Hydrogen isotope fractionation in freshwater and marine algae: II. Temperature and nitrogen limited growth rate effects

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ABSTRACT

Zhang and Sachs [Hydrogen isotope fractionation in freshwater algae: I. Variations among lipids and species. Organic Geochemistry 38, 582–608, 2007] demonstrated that algal lipid δD values track water δD values with high fidelity ($R^2 > 0.99$), but that D/H fractionation varied among lipids and algal species. Here we report on the influence of temperature and nitrogen limitation on D/H fractionation in lipids from cultured microalgae. Two species of freshwater green algae, Eudorina unicocca and Volvox aureus, were grown in batch culture at 15 °C and 25 °C. Increased D/H fractionation of $2-4‰/°C$ occurred at the higher temperature in all lipids analyzed. The marine diatom Thalassiosira pseudonana was grown in continuous culture under nitrogen replete (NR) and nitrogen limited (NL) conditions, resulting in a growth rate that was 4.4 fold lower under the latter conditions. The fatty acid content of NL cells was approximately 4 fold higher than in NR cells, whereas the sterol content was similar in both. While sterols from the NL culture were enriched in deuterium by 37‰ relative to the NR culture, fatty acids from both cultures had similar δD values, implying that D/H fractionation during isoprenoid (branched) lipid synthesis is affected by nitrogen limitation, but D/H fractionation during acetogenic (linear) lipid synthesis is not. Cross-talk of the precursor isopentenyl diphosphate between the cytosolic MVA and plastidic DOXP/MEP synthetic pathways is a plausible mechanism for the observed D/H differences between isoprenoid and acetogenic lipids. This preliminary study highlights the need to consider both the type of lipid and potential changes in growth conditions in paleoenvironmental studies using lipid D/H ratios.

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1. Introduction

Hydrogen isotope ratios in plant and algal lipids from sediments are increasingly used to reconstruct past climates. The water cycle is particularly amenable to study with this technique because changes in precipitation, evaporation and runoff alter the D/H ratio of water in lakes and oceans and all hydrogen in algae is derived from water. Furthermore, hydrogen atoms covalently bonded to carbon atoms, as is the case for most hydrogen in lipids, are stable over geologic time. Yet very little is known about the influence of environmental conditions on D/H fractionation in algae.

In a previous study we demonstrated that algal lipid δD values closely co-varied with water δD values, but that D/H fractionation varied between different classes of lipids and species of algae (Zhang and Sachs, 2007). Therefore, in theory, paleoenvironmental water δD values could be reconstructed with fractionation factors derived from culture experiments. But it is critical to know how environmental factors such as temperature, salinity, nutrient avail-

ability and light levels influence D/H fractionation during lipid biosynthesis. To date very few studies have addressed these effects, and those that did have reported contradictory results.

The effect of temperature on D/H fractionation in plants and algae remains controversial. White et al. (1994) indicated that the net biochemical fractionation (ε) in white pine trees appeared to change linearly with temperature at a rate of +1.3‰/°C (i.e., more positive δD values occurred at elevated temperature). However, Roden and Ehleringer (2000) reported that there was no influence of temperature on D/H fractionation in tree ring cellulose. But D/H fractionation in higher plants is also influenced by several other factors, including relative humidity, the isotopic composition of atmospheric water vapor, and airflow dynamics in the leaf boundary layer (Buhay et al., 1996). As a result, the direct influence of temperature on D/H fractionation in higher plants remains uncertain.

Fewer studies have addressed the effect of temperature on D/H fractionation in algae. Stiller and Nissenbaum (1980) observed an inverse relationship between temperature and δD values in phytoplankton. Estep and Hoering (1980) also observed an inverse relationship between temperature and the δD values of total organic matter in batch culture of the green alga Chlorella sorokiniana in which temperature was increased stepwise from

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20 to 42 °C over 11 days at increments of 3 to 9 °C. Though the temperature dependence was not systematic, δD values decreased by 9–10‰ as temperature increased from 23 to 26 °C and from 33 to 42 °C (Estep and Hoering, 1980).

Even less is known about the effect of growth rate on D/H fractionation in plants and algae. Schouten et al. (2006) analyzed the δD values of C_{37} alkenones in batch cultures of the marine coccolithophorids *Emiliania huxleyi* and *Gephyrocapsa oceanica* grown at different temperatures and salinities and reported that there was no significant correlation of the fractionation factor with temperature, but that there was a negative correlation with growth rate and a positive correlation with salinity.

In order to explore the effect of temperature on D/H fractionation in algal lipids we conducted batch culture experiments at 15 and 25 °C with two freshwater green algae, *Eudorina unicocca* and *Volvox aureus*. To investigate the influence of nitrogen induced growth rate changes on D/H fractionation in algal lipids we performed continuous culture experiments with the marine diatom *Thalassiosira pseudonana*. Here we report on how these environmental parameters influenced the D/H fractionation in individual lipids.

2. Methods

2.1. Temperature experiments

Eudorina unicocca (EU) and *V. aureus* (VV) inoculants were supplied by the Culture Collection of Algae and Protozoa (CCAP) in Cumbria, United Kingdom (now in Dunstaffnage Marine Laboratory, Oban, Scotland) and cultivated in batch cultures at the Woods Hole Oceanographic Institution in 2003 as described in Zhang and Sachs (2007).

Temperature influences on δD values of the algae species were assessed through culture experiments. Six cultures of *E. unicocca* were grown in total, with three at 15 °C (LT, low temperature) and three at 25 °C (HT, high temperature) (Table 1). Each of the three LT and HT cultures were grown in medium with water δD values of 299‰ (EULT1/EUHT1), 95‰ (EULT2/EUHT2) and −56‰ (EULT3/EUHT3) (Table 1). Four cultures of *V. aureus* were also grown under the same conditions, two at 15 °C (LT) and two at 25 °C (HT) with medium water δD values of 299‰ (VVLT1/VVHT1) and 93‰ (VVLT2/VVHT2) (Table 1).

Cell density was estimated by visible light absorption at 400, 500, 600 and 700 nm using an Agilent HP8452A Diode Array Spectrophotometer (Fig. 1). Growth rates (divisions d^{-1}, k) were determined using the endpoints of the exponential portion of the growth curves (by curve fitting) and the exponential growth equation (Eq. (1))

$$k = \log_2(N_1/N_0)/(T_1 - T_0) \tag{1}$$

where N_1 and N_0 are the absorption values at the end and beginning, respectively, and T_1 and T_0 are the time (days) at the end and beginning, respectively, of the exponential growth period (Adolf et al., 2003). Representative growth curves of VVLT2 and VVHT2 are shown in Fig. 1. The LT and HT cultures of *E. unicocca* and *V. aureus* were harvested within the exponential phase of growth by filtration through 293 mm diameter Whatman GF/F filters.

Water δD values were determined at the start and end of each culture experiment (Zhang and Sachs, 2007). The methods for lipid extraction, identification, purification and D/H analysis are described in Zhang and Sachs (2007).

2.2. Nutrient limited growth rate experiments

The marine centric diatom *T. pseudonana* (CCMP 1335) was acquired from the Provasoli–Guillard Center for the Culture of Marine Phytoplankton. This clone was collected in 1958 from Moriches Bay, Long Island Sound, New York, USA, and maintained in semi-continuous batch culture since that time. Two continuous cultures were conducted in order to achieve nitrogen replete (NR, control) and nitrogen limited (NL) growth at the School of Oceanography, University of Washington, in 2006.

Cultures were grown in 12 l polycarbonate culture vessels in the artificial seawater medium AQUIL, according to the recipe outlined in Price et al. (1988/89). Trace metal concentrations were buffered using 100 μM EDTA. For the NR culture, macronutrient concentrations in the supply medium were 17 μM PO_4^{3-}, 175 μM $Si(OH)_4$ and 270 μM NO_3^- and the dilution rate (defined as the volume of nutrient medium supplied per day divided by the volume of medium in the culture flask) was set at approximately 1.9 d^{-1} to support maximum growth rates (Table 2). For the NL culture, initial macronutrient concentrations were the same as for NR except that NO_3^- was reduced to 40 μM and the dilution rate was reduced to approximately 0.4 d^{-1}. All other parameters were kept constant, with temperature at 20 °C and light intensity (continuous light) at 200 ± 20 μmol m^{-2} s^{-1}. Cultures were stirred and bubbled with air that was passed through sterilized milli-Q water and a 0.2 μm (pore size) Acrodisc filter.

