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Seasonal changes in D/H fractionation accompanying lipid biosynthesis in *Spartina alterniflora*

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Abstract

To investigate potential variability in the biosynthetic fractionation of hydrogen isotopes between environmental water and plant lipids, the cord grass *Spartina alterniflora* was sampled from a single location in a coastal marsh over a period of 16 months. Values of δD for a variety of lipids were measured by gas chromatography/pyrolysis/isotope ratio mass spectrometry. *S. alterniflora* grows partially submerged in seawater, so it has a virtually unlimited supply of water with nearly unvarying isotopic composition. Temporal changes in the δD values of lipids can thus be interpreted as representing mainly variations in biosynthetic fractionation. Fatty acids, *n*-alkanes, and phytol extracted from *S. alterniflora* have nearly constant δD values from ~October through May, but exhibit marked decreases of up to 40‰ during summer months. These shifts in lipid δD values are interpreted as representing a change in the source of organic substrates, principally acetate, used for their biosynthesis. Lower summertime δD values for lipids are consistent with an increasing reliance on current photosynthate as feedstock for biosynthesis, whereas stored carbohydrate reserves are utilized more extensively during other times of the year. Regardless of the specific mechanism, the data emphasize that overall fractionations between water and plant lipids depend on biological as well as environmental variables, and that the biosynthetic fractionation is not necessarily constant even for a single plant. Because lipids such as fatty acids are present in all cells and turn over on timescales of weeks to months, measurements of δD values in fatty acids may also provide useful constraints for distinguishing biologic versus environmental controls on cellulose δD values in trees.

1. Introduction

The development of methods for measuring D/H ratios in individual lipids has lead to a surge of recent interest in using these data both as a tracer for sources of organic matter in the environment, and as a proxy for paleoclimatic variables. In the former case, useful application requires diagnostic differences in D/H ratios between different types of organisms, such as C3 versus C4 plants. Most previous research in this area has focused on describing the natural variability of D/H ratios in lipids between different species of organisms while seeking consistent patterns between related groups (Chikaraishi et al., 2004b; Bi et al., 2005; Chikaraishi and Naraoka, 2005). Clear differences between terrestrial and aquatic vegetation have been established (Chikaraishi et al., 2005), but uncertainties still exist regarding differences between other groups, such as phytoplankton versus macrophytes (Sessions et al., 1999) or C3 versus C4 plants (Bi et al., 2005).

In the second case (paleoclimate proxies), research has focused on surveys of modern environments spanning a range of environmental conditions in order to establish the relationship between D/H ratios of plant lipids and environmental water (Sauer et al., 2001; Huang et al., 2002, 2004; Sachse et al., 2004; Hou et al., 2006; Smith and Freeman, 2006). Studies focusing on particular lipids from terrestrial lakes in specific geographic regions have successfully documented strong correlations between δD values of lipids and environmental water (e.g., Huang et al., 2002). However, the correlations vary significantly between different studies (Sessions and Hayes, 2005)

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making it difficult to apply lipid δD values as a quantitative proxy for past environments.

Variability in the δD values of lipids, as well as other biochemicals, is controlled by both environmental and biological factors (Terwilliger and DeNiro, 1995). Climatic and hydrologic conditions influence the isotopic composition of environmental water (precipitation, groundwater, and surface water). That signal is then transferred to leaf water with some modification by biophysical processes, including uptake, transport and—most importantly—evapotranspiration (Roden et al., 2000; Barbour et al., 2004). Finally leaf water is used for the biosynthesis of plant lipids, a process accompanied by biochemical fractionations (Sternberg, 1988; Yakir and DeNiro, 1990). Differences in δD values between environmental water and plant lipids must reflect the sum of both biophysical and biochemical fractionations, and are referred to here simply as the net 'biological fractionation.' This biological fractionation is large, typically 150‰ or more, and is mainly due to fractionations associated with lipid biosynthetic pathways (Yakir and DeNiro, 1990; Sessions et al., 1999; Chikaraishi et al., 2004a).

