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Hydrogen isotope fractionation in freshwater algae: I. Variations among lipids and species

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Abstract

Five species of freshwater green algae, including three strains of *Botryococcus braunii* (two A Race, one B Race), *Eudorina unicocca* and *Volvox aureus*, were cultured under controlled conditions in media containing different concentrations of deuterium. The hydrogen isotopic ratios of lipids in the algae, including alkadienes, botryococcenes, heptadecenes, fatty acids, and phytadiene, were measured by gas chromatography–isotope ratio-mass spectrometry (GC–IRMS) and found to closely track water δD values. While correlation coefficients (R^2) in excess of 0.99 for all lipids in all species suggest that lipid δD values can be used to determine water δD values, hydrogen isotope fractionation was found to vary systematically between lipids and lipid homologues within a single alga, as well as for the same lipid between species of algae. Under similar growth conditions, two species of Chlorophyceae (*Eudorina unicocca* and *Volvox aureus*) and three species of Trebouxiophyceae (*Botryococcus braunii*) produced palmitic acid (C_{16} fatty acid) that differed by 90–100‰ relative to water. Ubiquitous lipids such as palmitic acid, with a multitude of aquatic and terrestrial sources, are therefore not good targets for D/H-based paleohydrologic reconstructions. In addition to the use of source-specific biomarkers that derive unambiguously from a single family or species, paleohydrologic applications of lipid D/H ratios will need to consider the as yet unstudied potential influence that environmental parameters such as nutrients, light and temperature, etc., may have on D/H fractionation during lipid synthesis.

1. Introduction

Hydrologic variations are difficult to reconstruct from the geologic record and are poorly reproduced by climate models, yet they are essential for understanding the natural variation of climate, especially in the tropics. Hydrogen and oxygen isotope ratios of lake and ocean water reveal hydrologic variations caused by the higher vapor pressure of HHO and $H_2{}^{16}O$ relative to HDO and $H_2{}^{18}O$. However, these ratios are often not preserved in the geologic record due to isotopic exchange or diagenesis or, as with mineral phases, are influenced by non-hydrologic processes. The 'holy grail' of paleohydrologic reconstructions is therefore a robust recorder of water

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isotopic variations that does not undergo alteration on the timescale of study.

The hydrogen isotopic composition of algal lipid biomarkers in lake sediments holds great potential for water isotopic reconstruction. All hydrogen in algae derives from water and algae do not transpire, a process that overprints δD values in land plants. Whereas hydrogen atoms covalently bound to organic nitrogen, sulfur, and oxygen are labile and exchange quickly and reversibly with hydrogen atoms in water (Werstiuk and Ju, 1989), hydrogen atoms in lipids are bound to carbon (Estep and Hoering, 1980) and are nonexchangable (Epstein et al., 1976; Sauer et al., 2001). Compared to bulk organic matter, individual lipid biomarkers are not subject to isotopic changes caused by preferential degradation of less stable compounds.

Despite the strong potential of algal lipids in D/H reconstructions technical challenges in continuous flow-isotope ratio mass spectrometry have limited their application. With these technical problems all but overcome in recent years (cf. Burgoyne and Hayes, 1998; Hilkert et al., 1999; Sessions et al., 1999) the next step in developing compound-specific hydrogen isotopic reconstructions of water D/H ratios is demonstrating that lipid biomarker δD values closely track water δD values. Several recent studies have addressed this issue (Sessions et al., 1999; Sessions, 2006; Sauer et al., 2001; Huang et al., 2002, 2004; Sachse et al., 2004; Chikaraishi et al., 2004a,b,c, 2005; Chikaraishi, 2006; Englebrecht and Sachs, 2005; Schouten et al., 2006). However, this is the first to systematically evaluate the fidelity with which δD values in a variety of lipid biomarkers from cultured freshwater microalgae record water δD values.

Estep and Hoering (1980, 1981) pioneered hydrogen isotopic biogeochemistry by measuring the δD values of total organic matter in axenic algal cultures that were $-90‰$ to $-110‰$ relative to the water. They concluded that (1) photosynthesis was the primary process causing hydrogen isotopic fractionation in plants, (2) some additional fractionation occurred in the dark reactions during the synthesis of malic or pyruvic acids and lipids, and (3) little hydrogen isotope fractionation occurred during respiration.

Englebrecht and Sachs (2005) cultured the marine microalga *Emiliania huxleyi* and demonstrated that alkenones closely tracked water δD values ($R^2 > 0.999$). Huang et al. (2004) showed that δD values of palmitic acid ($C_{16:0}$ fatty acid) in core-top sediments from lakes in eastern North America were well-correlated with both water δD values of the lakes and air temperature ($R^2 = 0.89$), and Sachse et al. (2004) showed that *n*-alkanes in core top sediments from lakes in a meridional transect through central Europe co-varied with δD values of precipitation.

To better constrain the extent to which algal lipids record water δD values and to address the species-dependence of hydrogen isotope fractionation in lipids we cultured five species of freshwater green algae – *Eudorina unicocca*, *Volvox aureus* (both belong to Chlorophyceae) and three strains of *Botryococcus braunii* (Trebouxiophyceae) – under controlled conditions in the laboratory, with each strain grown in five media containing different enrichments of deuterium. We measured δD values of several lipids from each culture, including alkadienes, botryococcenes, heptadecene, fatty acid methyl esters (FAMEs), phytadiene and free fatty acids. Reported here are the results of hydrogen isotopic fractionation in those lipids from the five freshwater algal species.

2. Methods

2.1. Algal cultures

2.1.1. Algal species

Five species of freshwater green algae were cultured: *Eudorina unicocca*, *Volvox aureus* and three strains of *Botryococcus braunii*. Relative to red and brown algae, green algae are closer in origin to land plants (Kirk, 1998).

Botryococcus braunii is a colonial member of Trebouxiophyceae (Senousy et al., 2004), characterized by high production of lipids and widely distributed in freshwater lakes and ponds. Interest in *Botryococcus braunii* arose in the 1960s when the algae were discovered to synthesize large amounts of hydrocarbons (cf. Maxwell et al., 1968; Douglas et al., 1969). Axenic inoculants of three strains of *Botryococcus braunii* were provided by Dr. Pierre Metzger of the Laboratoire de Chimie Bioorganique et Organique Physique in France. Two were from the A race, one from Morocco (a small pool in Oukaidem, Atlas) and the other from Lake Titicaca (Bolivia). The third was from the B race, from Martinique (French West Indies).

Eudorina unicocca and *Volvox aureus* both belong to Chlorophyta (green algae), chlorophyceae, volvocales and they are the most common single-celled green algae in natural water bodies. Each possesses

biflagellate cells held together in a coenobium of defined shape, usually spherical (Kirk, 1998). The inoculants of *Eudorina unicocca* and *Volvox aureus* were supplied by the Culture Collection of Algae and Protozoa (CCAP) in Cumbria, United Kingdom (now located in Dunstaffnage Marine Laboratory, Oban, Scotland). The two cultured strains were *Eudorina unicocca* G.M. Smith 1930 (CCAP 24/1C, originated from freshwater near Bloomington, Indiana, USA) and *Volvox aureus* Ehrenberg 1838 (CCAP 88/6, originated from freshwater in Malham Tarn, Yorkshire, England).

We conducted the culture experiments in the laboratory of Dr. Daniel Repeta at the Woods Hole Oceanographic Institution from July to November, 2003.

2.1.2. Preparation of deuterium-enriched water and the determination of water δD values

The reference standard for D/H ratios in this work is Vienna Standard Mean Ocean Water (VSMOW) with $D/H = 155.76 \pm 0.05 \times 10^{-6}$. δD is defined as

$$\delta D = [(D/H)_{\text{sample}} - (D/H)_{\text{VSMOW}}]/(D/H)_{\text{VSMOW}} \times 1000‰$$

In order to investigate the relationship between lipid δD and water δD over a wide range of values, we set up five cultures for each species in waters with different deuterium concentrations. We mixed varying amounts of Aldrich deuterium oxide (99.9% D) with distilled water ($\delta D = -65‰$) to produce medium with five different δD values, ranging from $-65‰$ to $+450‰$.

Water δD values were measured on an H-device-Thermo Finnigan Delta^{plus} XL mass spectrometer at Dartmouth College. The H-Device consists of a quartz reactor filled with chromium powder and a series of valves. The quartz reactor was sealed at one end by a septum and at the other end by a pneumatic valve. The reactor was held at 850 °C and the air evacuated by the vacuum system in the mass spectrometer. 1 μl of water was injected through the septum and flash evaporated. The water was reacted for one minute and the resulting gases admitted to the inlet system of the mass spectrometer. Precision of the water δD analyses was $0.5‰$.

2.1.3. Culture media

The medium for the culture experiments was autoclaved before algal inoculants were introduced. Glassware was acid-leached overnight and rinsed with deionized water, then autoclaved. Algal transfers were conducted in a laminar flow bench with sterilized labware.

B. braunii was grown on a modified CHU 13 medium (composition in mg/l): KNO_3, 200; $K_2HPO_4{\cdot}3\,H_2O$, 54; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 2H_2O$, 52; FeNaEDTA, 10; plus 5 ml of a solution of micronutrients (mg/l): H_3BO_3, 286; $MnSO_4 \cdot H_2O$, 154; $ZnSO_4 \cdot 7H_2O$, 22; $CuSO_4 \cdot 5H_2O$, 8; $Na_2MoO_4 \cdot 2H_2O$, 6; $CoSO_4 \cdot 7H_2O$, 9. The pH was approximately 7.4.

Cultures of *E. unicocca* and *V. aureus* were grown in Jaworski's Medium (JM). The stock composition in g/200 ml was: (1) $Ca(NO_3)_2 \cdot 4H_2O$, 4.0; (2) K_2HPO_4, 2.48; (3) $MgSO_4 \cdot 7H_2O$, 10.0; (4) $NaHCO_3$, 3.18; (5) FeNaEDTA, 0.45; Na_2EDTA, 0.45; (6) H_3BO_3, 0.496; $MnCl_2 \cdot 4H_2O$, 0.278 g; $(NH4)_4Mo_7O_{24} \cdot 4H_2O$, 0.20 g; (7) Cyanocobalamin, 0.008 g; Thiamine HCl, 0.008 g; Biotin, 0.008 g; (8) $NaNO_3$, 16.0 g; (9) $Na_2HPO_4 \cdot 12H_2O$, 7.2 g. Stock solutions 1–9 were diluted to 1 l with deionized water.

2.1.4. Light intensity and temperature control

Continuous light was provided by two banks of two 25 W cool-white fluorescent tubes. Light intensity was measured with a Biospherical Instruments QSL-100 probe equipped with a QSP-170 sensor. The center of the chamber was $\sim 20 \times 10^{15}$ quanta/scm^2, or 320 μmol/m^2 s. Illumination was on a 12 h light:12 h dark photo cycle.

The 15 cultures of *B. braunii* (A1W1-5, A2W1-5 and BBW1-5; A1: A race, Morocco strain; A2, A race, Titicaca strain; BB: B race, Martinique strain) were grown at summer room temperature (July–September, 2003), which averaged 28 °C but varied from 26.5 °C to 30 °C.

E. unicocca and *V. aureus* were grown at 15 °C in a closed chamber built from foam insulation and cooled with an air-conditioner (Haier, 5000 BTU). The temperature inside the chamber varied between 14.5–16 °C during the course of the experiment (EUW1-5, VVW1-5; EU: *E. unicocca*; VV: *V. aureus*).

2.1.5. Aeration

To expedite algal growth the culture medium was bubbled with 1% CO_2 in purified air that had been passed through two 0.2 μm membrane filters. Metzger et al. (1985a) reported that hydrocarbon levels of *Botryococcus brauni* were greatly affected by culture conditions; air augmented with 1% CO_2 increased the hydrocarbon contents from 5% in

unaerated cultures to 20–61% in aerated cultures, depending on the strain.

All plastic tubing and filters were autoclaved before use. Air was introduced into the medium near the bottom of the flask so that bubbling kept the algae in suspension. The flow rate was ~250 ml/min. 1% CO_2 air was made by mixing N_2 gas and gas from tanks of 95% O_2 and 5% CO_2 in a cell filled with glass beads.

Evaporation during the course of the experiments caused deuterium enrichment in the medium that we evaluated with a control experiment. A culture flask with 1.5 l water ($\delta D = -67.7\%$) was aerated with the algal cultures and sampled every five days. After 45 days the water was enriched in deuterium by 45‰. Aliquots of medium were therefore collected from every culture flask throughout the course of the experiments for D/H analysis. δD_{water} values at the start and end of each culture experiment are shown in Tables 1–3.

2.1.6. Cell density monitoring and harvest

Cell density was monitored daily by measuring a small aliquot of each culture on a HP8452A Diode Array Spectrophotometer. Because the aliquots were not returned to the culture flasks (in order to prevent contamination) they typically constituted the largest source of water loss during the experiments. Growth curves for each strain of *B. braunii,* and for *E. unicocca* and *V. aureus* were obtained by measuring the absorption of light at 400, 500, 600, and 700 nm. Absorption curves at different wavelengths are nearly identical. Representative absorption curves for 600 nm light are shown in SFig. 1 (supplementary material). The continuous increase in light absorption (and by extension, cell density) with time was partly caused by evaporative loss of water resulting from aeration. *B. braunii*, and *E. unicocca*/*V. aureus* cultures were harvested after 45 days and 31 days, respectively, during the exponential-phase on the growth curves.

Cultures were harvested by helium-pressurized filtration through 293 mm diameter Whatman GF/F filters (pore size 0.7 μm), and subsequently kept at −20 °C until analysis. A small aliquot of filtrate was filtered through a 0.2 μm membrane filter to obtain water for δD analysis.

2.2. Lipid extraction and isolation

Harvested algae on GF/F filters were freeze-dried, cut into 0.5 × 0.5 cm pieces, and extracted on a Dionex ASE-200 pressurized fluid extractor with dichloromethane (DCM) and methanol (MeOH) (9:1) at 1500 psi and 150 °C. The total lipid extract was fractionated on an aminopropyl cartridge-style SPE column (Burdick & Jackson, size 500 mg/4 ml) with DCM/isopropyl alcohol (IPA) (3:1). Retained fatty acids were recovered with 4% acetic acid in diethyl ether, methylated with 10% BF_3 in MeOH and purified by urea adduction. The DCM/IPA fraction was fractionated by column chromatography using 5% water-deactivated silica gel in a 29 cm × 1.2 cm glass column. Hydrocarbons were eluted with hexane, FAMEs and phytadienes with 10% ethyl acetate (EtOAc) in hexane, and alcohols (including phytol and sterols) with MeOH. FAMES and phytadienes were purified further by urea adduction.

Each fraction was analyzed by gas chromatography–mass spectrometry (GC–MS) to positively identify each lipid and determine its purity, then by gas chromatography with flame-ionization detection (GC-FID) to determine individual lipid concentrations. Agilent 6890 gas chromatographs were operated with programmable temperature vaporization (PTV) inlets, 60 m Varian Chrompac CP-Sil 5 capillary columns with 0.32 mm i.d. and 0.25 μm film thickness, and helium carrier gas. Oven temperature programs differed for each lipid class.

Alkadienes in the Titicaca strain and botryococcenes in the Martinique strain in *B. braunii* cultures were hydrogenated with Raney Nickel in order to elucidate their exact structures.

2.3. Determination of molecular D/H ratios

The hydrogen isotopic composition of each lipid fraction was measured by gas chromatography–isotope ratio-mass spectrometry (GC–IRMS) with a Finnigan Delta^{plus} XP mass spectrometer, equipped with a Trace GC and a Combustion III interface.

