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Light-dependent carbon isotope fractionation in the coccolithophorid *Emiliania huxleyi*

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Abstract

The carbon isotopic composition of marine phytoplankton varies significantly with growth conditions. Aqueous CO_2 concentration [CO_2] and algal growth rate (μ) have been suggested to be important factors determining isotope fractionation (ε_p). Here we examine ε_p of the coccolithophorid *Emiliania huxleyi* in relation to CO_2 concentration and light conditions in dilute batch cultures. Cells were incubated at different irradiance cycles, photon flux densities (PFDs), and [CO_2]. Isotope fractionation varied between 6.7 and 12.3‰ under 16 : 8 h light : dark cycle (L : D) and between 14.7 and 17.8‰ at continuous light. ε_p was largely independent of ambient [CO_2], varying generally by less than 2‰ over a range of [CO_2] from 5 to 34 μmol L^{-1}. Instantaneous carbon-specific growth rates (μ_C) and PFDs, ranging from 15 to 150 μmol m^{-2} s^{-1}, positively correlated with ε_p. This result is inconsistent with theoretical considerations and experimental results obtained under constant light conditions, suggesting an inverse relationship between ε_p and μ. In the present study the effect of PFDs on ε_p was stronger than that of μ and thus resulted in a positive relationship between μ and ε_p. In addition, the L : D cycle of 16 : 8 h resulted in significantly lower ε_p values compared to continuous light. Since the observed offset of about 8‰ could not be related to daylength-dependent changes in μ_C, this implies a direct influence of the irradiance cycle on ε_p. These findings are best explained by invoking active carbon uptake in *E. huxleyi.* If representative for the natural environment, these results complicate the interpretation of carbon isotope data in geochemical and paleoceanographic applications.

Photosynthetic carbon fixation discriminates against the heavier $^{13}CO_2$, causing the isotopic composition of organic material to be depleted in ^{13}C compared to the inorganic carbon source. Most of this isotope fractionation occurs during enzymatic CO_2 fixation by ribulose-1,5-bisphosphate-carboxylase/oxygenase (RubisCO). Several studies demonstrated that isotope fractionation of phytoplankton varies over a wide range as a function of the environmental conditions and physiological characteristics of the algal species. Degens et al. (1968) were the first to provide experimental evidence for a positive correlation between aqueous CO_2 concentration [CO_2] and carbon isotope fractionation (ε_p) in marine phytoplankton. More recently isotope fractionation was shown to be inversely correlated with growth rate (μ) (Fry and Wainright 1991). To account for the combined effects of μ and [CO_2], various authors used the ε_p versus μ/[CO_2] relationship for interpreting isotope data. In case of entirely diffusive CO_2 uptake, an inverse linear correlation between ε_p and μ/[CO_2] would be expected (Francois et al. 1993; Laws et al. 1995; Rau et al. 1996). Any deviation from linearity strongly suggests that processes other than uncatalyzed diffusive CO_2 influx are involved in carbon acquisition (Laws et al. 1997; Burkhardt et al. 1999*a*).

The relationship between [CO_2] and isotope fractionation suggests that the isotopic composition ($\delta^{13}C_{org}$) of sedimentary organic matter may be used as a proxy for ancient [CO_2] (Jasper and Hayes 1990). The applicability of $\delta^{13}C_{org}$ for paleo-CO_2 reconstruction strongly depends on the relative impact of factors other than [CO_2] on isotope fractionation. Species-specific differences in ε_p responses are known to complicate the interpretation of isotope data in the field (e.g., Popp et al. 1998; Burkhardt et al. 1999*a*). The use of species- or group-specific marine biomarkers instead of bulk organic matter can help to circumvent these complications and to exclude the influence of terrestrial input on sedimentary $\delta^{13}C_{org}$. Alkenones, a class of long-chain unsaturated ketones, are exclusively synthesized by haptophytes and are well preserved in sediments. They have been successfully applied in reconstructing paleo–sea surface temperatures (Müller et al. 1998 and references therein) and may also serve as a potent biomarker in paleo-pCO_2 reconstruction (Jasper and Hayes 1990).

In laboratory experiments isotope fractionation of phytoplankton cultures yielded inconsistent results with respect to μ and [CO_2]. Although a linear dependence between ε_p and μ/[CO_2] was obtained in some studies (e.g., Laws et al. 1995; Bidigare et al. 1997), others indicated relationships deviating from linearity (e.g., Laws et al. 1997; Burkhardt et al. 1999*a*). Comparison of results obtained in chemostat and batch cultures reveal significant differences in absolute ε_p responses (Riebesell et al. 2000*b*). While growth in a dilute batch culture can be controlled by light intensity, cells in a chemostat grow under nutrient limitation and are commonly light saturated. Differences in light conditions were found to influence isotope fractionation of microalgae (Thompson and Calvert 1995; Leboulanger et al. 1995). Deviation from a linear relationship between ε_p and μ/[CO_2] and light dependence in ε_p indicate active regulation of carbon acquisition in phytoplankton.

Here we investigate the isotope fractionation of the alke-

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none-producing coccolithophorid *Emiliania huxleyi* in relation to CO_2 concentration and light conditions. This marine phytoplankton species has a global distribution and regularly forms extensive blooms over large areas of the ocean (Brown and Yoder 1994). In addition to its importance for the oceanic carbon cycle (Westbroek et al. 1993), *E. huxleyi* is thought to be the predominant producer of alkenones (Volkman et al. 1980). The primary aim of this study is to investigate whether the light conditions during growth, i.e., photon flux density and irradiance cycle, have a direct effect on isotope fractionation of *E. huxleyi* and how this is related to the effect of CO_2 and μ.

Material and methods

Experimental setup—The coccolith-bearing strain *E. huxleyi* (PML B92/11) was grown in dilute batch cultures under different incident photon flux densities (PFDs), irradiance cycles (continuous light and light : dark [L : D] cycle of 16 : 8 h), and [CO_2] at a constant temperature of 15°C. The growth medium consisted of 0.2-μm filtered natural seawater enriched with metals and vitamins according to f/2 medium (Guillard and Ryther 1962) and nitrate and phosphate concentrations of 100 and 6.25 μmol L^{-1}, respectively. The salinity of the seawater batches was 31.5 (at continuous light) and 29.7 (at L : D cycle 16 : 8 h).