Most previous studies of lipids have implicitly assumed that the net biological fractionation between lipids and environmental water should be nearly constant for, and thus characteristic of, individual species (e.g., Chikaraishi et al., 2004b). Efforts to develop lipid paleoclimate proxies have typically sampled a large range of environmental conditions while minimizing biological variance. Conversely, studies aimed at using D/H ratios to identify organic matter sources have generally limited environmental variability while maximizing biological diversity. However, the assumption of nearly constant biological fractionation has not yet been tested in detail. Indeed, there are reasons to suspect that it might not hold true. For example, Yakir and DeNiro (1990) showed that carbohydrates become progressively enriched in D during their transformation into storage products such as starch. Yakir (1992) suggested that the overall level of D-enrichment in plants might be used as an indicator of metabolic status. Studies of the hydrogen-isotopic composition of plant cellulose already take detailed account of both environmental and biological factors (Roden et al., 2000; Terwilliger et al., 2001), an approach that should prove beneficial for lipids as well.

Here, I present results from the first compound-specific D/H study of lipids in which both environmental and biological (species) variability is minimized in order to examine the constancy of biochemical fractionations. Leaves from the C4 cord grass *Spartina alterniflora*, a species common in many tidal marshes, were sampled and analyzed from plants growing at a single location over the course of 16 months. Because these plants are partially submerged in seawater twice daily, they have a virtually unlimited supply of water with nearly unvarying isotopic composition. Changes in the δD values of lipids can thus be interpreted as representing variability in the net biologic fractionation between environmental water and plant lipids. The results suggest that future studies aimed at characterizing the hydrogen-isotopic composition of plant lipids will need to account for significant seasonal, and potentially annual, variability in this fractionation.

2. Methods

2.1. Sample collection

Samples of *S. alterniflora* were collected over a 16-month period in 2002 and 2003 from Woodneck Marsh, a tidal salt marsh on the Buzzard's Bay side of Cape Cod, Massachusetts (Fig. 1). All samples were collected from a single, monospecific stand of grass located within the intertidal zone near the mouth of the marsh. No significant streams or rivers empty into Woodneck Marsh, limiting the influx of freshwater to groundwater and runoff from precipitation events. Seasonal water samples from the marsh were collected, but were mistakenly discarded before they could be analyzed. Thus temporal variations in environmental water could not be directly measured in this study. They are assumed to be minimal, an assumption that is examined more closely in the discussion.

Leaf samples were collected by cutting plants at ground level with scissors, and were stored in Ziplock freezer bags at −20 °C until processing. Samples were generally collected in the early morning without regard to tidal phase. No samples were collected during January–March 2002 because of heavy snow and ice cover. A total of 53 samples were collected, of which 34 were analyzed for D/H isotopic composition (not including replicate extractions).

2.2. Lipid extraction

For most samples, 3–4 leaves from a single plant were homogenized to provide sample material. In some cases, single leaves or parts of a leaf were used (see below). Leaves were washed by hand in distilled water, cut into 5-mm pieces with solvent-cleaned scissors, lyophilized, and ground with a mortar and pestle under liquid N_2. Powders were next saponified in 0.5 M NaOH in H_2O at 70 °C for 4 h. No effort was made to distinguish free versus esterified lipids. Samples were extracted by shaking three times with methyl *t*-butyl ether, using centrifugation to break the strong emulsion that developed. The collected extract was dried over Na_2SO_4 and an aliquot was analyzed by gas chromatography/mass spectrometry (GC/MS) for lipid abundance. The remaining extract was separated into four fractions by solid-phase extraction (SPE) following the method described in Sessions et al. (1999). Briefly, SPE separations used 0.5 g of Discovery DSC-NH2 stationary phase (Supelco) hand-packed into 8 mL glass syringe barrels. Samples were eluted in four fractions: F1 (hydrocarbons), 4 mL hexane; F2 (ketones and ethers), 7 mL 4:1 hexane/dichloromethane (DCM); F3 (alcohols), 7 mL 9:1 DCM/acetone; F4 (carboxylic acids), and 8 mL 50:1 DCM/formic acid. Alcohols (F3) were derivatized as