The Trace GC was equipped with a PTV inlet operated in splitless mode, a 30 m DB-5 capillary column (J&W Scientific) with 0.25 mm i.d. and 0.25 μm film, and was operated at a constant helium flow rate of 1 ml/min. The oven temperature program differed for each lipid class. For hydrocarbons, the starting temperature was 90 °C, rising to 230 °C at 13 °C/min, then to 325 °C at 5.5 °C/min, followed by 13 min at 325 °C. Effluent from the GC entered the GC-C III interface, a graphite-lined ceramic tube at 1400 °C, where quantitative pyrolysis to graphite, hydrogen gas and carbon monoxide occurred (Burgoyne and Hayes, 1998). The hydrogen gas stream

Table 1
Hydrogen isotope ratios and *D*/*H* fractionation in hydrocarbons from three strains of *B. braunii*

Cultures	H_2O δD	H_2O δD	C_{27} alkadiene				C_{29} alkadiene				C_{29} alkatriene				C_{31} alkadiene			
	start	harvest	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε
Botryococcus brauni, A race, Morocco strain																		
A1W1	−64.5	−29.5	−184.9	3.7	0.840	−160.1	−235.7	1.0	0.788	−212.4	−232.6	3.2	0.791	−209.2	−225.5	1.9	0.798	−201.9
A1W2	88.4	117.1	−84.3	0.9	0.820	−180.2	−122.3	1.4	0.786	−214.3	−123.5	0.3	0.785	−215.4	−116.2	2.8	0.791	−208.9
A1W3	187.7	220.8	−35.8	2.1	0.790	−210.2	−58.1	3.4	0.772	−228.5	−64.1	3.1	0.767	−233.3	−51.5	1.3	0.777	−223.1
A1W4	285.6	316.9	32.6	2.4	0.784	−215.9	18.6	2.2	0.773	−226.5	10.2	2.9	0.767	−232.9	23.4	3.1	0.777	−222.8
A1W5	472.2	497.2	168.6	2.9	0.780	−219.5	162.5	1.1	0.776	−223.5	151.9	1.9	0.769	−230.6	169.4	3.2	0.781	−219.0
Average					0.803	−197.2			0.779	−221.0			0.776	−224.3			0.785	−215.1
Standard deviation					0.026	25.9			0.007	7.3			0.011	11.2			0.009	9.4

Cultures	H_2O δD	H_2O δD	C_{27} alkadiene				C_{29} alkadiene				C_{31} alkadiene			
	start	harvest	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε
Botryococcus brauni, A race, Titicaca strain														
A2W1	−64.0	−26.0	−224.0	1.4	0.797	−203.3	−244.9	2.2	0.775	−224.8	−243.7	1.9	0.776	−223.5
A2W2	87.6	128.6	−122.9	0.9	0.777	−222.9	−120.4	1.0	0.779	−220.6	−122.6	0.8	0.777	−222.6
A2W3	182.1	221.3	−55.0	2.2	0.774	−226.2	−57.6	2.8	0.772	−228.4	−56.2	2.9	0.773	−227.2
A2W4	280.7	321.1	20.4	4.9	0.772	−227.6	7.0	3.3	0.762	−237.7	7.3	3.4	0.762	−237.5
A2W5	458.0	499.7	135.7	4.9	0.757	−242.7	130.8	5.2	0.754	−246.0	131.8	6.8	0.755	−245.3
Average					0.775	−224.5			0.769	−231.5			0.769	−231.2
Standard deviation					0.014	14.1			0.010	10.3			0.010	9.8

Cultures	H_2O δD	H_2O δD	C_{30} botryococcene				C_{31} botryococcene				C_{34} botryococcene(4)				C_{34} botryococcene iso(5)+?[a]				$C_{32}+C_{33}$ botryococcene[a]				C_{34} botryococcene iso?[b]			
	start	harvest	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε	δD	σ	α	ε
Botryococcus braunii, B race, Martinique																									
BBW1	−64.0	−18.9	−288.7	1.4	0.725	−275.0	−310.3	3.9	0.703	−297.0	−334.4	4.1	0.678	−321.5	−325.5	4.6	0.687	−312.5	−331.1	3.5	0.682	−318.2	−373.4	1.5	0.639	−361.3
BBW2	102.2	161.1	−172.9	2.4	0.712	−287.6	−184.8	1.2	0.702	−297.9	−224.3	0.9	0.668	−331.9	−206.0	1.1	0.684	−316.2	−213.3	1.6	0.678	−322.4	−258.7	3.1	0.638	−361.6
BBW3	184.1	230.2	−102.1	2.0	0.730	−270.1	−125.8	2.1	0.711	−289.4	−162.7	1.2	0.681	−319.4	−148.7	2.8	0.692	−308.0	−151.3	2.9	0.690	−310.1	−205.8	2.2	0.646	−354.4
BBW4	273.7	327.6	−16.3	3.8	0.741	−259.0	−56.7	5.7	0.711	−289.5	−91.4	3.2	0.684	−315.6	−81.4	6.6	0.692	−308.0	−85.9	3.7	0.689	−311.5	−147.0	4.1	0.643	−357.4
BBW5	455.1	513.6	101.4	2.8	0.728	−272.3	76.8	6.1	0.711	−288.6	48.1	1.7	0.692	−307.5	58.4	2.1	0.699	−300.7	37.8	4.2	0.686	−314.3	−29.6	2.2	0.641	−358.8
Average					0.727	−272.8			0.708	−292.5			0.681	−319.2			0.691	−309.1			0.685	−315.3			0.641	−358.7
Standard deviation					0.010	10.3			0.005	4.6			0.009	8.9			0.006	5.8			0.005	5.1			0.003	3.0

Each strain was grown in five media containing different concentrations of deuterium. The δD value of each lipid was measured in triplicate and the average and standard deviation are reported.
The δD value of the culture medium at the start and harvest are also shown.
iso, isomer; σ, standard deviation.
Frationation factor, α, is defined as $(D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$.
Fractionation, ε, is defined as $(\alpha - 1)*1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1]*1000$.

[a] The peaks of C_{34} botryococcene (compound **5**), C_{32} botryococcene (compound **6**) and C_{33} botryococcene (compound **7**) coelute into two peaks and it is hard to distinguish among them. The peak of C_{34} botryococcene isomer (**5**) + ? is likely the coelution of C_{34} isomer (compound **5**) and the unidentified peak before it. The assigned $C_{32} + C_{33}$ botryococcene could even include the small peak of C_{34} botryococcene isomer which elutes after them.

[b] This peak could be another C_{34} botryococcene or another homologue with more carbon atoms.

Table 2
Hydrogen isotope ratios and D/H fractionation in free fatty acids, natural FAMEs and phytadienes in three strains of *B.braunii*

Cultures	H_2O δD at start	H_2O δD at harvest	C_{16} fatty acid				$C_{18:1}$ fatty acid				$C_{20:1}$ fatty acid				$C_{26:1}$ fatty acid				$C_{28:1}$ fatty acid				$C_{30:1}$ fatty acid			
			δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε
Botryococcus braunii, B race, Martinique strain																										
BBW1	−64.0	−18.9	−192.1	3.4	0.823	−176.6	−191.6	3.1	0.824	−176.0	−177.9	0.3	0.838	−162.1	−187.9	1.7	0.828	−172.3	−189.4	3.5	0.826	−173.8	−191.0	0.9	0.825	−175.4
BBW2	102.2	161.1	−37.8	0.5	0.829	−171.3	−31.1	0.2	0.835	−165.5	−11.0	4.0	0.852	−148.2	−33.0	4.0	0.833	−167.1	−30.9	1.5	0.835	−165.4	−27.1	2.4	0.838	−162.0
BBW3	184.1	230.2	15.9	2.0	0.826	−174.2	19.4	3.5	0.829	−171.3	33.2	5.8	0.840	−160.2	12.6	6.3	0.823	−176.9	20.4	2.2	0.829	−170.5	ND	ND	ND	ND
BBW4	273.7	327.6	95.4	3.6	0.825	−174.9	86.8	4.9	0.819	−181.3	115.3	1.3	0.840	−159.9	ND	ND	ND	ND	96.8	1.2	0.826	−173.8	ND	ND	ND	ND
BBW5	455.1	513.6	228.8	0.7	0.812	−188.1	233.7	3.5	0.815	−184.9	256.2	1.1	0.830	−170.0	ND	ND	ND	ND	242.7	3.0	0.821	−179.0	ND	ND	ND	ND
Average					0.823	−177.0			0.824	−175.8			0.840	−160.1			0.828	−172.1			0.828	−172.5			0.831	−168.7
Standard deviation					0.007	6.5			0.008	7.8			0.008	7.8			0.005	4.9			0.005	5.0			0.009	9.5
Botryococcus braunii, A race, Titicaca strain																										
A2W1	−64.0	−26.0	−206.5	1.3	0.815	−185.4	−205.6	1.9	0.816	−184.4	−175.9	4.4	0.846	−153.9	−167.2	5.8	0.855	−145.0	−184.2	4.9	0.838	−162.4	−175.6	0.2	0.846	−153.6
A2W2	87.6	128.6	−85.1	0.5	0.811	−189.4	−79.2	1.0	0.819	−181.4	−61.9	2.0	0.831	−168.8	−57.3	4.3	0.835	−164.8	−62.7	1.1	0.831	−169.5	−59.1	1.2	0.834	−166.3
A2W3	182.1	221.3	−25.3	1.5	0.798	−201.9	−6.6	2.5	0.813	−186.6	ND	ND	ND	ND	−10.2	6.0	0.810	−189.6	−2.2	1.1	0.817	−183.0	6.0	3.3	0.824	−176.3
A2W4	280.7	321.1	85.4	4.7	0.822	−178.4	72.5	2.1	0.812	−188.1	9739	5.5	0.831	−168.9	ND	ND	ND	ND	95.7	2.1	0.89	−170.6	109.1	5.0	0.840	−160.5
A2W5	485.0	499.7	189.4	5.5	0.793	−206.9	206.8	5.9	0.805	−195.3	228.4	3.0	0.819	−180.9	ND	ND	ND	ND	218.7	4.0	0.813	−187.4	229.0	2.8	0.820	−180.5
Average					0.808	−192.4			0.813	−187.2			0.832	−168.1			0.834	−166.4			0.825	−174.6			0.833	−167.4
Standard deviation					0.012	11.8			0.005	5.2			0.011	11.1			0.022	22.3			0.010	10.3			0.011	11.1
Botryococcus braunii, A race, Morocco strain																										
A1W1	−64.5	−29.5	−219.9	3.1	0.804	−196.2	−210.0	3.4	0.814	−185.9	−185.5	3.4	0.839	−160.7	−197.5	0.5	0.827	−173.0	−199.8	1.4	0.825	−175.4	−206.4	4.3	0.818	−182.0
A1W2	88.4	117.1	−103.0	1.6	0.803	−197.1	−91.0	2.8	0.814	−186.3	−67.2	1.1	0.835	−165.0	−77.5	3.6	0.826	−195.9	5.4	1.1	0.824	−176.5	0.2	4.4	0.819	−180.7
A1W3	187.7	220.8	−21.4	1.8	0.802	−198.4	−8.6	0.4	0.812	−187.9	12.4	5.3	0.829	−170.8	−18.3	7.4	0.804	−195.9	5.4	1.1	0.824	−176.5	0.2	4.4	0.819	−180.7
A1W4	285.6	316.9	69.2	1.2	0.812	−188.1	81.0	4.4	0.821	−179.1	ND	ND	ND	ND	ND	ND	ND	ND	81.3	2.2	0.821	−178.9	ND	ND	ND	ND
A1W5	472.2	497.2	198.0	4.3	0.800	−199.9	205.7	3.8	0.805	−194.7	240.2	7.3	0.828	−171.7	229.9	1.8	0.821	−178.5	220.3	3.2	0.815	−185.0	ND	ND	ND	ND
Average					0.804	−195.9			0.813	−186.8			0.833	−167.0			0.820	−180.4			0.823	−177.5			0.821	−178.9
Standard deviation					0.005	4.6			0.006	5.6			0.005	5.2			0.011	10.6			0.005	4.9			0.004	4.3

Cultures	H_2O δD at start	H_2O δD at harvest	Phytadlene				Naturally occuring FAME δD																			
			δD	Stdev	α	ε	C_{16}	Stdev	α	ε	$C_{18:1}$	Stdev	α	ε	$C_{20:1}$	Stdev	α	ε	$C_{28:1}$	Stdev	α	ε	$C_{30:1}$	Stdev	α	ε
Botryococcus braunii, B race, Martinique strain																										
BBW1	−64.0	−18.9	−291.2	7.8	0.722	−277.6	−175.5	1.6	0.840	−159.6	−182.3	0.1	0.833	−166.6	−162.4	5.7	0.854	−1469.3	−179.7	3.5	0.836	−163.9	−182.8	3.2	0.833	−167.0
BBW2	102.2	161.1	−197.5	1.6	0.691	−308.8	−49.1	1.0	0.819	−181.0	−46.1	3.3	0.822	−178.4	−22.5	3.9	0.842	−158.1	−45.2	6.3	0.822	−177.7	−49.4	2.4	0.819	−181.2
BBW3	184.1	230.2	−150.1	1.4	0.691	−309.2	7.0	2.7	0.819	−181.5	1.3	2.7	0.814	−186.0	25.0	5.4	0.833	−166.8	4.7	0.5	0.817	−183.3	ND	ND	ND	ND
BBW4	273.7	327.6	ND	ND	ND	ND	69.3	6.9	0.805	−194.5	79.2	6.1	0.813	−187.1	106.2	4.6	0.833	−166.7	78.8	2.6	0.813	−187.4	ND	ND	ND	ND
BBW5	455.1	513.6	ND	ND	ND	ND	205.8	8.5	0.797	−203.3	208.6	6.5	0.799	−201.5	ND	ND	ND	ND	217.7	0.7	0.804	−195.5	ND	ND	ND	ND
Average					0.702	−298.5			0.816	−184.0			0.816	−183.9			0.841	−159.5			0.818	−181.6			0.826	−174.1
Standard deviation					0.018	18.1			0.017	16.6			0.013	12.8			0.007	9.7			0.012	11.8			0.010	10.1
Botryococcus braunii, A race, Titicaca strain																										
A2W1	−64.0	−26.0	ND	ND	ND	ND	−202.1	4.2	0.819	−180.8	−214.3	1.2	0.807	−193.3	−192.7	1.9	0.829	−171.1	−208.0	3.3	0.813	−186.8	−204.7	5.5	0.817	−183.4
A2W2	87.6	128.6	ND	ND	ND	ND	−78.9	0.9	0.816	−183.8	−83.8	2.3	0.812	−188.2	−69.1	5.0	0.825	−175.1	−72.7	3.4	0.822	−178.3	ND	ND	ND	ND
A2W3	182.1	221.3	ND	ND	ND	ND	8.5	4.6	0.826	−174.3	−0.5	0.9	0.818	−181.6	27.8	4.7	0.842	−158.5	1.8	2.8	0.820	−179.7	ND	ND	ND	ND
A2W4	280.7	321.1	ND	ND	ND	ND	69.9	1.5	0.810	−190.1	72.7	1.6	0.812	−188.0	N.D	N.D	ND	ND	82.3	7.2	0.819	−180.8	ND	ND	ND	ND
A2W5	485.0	499.7	ND	ND	ND	ND	208.0	2.1	0.805	−194.5	206.2	1.0	0.804	−195.7	245.2	0.1	0.830	−169.7	ND	ND	ND	ND	ND	ND	ND	ND
Average									0.815	−184.7			0.811	−189.4			0.831	−168.6			0.819	−181.4			0.817	−183.4
Standard deviation									0.008	7.9			0.005	5.5			0.007	7.2			0.004	3.7			ND	ND

Each strain was grown in five media containing different concentrations of deuterium. The δD value of each lipid was measured in triplicate and the average and standard deviation are reported. δD values of free fatty acids were measured on methyl ester derivatives and corrected for isotopic composition of the added methyl group. The δD value of the culture medium at the start and harvest are also shown.

stdev, standard deviation; ND, not determined due to insufficient quantity of material.