In the experiments five different CO_2 concentrations, ranging from 5 to 34 μmol L^{-1}, were adjusted by addition of HCl or NaOH. The pH was measured potentiometrically with a pH meter (WTW-pH-3000), which was calibrated prior to each experiment. Immediately after pH adjustment, the 2.4-liter borosilicate bottles were closed with Teflon-lined screw caps to avoid further CO_2 exchange. Each treatment was incubated in triplicate. Photon flux density and irradiance cycles were controlled by a Rumed 1200 light thermostat with daylight fluorescent lamps providing a spectrum similar to that of sunlight. In the first experiment the cells were incubated in continuous light at four different PFDs (15, 30, 80, and 150 μmol photons m^{-2} s^{-1}). In the second experiment we investigated the effect of an L : D cycle (16 : 8 h) at three different PFDs (30, 80, and 150 μmol photons m^{-2} s^{-1}).

During the experiments the cells were allowed to divide 7–9 times. To avoid large changes in the carbonate system due to cellular carbon uptake, the cells were inoculated at low concentrations (<300 cells ml^{-1}) and harvested at cell concentrations of 30,000 ± 10,000 cells ml^{-1}. The drift in dissolved inorganic carbon (DIC) over the course of the experiment was generally <3%, ensuring only small changes in pH, [CO_2], nutrient concentrations, and $\delta^{13}C$ of DIC. Low cell densities also minimized changes in light intensity due to self-shading. To keep the cells in suspension, the culture bottles were gently rotated three times a day.

Sampling and analysis—The carbonate system was calculated from alkalinity, DIC, phosphate, temperature, and salinity using the dissociation constants of Goyet and Poisson (1989). Alkalinity samples were taken from the filtrate (0.6 μm; QMA filter) and subsequently fixed with $HgCl_2$ solution (140 mg L^{-1} final concentration), stored at 4°C, and measured within 4 weeks. Alkalinity was determined by potentiometric titration in duplicate with 0.05 N HCl solution (Brewer et al. 1986) and subsequent calculation from linear Gran plots (Gran 1952). The precision of the measurement was ±3 μmol L^{-1}. DIC samples were fixed with $HgCl_2$ solution (140 mg L^{-1} final concentration), tightly closed without headspace, and stored at 4°C. Shortly before extraction and measurements, the samples were gently filtered (0.45-μm; cellulose acetate filter). Subsequently, all forms of DIC were converted to CO_2 by acidification with H_3PO_4 and purged with N_2 carrier gas. CO_2 was detected coulometrically and titrated potentiometrically (UIC CM 5012). DIC was measured in duplicate at a precision of ±3 μmol L^{-1}. To determine isotopic composition of DIC ($\delta^{13}C_{DIC}$), 100 ml were fixed, stored, and filtered like the DIC samples and were subsequently extracted in a vacuum line, as described by Mackensen et al. (1996). Measurements of $\delta^{13}C_{DIC}$ were performed with a mass spectrometer (Finnigan MAT 252) at a precision of ±0.03‰. The isotopic composition of CO_2 ($\delta^{13}C_{CO_2}$) was calculated from $\delta^{13}C_{DIC}$ using the equation by Rau et al. (1996) based on Mook et al. (1974):

$$\delta^{13}C_{CO_2} = \delta^{13}C_{DIC} + 23.644 - (9701.5/T_K) \quad (1)$$

Samples for particulate organic carbon (POC) and total particulate carbon (TPC) were filtered onto precombusted QMA filters (500°C; 12 h) and stored at −25°C in precombusted Petri dishes (500°C; 12 h). Prior to the measurement, POC filters were fumed with HCl for 2 h to remove all inorganic carbon. TPC, POC, and relating $\delta^{13}C$ values were subsequently measured in duplicate on a mass spectrometer (ANCA-SL 20-20), with a precision of ±0.5 μg C and ±0.5‰, respectively. The isotopic composition is reported relative to the PeeDee belemnite standard (PDB):

$$\delta^{13}C_{Sample} = \left[\frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] \times 1000 \quad (2)$$

Isotope fractionation during POC formation (ε_p) was calculated relative to the isotopic composition of CO_2 in the medium (Freeman and Hayes 1992):

$$\varepsilon_p = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{POC}}{1 + \frac{\delta^{13}C_{POC}}{1000}} \quad (3)$$

Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. $\delta^{13}C_{PIC}$ was determined according to

$$\delta^{13}C_{PIC} = \frac{\delta^{13}C_{TPC} \times TPC - \delta^{13}C_{POC} \times POC}{PIC} \quad (4)$$

Isotope fractionation during calcite precipitation ($\varepsilon_{calcite}$) is reported relative to the isotopic composition of DIC in the medium

$$\varepsilon_{calcite} = \frac{\delta^{13}C_{DIC} - \delta^{13}C_{PIC}}{1 + \frac{\delta^{13}C_{PIC}}{1000}} \quad (5)$$

Samples for cell counts were fixed with formalin (0.4% final concentration, buffered with hexamethylenetetramine). At

low cell densities (10^1–10^3 cells ml^{-3}), cell concentrations were determined with an inverted microscope. At higher cell densities at the end of the experiment (10^3 cells ml^{-1}) a Coulter Multiziser was used for cell enumerations. The 24-h specific growth rate (μ) was calculated according to Eq. 6, where N_0 and N_{fin} represent the cell concentrations at the beginning and the end of the experiments, respectively, and Δt is the corresponding duration of incubation in days:

$$\mu = \frac{\ln N_{fin} - \ln N_0}{\Delta t} \quad (6)$$

The preadaptation of the cells to the experimental conditions for at least seven divisions ensured logarithmic growth after inoculation, which was confirmed by daily cell counts in control bottles. The sampling during the L : D cycle was conducted at the onset of the light period. Since carbon fixation only takes place during light, μ was corrected for the duration of the photoperiod. This instantaneous growth rate (μ_i) represents growth during the photoperiod, enabling us to compare growth rates at different L : D cycles.

$$\mu_i = \frac{\mu \times (L + D)}{L - D \times r} \quad (7)$$

L and D represent the length of the light and dark period, respectively, and r accounts for the respiratory carbon loss during the dark period, assuming r equals 0.15 (Laws and Bannister 1980). As long as the cellular organic carbon content (POC $cell^{-1}$) remains constant, μ_i can be directly compared between different treatments. In the case of varying carbon cell quota, μ_i should be expressed as carbon-specific growth rate (μ_C) representing carbon fixation during the photoperiod.

$$\mu_C = (\text{POC cell}^{-1}) \times \mu_i \quad (8)$$

Results

Growth rates—Growth rates (μ) of *E. huxleyi* ranged from 0.5 to 1.2 d^{-1} (Table 1). Over the investigated range of $[CO_2]$, μ was largely independent of $[CO_2]$ within PFD treatments. Light intensity had a strong effect on growth rate. At both irradiance cycles μ increased with increasing PFDs and appeared to level off above PFD 80 μmol m^{-2} s^{-1} at continuous light. Growth rates obtained under the L : D cycle were only slightly below those at continuous light.