Subsamples were taken daily for the analysis of *in vivo* fluorescence, dissolved nutrients, chlorophyll *a* (Chl *a*) concentrations and cell densities. Chl *a* concentrations were determined by fluorescence on a Turner Designs Trilogy fluorometer after extraction in 90% acetone at −20 °C for 24 h as described in Parsons et al. (1984). Cell growth was monitored by *in vivo* fluorescence using a Turner 10-AU fluorometer and reported in relative fluorescence unit (rfu; Fig. 2) (Brand et al., 1981). Cell enumerations were performed on 5 ml aliquots of the culture that had been preserved in glass vials with acidic Lugols fixative (final concentration ~5%) using a hemocytometer with a Nikon Labophot 2 compound microscope.

Dissolved $Si(OH)_4$, nitrate (NO_3^-) plus nitrite (NO_2^-), and PO_4^{3-} were measured with standard procedures using a Bran + Luebbe Autoanalyzer 3 (Barwell-Clarke and Whitney, 1996). Cells from a 10 ml aliquot of the culture were removed by filtration through a Whatman GF/F filter and the filtrate collected in acid-washed polypropylene tubes and then frozen at −20 °C until analysis. Nitrite concentrations were assumed negligible relative to NO_3^- and all NO_3^- plus NO_2^- measurements are reported as NO_3^-.

Cultures were initially grown in batch culture format to increase the biomass within each vessel. Once adequate cell densities were achieved (>1 × 10^5 cells ml^{-1}; day 3 for NR and day 4 for NL; Fig. 2) the continuous cultures were turned on and fresh medium was supplied continuously at the appropriate dilution rate (Table 2). Specific growth rates (d^{-1}) were calculated using the equation:

$$\mu = [(\ln(d_1) - \ln(d_0)]/(T_1 - T_0) + D \tag{2}$$

where d_1 and d_0 are the cell densities at the end and beginning, respectively, of the continuous cultures, T_1 and T_0 are the time (days) at the end and beginning, respectively, of the continuous cultures, and D is the dilution rate (Leonardos, 2008). Growth rates in divisions d^{-1} were determined by multiplying specific growth rates by $\log_2(e)$ and are reported in Table 2.

The cultures were considered to be in a near steady-state once *in vivo* fluorescence did not change by >10% in a 24 h period (Fig. 2). For the NR culture, minor, but steady increases in biomass indicated cells were growing at a slightly faster rate than that set by the dilution rate (>1.9 d^{-1}). In the NL culture, growth rate of *T. pseudonana* was controlled by the dilution rate and cell density was controlled by the concentration of nitrate in the medium (Droop, 1974). Following inoculation of the culture vessel, nitrate was in excess, allowing the algae to grow at their maximum growth rate and causing the NO_3^- concentration to decline. After

Table 1

Hydrogen isotope ratios and D/H fractionation in free fatty acids, FAMEs and phytadiene in *Eudorina unicocca* and *Volvox aureus* grown at 15 and 25 °C.

Culture[a]	Growing T(°C)	H$_2$O δD at start	H$_2$O δD at harvest	Fatty acids δD (corrected, ‰)								Heptadecene (‰)				Natural fatty acid methy esters (‰)							
				C$_{16}$	σ	α	ε	C$_{18}$ total[c]	σ	α	ε	C$_{17:1}$	σ	α	ε	C$_{16}$	σ	α	ε	C$_{18}$ total[d]	σ	α	ε
Eudorina unicocca																							
EULT1	15	297.7	306.9	223.1	2.6	0.936	-64.1	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
EULT2	15	95.4	109.9	42.3	0.8	0.939	-60.9	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
EULT3	15	-62.4	-50.5	-104.2	1.9	0.943	-56.6	-101.1	1.6	0.947	-53.3	-143.5	2.3	0.902	-98.0	-110.3	1.8	0.937	-63.0	-120.9	2.2	0.926	-74.2
Average						0.939	-60.5			0.947	-53.3			0.902	-98.0			0.937	-63.0			0.926	-74.2
Standard deviation						0.004	3.8																
EUHT1	25	299.0	307.1	185.1	3.7	0.907	-93.3	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	171.1	4.6	0.896	-104.0	159.8	1.2	0.887	-112.7
EUHT2	25	95.4	101.1	-10.5	0.6	0.899	-101.4	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
EUHT3	25	-55.7	-45.7	-127.3	0.8	0.914	-85.5	-117.6	0.7	0.925	-75.4	-176.5	1.2	0.863	-137.2	-140.2	1.2	0.901	-99.0	-147.0	4.1	0.894	-106.2
Average						0.907	-93.4			0.925	-75.4			0.863	-137.2			0.898	-101.5			0.891	-109.5
Standard deviation						0.008	7.9											0.004	3.5			0.005	4.5
Δε$_{HT-LT}$[b]						-0.033	-32.9			-0.022	-22.1			-0.039	-39.2			-0.039	-38.5			-0.035	-35.3
Volvox aureus																							
VVLT1	15	298.8	311.2	248.5	6.0	0.952	-47.9	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	220.2	1.7	0.931	-69.4	226.5	5.7	0.935	-64.6
VVLT2	15	93.7	99.0	53.1	3.5	0.958	-41.8	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	26.6	1.0	0.934	-65.9	N.D.	N.D.	N.D.	N.D.
Average						0.955	-44.8											0.932	-67.7			0.935	-64.6
Standard deviation						0.004	4.3											0.003	2.5				
VVHT1	25	299.8	306.4	191.6	1.9	0.912	-87.8	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	166.0	1.2	0.893	-107.4	N.D.	N.D.	N.D.	N.D.
VVHT2	25	93.2	103.4	15.2	6.5	0.920	-79.9	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	-9.9	4.7	0.897	-102.7	N.D.	N.D.	N.D.	N.D.
Average						0.916	-83.9											0.895	-105.1				
Standard deviation						0.006	5.6											0.003	3.4				
Δε$_{HT-LT}$[b]						-0.039	-39.1											-0.037	-37.4				

The fractionation factor, α, was calculated for each culture from the measured δD values of biomarkers and water at harvest according to the equation $\alpha = (D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$. Fractionation, ε, is defined as $(\alpha - 1)*1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1]*1000$.

[a] LT, low temperature; culture growing inside a chamber at 15–16 °C. HT, High temperature; culture growing inside a chamber at 25–26 °C. Each LT/HT pair started with same condition except growing temperatures.

[b] $\Delta\varepsilon_{HT-LT}$ is the difference in hydrogen isotope fractionation between cultures grown at 25 and 15 °C, i.e., $\varepsilon_{HT}-\varepsilon_{LT}$.

[c] All fatty acids with C$_{18}$ skeleton are merged into one peak on GCIRMS.

[d] All fatty acid methyl esters with C$_{18}$ skeleton are integrated as one peak on GCIRMS. N.D., not determined.
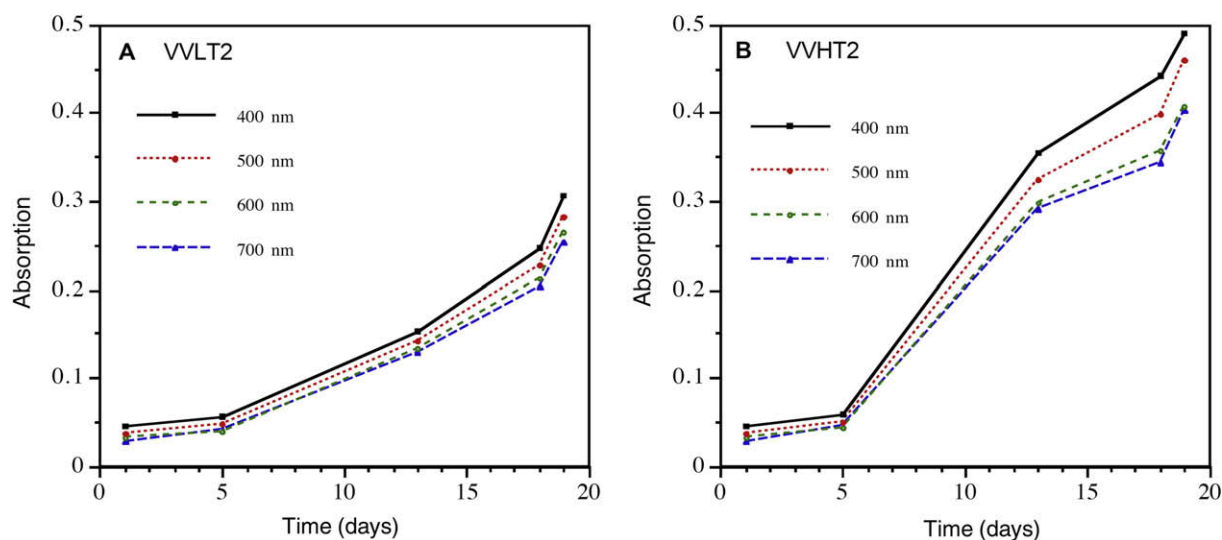
Fig. 1. Representative growth curves of *Volvox aureus* cultures grown at 15 °C (lower temperature, LT) and 25 °C (higher temperature, HT), showing the biomass accumulating at a slightly faster rate at the higher temperature. The *X*-axis indicates time in days. The *Y*-axis is light absorption in absorption units relative to distilled water. Absorption was measured at 400, 500, 600 and 700 nm wavelength. VV: *Volvox aureus*.