α is defined as $(D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$;$\varepsilon$ is defined as $(\alpha - 1)*1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1]*1000$.

Table 3
Hydrogen isotope ratios and *D/H* fractionation in free fatty acids, FAMEs and phytadiene in *E. unicocca* and *V. aureus*

Cultures	H_2O δD at start	H_2O δD at harvest	C_{14} fatty acid				C_{16} fatty acid				$C_{18unsat}$ fatty acid[a]				C_{18sat} fatty acid[a]			
			δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε	δD	Stdev	α	ε
Eudorina unicocca																		
EUW1	−62.4	−50.5	−135.7	0.9	0.910	−89.8	−104.2	1.9	0.943	−56.6	−97.3	1.6	0.951	−49.3	−116.5	4.8	0.931	−69.5
EUW2	78.1	84.1	−32.2	4.1	0.893	−107.3	35.3	2.1	0.955	−45.0	41.5	4.4	0.961	−39.3	9.4	5.4	0.931	−68.9
EUW3	218.6	220.7	110.4	0.4	0.910	−90.4	167.2	2.4	0.956	−43.9	162.4	4.1	0.952	−47.8	153.7	3.4	0.945	−54.9
EUW4	340.7	340.8	201.2	5.0	0.896	−104.1	273.4	0.2	0.950	−50.2	ND	ND	ND	ND	ND	ND	ND	ND
EUW5	458.8	463.7	298.5	3.8	0.887	−112.8	386.0	3.7	0.947	−53.0	411.8	8.7	0.965	−35.5	379.5	11.6	0.942	−57.5
Average					0.899	−100.9			0.950	−49.8			0.957	−42.9			0.937	−62.7
Standard deviation					0.010	10.3			0.005	5.3			0.007	6.7			0.008	7.6
Volvox aureus											$C_{18:3/1}$[b]							
VVW1	−62.5	−51.2	ND	ND	ND	ND	−87.3	3.3	0.962	−38.1	−77.0	0.9	0.973	−27.2	−90.0	2.4	0.959	−41.0
VVW2	90.8	106.5	ND	ND	ND	ND	23.2	4.2	0.925	−75.3	40.1	3.6	0.940	−60.1	ND	ND	ND	ND
VVW3	226.1	232.6	ND	ND	ND	ND	149.9	1.9	0.933	−67.1	158.5	2.1	0.940	−60.1	161.4	2.1	0.942	−57.7
VVW4	334.8	338.5	ND	ND	ND	ND	265.5	4.8	0.945	−54.5	ND	ND	ND	ND	ND	ND	ND	ND
VVW5	452.1	452.8	ND	ND	ND	ND	372.2	3.2	0.945	−55.5	397.2	4.0	0.962	−38.3	405.4	2.8	0.967	−32.7
Average									0.942	−58.1			0.954	−46.4			0.956	−43.8
Standard deviation									0.014	14.1			0.016	16.4			0.013	12.8

Culture	H_2O δD at start	H_2O δD at harvest	Phytadiene				Heptadecene				Naturally occurring FAME δD											
			δD	stdev	α	ε	δD	stdev	α	ε	C_{16}	stdev	α	ε	C_{18} unsat[c]	stdev	α	ε	C_{18} sat[d]	stdev	α	ε
Eudorina unicocca																						
EUW1	−62.4	−50.5	ND	ND	ND	ND	−143.5	2.3	0.902	−98.0	−110.3	1.8	0.937	−63.0	−106.0	1.7	0.942	−58.5	−120.9	2.4	0.926	−74.2
EUW2	78.1	84.1	ND	ND	ND	ND	−22.1	0.8	0.902	−98.0	22.7	4.3	0.943	−56.7	23.5	4.2	0.944	−55.9	−5.2	4.2	0.918	−82.4
EUW3	218.6	220.7	ND	ND	ND	ND	60.0	2.2	0.868	−131.7	121.7	2.4	0.919	−81.1	135.5	7.4	0.930	−69.8	ND	ND	ND	ND
EUW4	340.7	340.8	ND	ND	ND	ND	179.8	1.7	0.880	−120.0	243.7	2.9	0.928	−72.4	254.5	0.2	0.936	−64.4	ND	ND	ND	ND
EUW5	458.8	463.7	ND	ND	ND	ND	302.2	2.3	0.890	−110.3	354.9	0.5	0.926	−74.3	355.7	3.3	0.926	−73.8	ND	ND	ND	ND
Average									0.888	−111.6			0.930	−69.5			0.936	−64.5			0.922	−78.3
Standard deviation									0.015	14.6			0.010	9.7			0.008	7.5			0.006	5.8
Volvox aureus															$C_{18:3/1}$[e] Stdev				C_{18}[e]Stdev			
VVW1	−62.5	−51.2	−285.1	6.5	0.753	−246.6	−132.8	2.1	0.914	−86.1	−106.0	1.8	0.942	−57.8	−81.6	2.1	0.968	−32.1	−98.6	1.6	0.950	−50.0
VVW2	90.8	106.5	−178.1	7.9	0.743	−257.2	−11.5	2.9	0.893	−106.6	22.9	0.7	0.924	−75.6	26.9	0.9	0.928	−71.9	24.3	3.9	0.926	−74.3
VVW3	226.1	232.6	ND	ND	ND	ND	76.7	N/A[h]	0.874	−126.4	90.9	N/A[f]	0.885	−114.9	103.8	N/A[f]	0.896	−104.5	ND	ND	ND	ND
VVW4	334.8	338.5	ND	ND	ND	ND	ND	ND	ND	ND	224.0	1.0	0.915	−85.5	245.2	2.7	0.930	−69.7	259.1	7.2	0.941	−59.2
VVW5	452.1	452.8	ND	ND	ND	ND	ND	ND	ND	ND	317.2	6.8	0.907	−93.4	333.6	3.5	0.918	−82.1	358.8	6.8	0.935	−64.7
Average					0.748	−251.9			0.894	−106.4			0.922	−78.1			0.936	−63.9			0.938	−62.0
Standard deviation					0.008	7.5			0.020	20.2			0.015	15.3			0.022	21.9			0.010	10.2

Each strain was grown in five media containing different concentrations of deuterium. The δD value of each lipid was measured in triplicate and the average and standard deviation are reported. δD values of free fatty acids were measured on methyl ester derivatives and corrected for isotopic composition of the added methyl group. The δD value of the culture medium at the start and harvest is also shown.
ND, not determined due to insufficient amount; Stdev, standard deviation, based on triple measurements unless noted otherwise. α, is defined as $(D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$; ε, is defined as $(\alpha - 1)*1000 = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1]*1000$.

[a] All fatty acids with C_{18} skeleton are merged into two peaks only, unsaturated and saturated. Unsaturated are mainly $C_{18:1}$ and $C_{18:3}$, but possibly includes small peak of $C_{18:2}$.

[b] All fatty acids with C_{18} skeleton are merged into two peaks only, unsaturated and saturated. Unsaturated is predominated by $C_{18:3}$, followed by $C_{18:1}$ and, to a much less extent, $C_{18:2}$. In VVW5, unsaturated is one hump.

[c] For EUW1, 2 and 3, all fatty acids with C_{18} skeleton are merged into two peaks only, unsaturated and saturated. Unsaturated are mainly $C_{18:1}$ and $C_{18:3}$, but includes small peak of $C_{18:2}$; In EUW4 and 5, unstaruated is one hump.

[d] For EUW3, 4 and 5, saturated FAME does not have sufficient amount for δD measurement.

[e] All FAMEs with C_{18} skeleton are merged into two peaks only, unsaturated and saturated. Unsaturated is predominated by $C_{18:3}$, followed by $C_{18:1}$ and, to a much less extent, $C_{18:2}$. In VVW4 and 5, unstaruated is one hump.

[f] VVW3 natural FAME and heptadecene fraction has only one measurement due to limited amount after processing and not included in average calculation.

was introduced to the mass spectrometer via an open split, where a flow of helium carried the H_2 gas into the mass spectrometer.

Sensitivity of the instrument was monitored with six pulses of commercial H_2 gas (ultra high purity grade) via a second open split, four at the beginning and two at the end of each run (SFig. 2). Interference from H_3^+, which is formed in the ion source via ion-molecule reactions between H_2^+ and neutral H_2, impedes the accurate determination of HD^+ (Sessions et al., 1999). A calibration curve, or an "H_3^+ factor", was determined daily to correct for the H_3^+ interference. H_3^+ was determined by measuring the (m/z 3)/(m/z 2) response of 10 injections of H_2 reference gas. A low and stable value of less than seven was typically achieved.

To every sample was added a set of co-injection standards with known δD values that were chosen to bracket the peaks of interest in the chromatograms. All isotopic standards were obtained from Dr. Arndt Schimmelmann at the Biogeochemical Laboratory, Indiana University. For hydrocarbons, a mixture of C_{14} *n*-alkane, C_{16} *n*-alkane, 5α-androstane, and C_{40} *n*-alkane was used. C_{14} and C_{16} *n*-alkanes were used as "throw-away" peaks to avoid any possible hydrogen isotope memory effect, while 5α-androstane and C_{40} *n*-alkane were used as isotopic standards for the computation of lipid δD values with IsoDat 2.0 software (Thermo Finnigan) (SFig. 2). A different mixture of standards was prepared for each lipid fraction.

Each sample was run in triplicate and the standard deviation was usually less than 5‰ (Tables 1–3), similar to values reported by Sessions et al. (1999) and Englebrecht and Sachs (2005). A set of 15 *n*-alkanes with known δD values (Mixture A or B) acquired from Indiana University were injected every 6–9 runs to ensure the accuracy of our data. δD values are reported with reference to the VSMOW standard.

2.4. Correction of δD contribution from $-CH_3$ added during methylation

Fatty acid δD values were measured on methyl ester derivatives. The δD value of the three H atoms added during the methylation reaction with BF_3 in MeOH was determined by experimentation with phthalic acid (Dr. Arndt Schimmelmann, Indiana University) having a known δD value. Three experiments performed over the course of this study, each measured in triplicate, indicated a δD value for the three derivative H atoms of -115.7 ± 0.5‰. This value was used to correct the measured δD values of fatty acid methyl esters for the added hydrogen atoms by mass balance.

3. Results

3.1. Algal growth

Growth curves for all cultures indicated exponential growth and, by extension, healthy cultures (SFig. 1). With a relatively small (but uncertain) contribution to light absorption caused by evaporation, most of the progressive increase in light absorption can be attributed to an increasing biomass. Growth rates were determined using the endpoints of the exponential portion of the growth curves (by curve fitting, SFig. 1) as k (divisions/d) $= \log_2(N_1/N_0)/(T_1 - T_0)$, 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). Calculated growth rates for the Morocco, Titi–caca and Martinique strains of *B. braunii* were 0.128–0.138, 0.120–0.133 and 0.124–0.135 divisions/day, respectively. *E. unicocca* and *V. aureus* had growth rates of 0.156–0.166 and 0.159–0.170 divisions/day, respectively.

3.2. Hydrocarbon distributions within *B. braunii*, *E. unicocca* and *V. aureus*

3.2.1. *B. braunii*, A race (Titicaca and Morocco)

A race cultures contained substantial quantities of odd carbon-numbered hydrocarbons in the range of C_{23}–C_{33}, each with a terminal double bond (Metzger et al., 1985a, 1986; Metzger and Largeau, 1999). Hydrocarbons in the Titicaca strain consisted almost exclusively of C_{25}–C_{31} odd *n*-alkadienes: $C_{27}H_{52}$ (1, 18; *E*/*Z*), $C_{29}H_{56}$ (1, 20; *E*/*Z*), $C_{31}H_{60}$ (1, 22; *E*/*Z*) (SFig. 3a) (structure **1** in Appendix A), in accord with previous reports (Metzger et al., 1986). Minor alkenes included $C_{23}H_{44}$ (1, 14; *E*/*Z*), and $C_{25}H_{48}$ (1, 16; *E*/*Z*), and C_{29} alkatrienes (SFig. 3a). On GC-IRMS, the *Z*/*E* isomers co-eluted, resulting in a single isotopic value.

Hydrocarbons in the Morocco strain differed from those in the Titicaca strain in that only one isomer of each odd C_{25}–C_{31} *n*-alkadiene was observed and an appreciable concentration of the $C_{29}H_{54}$ alkatriene eluted between the C_{29} and C_{31} alkadienes (SFig. 4a), as previously reported by Metzger et al. (1986).

3.2.2. B. braunii, B race (Martinique)

3.2.2.1. Botryococcenes. Polymethylated triterpenes of the generalized formula C_nH_{2n-10}, where $30 \leqslant n \leqslant 37$, termed botryococcenes, are produced by the B race (Metzger et al., 1985a,b, 1988; Metzger and Largeau, 1999). Botryococcene mixtures exhibit a large range of molecular mass and isomerism related to genetic and physicochemical factors (Metzger et al., 1985b). Seven botryococcenes were identified by mass spectrometry and coinjection with authentic standards provided by Dr. Pierre Metzger (SFig. 5a and Appendix A), the most abundant of which were two isomers of the C_{34} botryococcene (structures **4** and **5** in Appendix A), with lesser quantities of C_{30}, C_{31}, C_{32} and C_{33} botryococcenes (structures **2**, **3**, **6** and **7** in Appendix A).

Positive identification of all botryococcenes during GC–IRMS was usually not possible due to inadequate peak separation caused by the large injections required for δD determinations. For example, in GC–IRMS chromatograms, compounds **5**–**7**, which were baseline-separated on the GC-FID (SFig. 5a), were not well-resolved, co-eluting in as few as two peaks. The last peak in GC–IRMS chromatograms may be another isomer of the C_{34} botryococcene or it may be a different homologue altogether. In either case, pending positive structural identification by GC–MS, we refer to that compound as C_{34} iso in Table 1 and SFig. 5a.

The C_{30} botryococcene is the precursor for the C_{31}–C_{34} botryococcenes which are synthesized by methylation on positions 3, 7, 16 and/or 20 of the C_{30} backbone (Metzger et al., 1987; Okada et al., 2004). The relative abundance of C_{30} botryococcene in a population results from a balance between its production and its loss via methylation to form C_{31}–C_{34} botryococcenes (Okada et al., 2004). As a culture ages and botryococcenes accumulate, the synthesis of botryococcenes is shifted toward the longer homologues, resulting in minor amounts of C_{30} botryococcene late in a culture cycle (Okada et al., 2004). Growing for 45 days, C_{34} botryococcenes were most abundant in our cultures and C_{30} abundances were low (SFig. 5a).

3.2.2.2. Phytadiene. Quantities of phytadiene sufficient for D/H analysis existed in only three cultures owing to their loss during urea adduction of FAMEs (the two co-eluted during column chromatography). Four isomers were observed, each with a parent ion at *m/z* 278. Structure determination was based on the comparison of our electron impact mass spectra and gas chromatographic retention times with published data (Fukushima et al., 1992).

