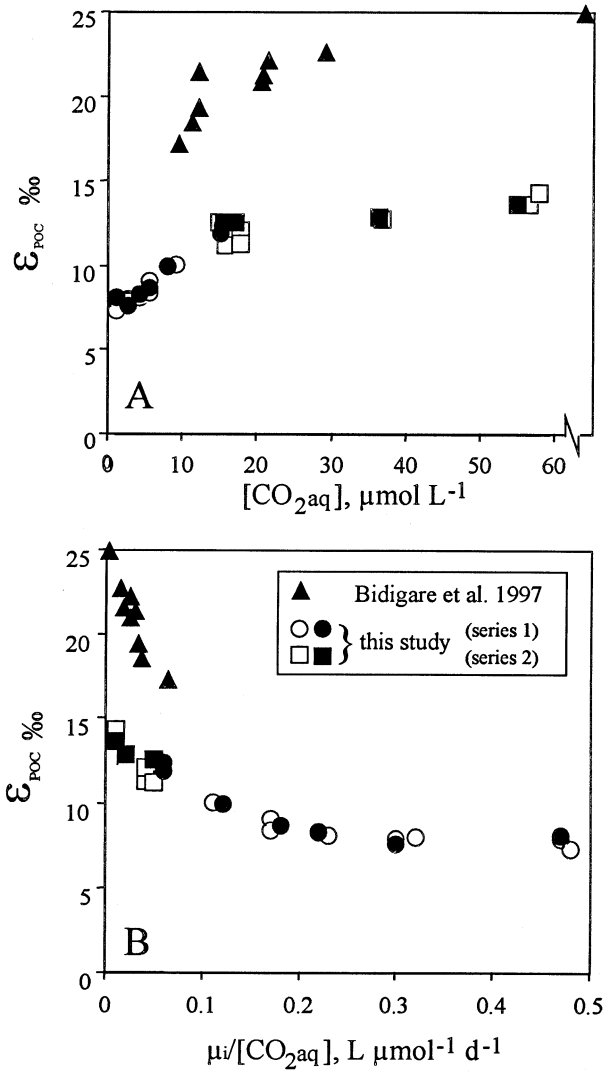

Fig. 2. Carbon isotope fractionation of (A) bulk organic matter (ε_{POC}) versus [CO_{2aq}], and (B): bulk organic matter (ε_{POC}) versus μ_i/[CO_{2aq}] in chemostat culture experiments with *E. huxleyi* (clone BT6 and B92/11) by Bidigare et al. (1997) and in batch culture incubations (clone B92/11) obtained in this study. Open symbols indicate incubations in which parallel measurements of lipid contents and isotope compositions were performed. CO_2 concentrations of Bidigare et al. (1997) were converted from μmol kg^{-1} to μmol L^{-1}.

distinct differences in this relationship exist with respect to the absolute values: (i) ε_{POC} estimates are up to 8‰ higher in the study of Bidigare et al. (1997) compared with our results for identical μ_i/[CO_{2aq}] values, and (ii) the slope of the ε_{POC} versus μ_i/[CO_{2aq}] relationship obtained by Bidigare et al. (1997) is considerably steeper than that found in our study (Fig. 2b). Accounting for the variability in cell carbon content and cell size between and within the data sets according to Burkhardt et al. (1999a) has no significant effect on the ε_{POC} relationships and the differences found between them.

These differences clearly indicate that the manner in which the alga is cultured can have a major effect on ε_{POC}, implying a substantial difference in the regulation of carbon uptake relative to carbon fixation under different growth conditions. In the experiments of Bidigare et al. (1997) the alga was grown in nitrate-limited chemostats under continuous light whereas our cultures were nutrient-replete and grown under a light:dark cycle (although both at saturating light intensities and similar temperature). An offset in ε_{POC} of 5–6‰ between cultures grown under continuous light relative to cultures maintained at a 16:8 light/dark cycle was found for various diatom species (Burkhardt et al., 1999b). This offset, if occurring also in *E. huxleyi*, may explain part of the difference in ε_{POC} between this and the study of Bidigare et al. (1997). In addition, differences in the growth-limiting resource (whether light, nutrients or some other environmental variable) may also play a role (Riebesell et al., 2000). Further side-by-side culturing experiments are required to fully elucidate the cellular mechanisms that could explain such differences.