Table 2
Growth characteristics, medium δD values and residual nitrogen concentrations in nitrogen replete (NR) and nitrogen limiting (NL) chemostat cultures of the diatom *Thalassiosira pseudonana*.

Growth condition	Medium δD (‰)[a]	Dilution rate (d^{-1})[a,b]	Growth rates (divisions d^{-1})[c]	Chl a content (pg cell^{-1})[a]	FA content[a,d] (pg cell^{-1})	Sterol content[a,d] (pg cell^{-1})	Residual nutrients (μM)		
							NO$_3^-$ [e]	PO$_4^{-3}$	Si(OH)$_4$
N-limited (NL)	−78.9 ± 4.1	0.40 ± 0.03	0.66 ± 0.04	0.020 ± 0.002	0.30	0.059	2.8	11.1	116.9
N-replete (NR)	−75.7 ± 2.2	1.88 ± 0.10	2.89 ± 0.14	0.110 ± 0.004	0.07	0.074	>200	7.9	86.1

[a] The values given here are the average of daily measurements since the continuous cultures were turned on.

[b] During chemostat cultivation, an equilibrium (steady-state) is established. The higher the dilution rate, the faster the organisms are allowed to grow. Above a given dilution rate the cells will not be able to grow any faster, and the culture will be washed out of the vessel.

[c] Specific growth rates (d^{-1}) were calculated using the equation: $\mu = [(\ln(d_1) - \ln(d_0)]/(T_1 - T_0) + D$ where d_1 and d_0 are the cell densities at the end and beginning, respectively, of the continuous cultures, T_1 and T_0 are the time (days) at the end and beginning, respectively, of the continuous cultures and D is the dilution rate (Leonardos, 2008). Growth rates in divisions d^{-1} were determined by multiplying specific growth rates by $\log_2(e)$.

[d] FA = C$_{16}$ fatty acid and sterol is 24-methyl-cholesta-5,24(28)-dien-3β-ol.

[e] Nitrate was the growth-limiting nutrient in this experiment, which was limited in NL and replete in NR, leading to the growth rate difference.
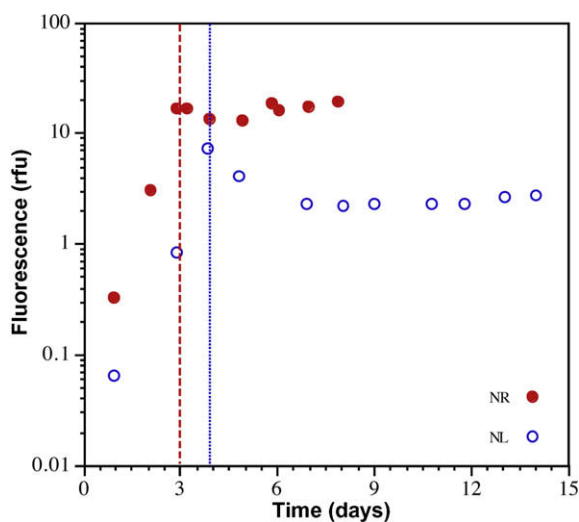

Fig. 2. Growth of the marine diatom *Thalassioisira pseudonana* in continuous cultures under nitrogen replete (NR) and nitrogen limiting (NL) conditions. Cell growth was quantified by measuring chlorophyll fluorescence, the results of which are presented in relative fluorescence units (rfu). The dashed and dotted lines indicate when the continuous cultures were started in the NR and NL treatments, respectively.

the continuous culture was turned on, as the NO$_3^-$ concentration declined the growth rate of the algae declined. *In vivo* fluorescence stabilized after day 7. The NR culture was harvested on day 8 whereas the NL culture was harvested on day 14. Cultures were concentrated onto 25 mm Millipore 0.2 μm filters and stored frozen at −80 °C.

Aliquots of the culture media were collected daily throughout the continuous growth of both NL and NR cultures. δD analyses were performed on a Delta V IRMS with sample introduction by a Thermo/Finnigan temperature conversion elemental analyzer (TC/EA) using a CTC Analytics GCPal liquid autosampler. External precision of the water δD analyses was ca. 1‰. The water δD values given in Table 2 are the averages of daily measurements.

Because the 0.2 μm polycarbonate filter would have dissolved in solvent, harvested *T. pseudonana* cells were thawed and transferred to 47 mm diameter Whatman GF/F filters with distilled water prior to freeze drying. Lipid extraction and purification procedures are described in detail in Zhang and Sachs (2007). In addition, a set of internal standards consisting of *n*-C$_{37}$ alkane, *n*-C$_{21}$ alkanol and *n*-C$_{21}$ fatty acid were added prior to lipid extraction. Individual lipid biomarkers were identified by GC-MS (Agilent 6890/5973 N GC/MSD) and quantified by GC-FID by comparing the peak area of analytes with that of *n*-C$_{21}$ alkanol and *n*-C$_{21}$ fatty acid for sterols and fatty acids, respectively.

The hydrogen isotopic composition of purified lipids was measured at the University of Washington on a Thermo Delta V Plus isotope-ratio mass spectrometer (IRMS) with sample introduction from a Thermo Trace GC II gas chromatograph connected to the IRMS via a Thermo GC/TC high-temperature conversion interface (GCC III). The GC was operated with a splitless inlet, a 30 m DB-5 capillary column (J&W Scientific) with 0.25 mm i.d. and 0.25 μm film thickness, and a constant helium flow rate of 1 ml/min. The GC oven was programmed from 80 to 130 °C at 10 °C/min, then to 325 at 4.5 °C/min, followed by 10 min at 325 °C. Sensitivity of the IRMS was monitored with six pulses of ultra high purity H_2 gas via a second open split, four at the beginning and two at the end of each run. A low and stable H_3^+ factor of less than 7.5 ppm/mV was typically achieved. A set of co-injection standards obtained from Indiana University (Dr. Arndt Schimmelmann) was added for calibration as described in Zhang and Sachs (2007).

Fatty acid δD values were measured on methyl esters produced by reaction of free acids with methanolic HCl (Sigma–Aldrich). The contribution to the fatty acid δD values from the added methyl group (three hydrogen atoms) was determined by methylation of a phthalic acid standard with a known δD value of its sodium salt (Dr. Arndt Schimmelmann, Indiana University).

Sterols were acetylated by reaction with acetic anhydride with a known δD value (Dr. Arndt Schimmelmann, Indiana University). A mass balance was used to deduce the δD values of fatty acids and sterols. δD values of sterols and fatty acids determined in this way do not include the carboxylic or hydroxyl hydrogen atoms, an acceptable omission given the relative lability of those hydrogen atoms.

Consistent with our previous study (Zhang and Sachs, 2007), the defined reference standard for D/H ratio is V-SMOW with $D/H = 155.76 \pm 0.05 \times 10^{-6}$. δD is defined as: $\delta D = [(D/H)_{sample} - (D/H)_{VSMOW}]/(D/H)_{VSMOW} \times 1000‰$. As is customary in biogeochemistry, we define the "fractionation factor" as $\alpha_{lipid-water} = (D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$ and the "isotope fractionation" (or the "enrichment factor") as $\varepsilon_{lipid-water} = (\alpha_{lipid-water} - 1) \times 1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1] \times 1000$. Because lipids are always depleted in deuterium relative to growth water, $\varepsilon_{lipid-water}$ is always negative. Larger fractionation simply means that the absolute value of this negative number is greater.