Phytadienes are degradation products of phytol, the ester-linked side-chain of chlorophyll-*a*. They can be produced (i) at elevated temperatures from chlorins, (ii) during GC analysis of underivatized phytol, and (iii) by acidic dehydration of phytol (Grossi et al., 1996). However, a controlled experiment in which we injected underivatized phytol (Sigma-Aldrich) onto the GC demonstrated that less than 1% was converted to phytadienes, and those produced had a different distribution of isomers. Furthermore, chlorophyll (Pfaltz & Bauer) subjected to identical ASE extraction conditions resulted in no production of phytadienes. We therefore conclude that phytadienes occurred naturally in the B Race of *B. braunii*, probably by dehydration of phytol (Volkman and Maxwell, 1986; Fukushima et al., 1992; Grossi et al., 1996). Here we report only the δD values of the most abundant isomer, neophytadiene (structure **8** in the Appendix A) (Table 2). Phytols were likely still present, along with sterols, in the alcohol fractions which were not analyzed.

3.2.3. Hydrocarbon distributions in Eudorina unicocca and Volvox aureus

Both *E. unicocca* and *V. aureus* had similar hydrocarbon distributions characterized almost exclusively by 8-heptadecene ($C_{17:1}$) (structure **9** in the Appendix A) with minor amounts of heptadecane (C_{17}) and 10-nonadecene ($C_{19:1}$) (SFig. 6a). This distribution of hydrocarbons differs from the short-to-mid chain length alkane distributions found in many algae (Gelpi et al., 1970). Because the hydrocarbon fraction contained a single abundant compound it was combined with the FAME fraction for δD analysis (SFigs. 6c and 7b). Though no published reports of lipid distributions in *E. unicocca* or *V. aureus* exist, and few reports of 8-heptadecene are in the literature, it is likely derived from glycerol monoolein in the cell membrane (Peterson, 1980).

Phytadienes occurred in *E. unicocca* and *V. aureus* (SFigs. 6c and 7b), but losses during sample workup prevented D/H analysis of all but the most abundant isomer (compound **8** in the Appendix A) in two cultures of *V. aureus* (Table 3).

3.3. Fatty acid distributions

The B race (Martinique) contained even numbered monocarboxylic acids ranging from *n*-C_{14} to *n*-C_{30}, with C_{16}, $C_{18:1}$ and $C_{28:1}$ predominating

(SFig. 5b). Except for the C_{16} fatty acid (palmitic), all other fatty acids were unsaturated. Linoleic acid ($C_{18:2}\omega6$) and linolenic acid ($C_{18:3}\omega3$) eluted closely with monounsaturated $C_{18:1}$. Of the longer homologues, $C_{28:1}$ was most abundant, followed by substantial quantities of $C_{20:1}$, $C_{26:1}$ and $C_{30:1}$, and trace amounts of $C_{22:1}$ and $C_{24:1}$. Such distributions are in agreement with previous reports (Douglas et al., 1969).

Unlike the hydrocarbons, fatty acid distributions were similar between A and B races. A race fatty acids were primarily even numbered, varying in length from C_{16} to C_{32}, with C_{16}, $C_{18:1}$ and $C_{28:1}$ predominating (SFig. 3b). The only major differences between the A and B race fatty acids were a higher abundance of C_{16} relative to $C_{18:1}$ and a more pronounced even-over-odd predominance in the A race.

Compared to fatty acids in *B. braunii*, *E. unicocca* and *V. aureus* produced fatty acids with substantially shorter chain lengths (SFig. 6b). The most abundant fatty acid in both species was palmitic acid, $C_{16:0}$. Other saturated fatty acids included C_{18}, C_{14}, and trace amounts of C_{17} (SFig. 6b). The primary unsaturated fatty acids in the two species were $C_{18:2}$, $C_{18:3}$ and $C_{18:1}$, with lesser amounts of $C_{20:1}$ (SFig. 7a). Differences between the fatty acid distributions in the two species included relatively larger amounts of C_{18} in *V. aureus*, and relatively larger quantities of $C_{20:1}$ in *E. unicocca*.

3.4. Naturally occurring fatty acid methyl esters (FAMES)

Though not previously reported, our cultured *B. braunii* contained large quantities of naturally occurring FAMEs. Both A (Titicaca) and B (Martinique) races had FAME distributions that closely followed their fatty acid (FA) distributions, with C_{16}, $C_{18:1}$ and $C_{28:1}$ predominating and a pronounced even-over-odd predominance (SFigs. 3c and 5c).

A control experiment was conducted to ensure that our lipid extraction procedure (DCM/MeOH (9:1 v/v), 150 °C, 1,500 psi) did not cause methylation of fatty acids. C_{16} and C_{18} fatty acids (Sigma-Aldrich) were subjected to ASE extraction using three solvent systems: 100% DCM, 10% MeOH in DCM (1:9 v/v) and 50% MeOH in DCM (1:1 v/v). Extractions with DCM and 10% MeOH in DCM produced no measurable fatty acid methyl esters. Extraction with 50% MeOH in DCM converted less than 0.1% of both C_{16} and C_{18} FAs to FAMEs, substantially less than observed in our cultures.

While we recognize that the control experiments with pure fatty acids lacked the potential catalytic and matrix effects other compounds and components of cultured algae (on a filter) may provide, we have no evidence to indicate that the FAMEs in the cultures were produced during sample workup. Furthermore, when the Martinique and Titicaca cultures were extracted at room temperature by ultrasonication in 100% DCM, a gentle extraction procedure, significant quantities of FAMEs were presented.

Fatty acid methyl/ethyl esters have been reported in the extracts of algae (Weete, 1976), fungus (Laseter et al., 1968; Laseter and Weete, 1971), mammalian tissues (Saladin and Napier, 1967), pollen (Fathipour et al., 1967), and protozoans (Chu et al., 1972). Hydroxy fatty acid methyl esters were found in marine algae (Sinninghe Damsté et al., 2003).

Though we cannot exclude the possibility that a small fraction of the FAMEs in our *B. braunii* were produced during sample preparation, the bulk of them are likely natural, begging the question why they have not previously been reported in algal cultures. We hypothesize that the common procedure of methylation of fatty acids or hydrolysis of total lipid extracts before column chromatography and GC–MS analysis destroyed or masked any natural FAMEs. The biosynthesis of FAMEs was investigated in the bacterium *Mycobacterium phlei*: *S*-adenosylmethionine as the most effective methyl donor and fatty acids as acyl acceptors (Akamatsu and Law, 1970).

E. unicocca and *V. aureus* also produced appreciable amounts of FAMEs. As with *B. braunii*, FAMEs in both species had very similar distributions to the free fatty acids, with C_{16} FAME most abundant, followed C_{18} and $C_{18:1/3/2}$ FAMEs, and a pronounced even-over-odd predominance (SFigs. 6c and 7b).

3.5. Hydrogen isotope fractionation in hydrocarbons

In quantifying D/H fractionation during lipid synthesis we adopt the traditional definition of "fractionation factor" for reactions under thermodynamic equilibrium, i.e., $\alpha_{\text{lipid–water}} = (\text{D/H})_{\text{lipid}}/(\text{D/H})_{\text{water}} = (\delta D_{\text{lipid}} + 1000)/(\delta D_{\text{water}} + 1000)$. Because the natural variability of α is small an approximation of "fractionation," termed an "enrichment factor", is often reported, with geochemists tending to use $\varepsilon_{A-B} \approx 10^3 \times \ln\alpha_{A-B}$ (Hoefs,

2004), and ecologists, $\varepsilon = (\alpha_{A-B} - 1) \times 1000$ (Lajtha and Marshall, 1994). Both quantities were calculated for each lipid in each culture and in the ensuing discussion we use the "ecologist's" definition, $\varepsilon_{\text{lipid–water}} = (\alpha_{\text{lipid–water}} - 1) \times 1000 = [(\delta D_{\text{lipid}} + 1000)/(\delta D_{\text{water}} + 1000) - 1] \times 1000$ (primarily because the ε values in five cultures calculated this way are more consistent). Because lipids are depleted in deuterium relative to water the value of fractionation is always negative. A "larger" isotope fractionation means the absolute value of ε is larger.

For continuity with recent literature we report the linear regression equations for five cultures of each species, $y = \text{slope} \times \delta D_{\text{water}} + \text{intercept}$. The observation that the intercept $\neq (\text{slope} - 1) \times 1000$, a point discussed by Sessions and Hayes (2005), will be discussed in detail in a subsequent paper. For now we simply note that the y-intercept of the linear regression of δD_{lipid} versus δD_{water} is the D/H fractionation when $\delta D_{\text{water}} = 0$.

δD values of C_{27}, C_{29} and C_{31} *n*-alkadienes in *B. braunii*, A Race (Titicaca and Morocco) closely tracked water δD values, with $R^2 > 0.99$ (Table 1; Fig. 1a and d). In spite of the large water δD range, α for C_{29} *n*-alkadiene in the Titicaca strain was nearly constant in five cultures, varying from 0.754 to 0.779, and averaging 0.769 with a standard deviation (σ) of 0.01. Similarly, α for the C_{29} *n*-alkadiene in the Morocco strain averaged 0.779 ± 0.007. Therefore, a simple approximation of $\alpha = (\text{D/H})_{\text{lipid}}/(\text{D/H})_{\text{water}} = (\delta D_{\text{lipid}} + 1000)/(\delta D_{\text{water}} + 1000)$ is suitable for the experiments reported here.

The ε values for the C_{29} *n*-alkadienes varied from −220.6‰ to −246.0‰ in the Titicaca strain, averaging -231.5 ± 10.3‰, and varied between −212.4‰ to −228.5‰, averaging -221.0 ± 7.3‰ in the Morocco strain (Table 1). Average α values were 0.769 and 0.779, respectively. The δD values of C_{27} alkadienes in two of the Morocco strain cultures (A1W1 and A1W2) differed substantially from the C_{29} alkadiene δD values (Table 1), an observation for which we have no explanation.

δD values of C_{30}–C_{34} botryococcenes in the B race, Martinique strain, of *B. braunii* closely tracked water δD values, with $R^2 > 0.99$ (Fig. 2a). The ε values of C_{30}, C_{31} and C_{34} averaged −273‰, −293‰, and −310‰ to −359‰, respectively (Table 1).

The δD values of 8-heptadecene in *E. unicocca* and *V. aureus* closely tracked water δD values, with $R^2 > 0.99$ (Fig. 3b and d). The ε values averaged −111.6‰ and −106.4‰, respectively (Table 3).

3.6. Hydrogen isotope fractionation in free fatty acids

3.6.1. Fatty acid δD values in *B. braunii*

Fatty acid δD values in all three A and B race *B. braunii* strains closely tracked water δD values, with $R^2 > 0.99$ (Figs. 1b, e and 2b). In the A race Titicaca strain, ε values of C_{16}, $C_{18:1}$ and $C_{28:1}$ fatty acids averaged −192.4‰, −187.2‰ and −174.6‰, respectively, while ε values of $C_{20:1}$ fatty acids averaged −168.1‰ (Table 2). In the A race Morocco strain, ε values of C_{16} and $C_{18:1}$ fatty acids averaged −195.9‰ and −186.8‰, respectively (Table 2), while ε values of $C_{20:1}$ and $C_{28:1}$ fatty acids averaged −167.0‰ and −177.5‰, respectively. In the B race (Martinique), ε values of C_{16}, $C_{18:1}$ and $C_{28:1}$ fatty acids averaged −177.0‰, −175.8‰ and −172.5‰, respectively, compared to −160.1‰ for the $C_{20:1}$ fatty acid. Taken together, the C_{16}, $C_{18:1}$ and $C_{28:1}$ fatty acids of A and B race *B. braunii* had ε values of −174‰ to −196‰, while the $C_{20:1}$ fatty acid was consistently enriched in deuterium by ~15‰.

3.6.2. Fatty acid δD values in *E. unicocca* and *V. aureus*

Fatty acid δD values in both *E. unicocca* and *V. aureus* closely tracked water δD values, with $R^2 > 0.99$ (Fig. 3a and c). The ε values of the C_{16} fatty acid were −49.8‰ in *E. unicocca* and −58.1‰ in *V. aureus*, significantly less than in *B. braunii* and, for *E. unicocca*, about 50‰ enriched in deuterium relative to the C_{14} fatty acid (Table 3).

Some uncertainty in the absolute δD values of C_{18} fatty acids exists as a result of partial co-elution of $C_{18:2}$, $C_{18:3}$, $C_{18:1}$, etc. (see notes in Table 3), at times requiring the integration of all unsaturated C_{18} acids as a single peak. Nevertheless, excellent linear relationships between C_{18} fatty acid (saturated and unsaturated) and water δD values, and similar ε values for the C_{18} and C_{16} fatty acids suggests that the uncertainty is relatively small.

3.7. Hydrogen isotope fractionation in FAMEs and phytadienes

3.7.1. FAME and phytadiene δD values in *B. braunii*

δD values of naturally occurring C_{16}, $C_{18:1}$ and $C_{28:1}$ FAMEs in *B. braunii* A (Titicaca) and B (Martinique) races closely tracked water δD values, with $R^2 > 0.99$ (Figs. 1c and 2c). The ε values of the A race (Titicaca) FAMEs were −181.4‰ to −189.4‰ for C_{16}, $C_{18:1}$ and $C_{28:1}$ FAMEs (Table 2). The $C_{20:1}$