A comparison of ε_{POC} values obtained in the field shows that these also cover a wide range (between 3 and 22‰; e.g., Bidigare et al., 1997; Dehairs et al., 1997; Pancost et al., 1997; Kukert and Riebesell, 1998). Eek et al. (1999) showed that growth conditions approaching nitrogen starvation can severely affect carbon isotope fractionation in natural population of haptophytes and speculated that cells operate in a different physiological role when nitrogen-containing nutrients are near-absent or replete. Since there are no growth rate measurements available for any of the field data sets, it is difficult to compare these data with those obtained under well-controlled laboratory conditions (e.g., see discussion in Kukert and Riebesell, 1998). Regarding the applicability of ε_{POC} responses observed in laboratory cultures to the natural environment, neither set of culturing conditions can really hope to be a good surrogate for the wide range of environmental variations found in the world's oceans, although one or the other may be more appropriate to some particular set of natural conditions. Of more importance is that a careful comparison of these and other results from cultured microalgae will ultimately provide the clues for identifying the reasons behind ε_{POC} variations in the field. Work in this direction is proceeding.

4.5. Effects of CO_2 Concentration on Isotope Composition Within and Between Lipid Classes

Our results suggest that there is a relatively small effect of [CO_{2aq}] on the isotope variations between different homologues within the lipid classes studied, at least over the [CO_{2aq}] range found in modern seawater (Table 4). Some deviation can be seen in the alkenone $\delta^{13}C$ values at very high [CO_{2aq}], but the reasons for this are unclear. Within the fatty acids there is clearly more variation in response, perhaps reflecting the fact that these lipids are synthesized at different sites and being used and recycled in a variety of cell functions.

The lipids fall into four groups with respect to their $\delta^{13}C$ offsets from POC, i.e., phytol, 14:0, 16:0, and 16:1 fatty acids in one group, ΣC_{18} PUFA and 36:2 fatty acids in another, 22:6 fatty acid and the alkenones in a third group with the sterol distinctly different from all other lipids measured. A one-way ANCOVA using the least squares difference (LSD) test (Fig. 3) confirmed the suggested groupings and demonstrated that the differences were highly significant. A *t*-test analysis (data not