3. Results

3.1. Algal growth rates

Representative growth curves for *V. aureus* grown at 15 °C (VVLT2) and at 25 °C (VVHT2) are shown in Fig. 1. Growth rate calculations for VVLT2 and VVHT2 were based on the 600 nm data shown in Fig. 1. Values were 0.171 and 0.205 divisions day^{-1}, respectively, indicating a relatively higher (20%) growth rate for the 25 °C culture than for the 15 °C culture. Though most of the observed increase in light absorption can be attributed to increasing phytoplankton biomass, a small contribution was caused by evaporation of 5–15% of the culture water during the experiment. Water δD values at harvest were within 5‰ for all LT and HT pairs, except for the EULT2 and EUHT2, which had final water δD values that differed by 8.8‰ (Table 1).

The growth rates of NR and NL cultures averaged 2.89 and 0.66 divisions day^{-1}, respectively (Table 2).

3.2. Lipid varieties and yields

Lipids in *E. unicocca* and *V. aureus* consisted primarily of fatty acids, naturally occurring fatty acid methyl esters (FAMEs) and 8-heptadecene (Zhang and Sachs, 2007). Sterol concentrations were

extremely low, and consequently, no D/H analyses were performed on those compounds. Lipid abundances were not quantified in either EU or VV cultures. However, the fatty acid composition for LT and HT cultures were very similar. For example, VVLT1 and VVHT1 had ratios of the two most abundant fatty acids, $C_{16:0}$ saturated fatty acid (palmitic) and $C_{18:1}$ fatty acid (oleic), of 6.8 and 8.4, respectively, while the ratios of $C_{16:0}/C_{18:1}$ in EULT1 and EUHT1 were 23 and 30, respectively.

Lipids in *T. pseudonana* consisted primarily of fatty acids and sterols. No hydrocarbons, *n*-alkanols or FAMEs were detected. Palmitic acid was the most abundant fatty acid, with much lesser amounts of *n*-C_{14} and *n*-C_{18} acids, and trace quantities of C_{12} and $C_{18:3}/C_{18:1}$ acids. The predominant sterol was 24-methyl-cholesta-5,24(28)-dien-3β-ol, confirmed by comparing the mass spectra of trimethylsilylated, acetylated and underivatized sterols with reports by Weete and Gandhi (1997), Kates et al. (1978) and Lin et al. (1982), respectively. A trace amount of 24-methylcholesterol eluted immediately after 24-methyl-cholesta-5,24(28)-dien-3β-ol, but was not sufficiently abundant to measure D/H ratios.

Thalassiosira pseudonana cell densities were almost twice as high in the NR cultures (7.64×10^5 cells ml^{-1}) as in the NL cultures (4.08×10^5 cells ml^{-1}), chlorophyll concentrations were five times higher in the NR cells (0.11 pg chl a cell^{-1}) than in the NL cells (0.02 pg chl a cell^{-1} in NL), and the sterol concentrations were 42% greater in the NR (0.074 pg cell^{-1}) relative to the NL cells (0.059 pg cell^{-1}) (Table 2). The ratio of sterols to chlorophyll a was thus 4.4 fold higher in NL cells (2.95) than in NR cells (0.67).

Fatty acids from *T. pseudonana* followed the opposite trend, with concentrations in the NL cells approximately four times higher than in the NR cells. Thus the concentration of palmitic acid in the NL cells was 0.30 pg cell^{-1} while those in the NR cells was 0.07 pg cell^{-1}. Furthermore, the ratio of palmitic acid to chlorophyll a was 23 fold higher in the NL cells (15) than in the NR cells (0.64).

3.3. Hydrogen isotope fractionation at different temperatures

Fractionation factors (α) and enrichment factors (ε) for each lipid in each culture are shown in Table 1. α values for the C_{16} fatty acid in *E. unicocca* were higher in all of the 15 °C cultures, which averaged 0.939 ± 0.004 (1σ), than in the 25 °C cultures, which averaged 0.907 ± 0.008, an average difference in α of 0.033 (Table 1). The associated ε values for the 15 and 25 °C cultures were −60.5 ± 3.8‰ and −93.4 ± 7.9‰, respectively, a difference of 32.9‰ (Table 1, Fig. 3A). As previously demonstrated by Zhang and Sachs (2007), changing the D/H composition of the water had little effect on D/H fractionation.

Sufficient quantities for isotopic analysis of the C_{18} fatty acid, the C_{16} FAME and heptadecene in *E. unicocca* were only available from the EULT3/EUHT3 experiment. As with the C_{16} fatty acid, the 15 °C cultures exhibited less isotope discrimination than the 25 °C cultures for each of these lipids (Table 1). Differences in α and ε values for the C_{18} fatty acid grown at 15 and 25 °C were 0.022 and 22.1‰, respectively, while α and ε values for the C_{16} FAME differed by 0.039 and 38.5‰ between the 15 and 25 °C culture experiments; α and ε values for heptadecene differed by 0.039 and 39.2‰, respectively (Table 1).

Lipids from *V. aureus* also exhibited less D/H fractionation at 15 °C than at 25 °C (Table 1). Differences in α and ε values for the C_{16} fatty acid were 0.039 and 39.1‰, respectively (Fig. 3B); α and ε values for the C_{16} FAME differed by 0.037 and 37.4‰, respectively.

3.4. Hydrogen isotope fractionation at different growth rates

The four fatty acids analyzed in *T. pseudonana* had very similar α and ε values in the NR and NL treatments (Table 3). The differences
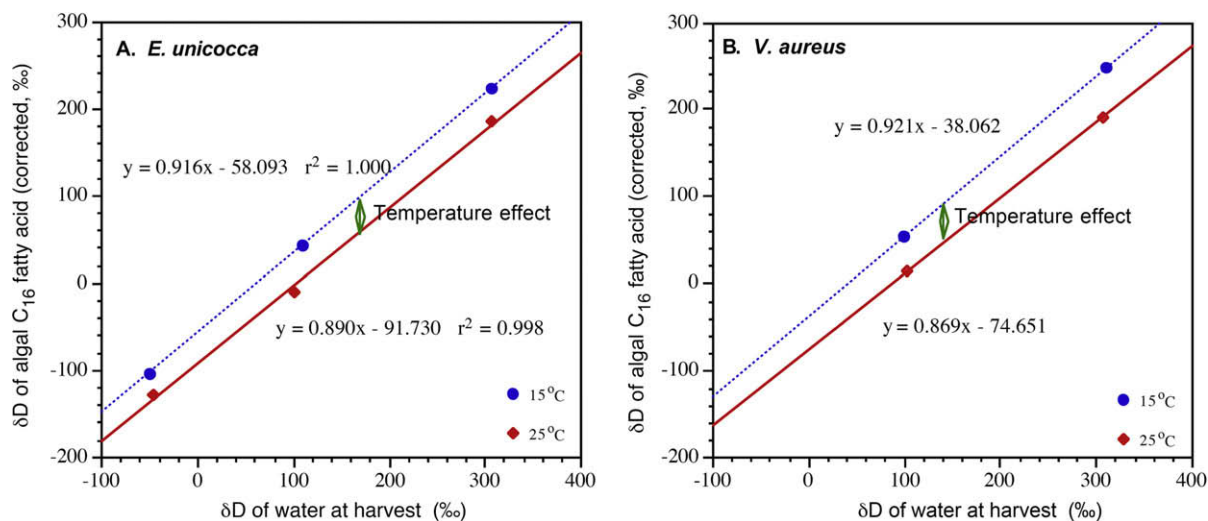
Fig. 3. D/H fractionation differences in C_{16} fatty acids in *Eudorina unicocca* (A) and *Volvox aureus* (B) cultures grown at 15 and 25 °C. (A) Relationships between C_{16} fatty acid and water δD values in cultures of *E. unicocca* at 15 and 25 °C were highly correlated ($r^2 > 0.99$). (B) Relationships between C_{16} fatty acid and water δD values in cultures of *V. aureus* at 15 and 25 °C. The C_{16} fatty acid in both species of green algae is depleted in deuterium at 25 °C relative to 15 °C.

in α and ε values for C_{14}, $C_{16:1}$, C_{16}, and C_{18} fatty acids in the two treatments averaged -0.001 ± 0.004 and $-0.93 \pm 4.10‰$, respectively (Table 3).

Nitrogen limitation had a far greater impact on the δD composition of 24-methyl-cholesta-5,24(28)-dien-3β-ol. Differences in fractionation factors averaged 0.037 and 37.3‰ for α and ε, respectively, between the NL and NR treatments of *T. pseudonana*. Lower δD, α and ε values in the sterol relative to the fatty acids from *T. pseudonana* are in accordance with the observation that isoprenoid lipids are significantly depleted in deuterium relative to acetogenic lipids (Zhang and Sachs, 2007, and references therein).