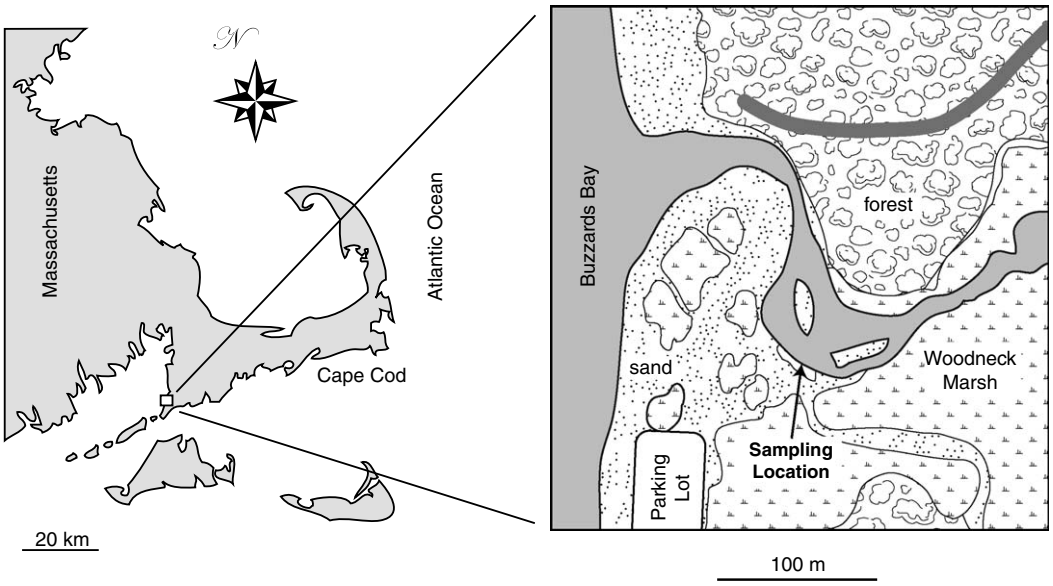

Fig. 1. Sample location map. The study site is located at 41°34′34.0″N, 70°38′24.4″W. The sampling location near the mouth of the marsh was chosen because it experiences maximal tidal flushing and hence minimizes the influence of evaporation and groundwater mixing on D/H ratios of water stored within in the marsh.

acetate esters by reaction with acetic anhydride/pyridine. Fatty acids (F4) were derivatized as methyl esters by reaction with BF_3/methanol.

2.3. GC/MS analyses

Ten percent of each total lipid extract was derivatized with bis-(trimethylsilyl)-triflouroacetamide (BSTFA) to form TMS–ethers, and was analyzed by GC/MS for lipid identity and abundance. Analyses were performed on a ThermoFinnigan TraceGC equipped with a DB-5ms column (30 m × 0.25 mm × 0.25 μm) and a PTV injector operated in splitless mode. The GC effluent was split (~3:1) between a ThermoFinnigan DSQ mass spectrometer and a flame-ionization detector (FID) for peak identification and quantitation, respectively. Compounds were identified by comparison of mass spectra and retention time to library data. Lipid abundances were calculated by reference to an internal standard (20 ng of either 5α-androstane or isobutyl palmitate) using FID peak areas. The *cis*/*trans* configuration of double bonds in C18:1 fatty acids were confirmed by comparison of retention times to authentic standards. Configuration of other unsaturated fatty acids could not be confirmed, but are presumed to be *cis*.