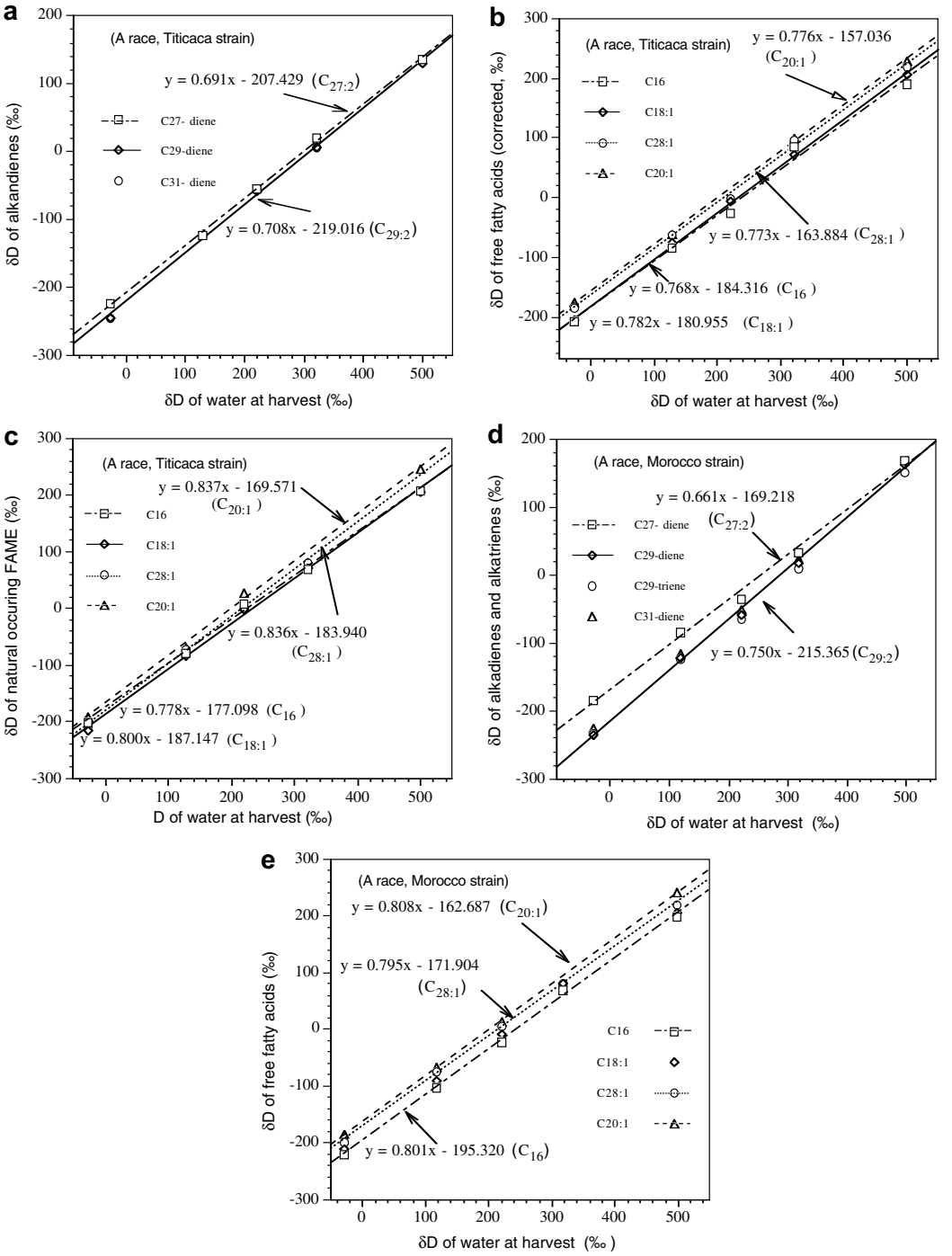

Fig. 1. Relationships between lipid and water δD values in cultures of *B. braunii*, A race, Titicaca (a–c) and Morocco (d–e) strains. All alkadiene, alkatriene, fatty acid and FAME δD values were highly correlated with water δD values ($R^2 > 0.99$). (a) C_{27}, C_{29} and C_{31} alkadienes in the Titicaca strain. C_{27} alkadienes were deuterium-enriched compared to other homologues. (b) C_{16}, $C_{18:1}$, $C_{20:1}$ and $C_{28:1}$ fatty acids in the Titicaca strain. $C_{20:1}$ was most deuterium-enriched, followed by $C_{28:1}$ and C_{16}. (c) C_{16}, $C_{18:1}$, $C_{20:1}$ and $C_{28:1}$ FAMES in the Titicaca strain. Chain-length-dependant δD variations were identical to the fatty acids. (d) C_{27}, C_{29} and C_{31} alkadienes and C_{29} alkatriene in the Morocco strain. C_{27} was most deuterium-enriched. (e) C_{16}, $C_{18:1}$, $C_{20:1}$ and $C_{28:1}$ fatty acids in the Morocco strain. $C_{20:1}$ was most deuterium-enriched, with $C_{28:1}$ slightly enriched compared to C_{16}.

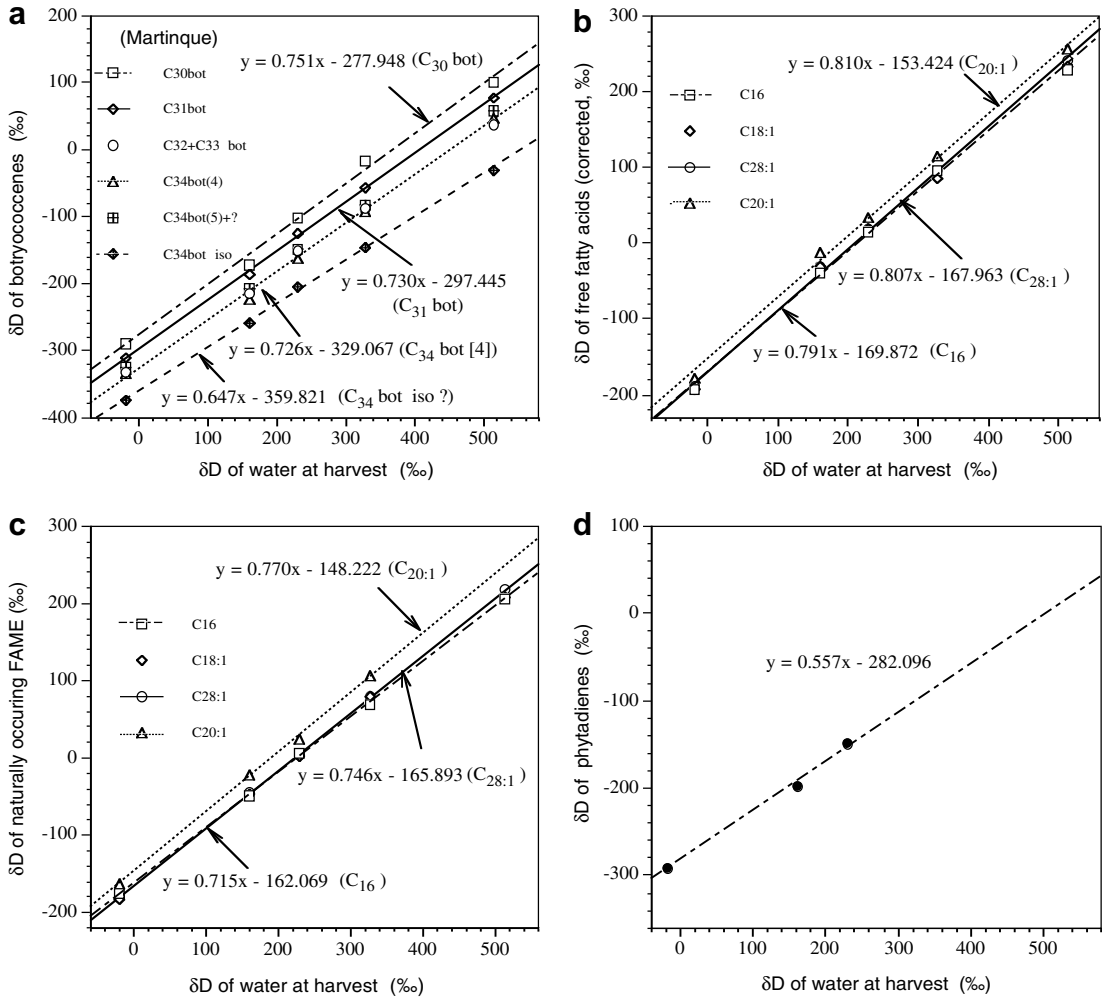

Fig. 2. Relationships between lipid and water δD values in cultures of *B. braunii*, B race, Martinique strain. All botryococcene, fatty acid, FAME and phytadiene δD values were highly correlated with water δD values ($R^2 > 0.99$). (a) C_{30}–C_{34} botryococcenes (see structures in Appendix A). Deuterium-depletion increased with increasing carbon number. The compound labeled "C_{34} bot iso?" (i.e., the last peak in SFig. 5a) may be an isomer of the C_{34} botryococcene, or possibly a longer homologue. (b) C_{16}, $C_{18:1}$, $C_{20:1}$ and $C_{28:1}$ fatty acids. $C_{20:1}$ was consistently the most deuterium-enriched homologue. (c) FAMEs. (d) Phytadiene.

FAME was ~20‰ enriched in deuterium relative to other homologues, as observed for the free fatty acids. The ε values of B race (Martinique) FAMEs were from −181.6‰ to −184.0‰ for C_{16}, $C_{18:1}$ and $C_{28:1}$ (Table 2). And, similar to the A race, the $C_{20:1}$ FAME was enriched in deuterium relative to other FAMEs by ~20‰, with an ε value of −159.5‰.

Phytadiene δD values were measured in three B race (Martinique) cultures that had sufficient material. δD values of phytadiene were linearly correlated with water δD values, with $R^2 > 0.99$ (Fig. 2d). The ε values averaged -298.5 ± 18.1‰ (Table 2), substantially greater than for fatty acids and FAMEs. We also note that the slope of the regression was low, 0.557, compared to 0.73–0.83 for fatty acids (Fig. 2d; Table 4). With only three cultures to define the slope, however, we are unable to evaluate its significance.

3.7.2. FAME and phytadiene δD values in *E. unicocca* and *V. aureus*

δD values of C_{16} and $C_{18:1}$ FAMEs from *E. unicocca* closely tracked water δD values, with $R^2 > 0.99$ (Fig. 3b). The ε values for two FAMEs in *E. unicocca* were −69.5‰ and −78.3‰, somewhat more negative than the corresponding FAs (Table 3).

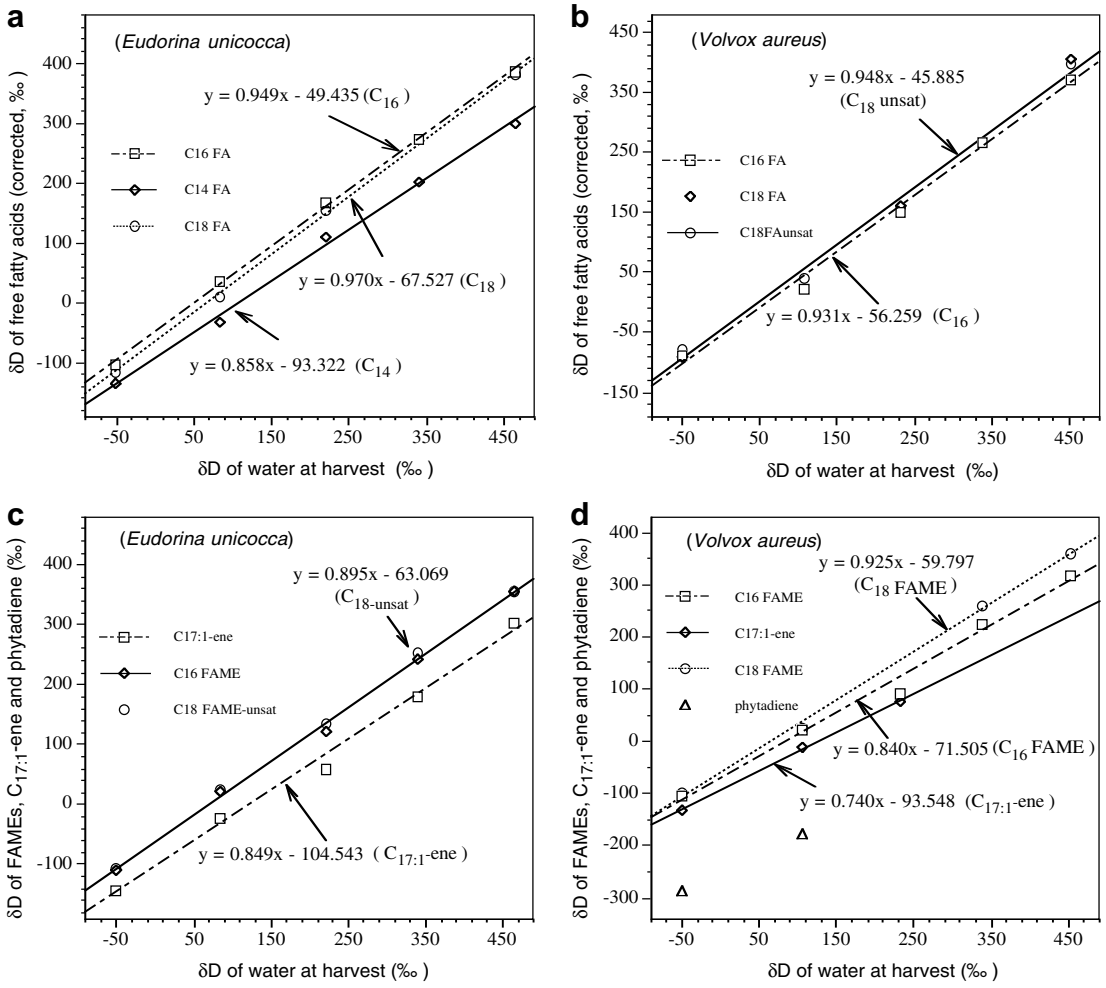

Fig. 3. Relationships between lipid and water δD values in cultures of *Eudorina unicocca* and *Volovx aureus*. All fatty acid, FAME and *n*-alkene δD values were highly correlated with water δD values ($R^2 > 0.99$). (a) C_{14}, C_{16} and C_{18} fatty *E. unicocca* cultures. C_{14} was deuterium-depleted compared to both C_{16} and C_{18}. (b) Heptadecenes and C_{16} and C_{18} FAMEs in *E. unicocca*. Heptadecene is substantially depleted in deuterium relative to fatty acids and FAMEs. (c) C_{16} and C_{18} fatty acids in *V. aureus*. (d) FAMEs, heptadecene and phytadienes in *V. aureus*. δD values of heptadecene (in three cultures for which we have data) were depleted in deuterium relative to fatty acids and FAMEs. Although only two analyses of phytadienes were performed both were significantly depleted in deuterium relative to all other lipids.

δD values of the C_{16} FAME in *V. aureus* also closely tracked water δD values, with $R^2 = 0.99$ (Fig. 3d). The ε value for the C_{16} FAME in *V. aureus*, $-78‰$, was more negative than in *E. unicocca*. The slope of the water–lipid δD regression for the $C_{18:1}$ FAME was larger than for the C_{16} FAME (Fig. 3d; Table 5).

Two *V. aureus* cultures had sufficient phytadiene for δD determination. The ε values of $-246.6‰$ and $-257.2‰$ indicate substantial deuterium depletion in phytadiene relative to FAs and FAMEs. A similar isotopic depletion in phytadiene relative to FAs and FAMEs was observed in *B. braunii* (Martinique) (Fig. 2d; Table 2).