Carbon-specific growth rates (μ_C) varied between 3.5 and 22.8 pg C $cell^{-1}$ d^{-1} (Fig. 1). Since cellular organic carbon content (POC $cell^{-1}$) generally increased with increasing $[CO_2]$ (Table 1), the same trend is observed between μ_C and $[CO_2]$. PFD-dependent variation in POC $cell^{-1}$ was more than twofold and caused the largest variation in μ_C. Since 24-h growth rates (μ) differed only slightly between irradiance cycles, carbon-specific growth rates during the photoperiod at the L : D cycle generally exceeded those under continuous light at comparable $[CO_2]$.

The inorganic carbon (calcite) content per cell (PIC $cell^{-1}$) increased with PFDs and decreased with increasing $[CO_2]$ in all treatments but at PFD 15 μmol m^{-2} s^{-1} (Table 1). Owing to the concomitant increase in POC $cell^{-1}$, the PIC/POC ratio significantly decreased with $[CO_2]$ in all treatments ($P < 0.001$ ANOVA, F-test).

Carbon isotope fractionation—Values of ε_p varied between 14.7 and 17.8‰ under continuous light and between 6.7 and 12.3‰ at the L : D cycle (Fig. 2). Isotope fractionation was largely independent of the ambient CO_2 concentration, i.e., variations in ε_p were generally less than 2‰ and showed no clear trend over the investigated $[CO_2]$ range. Only at PFD 150 μmol m^{-2} s^{-1} under the L : D cycle was a positive trend of ε_p with $[CO_2]$ obtained at concentrations <15 μmol CO_2 L^{-1}.

Considering each PFD treatment separately yields no consistent relationship between ε_p and μ_C (Fig. 3). Combining all PFD treatments of the same irradiance cycle, a positive correlation between μ_C and ε_p is observed ($P < 0.001$). In other words, *E. huxleyi* discriminates against ^{13}C more strongly at high compared to low light intensities, despite its higher rate of carbon fixation under high PFDs. Compared to continuous light, the L : D cycle of 16 : 8 h caused substantially lower ε_p values of about 8‰. This offset in ε_p between irradiance cycles cannot be explained by the day-length-dependent differences in μ_C, since for the same PFD treatment μ_C differences are comparatively small and their ranges partly overlap.

To examine the combined effects of $[CO_2]$ and μ_C we plotted ε_p versus $\mu_C/[CO_2]$ (Fig. 4). Although the ε_p versus $\mu_C/[CO_2]$ relationships of individual PFD treatments show no consistent trend, the slopes seem to change with light intensity. At high PFDs, the relationship was inversely correlated, whereas at low PFDs no clear dependence between ε_p and $\mu_C/[CO_2]$ existed. Moreover, the data of the different irradiance cycles still show a significant offset in ε_p and thus cannot be described by the same regression. Consequently, most of the variance in the data cannot be explained by changes in $[CO_2]$ and μ_C.

In addition to the effect of the irradiance cycle on isotope fractionation, a PFD effect is indicated, i.e., at high PFDs the isotope fractionation is higher than at low PFDs. In view of the small variability of ε_p within each PFD treatment, we pooled the data and plotted ε_p versus PFD (Fig. 5). In both irradiance cycles ε_p and PFDs were positively correlated ($P < 0.001$). At continuous light, the increase in ε_p with increasing light intensity leveled off above PFD 80 μmol m^{-2} s^{-1}.

The carbon isotope composition of the coccolith calcite ($\delta^{13}C$-PIC) increased by 3 to 4‰ with increasing PFDs (Table 1). Owing to the large variability in the data, however, this trend is statistically not significant ($P > 0.05$). $\varepsilon_{calcite}$ values (expressed relative to $\delta^{13}C$-DIC) varied between -1.5 and 4.2‰ and showed no trend with $[CO_2]$, ε_p, and PIC/POC.

Discussion

CO_2 concentration and growth rates—Phytoplankton carbon isotope fractionation (ε_p) has been a subject of considerable research over the last decade. Although a number of factors could be identified to influence ε_p, their relative importance to the overall isotopic signal is still uncertain.

Table 1. Experimental conditions, carbon quota, growth rates, and isotope measurements in dilute batch culture incubation: irradiance cycle (*L*:*D*; in h:h), PFD (μmol m^{-2} s^{-1}), [CO_2] (μmol L^{-1}), pH, POC/cell (pg C cell^{-1}), PIC/cell (pg C cell^{-1}), PIC/POC, μ (d^{-1}), μ_i (d^{-1}), μ_c (pg C cell^{-1} d^{-1}), $\delta^{13}C_{DIC}$ (‰), $\delta^{13}C_{CO_2}$ (‰), $\delta^{13}C_{POC}$ (‰), $\delta^{13}C_{PIC}$ (‰), ε_p (‰), and $\varepsilon_{calcite}$ (‰) (*see text for definition of variables*). Values represent the mean of triplicate incubations (n = 3).