2.4. D/H analyses

Because hydrogen-isotopic analyses require >10-fold larger samples than do GC/MS analyses, only the most abundant compounds in each fraction were analyzed for D/H ratio. All of the unsaturated C_{18} fatty acids coeluted to some degree in these isotopic analyses, so a single δD value was measured for all three compounds. The D/H ratios of individual lipids were measured using a ThermoFinnigan TraceGC coupled to a Delta + XP mass spectrometer via a pyrolysis interface (Finnigan GC/TC) operated at 1440 °C. Separations were performed using the same conditions (injector, column, and temperature program) as for the GC/MS. Typically 4–6 *n*-alkanes or fatty acid methyl esters (FAMEs) of known D/H ratio were coinjected with each sample. Two of these compounds were used as reference peaks for the calibration of isotopic analyses, while the remainder were treated as unknowns to test accuracy. Data were then normalized to the SMOW/SLAP isotopic scale by comparison to an external standard containing 15 *n*-alkanes with δD values ranging from −41 to −256‰ (Sessions et al., 2001). Correction for H added by both methylation and acetylation reagents was performed by analyzing dimethyl phthalate and acetic anhydride as described by Sessions et al. (2002). All isotopic data are reported in the typical δD notation, in units of permil (‰) relative to the V-SMOW standard.

The mean precision for replicate analyses of the external *n*-alkane standard compounds was 2.1‰ (1σ), and the root-mean-square (RMS) error for all external standards was 3.1‰ ($n = 2178$) during the period when these samples were analyzed. For the internal standards (i.e., compounds coinjected with samples that were not treated as isotopic reference peaks) precision averaged 5.7‰ (1σ), while the absolute error in δD values for these compounds varied from 1‰ to 16‰. The RMS error for all internal standards was 14.8‰. Because results for these internal standards are (i) less accurate than for external standards, (ii) much more precise than they are accurate, and (iii) not systematically biased with respect to 'true' δD values (the average error for internal standards, considering both positive and negative deviations, was −0.15‰), this result can be attributed to the coelution of minor sample components with some

internal standards. In summary, the uncertainty associated with repeated analyses of the same compounds in multiple samples is likely <6‰. Because of the systematic offsets described above, comparison of these data with other studies may be subject to uncertainties as large as ~15‰.

3. Results and discussion

3.1. Lipid abundance

The lipid composition of five representative samples, and the average for all 34 samples, is given in Table 1. Because the dataset contains more samples collected during summer months than winter, these average values are biased towards plants harvested during the summer. Complete compositional data are provided in the electronic annex accompanying this manuscript. Prominent lipid components included C_{27}, C_{29}, and C_{31} *n*-alkanes, C16:0, 18:1, 18:2, and 18:3 fatty acids, phytol, β-sitosterol (24-ethylcholest-5-en-3β-ol), and the pentacyclic triterpenoids lupenone and lupenol. The *trans*-C18:1 and C18:3 (unknown configuration) fatty acids could not be separated (either as TMS ethers or as methyl esters) on the GC columns used in this study, and are combined into a single value in Table 1. The abundance and distribution of lipids in *S. alterniflora* measured here is broadly consistent with results previously described by Schultz and Quinn (1973) and Canuel et al. (1997). Minor amounts of ergosterol (24-methylcholest-5,7,22-trien-3β-ol) in samples collected in winter and early spring likely represent a small input from fungi (Davis and Lamar, 1992). However, there was no observable correlation between concentrations of fatty acids and ergosterol, suggesting that fungal growth did not contribute significantly to the fatty acids. Fatty acids averaged 78% of the total lipid extract (range 40–90%), triterpenoids averaged 13%, *n*-alkanes averaged 3.5%, and phytol averaged 4.5%.