4. Discussion

4.1. Influence of growth rate on lipid composition

There is compelling evidence that cellular fatty acid concentrations increase in many algae in response to nitrogen limitation (e.g., Miller, 1962; Shifrin and Chisholm, 1981; Piorreck et al., 1984; Suen et al., 1987; Livne and Sukenik, 1992). Piorreck et al. (1984) reported that at low nitrogen levels green algae contained a high abundance of lipids (45% of biomass) and that more than 70% of those lipids were the $C_{16:0}$ and $C_{18:1}$ fatty acids. At high nitrogen levels the cellular abundance of total lipids dropped to about 20% of the biomass (Piorreck et al., 1984). Livne and Sukenik (1992) measured the fatty acid content and the rate of lipid synthesis in the marine prymnesiophyte *Isochrysis galbana* grown under nitrogen limited conditions and attributed an increase in total fatty acid content to a reduction in cell division and an increased rate of lipid synthesis.

Nitrogen limitation imposes a reduction of carbon flow into nitrogen containing compounds (Livne and Sukenik, 1992) such as chlorophyll, proteins, nucleic acids, etc., and is the likely cause of the lower concentration of chlorophyll *a* in the NL treatment of *T. pseudonana* (0.020 ± 0.002 pg cell^{-1}) than in the NR treatment (0.110 ± 0.004 pg cell^{-1}) (Table 2). Storage lipids are devoid of nitrogen, and may therefore continue to be synthesized in nitrogen limited cells. Although cell division was reduced in the NL treatment, anabolic fatty acid synthesis may have remained high, resulting in four times the C_{16} fatty acid concentration in NL cells (0.30 pg cell^{-1}) compared to NR cells (0.07 pg cell^{-1}) (Table 2).

Unlike cellular abundances of fatty acids, sterol abundances are relatively stable at different growth rates (Hallegraeff et al., 1991; Mansour et al., 2003), likely owing to their role as components of the cell membrane lipid bi-layer that regulates membrane fluidity and permeability. Our observation of similar sterol concentrations in NL and NR cells supports this paradigm (Table 2).

The different production rates of fatty acids and 24-methyl-cholesta-5,24(28)-dien-3β-ol under NR and NL conditions implies that growth rate, and the rate of synthesis of individual lipids are not necessarily coupled. It is conceivable, if not likely, that a change in either the growth rate or the synthesis rate of individual lipids could influence the D/H ratio of those lipids. The following discussion of lipid D/H fractionation under varying degrees of nitrogen limitation focuses on the production rate of individual lipids rather than the growth rate of cells, the former presumably being a consequence of the latter.

4.2. Influence of nitrogen limited growth rate on D/H fractionation in lipids

4.2.1. Fatty acids

In order for fatty acid δD values to remain constant at different growth rates the isotopic composition of the hydrogen source(s) for fatty acid synthesis (i) must either be unaffected by growth rate changes, (ii) must have stable D/H ratios, such as might occur with a large reservoir of hydrogen, or (iii) must compensate in a way that D-depleted hydrogen is added in some steps and D-enriched hydrogen is added in others. As reviewed in Zhang and Sachs (2007), fatty acid synthesis can be divided into three steps: the activation of acetyl-CoA to malonyl-CoA, chain initiation from a unit of acetyl-CoA plus a unit of malonyl-CoA and subsequent elongation with malonyl-CoA by a fatty acid synthase (FAS) complex, and desaturation by desaturase enzyme (Duan et al., 2002).

At the odd numbered carbon positions, carbon comes from the carboxyl group (−C=O−) of acetate, and hydrogen is derived entirely from NADPH. At the even numbered carbon positions one hydrogen atom is derived from acetate and the other from water during the enoyl-ACP reductase step (Duan et al., 2002; Schmidt et al., 2003). Presently the precise mechanism of enzyme mediated exchange of carbon bound hydrogen with intracellular water is not known. If substantial hydrogen exchange occurs between fatty

Table 3
Hydrogen isotope ratios and D/H fractionation in fatty acids and a sterol in *Thalassiosira pseudonana* chemostat cultures grown under nitrogen replete (NR) and nitrogen limited (NL) conditions.

Cultures	Water δD (‰)	δD of fatty acids (corrected, ‰)																δD of sterols (corrected, ‰)			
		C14	σ	α	ε	C16:1	σ	α	ε	C16	σ	α	ε	C18	σ	α	ε	Sterol	σ	α	ε
NL	−78.9	−197.3	2.5	0.871	−128.5	−207.6	2.1	0.860	−139.8	−211.2	1.2	0.856	−143.6	−208.0	2.3	0.860	−140.2	−350.4	1.3	0.705	−294.7
NR	−75.7	−191.5	3.1	0.875	−125.3	−201.2	2.6	0.864	−135.8	−206.9	2.8	0.858	−142.0	−210.0	2.4	0.855	−145.3	−382.6	2.5	0.668	−332.0
Δε_NR-NL				0.003	3.2			0.004	3.9			0.002	1.6			−0.005	−5.1			−0.037	−37.3
All fatty acid Δα and Δε average				0.001	0.93																
All fatty acid Δα and Δε standard deviation				0.004	4.10																

σ = standard deviation, based on triple measurements.
The fractionation factor, α, was calculated for each culture from the measured δD values of biomarkers and water at harvest according to the equation $\alpha = (D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$.
Fractionation, ε, is defined as $(\alpha - 1)*1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1]*1000$. $\Delta\varepsilon_{NR-NL}$ is the difference in hydrogen isotope fractionation between NR and NL continuous cultures, i.e., $\varepsilon_{NR}-\varepsilon_{NL}$.

acids and their intermediates with a large pool of intracellular water it could explain the insensitivity to growth rate of fatty acid δD values.

Kreuzer-Martin et al. (2006) reported that $C_{14:0}$ and $C_{16:0}$ fatty acids exhibited larger deuterium depletions in the log phase of growth than in the stationary phase of growth in the bacterium *Escherichia coli*, and attributed the greater isotopic depletion to a larger contribution of hydrogen from intracellular water during log phase growth. As a heterotroph *E. coli* maintains metabolic water that is more D-depleted than its extracellular water. As a photoautotroph, *T. pseudonana* maintains intracellular (or metabolic) water enriched in deuterium because isotopically depleted hydrogen is continuously removed for NADPH production (Schmidt et al., 2003), leaving D enriched metabolic water. Furthermore, there is no evidence to suggest that the enzymes involved in fatty acid synthesis differ between log and stationary growth phases (Heath and Rock, 1996; Rock and Jackowski, 2002). If the hypothesis of Kreuzer-Martin et al. (2006) applies, fatty acids in *T. pseudonana* NR cells ought to be less D-depleted than those in NL cells, a prediction not supported by our data. Thus, either hydrogen exchange occurs between intracellular water and fatty acids or their intermediates, and/or intracellular water δD values are insensitive to growth rates.

4.2.2. Sterols

D/H fractionation in sterols from *T. pseudonana* varied widely with nitrogen nutritional status. Values of ε for 24-methyl-cholesta-5,24(28)-dien-3β-ol were 37‰ lower in rapidly growing nitrate replete cells than in slow growing nitrate limited cells (Table 3). Sterols, like other isoprenoid lipids, originate from a branched isoprene C_5 unit, isopentenyl diphosphate (IPP), or its isomer, dimethylallyl diphosphate (DMAPP). The "eukaryotic" acetate mevanolic acid (MVA) pathway starts from three acetyl-CoAs and requires six enzymes, two NADPHs and three ATPs to produce IPP, which is subsequently converted to DMAPP by IPP isomerase. In contrast, the non-MVA, "prokaryotic" 1-deoxyxylulose 5-phosphate/2-C-methyl-D-erythritol 4-phospate (DOXP/MEP) pathway of IPP synthesis begins with pyruvate and glycerinaldehyde-3-phosphate, and involves seven enzymes, three ATP equivalents, and three NADPHs to produce both IPP and DMAPP (Lichtenthaler, 2004).

In higher plants and many algae, such as the Bacillariophyta to which *T. pseudonana* belongs, the two IPP producing biochemical pathways operate simultaneously, with the MVA pathway restricted to the cytoplasm and the DOXP/MEP pathway restricted to the chloroplast (Fig. 4; Schwender et al., 2001; Bick and Lange, 2003; Hemmerlin et al., 2003; Lichtenthaler, 2004; Armbrust et al., 2004). Although sterol synthesis in most plants and algae occurs via the MVA pathway, some algae, including certain chlorophytes, can use the DOXP/MEP pathway to synthesize sterols and other isoprenoid lipids such as phytol and carotenoids (Fig. 4) (Lichtenthaler, 1999; Schwender et al., 2001; Sato et al., 2003).