L:*D*	PFD	[CO_2]	pH	POC/ cell	PIC/ cell	PIC/ POC	μ	μ_i	μ_c	$\delta^{13}C_{DIC}$	$\delta^{13}C_{CO_2}$	$\delta^{13}C_{POC}$	$\delta^{13}C_{PIC}$	ε_p	$\varepsilon_{calcite}$
24:0	15	33.8	7.84	7.23	2.46	0.34	0.54	0.54	3.89	0.17	−9.85	−24.66	−0.95	15.18	1.13
	15	30.4	7.89	6.60	2.57	0.39	0.54	0.54	3.58	−0.45	−10.47	−24.97	−2.14	14.87	1.71
	15	19.2	8.07	6.73	2.95	0.44	0.53	0.53	3.54	−0.42	−10.44	−24.76	−4.24	14.68	3.84
	15	19.9	8.06	6.74	2.73	0.41	0.54	0.54	3.63	−0.32	−10.35	−24.57	−2.97	14.58	2.65
	15	13.2	8.23	6.63	2.79	0.42	0.53	0.53	3.51	−0.18	−10.21	−25.69	−4.40	15.89	4.24
	30	27.3	7.92	9.91	5.02	0.51	0.81	0.81	8.02	0.01	−10.01	−24.95	0.10	15.32	−0.08
	30	23.2	7.99	9.18	5.43	0.59	0.76	0.76	7.02	0.05	−9.98	−25.54	−0.48	15.97	0.53
	30	18.6	8.08	7.79	5.25	0.67	0.81	0.81	6.28	−0.34	−10.36	−25.51	−0.85	15.55	0.51
	30	16.9	8.12	8.16	5.62	0.69	0.83	0.83	6.80	−0.15	−10.17	−25.60	−2.67	15.84	2.53
	30	11.9	8.27	7.21	5.65	0.78	0.77	0.77	5.52	−0.44	−10.46	−26.30	−3.13	16.27	2.70
	80	29.2	7.90	14.55	7.62	0.52	1.16	1.16	16.92	−0.16	−10.18	−26.60	0.18	16.87	−0.34
	80	27.3	7.93	12.27	8.03	0.65	1.12	1.12	13.72	0.05	−9.98	−26.61	0.00	17.09	0.05
	80	19.5	8.07	12.65	8.85	0.70	1.03	1.03	12.98	0.55	−9.47	−26.78	−0.34	17.78	0.90
	80	19.3	8.07	10.63	8.42	0.79	1.08	1.08	11.50	−0.42	−10.45	−26.80	0.04	16.81	−0.46
	80	11.7	8.27	10.30	8.55	0.83	1.02	1.02	10.53	−0.70	−10.73	−26.56	0.04	16.27	−0.75
	150	30.0	7.88	14.30	7.96	0.56	0.93	0.93	13.26	0.02	−10.01	−27.20	0.58	17.67	−0.56
	150	22.6	8.00	12.04	8.02	0.67	0.98	0.98	11.76	0.37	−9.65	−26.31	1.70	17.11	−1.32
	150	17.9	8.09	12.81	8.61	0.67	1.10	1.10	14.03	−0.36	−10.38	−27.03	1.76	17.11	−2.11
	150	16.8	8.12	10.99	8.00	0.73	1.15	1.15	12.59	−0.24	−10.26	−27.30	0.92	17.51	−1.15
	150	11.7	8.27	10.20	8.98	0.88	1.09	1.09	11.08	−0.11	−10.14	−26.58	0.48	16.89	−0.60
16:8	30	32.1	7.84	5.90	3.70	0.63	0.66	1.07	6.34	0.45	−9.58	−17.19	−1.39	7.74	1.84
	30	23.8	7.97	5.66	3.71	0.66	0.66	1.08	6.10	0.31	−9.71	−16.29	−0.15	6.68	0.46
	30	16.2	8.13	9.15	5.73	0.63	0.74	1.20	10.99	0.18	−9.85	−17.28	−1.73	7.56	1.91
	30	10.9	8.29	7.48	5.07	0.68	0.68	1.10	8.23	0.14	−9.88	−16.43	−1.14	6.66	1.28
	30	5.3	8.57	6.29	5.14	0.82	0.65	1.06	6.67	0.10	−9.92	−17.45	−1.74	7.66	1.84
	80	32.2	7.84	13.42	8.51	0.64	0.94	1.52	20.41	0.43	−9.60	−19.15	−0.34	9.74	0.77
	80	24.2	7.96	14.67	8.75	0.60	0.96	1.55	22.75	0.16	−9.86	−19.93	−3.36	10.27	3.54
	80	16.3	8.12	10.74	8.39	0.78	1.06	1.71	18.39	−0.22	−10.24	−18.01	0.09	7.91	−0.31
	80	12.5	8.23	11.03	8.18	0.74	0.99	1.61	17.72	0.14	−9.89	−19.43	−1.15	9.73	1.29
	80	5.5	8.55	8.29	8.50	1.03	0.98	1.59	13.19	0.82	−9.21	−17.74	−0.23	8.69	1.05
	150	27.2	7.93	9.07	7.01	0.77	1.08	1.75	15.86	−0.30	−10.32	−21.99	−2.17	11.93	1.88
	150	21.3	8.03	9.69	7.76	0.80	1.10	1.78	17.29	−0.32	−10.35	−22.05	−1.53	11.97	1.21
	150	18.4	8.09	8.59	7.28	0.85	1.10	1.78	15.32	−0.20	−10.22	−21.86	−0.78	11.90	0.58
	150	12.4	8.25	8.48	8.08	0.95	1.15	1.86	15.82	−0.24	−10.27	−20.85	−1.15	10.81	0.91
	150	5.6	8.56	8.28	8.49	1.02	1.09	1.77	14.63	−0.70	−10.72	−19.73	−0.92	9.19	0.22

While some authors attributed much of the variation in carbon isotopic composition of phytoplankton to ambient [CO_2] (e.g., Deuser et al. 1968; Popp et al. 1998), more recent investigations indicate only a weak dependence of ε_p on [CO_2]. Burkhardt et al. (1999*a*) investigated isotope fractionation of various microalgae under high PFDs and nutrient replete conditions and found the CO_2-dependent responses in ε_p to be smaller than 3‰ over a range of 3 to 25 μmol CO_2 L^{-1}. In the present study, variations in ε_p were generally smaller than 2‰ and showed no systematic trend over the investigated range of 5 to 35 μmol CO_2 L^{-1} (Fig. 2).