Seasonal changes in lipid abundance are shown in Fig. 2. The most prominent seasonal change observed by Schultz and Quinn (1973), a shift from C18:1 to C18:3 as the most abundant fatty acid during the summer, could not be duplicated here because of coelution of the two

Table 1
Abundance of lipid compounds for five representative samples and for the average of all samples

Sample, collection date:	Sp-20, 5/6/03[a]	Sp-19, 5/6/03[a]	Sp-53, 7/26/03	Sp-46, 10/8/03	Sp-15, 12/19/02	Average[b]	Range[b]
Hydrocarbons (% of total lipids)							
n-C_{27}	2.5	0.5	0.3	0.5	1.3	0.7	3.7
n-C_{29}	5.7	0.7	0.5	1.2	3.4	2.1	11.1
n-C_{31}	2.0	0.8	0.2	0.7	0.7	0.7	4.0
Diterpenoids (% of total lipids)							
Phytol	1.3	4.5	5.8	4.9	3.5	4.5	6.8
Phytanoic acid	1.5	nd[c]	nd	nd	3.0	0.3	3.0
Triterpenoids (% of total lipids)							
24-Ethylcholesta-3,5-diene	2.4	0.2	nd	nd	0.7	0.2	2.4
Ergosterol	1.6	nd	nd	nd	0.8	0.3	3.2
Pentacyclic triterpenol[d]	4.3	nd	nd	1.9	5.1	2.8	11.0
β-Sitosterol	2.2	4.2	4.1	2.1	2.7	3.5	12.9
Lupenone	2.9	0.5	1.3	2.1	1.3	1.5	5.8
Lupenol	6.4	0.4	0.3	1.9	5.6	4.1	25.2
Fatty acids (% of total lipids)							
12:0	4.6	0.2	0.1	0.3	3.7	0.7	4.6
14:0	3.7	0.3	0.3	0.3	3.6	0.7	3.7
16:0	14.5	21.4	18.2	16.5	18.3	16.8	13.4
16:1	1.2	nd	1.6	0.7	nd	0.7	1.7
18:0	5.9	2.4	1.4	1.9	4.8	2.4	7.3
Cis-18:1	2.4	nd	nd	nd	0.2	0.2	2.4
Trans-18:1 + 18/3[e]	10.4	46.7	49.3	49.3	20.8	42.0	47.8
18:2	15.7	15.2	13.9	12.9	14.7	12.8	17.9
20 :0	1.0	0.4	0.4	0.1	0.8	0.3	1.0
22 :0	2.6	0.4	0.3	0.2	1.1	0.5	2.6
24 :0	1.7	0.3	0.3	1.0	0.5	0.4	2.1
Total lipids (μg/g dry weight)	780	8944	10,417	3824	2386	6038	16,577

[a] Samples Sp-19 and -20 were collected from the same location at the same time. Sp-20 consisted of dead leaves from the previous year's growth, while Sp-19 consisted of new (green) growth.

[b] Values are the arithmetic mean and total range for all samples collected during this study, including multiple leaves and plants collected on a single day but excluding replicate extracts of a single sample.

[c] Not detected.

[d] Specific identity of this triterpenol could not be confirmed from the mass spectral data.

[e] *Trans*-C18:1 and C18:3 fatty acids coeluted and their combined abundance is reported as a single value. The C18:3 fatty acid is believed to possess the *cis*-double bond configuration, though this could not be confirmed.