4. Discussion

4.1. Hydrogen isotopes in lipids from B. braunii

Lipids within a single class generally fell within a narrow δD range of $<50‰$. Between lipid classes hydrogen isotopic differences of 50–200‰ were

Table 4
Summary of D/H fractionation parameters in lipids from *B. braunii*

Strain	Analyte	R^2	Slope[a]	Intercept[a]	α[b]	Stdev[c]	ε[d]
Hydrocarbon							
Titicaca Strain	C_{27}-alkadiene	0.999	0.691	−207.4	0.7755	0.0141	−224.5
	C_{29}-alkadiene	0.999	0.708	−219.0	0.7685	0.0103	−231.5
	C_{31}-alkadiene	0.999	0.709	−219.0	0.7688	0.0098	−231.2
Morocco strain	C_{27}-alkadiene	0.995	0.661	−169.2	0.8028	0.0259	−197.2
	C_{29}-alkadiene	0.998	0.750	−215.4	0.7790	0.0073	−221.0
	C_{29}-alkatriene	0.998	0.723	−213.9	0.7757	0.0112	−224.3
	C_{31}-alkadiene	0.998	0.744	−207.1	0.7849	0.0094	−215.1
Martinique strain	C_{30} botryococcene	0.994	0.751	−277.9	0.7272	0.0103	−272.8
	C_{31} botryococcene	1.000	0.730	−297.4	0.7075	0.0046	−292.5
	C_{34} botryococcene	0.997	0.726	−329.1	0.6808	0.0089	−319.2
	C_{34} bot isomer + ?[e]	0.999	0.724	−316.3	0.6909	0.0058	−309.1
	$C_{32} + C_{33}$ bot (?)[e]	0.999	0.699	−318.3	0.6847	0.0051	−315.3
	C_{34} bot (?)[e]	0.999	0.647	−359.8	0.6413	0.0030	−358.7
Fatty acids							
Titicaca strain	C_{16} fatty acid	0.992	0.768	−184.3	0.8076	0.0118	−192.44
	$C_{18:1}$ fatty acid	0.999	0.782	−181.0	0.8128	0.0052	−187.2
	$C_{20:1}$ fatty acid[f]	0.999	0.776	−157.0	0.8319	0.0111	−168.1
	$C_{28:1}$ fatty acid	0.997	0.773	−163.9	0.8254	0.0103	−174.6
Morocco strain	C_{16} fatty acid	0.999	0.801	−195.3	0.8041	0.0046	−195.9
	$C_{18:1}$ fatty acid	0.998	0.797	−183.5	0.8132	0.0056	−186.8
	$C_{20:1}$ fatty acid[f]	1.000	0.808	−162.7	0.8330	0.0052	−167.0
	$C_{28:1}$ fatty acid	0.999	0.795	−171.9	0.8225	0.0049	−177.5
Martinique strain	C_{16} fatty acid	0.998	0.791	−169.9	0.8230	0.0065	−177.0
	$C_{18:1}$ fatty acid	0.998	0.790	−168.2	0.8242	0.0078	−175.8
	$C_{20:1}$ fatty acid	0.997	0.810	−153.4	0.8399	0.0078	−160.1
	$C_{28:1}$ fatty acid	0.999	0.807	−168.0	0.8275	0.0050	−172.5
Titicaca strain	C_{16} FAME	0.998	0.778	−177.1	0.8153	0.0079	−184.7
	$C_{18:1}$ FAME	0.998	0.800	−187.1	0.8106	0.0055	−189.4
	$C_{20:1}$ FAME[f]	0.998	0.837	−169.6	0.8314	0.0072	−168.6
	$C_{28:1}$ FAME[f]	0.999	0.836	−183.9	0.8186	0.0037	−181.4
	C_{16} FAME	1.000	0.715	−162.1	0.8160	0.0166	−184.0
	$C_{18:1}$ FAME	1.000	0.736	−166.4	0.8161	0.0128	−183.9
	$C_{20:1}$ FAME[f]	0.999	0.770	−148.2	0.8405	0.0097	−159.5
	$C_{28:1}$ FAME	1.000	0.746	−165.9	0.8184	0.0118	−181.6
Phytadiene							
Martinique strain	Phytadiene[g]	0.996	0.557	−282.1	0.7015	0.0181	−298.5

[a] The values of slopes and intercepts are derived from the linear regressions of δD values of biomrakers and δD values of waters at harvest; n is typically 5, unless noted otherwise.
[b] Fractionation factor, α, was calculated for each culture from the measured δD values of biomarkers and water at harvest according to the equation: $\alpha_{lipid-water} = (D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$. Reported is average value of five cultures with standard deviation given $n = 5$ unless otherwise noted.
[c] Stdev, standard deviation of measured fractionation factors $n = 5$ unless otherwise noted.
[d] Fractionation, ε, is defined as $1000 \times (\alpha - 1) = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1] \times 1000$, where α is the fractionation factor.
[e] Peak identification with certain uncertainty. See text for details.
[f] Where $n = 4$.
[g] Where $n = 3$.

common (Tables 1–3). In *B. braunii* A (Titicaca) and B (Martinique) races fatty acids and FAMEs with the same carbon skeleton had similar δD values (Fig. 4a and c), suggesting that the hydrogen atoms on the FAME were derived from the same pool of hydrogen from which fatty acids were produced and that the esterification reaction imparted little or no hydrogen isotope fractionation.

Table 5
Summary of D/H fractionation parameters in lipids from *E. unicocca* and *V. aureus*

Strain	Analyte	n[a]	R^2	Slope[b]	Intercept[b]	α[c]	Stdev[d]	ε[e]
Hydrocarbon								
Eudorina unicocca	8-heptadecene	5	0.993	0.849	−104.5	0.8884	0.0146	−111.6
Fatty acids								
Eudorina unicocca	C_{14} fatty acid	4	0.997	0.858	−93.3	0.8991	0.0103	−100.9
	C_{16} fatty acid	5	0.999	0.949	−49.4	0.9502	0.0053	−49.8
	C_{18} FA-unsaturated	4	0.999	0.984	−47.0	0.9571	0.0067	−42.9
	C_{18} fatty acid	4	0.999	0.970	−67.5	0.9373	0.0076	−62.7
Volvox aureus	C_{16} fatty acid	5	0.994	0.931	−56.3	0.9419	0.0141	−58.1
	C_{18} FA-unsat	4	0.992	0.948	−45.9	0.9536	0.0164	−46.4
	C_{18} fatty acid	3	0.996	0.978	−47.9	0.9562	0.0128	−43.8
Natural occurring fatty acid methyl esters (FAME)								
Eudorina unicocca	C_{16} FAME	5	0.998	0.895	−63.1	0.9305	0.0097	−69.5
	C_{18} FAME-unsat	5	0.999	0.898	−57.6	0.9355	0.0075	−64.5
Volvox aureus	C_{16} FAME	5	0.988	0.840	−71.5	0.9146	0.0212	−78.1
	C_{18} FAME-unsat	5	0.982	0.838	−55.4	0.9280	0.0262	−63.9
	C_{18} FAME	4	0.998	0.925	−59.8	0.9380	0.0102	−62.0

Notes:
[a] n indicates the number of cultures in which δD values of a biomarker were measured.
[b] The values of slopes and intercepts are derived from the linear regressions of δD values of a biomarkers and δD values of waters at harvest.
[c] Fractionation factor, α, was calculated for each culture from the measured δD values of biomarkers and water at harvest according to the equation: $\alpha_{lipid-water} = (D/H)_{lipid}/(D/H)_{water} = (\delta D_{lipid} + 1000)/(\delta D_{water} + 1000)$. Reported is average value of five cultures with standard deviation given $n = 5$ unless noted otherwise.
[d] Stdev, standard deviation of measured fractionation factors $n = 5$ unless noted otherwise.
[e] Fractionation, ε, is defined as $1000 \times (\alpha - 1) = [(\delta D_{lipid} + 1000)/(\delta D_{water} + 1000) - 1] \times 1000$, where α is the fractionation factor.

Hydrocarbons were depleted in deuterium relative to fatty acids in all cultures: Alkadienes in the A race (Titicaca and Morocco) by about 40‰, and botryococcenes in the B race (Martinique) by 100–180‰ (Fig. 4a–c; Tables 1 and 2). While in agreement with Estep and Hoering (1980), who reported deuterium enrichments in fatty acids relative to hydrocarbons in higher plants, these findings are at odds with Sessions et al. (1999) who reported deuterium depletions of ~50–100‰ in fatty acids relative to hydrocarbons in both a higher plant *Daucus carota* (carrot) and a microalga *Isochrysis galbana* (haptophyte).

Carbon chain length had a minor and variable influence on δD values of fatty acids, FAMEs and alkadienes in *B. braunii*, but a significant influence on δD values of botrycoccenes. In the Martinique strain, δD values of C_{16} and $C_{18:1}$ fatty acids were very similar while in both the Titicaca and Morocco strains δD values of $C_{18:1}$ fatty acids were slightly elevated compared to those of C_{16} fatty acids. However, in all three strains, δD values of $C_{28:1}$ were about 10‰ more positive than those of C_{16} and $C_{18:1}$. The $C_{20:1}$ fatty acid, a minor component, was always the most deuterium-enriched fatty acid, typically by ~20‰ relative to C_{16}, $C_{18:1}$ and $C_{28:1}$ FAs. Similar isotopic relationships were observed for FAMEs (Table 2).

The trend toward deuterium enrichment with increasing fatty acid chain length reported by Sessions et al. (1999) is not borne out by our data. Nor are the large deuterium enrichments of 112–163‰ in C_{18} relative to C_{16} FA in marine red and brown macroalgae reported by Chikaraishi et al. (2004c). Chikaraishi et al. (2004c) also reported a progressive deuterium depletion of −117‰ to −181‰ with increasing degree of unsaturation in $C_{18:0}$ to $C_{18:4}$ FAs. One possible explanation for the discordant findings might be isotopic fractionation during lipid purification when argentation chromatography was used. Chikaraishi et al. (2004c) converted FAs into FAMEs and then used $AgNO_3$-impregnated silica gel to separate unsaturated from saturated FAs, a procedure likely to cause D/H fractionation in carbon double bonds, particularly when multiple unsaturations exist (de Ligny, 1976). Alternatively, there might be real differences in isotopic fractionations during fatty acid

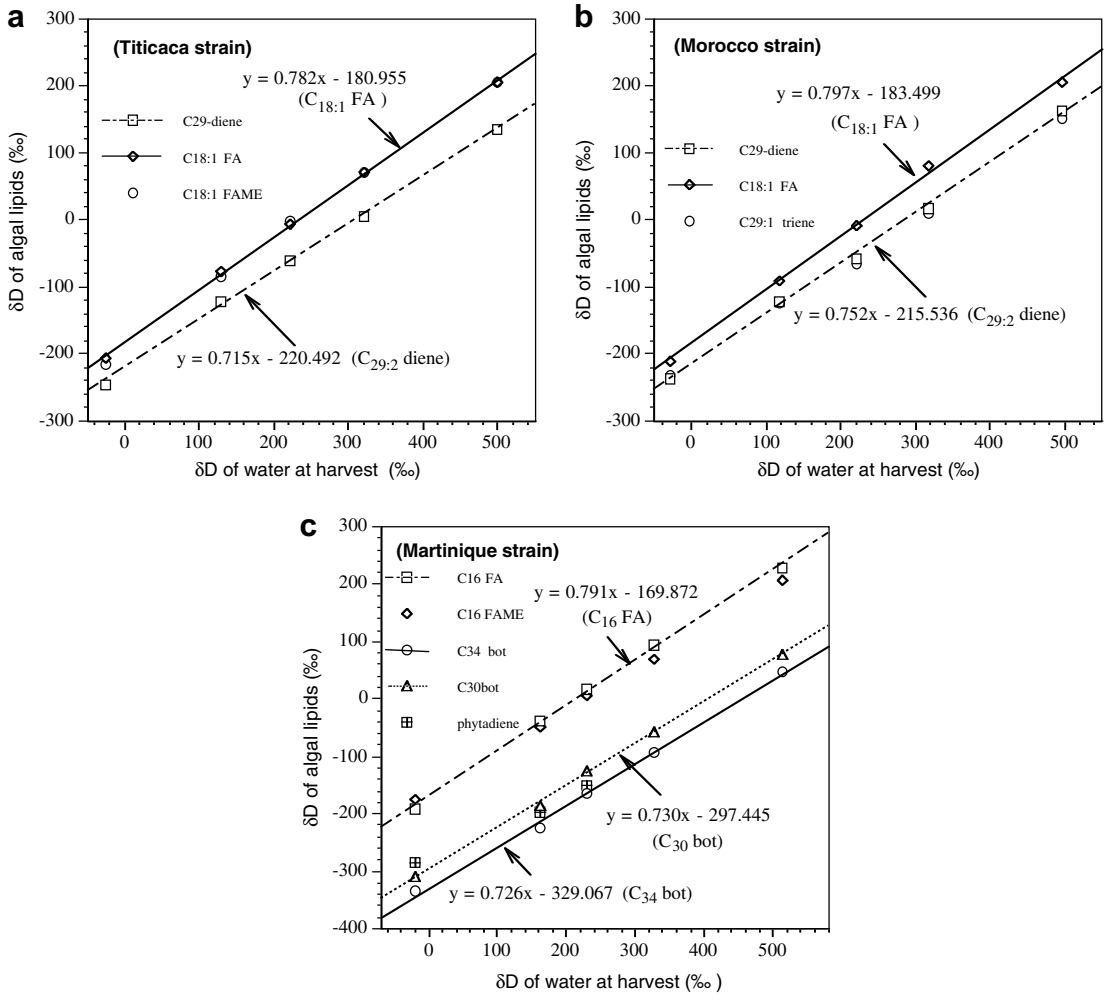

Fig. 4. D/H fractionation in different lipid classes within three strains of *B. braunii*. (a) A race, Titicaca strain. Alkadienes were ~40‰ depleted in deuterium relative to fatty acids. (b) A race, Morocco strain. Alkadienes and alkatrienes were ~40‰ depleted in deuterium relative to fatty acids. (c) B Race, Martinique strain. Botryococcenes and phytadienes were >110‰ depleted in deuterium relative to fatty acids and FAMEs.

synthesis between the green algae we cultured and the red/brown algae Chikaraishi et al. (2004c) investigated. Additional culture experiments and standardized lipid purification procedures are required to address this discrepancy.

Hydrogen isotope fractionation in alkadienes from the A race may have a slight carbon-chain-length dependence. In the Titicaca strain, δD values of C_{29} and C_{31} alkadienes were almost identical, while the C_{27} alkadiene was enriched in deuterium by ~7‰ relative to C_{29} and C_{31} (Fig. 1a; Table 1). In the Morocco strain δD values of C_{27} alkadienes were enriched by ~24‰ relative to C_{29} (Table 1). The one alkatriene analyzed, $C_{29:3}$, had a δD value very close to the $C_{29:2}$-diene (Table 1), suggesting further desaturation did not cause D/H fractionation.

Botryococcene δD values had a discernible carbon-chain-length dependence, with longer chain-lengths generally associated with more negative δD values (Fig. 2a; Table 1). As such, the C_{30} botryococcene—the precursor to all other botryococcenes—had the least negative δD value (−272.8‰), followed by the C_{31}(−292.5‰), and C_{34} botryococcenes (−319.2‰) (Table 1). We hypothesize that the methyl donor in botryococcene synthesis is depleted in deuterium relative to the C_{30} botryococcene, resulting in increasingly negative δD values with increasing carbon number. Nevertheless, our con-

clusion must be considered tentative due to co-elution of the C_{32} and C_{33} botryococcenes on the GC–IRMS (see Section 3.2.2.1).

Phytadienes in the B race had ε values that averaged −298.5‰, significantly deuterium-depleted relative to fatty acids (Table 2), consistent with prior studies of D/H fractionation in phytol from marine algae (Estep and Hoering, 1980; Sessions et al., 1999) and vascular plants (Chikaraishi et al., 2004a). Hydrogen isotope fractionation between phytadiene and water was similar to that between C_{34} botryococcenes and water (Fig. 4c; Tables 1 and 2).

4.2. Hydrogen isotopes in lipids from E. unicocca and V. aureus

FAMEs in both *E. unicocca* and *V. aureus* cultures were 20–27‰ depleted in deuterium relative to fatty acids (Table 3). The only dominant hydrocarbon in both species, 8-heptadecene, was 48–62‰ more negative than the C_{16} FAs.

A chain length effect may exist for fatty acids in *E. unicocca* cultures in which the C_{14} FA was 50‰ more negative than the C_{16} and C_{18} fatty acids (Table 3).

Two analyses of phytadiene δD values were made in *V. aureus*, yielding deuterium depletions of −246‰ and −257‰ relative to water (Table 3), similar to the deuterium depletions in *B. braunii* (Martinique).

4.3. Hydrogen isotope fractionation during lipid synthesis

During photosynthesis water is oxidized to O_2 and $NADP^+$ is reduced to NADPH (Raven et al., 1999), with the latter associated with a large D/H fractionation (Estep and Hoering, 1980; Luo et al., 1991). 3-Phosphoglyceric acid (PGA) (also called glycerate 3-phosphate) is produced when ribulose-1,5-bisphosphate and CO_2 are catalyzed by the Rubisco enzyme. The PGA is then converted to glyceraldehydes-3-phosphate (G3P) using ATP as energy and NADPH as reductant. During this conversion, NADPH yields hydrogen that is incorporated into organic matter.