Several authors have emphasized an effect of growth rate on isotope fractionation (e.g., Fry and Wainright 1991; Laws et al. 1995, 1997; Rau et al. 1996). In this study, ε_p was positively correlated with carbon-specific growth rates (μ_C) in both irradiance cycles (Fig. 3). This result contradicts the inverse correlation between ε_p and growth rate as proposed in various models of isotope fractionation (e.g., Francois et al. 1993; Laws et al. 1995; Rau et al. 1996). Since both μ_C and [CO_2] varied in our experiments, their combined effects may be responsible for some of the variations in ε_p. If these two parameters are the dominant factors determining isotope fractionation, the different treatments should follow more or less the same ε_p versus μ_C/[CO_2] relationship. This, however, is not indicated by the data of our study, which yield different trends between PFD treatments and a significant offset between irradiance cycles (Fig. 4). These results demonstrate that in addition to [CO_2] and μ_C, other factors must be responsible for much of the variation in ε_p.

Light intensity and irradiance cycles—Isotope fractionation positively correlated with photon flux density (Fig. 5). Since growth rates increased with PFDs, this finding is inconsistent with theoretical considerations and experimental

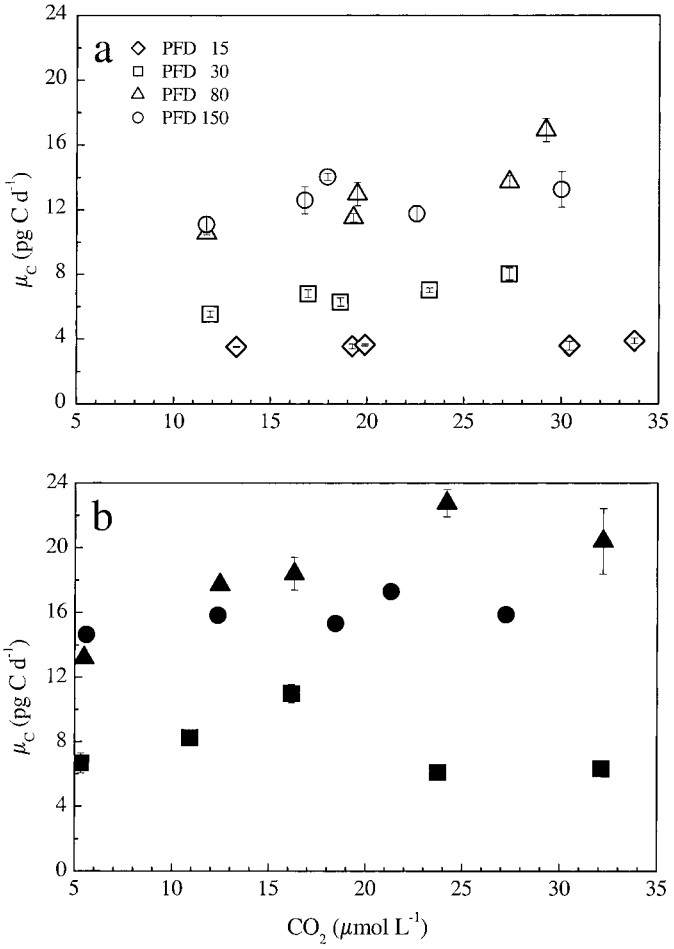

Fig. 1. Carbon-specific growth rates (μ_C) as a function of [CO_2] at different light conditions. Open symbols and closed symbols represent the incubation at (a) continuous light and (b) the 16:8 h light:dark cycle. Symbols denote for light intensities PFD 15, PFD 30, PFD 80, and PFD 150. Error bars represent ±1 standard deviation of triplicate incubations (n = 3).

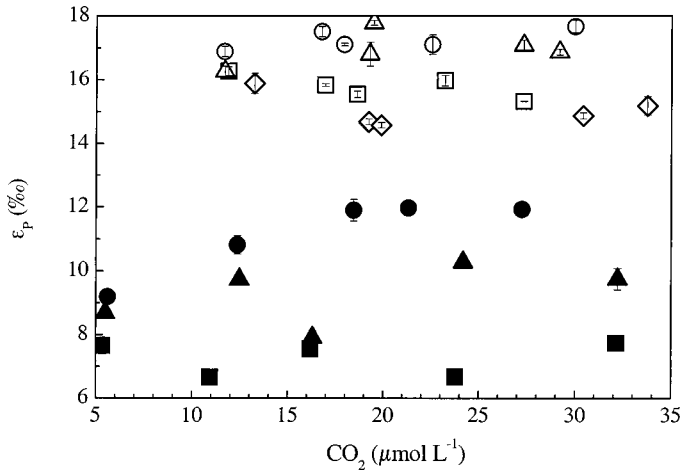

Fig. 2. Isotope fractionation (ε_p) as a function of [CO_2]. Symbols and error bars as in Fig. 1.

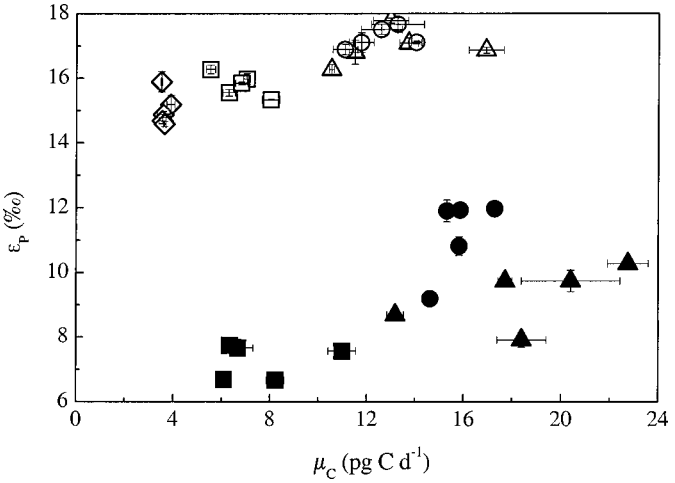

Fig. 3. Isotope fractionation (ε_p) as a function of carbon-specific growth rate (μ_C). Symbols and error bars as in Fig. 1.

results obtained under constant light conditions, suggesting an inverse relationship between ε_p and μ (e.g., Laws et al. 1995, 1997; Bidigare et al. 1997). A direct effect of PFDs on ε_p is also indicated in Fig. 3, showing ε_p values increasing with PFDs, despite a concomitant increase in growth rate. Thus, light intensity has a stronger effect on ε_p than growth rate under these experimental conditions. Thompson and Calvert (1995) investigated the effect of PFDs and daylength on the isotope fractionation of *E. huxleyi*. In accordance with their data we found that variations in ε_p were more closely related to irradiance than to growth rate.