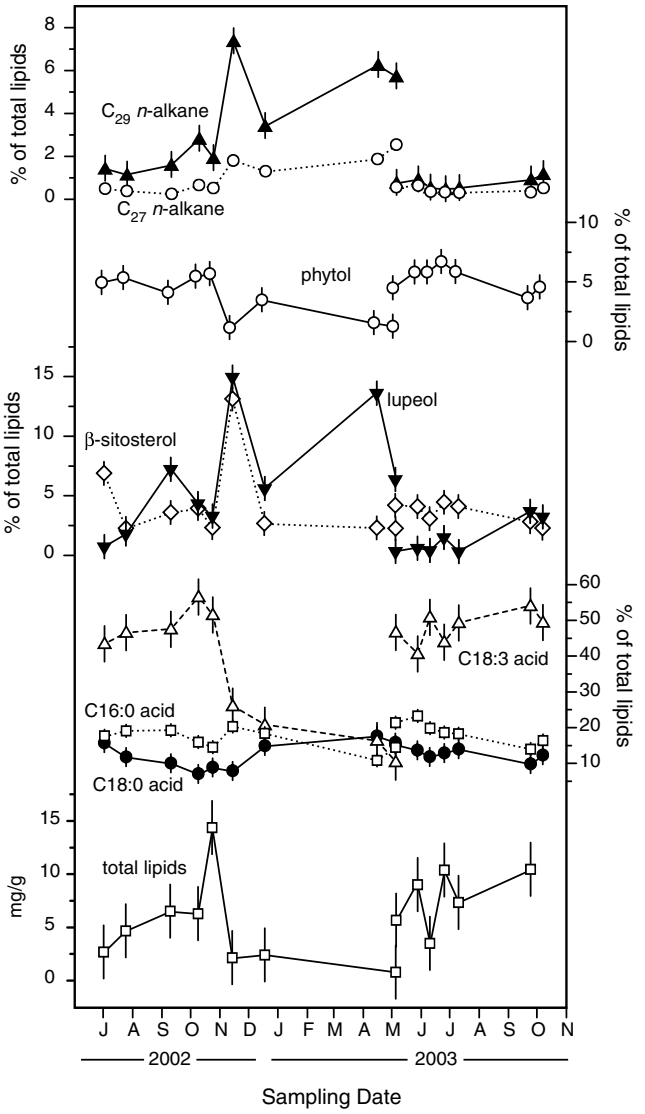

Fig. 2. Seasonal changes in relative lipid abundance. Where multiple samples were collected on a single day, average values are plotted. Error bars represent the standard deviation (1σ) for each compound obtained for replicate extractions of a single homogenized sample. They are approximately the size of the symbols for C_{27} *n*-alkane and C16:0 fatty acid. The break in lines connecting symbols at May, 2003 demarks the emergence of a new year's growth. Values for total lipid abundance are reported on a dry weight basis.

compounds. However, their relative abundances, based on selected-ion chromatograms, were consistent with this pattern. Fatty acid concentrations as a whole declined during the winter months, though this appears to reflect primarily a loss of the C18:1 and C18:3 fatty acids. Phytol concentrations also were lower during winter months, while C16:0 and C18:2 fatty acids remained at nearly constant concentration year-round. In contrast, both the absolute concentration and relative abundance of *n*-alkanes increased gradually throughout the year, returning to low values with the onset of a new season's growth. Lupenol concentrations increased throughout the year while those of β-sitosterol decreased slightly. The observed patterns of abundance are consistent with the rapid metabolic turnover of components such as C_{18} fatty acids and phytol, and their subsequent loss once biosynthesis ceased in the fall. The gradual increase in concentrations of *n*-alkanes might be attributed to a generally slower turnover rate, or to their greater resistance to microbial degradation. This question is examined in more detail below.

3.2. Lipid D/H ratios

Values of δD for lipids from five representative samples and the annual average are reported in Table 2. Complete isotopic data are provided in the electronic annex. In general, *n*-alkane δD values ranged from −125‰ to −192‰ with average values near −160‰. No significant differences between C_{27}, C_{29}, or C_{31} *n*-alkanes were discernible. Fatty acid δD values were systematically more enriched, averaging −145‰ and −126‰ for the C_{16} and C_{18} acids, respectively. A slight but persistent offset between C_{16} and C_{18} fatty acids was observed. Values of δD for the triterpenoids β-sitosterol and lupenol ranged from −122‰ to −191‰, and the diterpenoid phytol was strongly depleted in D, averaging −272‰.