Other evidence also suggests that under certain conditions isoprenoid intermediates can be transferred between the cytosolic MVA pathway and the plastidic DOXP/MEP pathway. Laule et al. (2003) provided indirect support for the presence of such an export mechanism in the vascular plant *Arabidopsis thaliana*. Hemmerlin et al. (2003) reported that sterols could be synthesized via the DOXP/MEP pathway when the MVA pathway in Bright Yellow-2 cells was inhibited, and that isoprenoids normally produced in the plastid by the DOXP/MEP pathway could be produced in the cytosol via the MVA pathway when the former was inhibited (Hemmerlin et al., 2003; Hartmann and Wentzinger, 2004). This suggested that significant exchange of isoprenoid intermediates occurred across the plastid envelope (Hemmerlin et al., 2003). Furthermore, Bick and Lange (2003) proposed that plastid membranes

possess a unidirectional proton/IPP coupled transport system for the export of IPP from the plastid to the cytosol.

We thus hypothesize that in fast growing (NR) cells, IPP and DMAPP monomers from both the MVA and DOXP/MEP pathways are mixed to produce sterols (Fig. 4). For instance, if the supply of IPP from the cytosolic MVA route is insufficient for sterol synthesis, additional IPP may come from the DOXP/MEP pathway. The metabolic state of the cells could dictate the relative contribution from the two pathways. Because the last reduction step during IPP synthesis via the DOXP/MEP pathway is characterized by a particularly large hydrogen isotope effect (Zhang and Sachs, 2007), and thus IPPs produced via that pathway are substantially D depleted relative to IPPs produced via the MVA pathway, a higher proportion of IPP derived from the DOXP/MEP pathway would result in the production of more deuterium depleted sterols (Fig. 4). This could explain the 37‰ depletion in deuterium in the fast growing NR *T. pseudonana* cells relative to the slow growing NL cells (Table 3).

Consistent with our findings, Sessions et al. (1999) reported that sterols from dormant plants, which may be analogous to our slow growing, NL *T. pseudonana* cells, were deuterium-enriched by 50–100‰ relative to actively growing plants, whereas fatty acids were substantially less enriched in deuterium, by 0–30‰. Cells in actively growing plants may incorporate some IPPs into sterols from the DOXP/MEP pathway resulting in more deuterium depletion than in sterols synthesized in dormant plants if the MVA pathway alone produces sterols. As we found, D/H fractionation in fatty acids observed by Sessions et al. (1999) was not greatly affected by growth rates.

Sessions (2006) attributed the larger D depletion in lipids from actively growing plants to their faster metabolism. This might explain why sterols in faster growing cells are more depleted in deuterium but does not explain why fatty acid D/H ratios were minimally affected by growth rate in our experiments. Because isoprenoid lipids alone expressed increased D/H fractionation at higher growth rates it suggests that the exchange of isoprenoid precursors (IPPs) between the cytosol and the plastid occurs in rapidly growing cells, but not the exchange of acetogenic precursors.

4.3. Influence of temperature on D/H fractionation

Cells grown at 25 °C exhibited substantially larger D/H fractionation than those grown at 15 °C. Six different acetogenic lipids, four in *E. unicocca* and two in *V. aureus*, had α values that averaged 0.034 ± 0.006 lower at 25 °C than at 15 °C, and ε values that averaged 34.4 ± 6.6‰ lower at 25 °C than at 15 °C (Table 1, Fig. 3). The larger change in fractionation in the opposite direction from what would be expected for equilibrium fractionation resulting from extensive hydrogen exchange rules out the possibility of any large scale hydrogen exchange during lipid synthesis.

Algal growth rates increase exponentially with increasing temperature until an optimum temperature is reached (Richard, 1986), after which a rapid decrease in growth rate occurs (Kruger and Eloff, 1978; Payer et al., 1980). Isolating the effect of temperature from that of growth rate is therefore necessary in order to evaluate the direct influence of temperature on D/H fractionation in algal lipids. Two lines of reasoning suggest that the growth rate independent temperature effect can be discerned from our experiments. First, there was no significant difference in D/H fractionation (α or ε) in fatty acids from *T. pseudonana* grown at rates that differed by a factor of 5 (Tables 2 and 3) suggesting little influence of growth rate on D/H fractionation in acetogenic lipid synthesis. Second, in our temperature experiments with *E. unicocca* and *V. aureus*, growth rates varied by only ~20%. Together these observations indicate that differences in D/H ratios between the

15 and 25 °C cultures most likely resulted from the different temperatures not different growth rates.

Both EU and VV cultures showed very similar fatty acid compositions for LT and HT cultures, i.e., a nearly constant ratio of $C_{16:0}$ vs. $C_{18:1}$ fatty acids. In addition, both $C_{16:0}$ vs. $C_{18:1}$ fatty acids displayed similar D depletion at the higher temperature. Thus the greater D depletions in fatty acids at increased temperature were unlikely caused by changes in the fatty acid composition within cells grown at different temperatures.

Temperature may influence D/H fractionation during lipid synthesis by at least three different mechanisms: its influence on enzyme activities, kinetic isotope effects, and hydrogen tunneling. Only the temperature influence on enzyme activities and hydrogen tunneling will be discussed here since kinetic effects are explored in a subsequent paper.

Lipid biosynthesis is catalyzed by a variety of enzymes whose conformation facilitates reaction with substrates (Chang, 2005). When the enzyme is in the native (active) state, the reaction rate increases with temperature. Temperature changes may induce the (partial) replacement of an enzyme by an isoenzyme with better heat or cold tolerance (Steele and Fry, 2000). Jahnke et al. (1999) reported that the sMMO and pMMO isozymes expressed different carbon isotopic fractionation factors (5% difference) in methanotrophic bacteria using the ribulose monophosphate pathway for carbon assimilation. However, after passing some optimum temperature that is specific to each enzyme, denaturalization can occur, making the enzyme lose its effectiveness as a catalyst. In this way the set of enzymes involved in the synthesis of a particular lipid, their sensitivity to temperature, and any D/H fractionation they impart will influence the δD value of the lipid.

Isotope fractionation in biochemical processes arises from unequal zero-point energies of bonds between heavy and light isotopes, resulting in different activation energies (Bigeleisen and Wolfsberg, 1959), which in turn are influenced by temperature. Fractionation factors are thus a function of both the temperature and the activation energy of the individual enzyme catalyzed reactions that comprise the lipid biosynthetic pathway.

Yet the greater D/H fractionation we observed at higher temperature is at odds with theory that indicates that equilibrium fractionation should decrease as temperature increases (Clark and Fritz, 1997). In any complex organism, changing growth temperature could lead to a whole suite of metabolic changes, any one of which could result in the observed isotopic differences. For example, different enzymes (isoenzymes or isozymes) may be used to synthesize lipids at different temperatures, each with its own fractionation (Jahnke et al., 1999). In one study by Steele and Fry (2000) two isoenzymes of xyloglucan endotransglycosylase (XET) isolated from *Arabidopsis* (i.e., cauliflower florets) were found to be temperature dependent, one with an optimum temperature of ca. 12 °C and exhibiting 55% of its maximal catalytic rate at −5 °C, total XET activity of mixed isoenzymes with a temperature optimum of ca. 30 °C (Steele and Fry, 2000) Assuming those two enzymes exhibit different isotope effects, the temperature at which the organism exists would cause different D/H ratios in the lipid product.

Other possible causes for a temperature influence on D/H fractionation in lipid synthesis include different mechanisms for synthesizing NADPH at different temperatures, such as photosynthesis versus the pentose phosphate pathway (Kruger and von Schaewen, 2003), and hydrogen tunneling due to strengthened substrate-enzyme-complex vibration at elevated temperatures (Kohen et al., 1999).