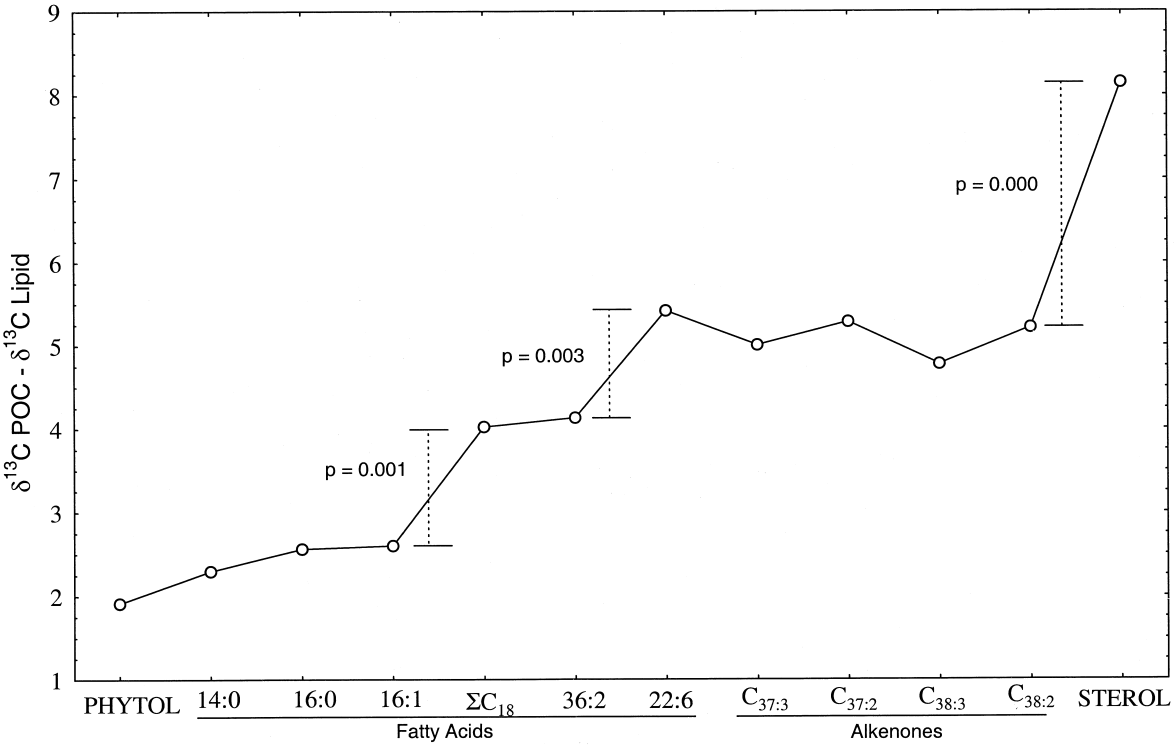

Fig. 3. One-way ANCOVA test using the least square differences (LSD) of the isotopic offsets between POC and individual lipids ($\Delta\delta^{13}C_{POC\text{-}lipid}$) at the different CO_{2aq} concentrations.

shown) gave the same groupings, but this is a less appropriate statistical test for data of this type.

Interestingly, the isotope fractionation difference between the 36:2 fatty acid (measured as the methyl ester, but isotopically corrected) and the alkenones is statistically significant. The biosynthesis of this fatty acid is thought to be closely related to that of the alkenones, so it is surprising that there should be a difference in $\delta^{13}C$ values. Either this assumption is not true or there is a significant isotope fractionation in one or more of the biosynthetic steps linking these compounds.

The 6‰ difference between phytol and the sterol is intriguing since both compounds are examples of isoprenoid biosynthesis. It seems likely that the two compounds are formed by mevalonate and nonmevalonate pathways and in different cellular compartments (Schwender et al., 1996). It is now known that in plants the biosynthesis of prenyl lipids and isoprenoids proceeds *via* two independent pathways (a) the classical acetate/mevalonate pathway for the biosynthesis of sterols, sesquiterpenes, triterpenoids in the cytosol and (b) the nonmevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway for the biosynthesis of isoprenoids such as carotenoids, phytol, plastoquinone-9, isoprene, mono-, and diterpenes in the plastid (Lichtenthaler, 1999).

Microalgae are thought to possess all of the oxygen-dependent fatty-acid desaturases found in higher plants, but too few studies have been carried out with microalgae to identify all the enzymes that are present and where in the cell they are active (Tocher et al., 1998). Differences in the fatty-acid $\delta^{13}C$ values could be explained by synthesis of some of the fatty acids (such as saturated and mono-unsaturated fatty acids) in the plastids (so-called prokaryotic synthesis) followed by their translocation, conjugated usually as glycerolipids, to the endoplasmic reticulum where they are acted upon by the eukaryotic enzymes leading to chain elongation and further desaturation (Tocher et al., 1998). In higher plants, the desaturation pathways that lead to polyunsaturated fatty acids are carried out in both microsomes and chloroplast, but there is some evidence that only a single pathway might operate in some microalgae. Evidence from radiolabelling studies has shown that the polyunsaturated fatty-acid desaturases are not present in the chloroplast of the diatom *Phaeodactylum tricornutum* (Arao et al., 1994). In the eustigmatophyte *Nannochloropsis*, $20{:}5n-3$ is only formed in an extrachloroplastidic location (Schneider et al., 1995). Stable isotope analyses combined with isolation of different polar lipids from plastid and cytosol compartments in the cell could provide a useful method for studying fatty-acid biosynthesis in microalgae such as *Emiliania*.

The isotopic offset between POC and the 37:2 methyl alkenone ($\Delta\delta^{13}C_{POC\text{-}lipid}$) of 5.4 ± 0.5‰ (Table 5) differs significantly from the value of 3.8‰ used by Jasper and Hayes (1990) and Jasper et al. (1994) in their application of alkenones as a paleobarometer and from the range of 4.0–4.3 for a noncalcifying strain and 3.9–5.4 for a calcifying strain by Popp et al. (1998a). No systematic change in the offset was observed with changing growth rates (Popp et al., 1998a) as well as over a wide range of CO_2 concentrations (this study). As suggested for the difference in ε_{POC} versus $\mu_i/[CO_{2aq}]$ responses discussed above, the observed discrepancy in the POC-alkenone isotopic offset may reflect the difference in experimental growth conditions between this and the study of Popp et al.

Table 5. Differences in $\delta^{13}C$ values between POC and individual lipids ($\Delta\delta^{13}C_{POC\text{-}lipid}$).