Burkhardt et al. (1999*b*) investigated the effect of different irradiance cycles on instantaneous growth rates (μ_i) and ε_p. One group of diatoms showed up to 6‰ lower ε_p values in an L:D cycle of 16:8 h compared to algae growing under continuous light. In our investigation the effect of the irradiance cycle was even stronger, yielding approximately 8‰ lower ε_p values for the L:D cycle compared to continuous light. Burkhardt et al. (1999*b*) attributed this response in ε_p

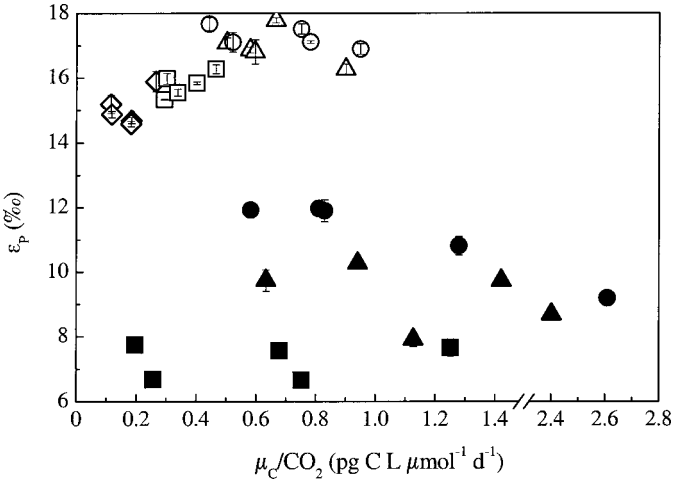

Fig. 4. Relationship between μ_C/[CO_2] and isotope fractionation (ε_p). Symbols and error bars as in Fig. 1.

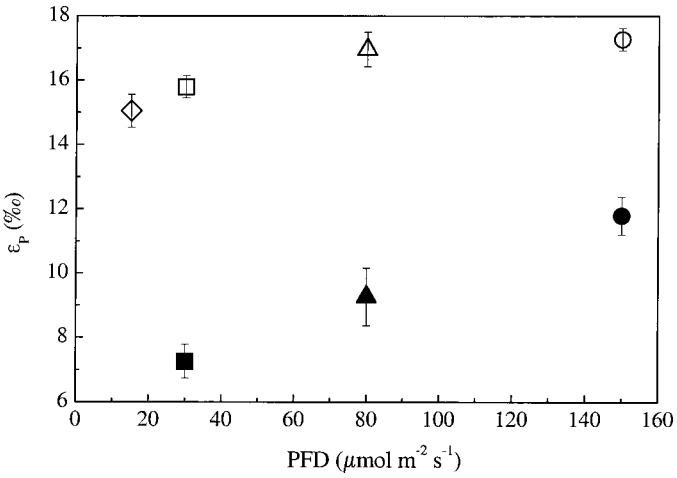

Fig. 5. Isotope fractionation (ε_p) as a function of photon flux density (PFD). Symbols as in Fig. 1, error bars represent ±1 standard deviation of the pooled data (n = 15).

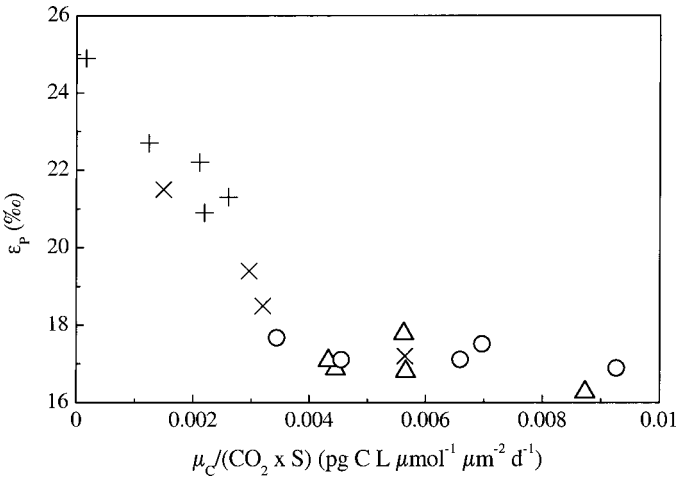

Fig. 6. Comparison of the relationship between $\mu_C/(CO_2 \times S)$ and isotope fractionation (ε_p) obtained by Bidigare et al. (1997) and in the present study. Chemostat data of the calcifying strain B92/11 (×) and noncalcifying strain BT6 (+) incubated under continuous light at ca. 250 μmol photons m^{-2} s^{-1}. Symbols of the dilute batch culture as in Fig. 1.

to an increase in instantaneous growth rates with decreasing daylength. In our study, however, daylength-dependent changes in μ_C cannot explain the magnitude of this difference in ε_p because carbon-specific growth rates largely overlap between irradiance cycles (Fig. 3). This is partly due to the lower cellular carbon quota at the L : D cycle (Table 1). The offset in ε_p between continuous light and the L : D cycle still persists in a ε_p versus $\mu_C/[CO_2]$ plot (Fig. 4). Consequently, our results suggest an effect of irradiance cycle on isotope fractionation, which is independent of its effect on growth rate.

If this effect is representative for the natural environment, it may explain some of the variability in $\delta^{13}C$ of suspended organic matter in the ocean. For instance, distinct latitudinal differences in $\delta^{13}C_{org}$ were previously attributed to the temperature-dependent increase in CO_2 concentration (Rau et al. 1989). Based on the results of this study it may also be related to the dependence of ε_p on the duration of the photoperiod, which increases with latitude during the phytoplankton growth season. This could be due to the effect of both daylength-dependent differences in instantaneous growth rate (Burkhardt et al. 1999*b*) and the direct effect of the irradiance cycle on ε_p as found in this study.