Our observation of a temperature effect on D/H fractionation is also at odds with a batch culture study by Schouten et al. (2006) with the marine coccolithophorids *Emiliania huxleyi* and *Gephyro-*
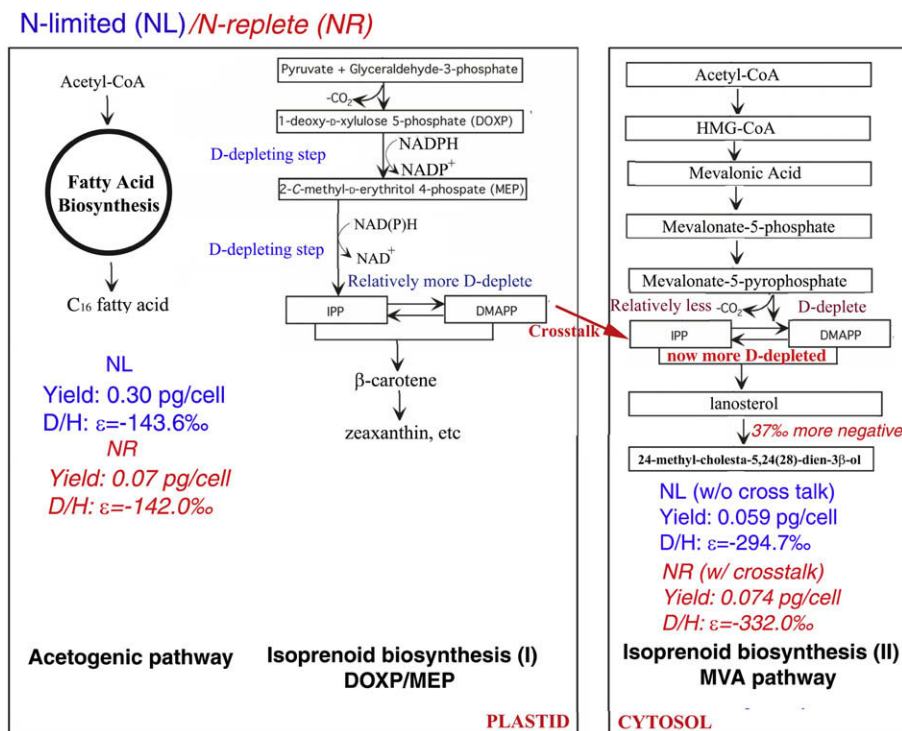
Fig. 4. Schematic diagram showing the cross-talk of IPPs between the plastidic DOXP/MEP pathway and the cytosolic MVA pathway in the marine diatom *Thalassiosira pseudonana*. The two IPP-producing biochemical pathways operate in parallel, with the MVA pathway housed in the cytoplasm (synthesizing isoprenoids such as β-carotene) and the DOXP/MEP pathway housed in the chloroplast (synthesizing isoprenoids such as sterols). Fatty acid concentrations were 4 fold higher in NL (0.30 pg/cell) than in NR (0.07 pg/cell) cells owing to their role as energy storage compounds. Sterol concentrations were similar in both NL (0.059 pg/cell) and NR (0.074 pg/cell), perhaps owing to their role as components of cell membranes. Under faster growing conditions the IPPs from the plastidic DOXP/MEP pathway that produces highly D-depleted products, might cross into the cytosol, providing additional IPPs to the MVA-pathway-products that are less D-depleted. As a result, sterols synthesized in the NR culture had δD values that were 37‰ lower (−332.0‰ vs. −294.7‰ in the NL culture). Acetogenic compounds such as fatty acids are synthesized exclusively in the plastid. As a result they had similar δD values (−143.6‰ in NR vs. −142.0‰ in NL) despite a 4 fold higher concentration in the NL culture. CoA, coenzyme A; DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-ᴅ-xylulose 5-phosphate; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-ᴅ-erythritol 4-phosphate.

capsa oceanica in which temperature did not influence D/H fractionation in alkenones, while salinity and growth rate did. A reanalysis of the Schouten et al. (2006) data, however, suggests that changing temperatures likely influenced both the growth rate and the hydrogen isotope fractionation (ε) in alkenones. Increasing temperature from 15 to 21 °C in the *G. oceanica* cultures, and from 10 to 15 °C in the *E. huxleyi* cultures, resulted in higher growth rates in both species (Fig. 5A and B). Above 15 °C *E. huxleyi* growth rates declined (Fig. 5B), implying that 15 °C may have been the optimum growth temperature (OGT). Pronounced inhibitory effects are observed in phytoplankton when the temperature exceeds the optimum by as little as 2–3 °C (Kruger and Eloff, 1978; Payer et al., 1980; Richard, 1986). We therefore limit our discussion to the range in which a temperature increase appeared to enhance growth.

Schouten et al. (2006) found that δD values in *G. oceanica* were lower by 16−26‰ at 21 °C relative to 15 °C, amounting to a 3‰ decrease per °C (Table 4; Fig. 5C), similar to the temperature dependency in our *E. unicocca* and *V. aureus* cultures (Table 1; Fig. 3). In addition, δD values in *E. huxleyi* were lower by 6−32‰ at 15 °C relative to 10 °C, but that trend did not continue above 15 °C (Table 4, Fig. 5D). Moreover, ratios of fractionation to growth rate (ε/μ, designed to isolate the temperature effect by normalizing the growth rate contribution) were significantly lower at higher temperature, averaging −366‰ and −262‰ day divisions^{-1} in *G. oceanica* at 15 and 21 °C, respectively, and −410‰ and −205‰ day divisions^{-1} in *E. huxleyi* at 10 and 15 °C, respectively (Table 4, Fig. 5E and F). If growth rate were the primary influence on D/H fractionation we would expect similar ε/μ values at different temperatures. It is

therefore possible that the effect of temperature on D/H fractionation in alkenones from the cultured coccolithophorids exceeded any effect from growth rate. Controlled experiments with chemostat cultures will be required to evaluate that possibility.

4.4. Implications for paleoclimate reconstructions based on lipid δD values

This preliminary study highlights the need to consider environmental influences on D/H fractionation when interpreting sedimentary lipid δD values, particularly if a large range in temperature and/or growth rate may have occurred. Though increased D/H fractionation at elevated temperatures was observed in lipids from *E. unicocca* and *V. aureus* in this study, and in *G. oceanica* in Schouten et al. (2006), it is too early to quantify the magnitude or universality of this effect. Locations where historical temperature changes were likely to have been small ought to be targeted until additional laboratory and field experiments are performed.

The complexity of the temperature effect on D/H fractionation is due, in part, to the fact that enzymes in different organisms may have different responses to temperature, and that the response may be non-linear. D/H fractionation responses to temperature are therefore also likely to be species specific, and ubiquitous algal and plant lipids in sediments, such as most fatty acids, *n*-alkanes, *n*-alcohols and *n*-acids (i.e., the leaf wax lipids), are likely to have D/H ratios reflecting multiple organism-specific temperature responses.
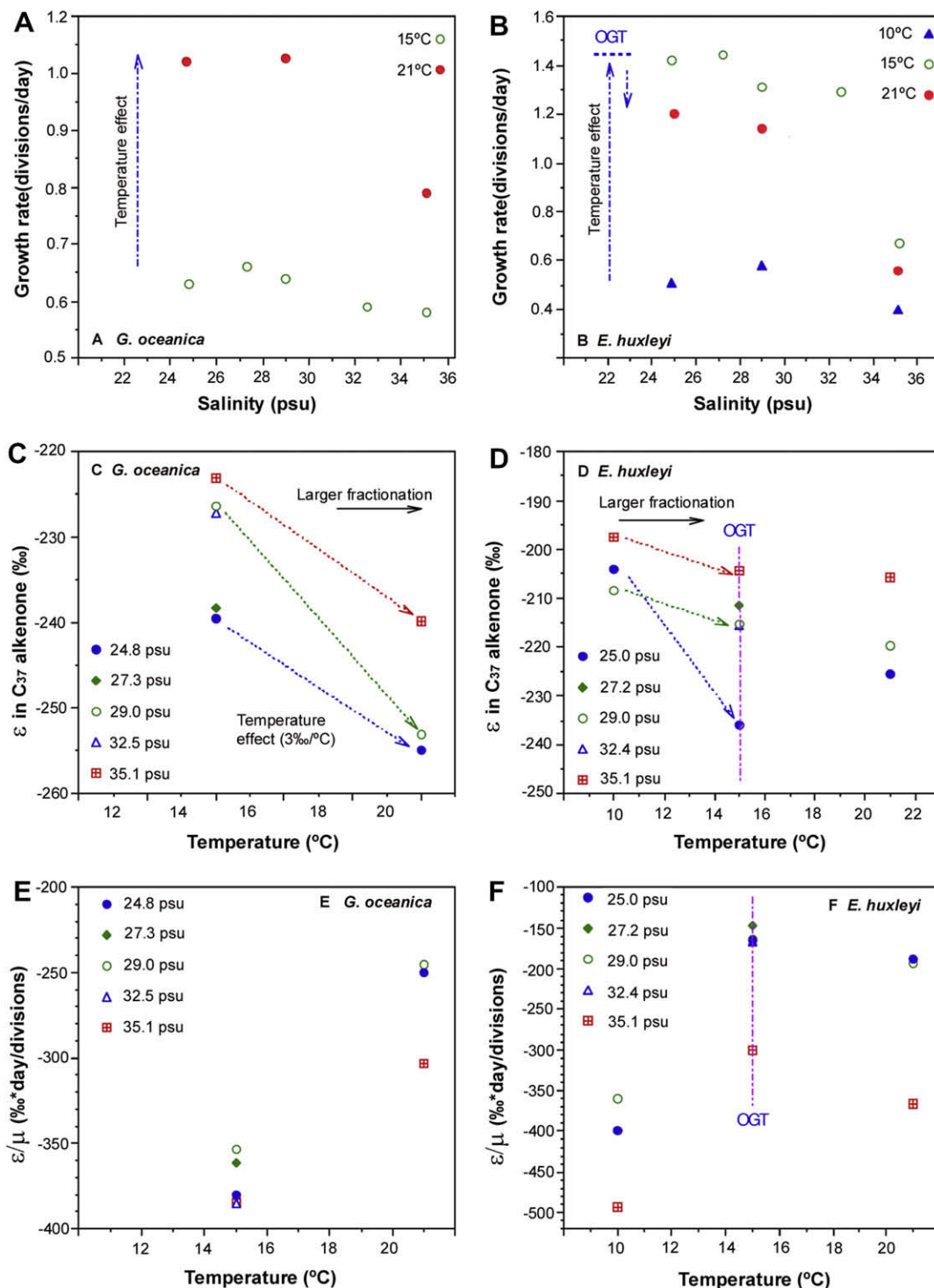
Fig. 5. Reanalyses of the Schouten et al. (2006) data on the temperature and growth rate effect on *D/H* fractionation in C_{37} alkenones from *Emiliania huxleyi* and *Gephyrocapsa oceanica*. (A) Relationship between growth rate (μ) and temperature (*T*) in *G. oceanica*. (B) Relationship between μ and *T* in *E. huxleyi*. (C) Relationship between ε and *T* in *G. oceanica*. (D) Relationship between ε and *T* in *E. huxleyi*. (E) Relationship between ε normalized by growth rate (ε/μ) and *T* in *G. oceanica*. (F) Relationship between ε normalized by growth rate (ε/μ) and *T* in *E. huxleyi*.