$[CO_{2aq}]$ (μmol L^{-1})	Phytol	Fatty acids						Sterol	Alkenones			
		14:0	16:0	16:1	ΣC_{18}*	22:6	36:2		37:3	37:2	38:3	38:2
1.1	1.3	3.7	3.4	4.1	4.3	5.8	5.3	6.8	5.7	5.9	5.9	5.6
2.7	2.1	3.2	3.3	4.2	4.2	5.2	4.7	7.7	5.7	5.7	5.5	5.2
4.0	2.6	2.5	3.2	4.6	4.3	4.5	4.6	9.1	5.6	5.2	6.1	6.1
5.3	2.5	3.4	3.4	4.2	4.8	7.4	6.0	8.2	5.7	5.5	5.6	5.4
7.6	2.0	2.5	1.4	2.2	4.0	4.1	4.2	9.0	5.4	5.2	5.2	5.4
14.5	1.9	1.8	1.6	1.9	3.8	5.8	3.4	7.7	4.6	5.0	4.5	4.9
15.0	1.9	1.2	1.7	1.6	3.4	4.4	4.1	7.1	3.7	4.9	3.1	3.2
16.3	1.7	0.8	1.7	−0.4	2.9	4.3	2.0	9.4	5.4	5.5	4.3	5.6
36.4	1.5	2.0	1.9	0.7	4.0	7.5	3.8	10.2	5.3	5.9	4.2	6.4
53.5	2.3	1.5	3.7	2.4	4.0	4.7	2.8	8.0	4.3	5.2	3.2	5.4
Mean	2.0	2.3	2.5	2.6	4.0	5.4	4.1	8.3	5.2	5.4	4.8	5.3
SD	0.3	0.8	0.9	1.4	0.4	1.0	0.9	0.9	0.6	0.3	0.9	0.5

* ΣC_{18} includes all polyunsaturated C_{18} fatty acids since these were not adequately separated on the capillary column used for isotope analysis.

(1998a). As in Bidigare et al. (1997), experiments by Popp et al. (1998a) were conducted in N-limited chemostats. In contrast, the batch cultures used in the present study were kept under nutrient-replete conditions. The availability of nitrogenous nutrients affects both the abundance and composition of microalgal lipids (Roessler, 1990). Thus, higher cellular lipid contents relative to proteins and carbohydrates are found under *N*-limitation, which could explain the lower isotopic offset between lipids and total cell carbon in *N*-limited chemostats compared to *N*-replete batch cultures. This interpretation is consistent with results of Schouten et al. (1998), which showed lipids to be more depleted in ^{13}C relative to the total cell material in batch compared with chemostat cultures.

The sometimes large and consistent differences between lipid classes must be taken into account when using isotopic compositions to assign origins to biological marker compounds in the sedimentary record. Much more study is needed of the isotopic fractionations associated with lipids in different algal classes before such data in sediments can be reliably used to infer different algal sources of organic matter.

4.6. Is it Useful to Reference $\delta^{13}C$ Values to a Common Lipid?

In view of possible effects of growth conditions on biochemical and isotopic composition of microalgae, Schouten et al. (1998) recently proposed to relate isotope fractionations of lipids to the isotope value of a particular biochemical rather than to POC. They suggested the 16:0 fatty acid as the internal reference marker because it is biosynthesized in relatively high amounts by all algae and its $\delta^{13}C$ value is easily measured since it usually does not coelute with other components on GC–MS analysis. Since growth rate remained relatively constant in our experiments (Table 1), except for the incubation at the lowest CO_2 concentration, we can directly compare the merits of relating $\delta^{13}C$ values to POC or to an internal marker such as the 16:0 fatty acid.

Calculation of the isotope fractionation between each lipid and the 16:0 fatty acid (Table 6) indicates that in general the standard deviation of the data becomes slightly larger compared to that between lipids and POC. This is consistent with the observation that the 16:0 fatty acid does not follow a similar

Table 6. Differences in the $\delta^{13}C$ values between the 16:0 fatty acid and individual lipids ($\Delta\delta^{13}C_{16:0\text{-}lipid}$).