Hydrogen isotopic variations within and between lipids in a single cell, and between lipids in different species, come from three sources: (1) the isotopic composition of biosynthetic precursors, (2) isotope effects during biosynthetic reactions, and (3) the isotopic composition of added hydrogen, primarily from NADPH and NADH (Smith and Epstein, 1970). The third source of isotopic variation is not related to the flow of substrates used in the assembly of carbon skeletons, providing a distinct difference from carbon isotope studies (Sessions et al., 1999).

Lipids in plants derive from three primary biosynthetic pathways (Chikaraishi et al., 2004a). Straight-chain (*n*-alkyl) lipids are produced via the acetogenic pathway using acetyl coenzyme-A (acetyl-CoA). Isoprenoid (i.e., branched) lipids are synthesized via the mevalonic acid (MVA) or non-mevalonic-acid pathway (DOXP/MEP, or 1-deoxy-D-xylulose 5-phosphate/2-*C*-methylerythritol 4-phosphate) using isopentenyl pyrophosphate (IPP).

4.3.1. D/H fractionation in acetogenic lipids: fatty acids

Fatty acids are the precursor to all other acetogenic lipids and acetyl-CoA is the direct precursor to fatty acids (Harwood, 1988). The principle photosynthate in the plant cell, sucrose, is converted to glucose 1-phosphate and further degraded in the cytoplasm to either malic or pyruvic acid (Stumpf, 1980). These two respiratory substrates enter the mitochondria where pyruvate is oxidatively decarboxylated to form acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (Fig. 5a).

Palmitic acid (C_{16}) is synthesized from acetyl-CoA and malonyl-CoA via several enzymatic reactions involving acyl carrier protein (ACP), NADPH and NADH (Fig. 5a) (Stumpf, 1980; Ohlrogee, 1987). Its immediate precursor is palmitoyl-ACP, which can have three fates: elongation to stearoyl-ACP, use in glycerolipid synthesis, or hydrolysis to palmitic acid. Hydrogen isotope fractionation in C_{16} fatty acid in algae is the sum of these processes.

Elongation of palmitoyl-ACP to stearoyl-ACP, and the subsequent desaturation of stearoyl-ACP to form oleoyl-ACP do not, in all likelihood, cause much hydrogen isotope fractionation, as evidenced by similar δD values in C_{16} and $C_{18:1}$ fatty acids in three strains of *B. braunii* (Table 2). This is consistent with the findings of Behrouzian et al. (2001) who reported that desaturation by stearoyl-ACP Δ^9 desaturase proceeded without a measurable kinetic isotope effect. Stearoyl-ACP desaturase has such a high activity that stearoyl-ACP rarely accumulates, with near complete conversion to oleate (Harwood, 1997). Supporting that notion is our observation of much higher concentrations of $C_{18:1}$ than $C_{18:0}$ fatty acids in *B. braunii* (SFigs. 3b, 4b, and 5b).

Longer FA homologues are synthesized from oleic acid by two different elongases, one in the

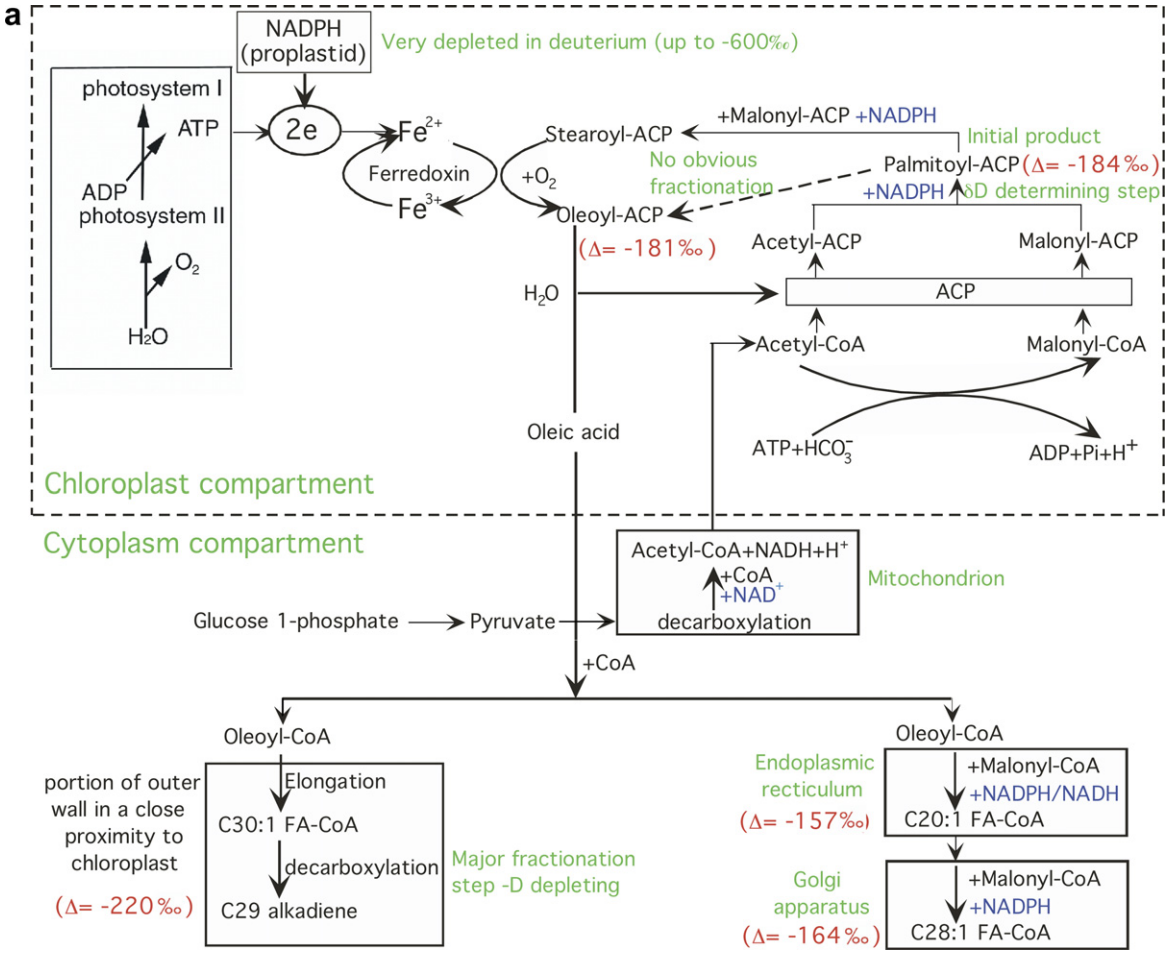

Fig. 5. Lipid biosynthetic pathways in *B. braunii* with estimated D/H fractionation. (a) Fatty acids and alkadienes in the A race (Titicaca) are produced via the acetogenic pathway using acetyl coenzyme-A as a precursor. (b) Botryococcenes and phytadiene in the B race (Martinique) are isoprenoid (i.e., branched) lipids synthesized via the DOXP/MEP pathway using isopentenyl pyrophosphate as a precursor (IPP). Δ values shown represent D/H fractionation when $\delta D_{water} = 0$. Acronyms are as follows, Co-A: coenzyme A; ACP: acyl carrier protein; DMAPP: dimethylallyl diphosphate; DOXP: 1-deoxy-D-xylulose 5-phosphate; FPP: farnesyl diphosphate; GPP: geranyl diphosphate; GGPP: geranylgeranyl diphosphate; IPP: isopentenyl diphosphate; MEP: 2-*C*-methyl-D-erythritol 4-phosphate; PSPP: presqualene diphosphate. (Referred from Pollard et al., 1979; Stumpf, 1980, 1987; Templier et al., 1984, 1991; Agrawal and Stumpf, 1985; Harwood, 1988; Schwender et al., 1997, 2001; Lichtenthaler, 1999; Rohmer, 1999; Charon et al., 1999, 2000; Szkopińska, 2000; Wanke et al., 2001; Okada et al., 2004; Sato et al., 2004).

endoplasmic reticulum (C_{18}-CoA elongase, catalyzing $C_{18:1} \rightarrow C_{20:1}$), the other in the Golgi apparatus (C_{20}-CoA elongase, catalyzing $C_{20:1} \rightarrow C_{22:1} \rightarrow C_{30:1}$) (Agrawal and Stumpf, 1985a,b; Lessire et al., 1985). Agrawal and Stumpf (1985b) further demonstrated that both the $C_{18:1} \rightarrow C_{20:1}$ and $C_{20:1} \rightarrow C_{22:1}$ elongations can proceed with NADPH as a reductant, but that only NADH can be used for the $C_{18:1} \rightarrow C_{20:1}$ elongation (Fig. 5a).

We hypothesize that consistently higher δD values of $C_{20:1}$ relative to all other fatty acids (Table 2; Figs. 1b, e and 2b) resulted from either or both its synthesis from $C_{18:1}$ in the endoplasmic reticulum or NADH serving as the sole reductant. Likewise, similar δD values of both $C_{28:1}$ and $C_{30:1}$ fatty acids (Table 1) may result from their synthesis in the Golgi apparatus. The hydrogen isotope data thus support the notion that the $C_{20:1}$ fatty acid is synthesized from $C_{18:1}$ in a different location than that in which subsequent elongations occur.

4.3.2. D/H fractionation in acetogenic lipids: alkadienes

The observed ~40‰ deuterium depletion of alkadienes relative to fatty acids (Fig. 4a, b) can be attributed to D/H fractionation during elongation

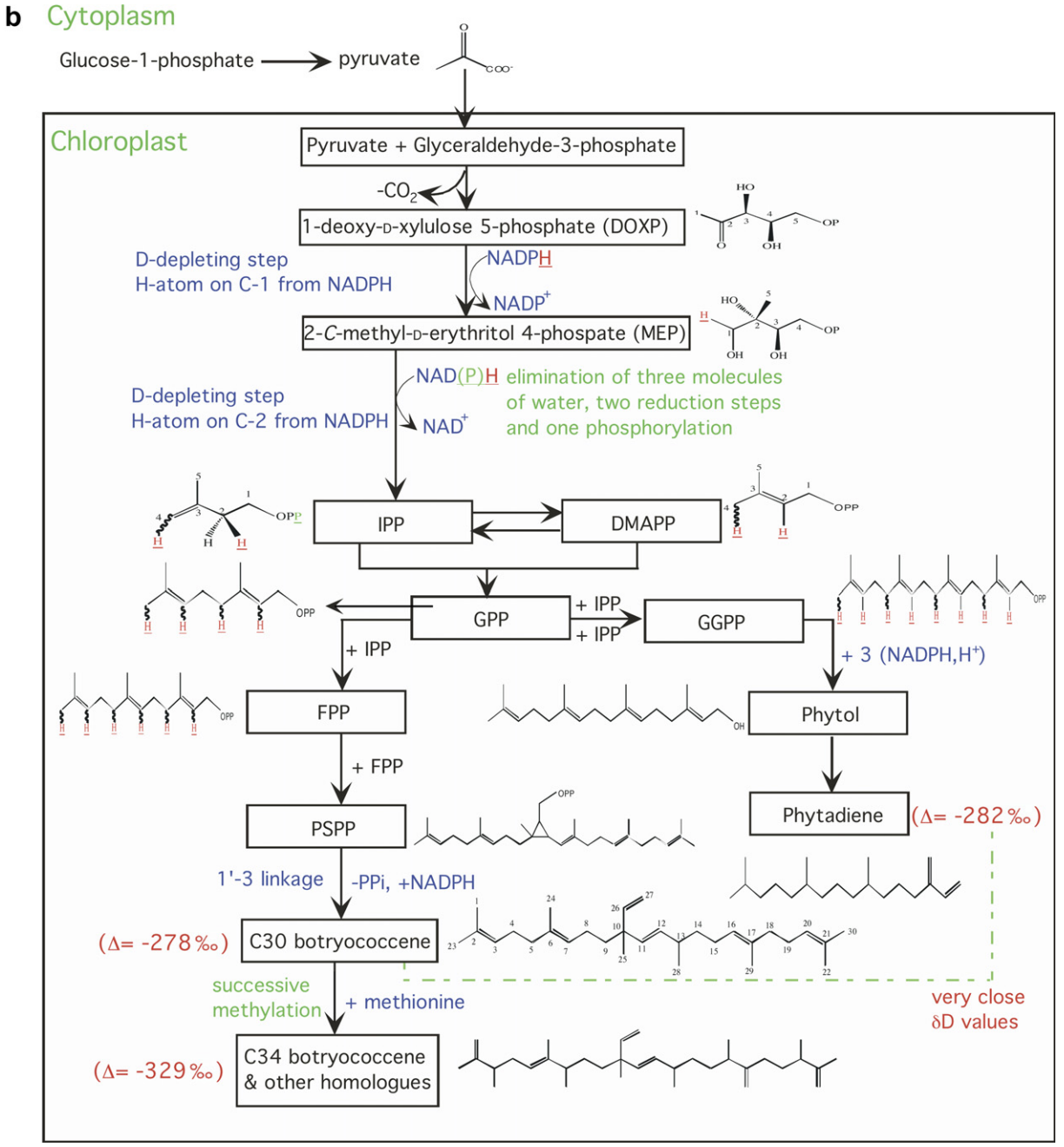

Fig. 5 (*continued*)

and decarboxylation of $C_{18:1}$ fatty acids during alkadiene (and alkatriene) biosynthesis (Templier et al., 1984, 1991). Considering that D/H fractionation during elongation of fatty acids is significantly less than 40‰ it is possible that the decarboxylation process is characterized by a large kinetic isotope effect (Fig. 5a).

Furthermore, we note that the C_{29} and C_{31} alkadienes had similar δD values in both (Titicaca and Morocco) A race strains, whereas the C_{27} alkadiene was enriched in deuterium (Fig. 1a and d; Table 1), suggesting a common biosynthetic route for C_{29} and C_{31} that differed from that for C_{27}. The C_{29} alkatriene δD values were similar to the C_{29} alkadiene

values suggesting that further desaturation resulted in little or no D/H fractionation.

4.3.3. D/H fractionation in isoprenoid lipids

δD values of isoprenoid (i.e., branched) lipids, such as botryococcenes and phytadienes, were much lower than δD values of acetogenic lipids (Tables 1–3). This pattern has been observed previously by several researchers and attributed to the different biosynthetic pathways for those two classes of lipids (c.f., Sessions et al., 1999; Hayes, 2001).

Green algae use the DOXP/MEP pathway exclusively for isoprenoid synthesis (Lichtenthaler, 1999; Schwender et al., 2001; Sato et al., 2003). Hydrogen atoms in isoprenoids have two sources. The decarboxylation of pyruvate and G3P yields DOXP (Schwender et al., 1997; Charon et al., 1999, 2000). A rearrangement followed by a reduction leads to the formation of MEP, which possesses the C_5 isoprene backbone (Fig. 5b) (Charon et al., 1999, 2000). That step uses NADPH as a hydrogen donor, thus incorporating deuterium-depleted hydrogen to C1 of MEP (corresponding to C-4 of IPP) (Fig. 5b). Conversion of MEP into IPP results in the elimination of three water molecules via two reductions and one phosphorylation (Lichtenthaler, 1999; Rohmer, 1999). Hydrogen atoms on C2 and C4 of IPP and/or DMAPP (dimethylallyl diphosphate) comes from NADH or NADPH (Charon et al., 1999), while the remaining hydrogen atoms comes from DOXP/MEP. It is likely that the IPP synthesis is responsible for much of the deuterium-depletion in isoprenoids from green algae (Fig. 5b).