Conversely, it is likely that *D/H* fractionation in acetogenic (linear) lipids, such as normal alkanes, ketones, aldehydes, acids, and their unsaturated analogs, is not influenced by growth rate. Alkenones, alkadienes, *n*-alkanes, fatty acids and other linear lipids are therefore good targets for *D/H*-based hydrologic reconstructions. Owing to the possible sensitivity of *D/H* fractionation to nitrate limited growth rate changes in branched (isoprenoid) lipids – especially those from plants and algae that contain two synthetic routes for the synthesis of IPP, as is the case in most higher plants – isoprenoids may not be good targets for paleohydrologic reconstructions. Since all chlorophytes use the DOXP/MEP pathway exclusively for isoprenoid synthesis (Lichtenthaler, 1999; Schwender et al., 2001) their isoprenoid lipids (e.g., botryococcenes) are unlikely to be influenced by nutrient induced growth rate effects on *D/H* fractionation and ought to be good targets for paleoclimate reconstructions.

Table 4
Schouten et al (2006) data on hydrogen isotope fractionation in C_{37} alkenones from *Gephyrocapsa oceanica* and *Emiliania huxleyi*.

Salinity	Temperature (°C)	Growth rate (μ)	α	$\Delta\alpha_{HT-LT}$	ε	$\Delta\varepsilon_{HT-LT}$	ε/μ
G. oceanica							
24.8	15	0.63	0.758		−242.4		−384.7
24.7	21	1.02	0.741	−0.016	−258.6	−16.3	−253.6
27.3	15	0.66	0.761		−239.4		−362.7
29.0	15	0.64	0.777		−222.8		−348.1
29.0	21	1.03	0.751	−0.026	−249.0	−26.2	−241.8
32.5	15	0.59	0.782		−217.9		−369.2
35.1	15	0.58	0.788		−211.9		−365.4
35.1	21	0.79	0.771	−0.017	−229.1	−17.2	−290.0
Average ε/μ at 15 °C=			−366.1		standard deviation=		18.3
Average ε/μ at 21 °C=			−261.8		standard deviation=		25.2
E. huxleyi							
24.9	10	0.51	0.791		−209.2		−410.2
24.9	15	1.42	0.759	−0.032	−241.2	−32.0	−169.9
25.0	21	1.20	0.772		−228.0		−190.0
27.2	15	1.44	0.788		−212.2		−147.4
29.0	10	0.58	0.792		−207.6		−358.0
29.0	15	1.31	0.786	−0.006	−214.0	−6.4	−163.4
29.0	21	1.14	0.783		−217.3		−190.6
32.4	15	1.29	0.793		−206.5		−160.1
35.1	10	0.40	0.815		−184.9		−462.3
35.1	15	0.68	0.808	−0.007	−192.4	−7.4	−282.9
35.1	21	0.56	0.811		−189.5		−338.4
Average ε/μ at 10 °C=			−410.2		standard deviation=		52.2
Average ε/μ at 15 °C=			−205.4		standard deviation=		67.2

α, ε data are from Schouten et al. (2006). The shaded areas highlight the temperature range in which temperature increase favors growth and salinities are the same.
$\Delta\varepsilon_{HT-LT}$ is the ε difference between cultures grown at 15 and 21 °C for *G. oceanica*, and at 10 and 15 °C for *E. huxleyi*.

When evaluating the effect of temperature on lipid δD records from lake sediments two processes need to be considered. The first is the aforementioned temperature effect on the D/H fractionation during lipid biosynthesis. The second is the effect of temperature on precipitation δD values that amounts to approximately a 4−6‰ change in δD per °C change in temperature (Dansgaard, 1964).

Huang et al. (2002) showed that the δD value of palmitic acid in Crooked Pond, Massachusetts, USA ranged from −230‰ to −160‰ during the last 12,000 yrs. This 70‰ range is probably larger than could reasonably explained by a temperature change alone, which would have to have been approximately 13 °C given the −3‰/°C sensitivity to temperature in our cultures. In addition to a temperature contribution, the 70‰ range in palmitic acid δD from Crooked Pond thus either indicates a change in precipitation (source, amount, evaporation, etc.) or a variable source of palmitic acid over time from vascular plants to aquatic plants and algae, or between different aquatic plants and/or algae (c.f., Zhang and Sachs, 2007), or some combination of precipitation and source changes.

5. Conclusion

Cells grown at 25 °C exhibited substantially higher D/H fractionation than those grown at 15 °C, with the mean difference in α being 0.034 ± 0.006 and that of ε being 34.4 ± 6.6‰ for six different acetogenic lipids, four in *E. unicocca* and two in *V. aureus*. D/H fractionation increased with increasing water temperature in C_{16} and C_{18} fatty acids, heptadecene, and naturally occurring FAMEs. The complex role temperature plays in enzyme activity and metabolism make it difficult to conclude that any one mechanism is responsible for the temperature dependence of D/H fractionation at this time.

Higher growth rates were associated with greater D/H fractionation in isoprenoid lipids (sterols), but had no effect on acetogenic lipids (fatty acids) in the marine diatom *T. pseudonana*. A 4.4 fold difference in growth rate due to nitrogen limitation resulted in a 37‰ increase in D/H fractionation (ε) in 24-methyl-cholesta-5,24(28)-dien-3β-ol, possibly the result of an increased supply of deuterium depleted isoprenoid intermediates from the plastidic DOXP/MEP synthetic pathway to the cytosolic MVA pathway when growth rates were high.

The content of fatty acids in faster growing (nitrogen replete) *T. pseudonana* cells was less than ¼ of that in slower growing (nitrogen limited) cells, while sterol concentrations were constant. This difference likely resulted from a shift of carbon flows into nitrogen containing compounds and away from fatty acids in the faster growing cells.

Additional controlled experiments with several species of microalgae are required to further characterize the role of temperature and nutrient limitation on D/H fractionation in lipids, and to discern the underlying mechanisms responsible for the isotopic differences. For example, culturing various families of algae over a small range of temperatures would help elucidate the role of different enzyme systems. Tracer experiments with ^{13}C or ^{14}C labelled precursors would help to elucidate to what extent the IPPs from DOXP pathway cross into MVA pathway under different growth rates.

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