$[CO_{2aq}]$ (μmol L^{-1})	Phytol	Fatty acids						Sterol	Alkenones		
		14:0	16:1($n-7$)	ΣC_{18}*	22:6($n-3$)	36:2 FA	37:3		37:2	38:3	38:2
1.1	−2.1	0.3	0.7	0.9	2.4	1.9	3.4	2.3	2.5	2.5	2.2
2.7	−1.2	−0.1	0.9	0.9	1.9	1.4	4.4	2.4	2.4	2.2	1.9
4.0	−0.6	−0.7	1.4	1.1	1.3	1.4	5.9	2.4	2.0	2.9	2.9
5.3	−0.9	0.0	0.8	1.4	4.0	2.6	4.8	2.3	2.1	2.2	2.0
7.6	0.6	1.1	0.8	2.6	2.7	2.8	7.6	4.0	3.8	3.8	4.0
14.5	0.3	0.2	0.3	2.2	4.2	1.8	6.1	3.0	3.4	2.9	3.3
15.0	0.2	−0.5	−0.1	1.7	2.7	2.4	5.4	2.0	3.2	1.4	1.5
16.3	0.0	−0.9	−2.1	1.2	2.6	0.3	7.7	3.7	3.8	2.6	3.9
36.4	−0.4	0.1	−1.2	2.1	5.6	1.9	8.3	3.4	4.0	2.3	4.5
53.5	−1.4	−2.2	−1.3	0.3	1.0	−0.9	4.3	0.6	1.5	−0.5	1.7
Mean	−0.6	−0.3	0.0	1.4	2.8	1.6	5.8	2.6	2.9	2.2	2.8
SD	0.7	0.6	1.0	0.8	1.1	0.8	1.3	0.7	0.8	0.7	0.9

* ΣC_{18} includes all polyunsaturated C_{18} fatty acids since these were not adequately separated on the capillary column used for isotope analysis.

curve, with respect to isotopic fractionation and CO_2 concentration, as the majority of the lipids (Fig. 1). While in principle the use of an internal marker for data normalization appears attractive, we feel that it is more profitable to consider the biochemical processes giving rise to isotopic differences and to look for regularities within data sets as a means of identifying likely biosynthetic pathways operating in the different classes of microalgae.

4.7. Can the $\delta^{13}C$ Values of Alkenones be Used as a Paleobarometer?

The results of this study demonstrate an inverse correlation of both $\delta^{13}C_{POC}$ and $\delta^{13}C_{alkenones}$ with $[CO_{2aq}]$ and a constant offset in the isotopic signal between POC and alkenones over the entire $[CO_{2aq}]$ range in this high-calcifying strain of *E. huxleyi*. This confirms the general trend obtained in a previous study using the same strain (Bidigare et al., 1997) and substantiates the concept, at least, of using alkenone isotope data for paleo-CO_2 reconstructions. When confined to the range of CO_2 concentrations typically found in ocean surface waters (ca. 8–25 μmol L^{-1}), however, CO_2-dependent differences in ε_{POC} cover a range of less than 3‰ in our data set and approximately 3–4 ‰ in the data of Bidigare et al. (1997). This suggests that the effect of $[CO_{2aq}]$ on isotope fractionation in *E. huxleyi* is small compared to potential changes of ε_{POC} due to growth rate (Bidigare et al., 1997) and factors that affect growth rate (Johnston, 1996; Thompson and Calvert, 1994). If this is representative for coccolithophorids under natural conditions, it would mean that the CO_2 sensitivity of an alkenone-based paleobarometer is low and that other environmental factors may easily mask the CO_2-related isotopic signal.

A possible complication in the use of the paleobarometer is also indicated in the observed difference in ε_{POC} versus $\mu_i/[CO_{2aq}]$ responses obtained in *N*-limited chemostats versus *N*-replete batch cultures. This can be interpreted as an additional indication that factors other than $[CO_{2aq}]$ and growth rate can be just as important in determining the isotopic signal of *E. huxleyi*. It may be too early to conclude, however, that the observed inconsistency in lab-derived ε_{POC} responses necessarily obscures the use of the paleobarometer. The bulk of coccolithophorid-produced organic matter accumulating in the sediments probably originates under conditions which are to some degree uniform with respect to ε_{POC}-relevant environmental factors. For example, half of the biomass accumulated in the upper water column during a phytoplankton bloom is produced during the last cell doubling and thus closely reflects the conditions present at the end of the bloom. In areas where bloom-derived organic matter constitutes the dominant fraction of coccolithophorid-based export production, a more or less uniform ε_{POC} vs $[CO_{2aq}]$ relationship may be applicable. Addressing these questions clearly requires detailed study both in the field and under well-constrained experimental conditions.

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