IPP and farnesyl diphosphate (FPP), the direct precursors C_{30} botryococcene, are synthesized via the DOXP/MEP pathway in *B. braunii* (B Race) (Sato et al., 2003) via a 2-step reaction in the chloroplast (Okada et al., 2004) (Fig. 5b). In the first step two molecules of FPP are condensed to form presqualene diphosphate (PSPP). In the second step, the cycloprapane ring in PSPP is cleaved, followed by reduction with NADPH (Fig. 5b) (Okada et al., 2004).

C_{30} botryococcene is rapidly converted to higher molecular weight (C_{31}–C_{34}) botryococcenes by successive methylation reactions (Metzger et al., 1987). The source of the additional methyl groups is presumably methionine, the origin of which remains unknown (Metzger et al., 1987). The fact that we observed progressively more deuterium-depletion with increasing carbon number (i.e., C_{30}–C_{34}) in botryococcenes suggests either that the methionine is depleted in deuterium relative to the C_{30} botryococcene and/or there is a kinetic isotope effect associated with the methylation reaction. The position of methylation on the C_{30} botryococcene may also influence the δD values of different isomers.

Phytadiene (specifically, neophytadiene (Compound **8** in the Appendix A), the most abundant isomer) is produced by dehydration of phytol (Volkman

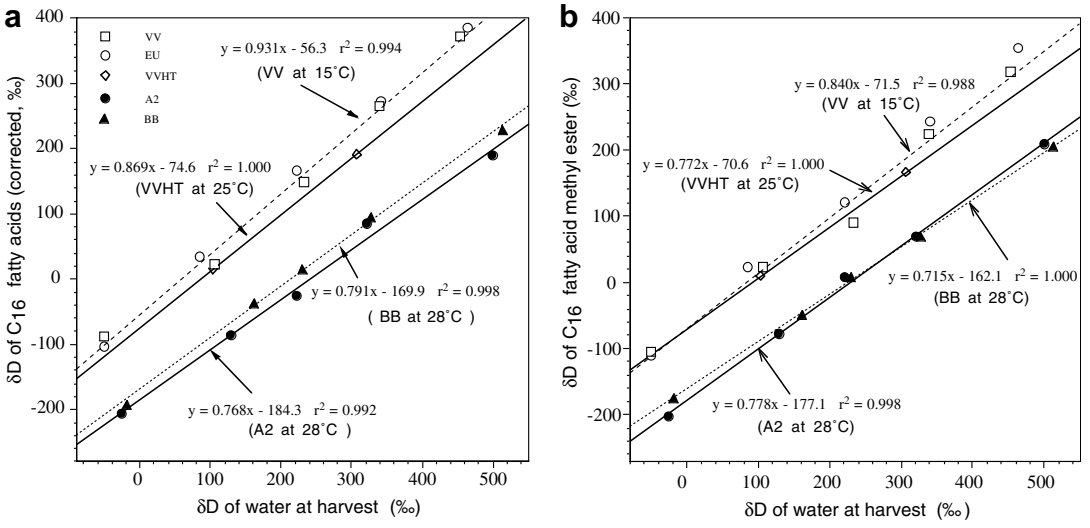

Fig. 6. Species and family dependence of D/H fractionation during synthesis of (a) palmitic acid ($C_{16:0}$) and (b) C_{16} FAME. Large (~100‰) differences in D/H fractionation in a single lipid are observed between families of green algae. The effect is much larger than for temperature, which has a small influence (see, for example, the lower slope for the high-temperature (VVHT, 25 °C) experiment with *V. aureus* compared to the 15 °C experiment (VV)). Acronyms are as follows: EU: *E. unicocca* grown at 15 °C. VV: *V. aureus* grown at 15 °C. VVHT1 and VVHT2: *V. aureus* grown at 25 °C. BB: *B. braunii*, B race, Martinique strain grown at 28 °C. A2: *B. braunii*, A race, Titicaca strain, grown at 28 °C.

and Maxwell, 1986; Grossi et al., 1996), which in turn is synthesized from GGPP (geranylgeranyl diphosphate) during three hydrogenation reactions using NADPH (Chikaraishi et al., 2004a). IPP and DMAPP serve as precursors to GGPP (Fig. 5b). Though we did not measure the δD value of phytol we did observe ε values of −251‰ to −298‰ in phytadienes, relative to water, in three species (*B. braunii* (B race), *E. unicocca* and *V. aureus*), in accord with Chikaraishi et al. (2004a).

4.3.4. Summary of D/H fractionation during lipid synthesis

Though fatty acids, botryococcenes and phytadienes in the Martinque strain of *B. braunii*, and fatty acids and phytadiene in *V. aureus* are all synthesized in the chloroplast (cf. Sato et al., 2003), presumably with the same pool of NADPH, significant hydrogen isotopic differences exist between botryococcenes/phytadienes and fatty acids in *B. braunii*, and phytadiene and fatty acids in *V. aureus*. These hydrogen isotopic differences between acetogenic and isoprenoid lipids result from different biosynthetic pathways for the two types of lipids.

4.4. Species-dependence of hydrogen isotope fractionation in green algae

A small but significant difference of ~10–15‰ in D/H fractionation during lipid synthesis was observed in different species, while a large difference of ~90–100‰ was observed between families of green algae (*B. braunii*, from the Trebouxiophyceae; *E. unicocca* and *V. aureus* from the Chlorophyceae) (Fig. 6a and b).

The water–lipid isotopic difference for two compounds (C_{16} FA and FAME) from four species and two families was compared (Fig. 6a and b). Notwithstanding a small influence of temperature on D/H fractionation during C_{16} FA and FAME synthesis of ~–3‰/°C (Zhang and Sachs, unpublished results), deuterium depletion in C_{16} FAs (Fig. 6a) and FAMEs (Fig. 6b) was much greater (i.e., >110‰) in *B. braunii* (Trebouxiophyceae) compared to either *V. aureus* or *E. unicocca* (Chlorophyceae), suggesting a large inter-family difference in D/H fractionation.

Between species the water-C_{16} FA isotopic difference was much smaller, amounting to 15‰ between the B (Martinique) (−177‰) and A (Titicaca) (−192‰) races of *B. braunii* (Table 2), and 8‰ between *E. unicocca* (−50‰) and *V. aureus* (−58‰) (Table 3). Similarly, the water-C_{16} FAME isotopic difference was 1‰ between Martinique (−184‰) and Titicaca (−185‰) strains of *B. braunii*, and 8‰ between *E. unicocca* (−70‰) and *V. aureus* (−78‰) (Tables 2 and 3).

Schouten et al. (2006) studied the effect of growth rate on D/H fractionation in alkenones from marine coccolithophorids. In a subsequent paper, we will explore the role that growth rate may play in D/H fractionation of lipids. Here, we note that a 5-fold difference in growth rate (at constant temperature) caused little change in ε. So although the large difference in D/H fractionation during C_{16} FA and FAME synthesis in *B. braunii* vs. *E. unicocca* and *V. aureus* may result in part from differing growth rates, there are likely to be large differences in the hydrogen isotopic fractionation during synthesis of a single lipid by different families of algae.

4.5. Implications for paleohydrologic reconstructions from lipid D/H

The near-perfect linear correlation ($R^2 > 0.99$) between all lipids studied and water δD values in five species of freshwater microalgae, and in alkenones from the marine phytoplankton *Emiliania huxleyi* (Englebrecht and Sachs, 2005), provides a sound basis for using sedimentary algal lipid δD values to reconstruct water δD values through time. An important caveat to doing so, however, is that very little is known yet about the role environmental parameters such as nutrients, light, temperature and salinity may play in influencing D/H fractionation during lipid synthesis. Studies we and other researchers are conducting ought to shed light on these potential influences in the near future.

Tables 4 and 5 list the linear regression equations for all lipids we have studied as well as α and ε values. Though the empirical linear regression equations can usually be used to derive water δD values from lipid δD values, neither the slope nor the intercept are equivalent to D/H fractionation as expressed by α and ε. Furthermore, simply adding ε to lipid δD values to reconstruct water δD values could be inappropriate if water δD values span a large range. We therefore recommend reconstructing water δD values from lipid δD values using α, which accounts for the D/H discrimination between lipid and water, using the equation: $\delta D_{water} = (\delta D_{lipid} + 1000)/\alpha - 1000$.

To demonstrate the utility of this approach we calculated water δD values from palmitic acid δD values in five *B. braunii* (Martinique) cultures using the average α value (0.816) reported in Table 4, and

compared this value to the measured δD_{water} values in those cultures at the time of harvest. The differences were 0.5‰, 8.1‰, 4.2‰, 3.4‰, and −20.4‰ for BBW1 to BBW5, respectively. Except for BBW5, which had $\delta D_{water} = 500‰$, the reconstructed δD_{water} values are satisfactory.

Furthermore, if non source-specific lipids, such as most fatty acids and sterols, are used to reconstruct water δD values, the influence of changing organic matter inputs to the particular lake or ocean sediment must be considered. Compounds such as palmitic acid (C_{16}) and oleic acid ($C_{18:1}$) are ubiquitous, being found in higher plants, algae and bacteria. Because large (>90‰) differences in D/H fractionation were observed in palmitic acid in different families of green algae (Fig. 6a) it is prudent to assume similarly large differences occur in other types of algae. Consequently, a change in the proportion of different algae in a lake would be expected to alter the δD value of palmitic acid deposited in sediment, absent any change in the lake water D/H ratio.

Palmitic acid and other lipids that derive from both aquatic and terrestrial plants and algae further confound the interpretation of down-core changes in δD measured on a non source-specific (i.e., ubiquitous) lipid because higher plant lipids are enriched in deuterium by ~30–60‰ relative to aquatic lipids (Sachse et al., 2004). Thus any change in the proportion of palmitic acid derived from aquatic algae and higher plants would be expected to alter the δD value of sedimentary palmitic acid in the absence of any change in lake or meteoric water δD values.

Clearly the use of non-specific lipids in lake sediment, such as palmitic acid, for reconstructing lake water δD values and paleohydrology is susceptible to misinterpretation (cf Huang et al., 2002). A better approach for down-core lake water δD reconstructions is the use of a lipid biomarker specific to a family of plants or algae, if not a species (the interspecies differences being rather small at ~5–15‰), and for which the fractionation factor, α, or an empirical water–lipid δD calibration has been developed. An example would be the C_{34} botryococcene, produced solely by the B race of *B. braunii*.

5. Conclusions

Here we have shown that lipids from five species of cultured green algae, including *E. unicocca*, *V. aureus* and three strains of *B. braunii*, were near-perfect recorders ($R^2 > 0.99$) of water D/H ratios. Barring any as yet unknown environmental influences on D/H fractionation during lipid synthesis, algal lipid δD values can therefore be used as surrogates for water δD values when the lipids derive from a single species or family.

All algal lipids were highly depleted in deuterium relative to environmental water, the result of kinetic isotope effects during enzymatic processes. In the green algae we studied, deuterium depletion in lipids varied with the biosynthetic pathways that produced them. As observed previously in other plants and algae, isoprenoid lipids (botryococcenes and phytadiene), which are synthesized via the DOXP/MEP pathway, were highly depleted in deuterium relative to acetogenic lipids (straight-chain fatty acids, alkadienes, etc.), which are synthesized via the acetogenic pathway.

Systematic hydrogen isotopic differences were associated with carbon chain length. The $C_{20:1}$ fatty acid was consistently deuterium-enriched relative to the $C_{28:1}$ and $C_{30:1}$ fatty acids, both of which were enriched in deuterium relative to the C_{16} and $C_{18:1}$ fatty acids. We attribute these differences to the site of elongation, which differs for $C_{18:1}$–$C_{20:1}$, and for $C_{20:1}$–$C_{28:1}$/$C_{30:1}$ fatty acids. Similarly, the C_{30} botryococcene is enriched in deuterium relative to longer (C_{31}–C_{34}) homologues, implicating the methylation reactions as an additional source of hydrogen isotopic fractionation.

Alkadienes, the products of elongation and decarboxylation of the $C_{18:1}$ fatty acid, are depleted in deuterium by 40‰ relative to that precursor, presumably the result of isotopic discrimination during the decarboxylation reaction.

D/H fractionation in lipids also varies between species of green algae. Deuterium depletion in a single compound, such as the C_{16} fatty acid (palmitic acid), was approximately 100‰ greater in *B. braunii* than in both *E. unicocca* and *V. aureus*.

Attempts at reconstructing water δD values through time using non-source-specific lipids, such as palmitic acid, therefore run the risk of misinterpreting changes in the source of sedimentary lipids for changes in water D/H ratios. Lipid biomarkers unique to a family (or genus) should be targeted for down-core reconstructions of water D/H ratios using empirically derived water–lipid fractionation factors (α) established from culture experiments.

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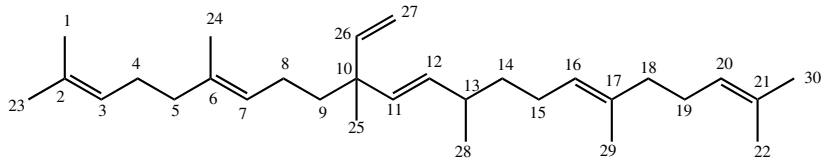
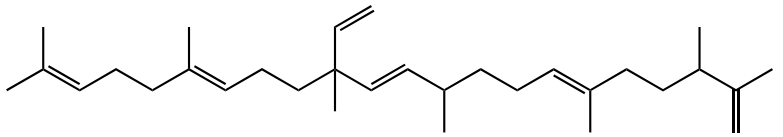
Appendix A

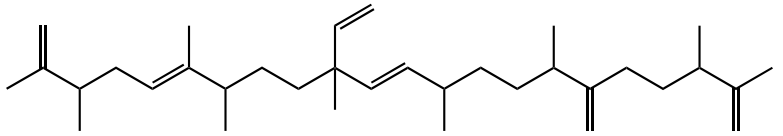
Molecular structures of hydrocarbons discussed in this paper. Compounds **2**–**7** are botryococcenes containing 30–34 carbon atoms.

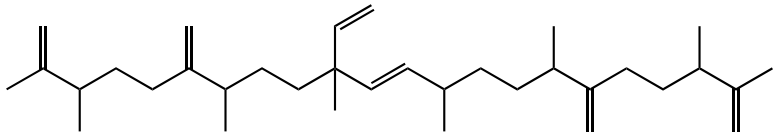
1. $CH_3(CH_2)_7CH{=}CH(CH_2)_xCH{=}CH_2$ Where x=15, 17 or 19

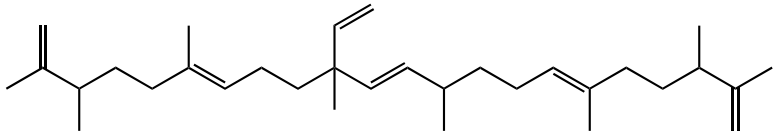
2. 

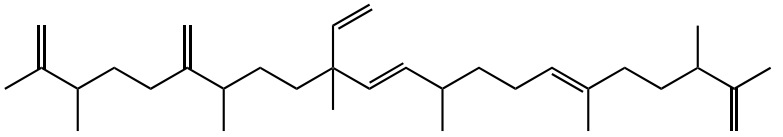
C30 bot

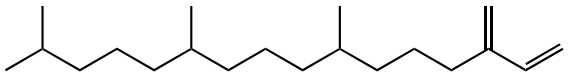
3. 
C31 bot

4. 
C34 bot

5. 
C34 bot-iso

6. 
C32 bot

7. 
C33 bot

8. 
Phytadiene

9. $CH_3(CH_2)_6CH{=}CH(CH_2)_7CH_3$

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.orggeochem.2006.12.004.

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