Nitrate- versus light-controlled growth—Various studies using different experimental approaches yielded inconsistent ε_p responses in relation to $[CO_2]$ and/or growth rate. Isotope fractionation by the calcifying strain (B92/11) and a naked strain (BT 6) of *E. huxleyi* was previously investigated by Bidigare et al. (1997). In their experiments cells were incubated in nitrate-limited chemostats under continuous light with PFDs of ca. 250 μmol m^{-2} s^{-1} and a constant temperature of 18°C. According to our results isotope fractionation of *E. huxleyi* strongly depends on the light conditions during growth. A comparison of our results with those of Bidigare et al. (1997), therefore, should encompass only data obtained at high PFDs and continuous light. Direct comparison of these two data sets indicates higher ε_p values and a considerably steeper slope in ε_p versus $\mu_C/[CO_2]$ in the study of Bidigare et al. (1997) (not shown). Significant differences in ε_p responses of various algal species were related to differences in the ratio of cellular organic carbon content to surface area (Rau et al. 1996; Popp et al. 1998). Although the same algal species was used, cellular carbon content varied nearly threefold in our experiments and was reported to remain constant in the study of Bidigare et al. (1997). We therefore calculated the surface area (S) of *E. huxleyi* by using the relationship between carbon cell quota and cell volume (Montagnes et al. 1994) and compared the two data sets in an ε_p versus $\mu_C/(CO_2 \times S)$ plot (Fig. 6). Accounting for cell size, however, did not significantly reduce the differences in ε_p responses between Bidigare et al. (1997) and our study.

If the factors $[CO_2]$, μ_C, irradiance, and cell size do not explain the apparent differences in ε_p responses between nitrate-limited chemostat and light-controlled batch cultures, other factors associated to the culture conditions may be responsible. In a chemostat, algal cells usually grow under light saturation and continuous light. Growth rates in chemostat incubations of Bidigare et al. (1997) were controlled by nutrient supply, i.e., by the dilution rate, and ranged from 0.2 to 0.6 d^{-1}. Higher growth rates could not be attained in their experiments as higher dilution rates apparently resulted in wash out of the cells. In our light-controlled batch cultures nutrients were replete and high PFDs consequently led to high growth rates. Owing to these differences in growth rates, the $\mu_C/(CO_2 \times S)$ values of the chemostat incubations were generally smaller than in the comparable PFD treatment of our batch cultures (Fig. 6). It cannot be ruled out that the trends converge and thus may be described by the same function. In that case, the resulting nonlinear relationship between ε_p and $\mu_C/(CO_2 \times S)$ would indicate a change in inorganic carbon acquisition of *E. huxleyi* over the range of $\mu_C/(CO_2 \times S)$ values covered by these two studies.

Mechanisms of isotope fractionation—Isotope fractionation models based on diffusive CO_2 supply predict a negative linear correlation between ε_p and $\mu_C/[CO_2]$ with a *y*-intercept close to the isotope fractionation of RubisCO (Francois et al. 1993; Laws et al. 1995; Rau et al. 1996). Since our data show significant deviations from these predictions, an exclusive CO_2 uptake by diffusion is not indicated for *E. huxleyi*. In fact, increasing isotope fractionation with increasing growth rates requires active carbon uptake, suggesting the operation of a carbon concentrating mechanism (CCM) in *E. huxleyi* (Nimer and Merret 1996; Laws et al. 1998). Light has been suggested to influence the CCM of microalgae (e.g., Badger and Price 1992; Sültemeyer et al. 1993). The mechanism underlying the observed light effect on ε_p is therefore most likely related to the CCM of *E. huxleyi*. According to theoretical considerations, active carbon uptake can affect isotope fractionation in different ways, as will be discussed below.

Sharkey and Berry (1985) developed a model in which isotope fractionation is ultimately determined by the isotopic composition of the inorganic carbon source and the leakage, defined as the ratio of carbon efflux (F_{out}) to carbon influx (F_{in}):

$$\varepsilon_p = a\varepsilon_S + \varepsilon_f \frac{F_{out}}{F_{in}} \quad (9)$$

where ε_S represents the equilibrium discrimination between the carbon sources CO_2 and HCO_3^- (ca. −10‰; Mook et al. 1974) and ε_f is the kinetic fractionation by RubisCO (ca. 28‰; *see* review by Raven and Johnston 1991). Since the original model allowed for only one inorganic carbon source, Burkhardt et al. (1999*b*) extended the model by including a factor *a*, which is the fractional contribution of HCO_3^- to total carbon uptake. Since HCO_3^- is enriched in ^{13}C relative to CO_2, an increasing proportion of HCO_3^- uptake decreases the apparent isotope fractionation ε_p, which is defined relative to CO_2 as the carbon source. Assuming no change in the inorganic carbon source, ε_p increases with increasing leakage. In case of CO_2 as the only carbon source and leakage being high (F_{out}/F_{in} approaching 1) ε_p approaches ε_f. At low leakage most of the CO_2 entering the cell is fixed into organic compounds, and ε_p approaches the isotopic composition of the inorganic carbon source.

If inorganic carbon is taken up in an energy-dependent process, the observed effect of PFDs and irradiance cycle on ε_p of *E. huxleyi* reflects changes in leakage and/or in the inorganic carbon source. The PFD dependence on ε_p could be the result of F_{in} increasing with PFDs at a rate higher than carbon fixation. Alternatively, it could indicate a change from CO_2 to HCO_3^- uptake at low PFDs. Based on results of Thompson and Calvert (1995), Laws et al. (1998) concluded that *E. huxleyi* uses HCO_3^- as the primary carbon source at low growth rates (i.e., low PFDs) and changes to active CO_2 uptake at high growth rates (i.e., high PFDs). However, an increasing proportion of HCO_3^- uptake at low growth rate seems unlikely since the relative contribution of diffusive CO_2 uptake to overall carbon acquisition should be highest under conditions of reduced ''carbon demand.'' Moreover, energy limitation at low PFDs may restrain energy-dependent uptake of HCO_3^-.

Owing to its high apparent half-saturation ($K_{1/2}$) values for photosynthetic CO_2 fixation, *E. huxleyi* is thought to rely on diffusive CO_2 uptake (Raven and Johnston 1991). In this regard it is noteworthy that CO_2 had little effect on growth rates within each PFD treatment, i.e., cell division was not stimulated by increasing $[CO_2]$ (Table 1). Carbon-specific growth rates, however, showed a CO_2-dependent increase under high PFDs (Fig. 1). It seems that although *E. huxleyi* is able to control its carbon acquisition to some extent, this mechanism is not efficient enough to reach carbon saturation under high PFDs. These results are consistent with Nimer and Merret (1993), who showed that at PFDs of 50 μmol m^{-2} s^{-1} photosynthetic $^{14}CO_2$ fixation of *E. huxleyi* was carbon saturated at 1 mM DIC, but at PFDs of 300 μmol m^{-2} s^{-1} it was not saturated at DIC concentrations of 2 mM (*see also* Paasche 1964).

The dependence of ε_p on the irradiance cycle could be caused by a higher proportion of HCO_3^- uptake at the L : D cycle compared to continuous light and/or lower leakage due to daylength-dependent increase in μ_C. The latter process can only account for part of the observed offset due to the small difference in μ_C between irradiance cycles (*see above*). However, this finding supports the alternative explanation that *E. huxleyi* increasingly uses HCO_3^- with decreasing daylength. HCO_3^- can either be used by direct uptake or by extracellular conversion by carbonic anhydrase (CA) followed by diffusion or active uptake of CO_2. In the latter case the isotope fractionation associated with the conversion by CA would eliminate the isotopic difference between HCO_3^- and CO_2 and make the two carbon sources isotopically indistinguishable (Riebesell and Wolf-Gladrow 1995). Hence the lower ε_p values in our study are therefore consistent with a direct rather than a CA-mediated uptake of HCO_3^-. This interpretation is in accordance with the results of previous studies, finding little or no external CA activity for *E. huxleyi* (Sikes and Wheeler 1982; Nimer et al. 1994).

The apparent difference in HCO_3^- use of *E. huxleyi* as a function of daylength bears an interesting resemblance to macroalgae growing in different areas of the tidal zone. Owing to the tidal cycle, intertidal species are restricted in the length of time they can photosynthesize. To overcome this shortcoming they seem to have developed a very efficient form of photosynthesis based on HCO_3^- use with high affinities for CO_2 and HCO_3^- and low ε_p values (Johnston et al. 1992). In contrast, subtidal species tend to rely on CO_2 and consequently have higher ε_p values.

It has been proposed that *E. huxleyi* is able to use HCO_3^- through the process of calcification, suggesting a coupling between calcification and photosynthesis (Sikes et al. 1980; Nimer and Merret 1993). In that case, the ratio of inorganic to organic carbon production (PIC/POC) should have an effect on ε_p. HCO_3^- was shown to be the main carbon source for calcification (Sikes et al. 1980). This is also indicated by $\delta^{13}C$-PIC values in our study (Table 1), which are close to the $\delta^{13}C$ of DIC (and hence HCO_3^-). At increasing PIC/POC ratios the impact of calcification-mediated HCO_3^- use on photosynthetic CO_2 fixation should increase and therefore cause a decrease in ε_p. In our experiments, however, ε_p values rather increased with PIC/POC (Table 1).

Based on the present data, a close coupling between calcification and photosynthetic CO_2 fixation cannot be verified.

Implications for paleoreconstructions—The use of the carbon isotope signal in bulk and compound-specific marine organic matter for geochemical and paleoceanographic applications strongly relies on the premise that isotope fractionation in phytoplankton is largely determined by a small number of environmental and cellular parameters. If representative for the natural environment, the results of this study severely compromise this premise. They suggest that the effect of light intensity and the irradiance cycle on carbon isotope fractionation in *Emiliania huxleyi* is equally strong or stronger than that of CO_2 concentration and algal growth rate. This finding further complicates the use of alkenone isotope data for reconstructing paleo-CO_2 (Rau et al. 1989; Jasper and Hayes 1990) and/or paleogrowth rates (Laws et al. 1995). The interpretation of marine carbon isotope data thus requires knowledge of the light conditions prevailing during organic matter production.

Light levels in the ocean's upper mixed layer differ strongly on both temporal and spatial scales due to seasonal as well as latitudinal/regional differences in vertical mixing, incident light intensities, and duration of the photoperiod. This precludes the use of a single ε_p versus $\mu/[CO_2]$ relationship for the entire ocean but necessitates an account of regional differences in light conditions for ε_p calibrations. A further complication may arise from ε_p dependence on the growth-limiting resource, i.e., the factor ultimately controlling phytoplankton growth rate. Such an effect has been suggested by Riebesell et al. (2000*a*) for the marine diatom *Phaeodactylum tricornutum* and may also be present in *E. huxleyi* as indicated by a comparison of ε_p responses obtained in nutrient-limited chemostat and light-controlled batch cultures (Fig. 6). Algal growth conditions may also influence the isotopic offset between total cell material and individual cellular compounds such as alkenones (Riebesell et al. 2000*b*). If applicable to the natural environment, these findings imply that explaining the observed variability in organic matter $\delta^{13}C$ in the modern ocean as well as in marine sediments requires extensive knowledge of the environmental conditions determining phytoplankton growth.

The physical forcing controlling surface ocean nutrient and mixing regimes, and hence phytoplankton growth conditions, shows large-scale geographical patterns. This has led Longhurst (1998) to partition the ocean into biogeochemical provinces, areas that share a common physical forcing. The environmental conditions controlling phytoplankton growth dynamics strongly differ between these provinces, supporting a wide range of pelagic ecosystems from high-biomass new production systems to low-biomass recycling systems. Within provinces, growth conditions are more or less uniform in space and to a certain degree predictable in time with respect to seasonal variations. Once a solid understanding of the multiple factors and processes determining isotope fractionation in key phytoplankton species has been achieved, it may become feasible to develop and apply ε_p calibrations for individual Longhurst provinces. Since the geographical boundaries between biogeochemical provinces are expected to have shifted over geological time, however, applying this approach in paleoreconstructions may still prove difficult. Variation in the carbon isotope signature in down-core records at any given location may therefore reflect shifts in the geographical distribution of biogeochemical provinces as well as large-scale changes in environmental conditions. The above discussion implies that any sensible application of this proxy requires a much better understanding of the relevant factors and processes involved in phytoplankton carbon isotope fractionation.

This study indicates that a single uniform relationship for ε_p versus $\mu/[CO_2]$ does not exist in the alkenone-producing coccolithophorid *Emiliania huxleyi*, even if differences in cellular carbon content and cell surface area are accounted for. Highest sensitivity in ε_p was obtained in response to changes in photon flux densities and irradiance cycles. These ε_p responses were independent of the associated changes in growth rates and imply active carbon acquisition in *E. huxleyi*. These findings may compromise the use of alkenone $\delta^{13}C$ as a proxy in paleoreconstructions.

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