To assess the spatial variability of δD values in *S. alterniflora*, several types of samples were analyzed in triplicate (Table 3). The results indicate that for all lipid compounds except fatty acids, the spatial variability—between plants, leaves, and parts of leaves—is no greater than the analytical uncertainty attributable to multiple extractions of the same sample, roughly 4–7‰. For the fatty acids, variability was highest between different parts of a single leaf, and was significantly greater than that for replicate extractions. This increased variability reflects a pattern of increasing δD value towards the tip of each leaf (Fig. 3) with an average 20‰ enrichment of D in the top third of a leaf relative to the bottom third. Data for *n*-alkanes suggest a similar pattern, though the enrichment is not statistically significant for those compounds. This pattern of isotopic enrichment from stem to leaf tip is qualitatively similar to the ~50‰ enrichment observed in leaf-water $\delta^{18}O$ by Helliker and Ehleringer (2000) for greenhouse-grown *Miscanthus sinensis*, another C4 grass. They attribute the pattern to a combination of evaporative isotopic enrichment, back-diffusion within leaf tissues, and transport of water through veins. The smaller D-enrichment in *S. alterniflora* is likely due to the much higher humidity in Woodneck Marsh relative to the greenhouse conditions utilized in the former study (35% relative humidity). Given that a measurable D-enrichment in leaf water exists even in high-humidity conditions that should minimize evaporation, these data provide support for the hypothesis of Smith et al. (2006) that lipids from C4 grasses will, on average, be enriched in D relative to those from C3 grasses because of increased evaporative enrichment of leaf water.

Seasonal changes in δD values are shown in Fig. 4. The most prominent feature of these data is a significant decrease in δD values of *n*-alkanes, fatty acids, and to a lesser extent phytol during the summer months. The most strongly

Table 2
Values of δD (‰) for selected lipid compounds in five representative samples, and for the average of all samples

Sample, collection date:	Sp-20, 5/6/03[a]	Sp-19, 5/6/03[a]	Sp-53, 7/26/03	Sp-46, 10/8/03	Sp-15, 12/19/02	Average[b]	Range[b]
Hydrocarbons							
n-C_{27}	−157	−151	−178	−152	−156	−160	42
n-C_{29}	−161	−161	−192	−160	−166	−168	41
n-C_{31}	−136	−125	−133	−166	na[c]	−156	45
Diterpenoids							
Phytol	na	−274	−275	−275	−266	−272	38
Triterpenoids							
β-Sitosterol	−161	−183	−177	−174	−186	−174	46
Lupenol	−139	na	−135	−133	−151	−139	31
Fatty acids							
16:0	−142	−162	−169	−136	−128	−145	56
18:1-3[d]	−113	−144	−145	−120	−118	−126	54

[a] Samples Sp-19 and -20 were collected from the same location at the same time. Sp-20 consisted of dead leaves from the previous year's growth, while Sp-19 consisted of new (green) growth.
[b] Values are the arithmetic mean and total range (max–min) for all samples collected during this study, including multiple leaves and plants collected on a single day but excluding replicate extracts of a single sample.
[c] Data are not available because the lipid component was not sufficiently abundant for isotopic analysis.
[d] The mono-, di-, and tri-unsaturated fatty acids partially coeluted during isotopic analysis, and a single δD value was determined for all three compounds.

Table 3
Summary of variance for δD values in different sample populations[a]

Population tested	Collection date	C_{29} alkane	Phytol	β-Sitosterol	C16:0 fatty acid	C18 fatty acids
Replicate analyses of single extract	All[b]	3.5	4.1	3.5	1.8	1.7
Replicate extracts of single sample	April 2003	7.7	3.5	3.6	2.2	1.8
	June 2003	5.5	4.6	6.4	0.6	2.1
Different parts of single leaf[c]	July 2002	5.7	13.3	6.1	10.9	10.7
	October 2003	6.3	5.1	5.6	14.7	12.2
Different leaves from single plant	July 2002	4.2	4.7	9.5	15.4	9.8
	October 2003	7.9	5.2	1.8	5.5	2.1
Different plants from same location	October 2002	3.4	6.9	5.8	1.7	1.3
	October 2003	0.7	7.7	4.5	8.1	7.7

[a] Reported values are the standard deviations (1σ, ‰) of δD values for 3 or more samples collected on the same day.
[b] Reported values are the pooled standard deviation (1σ, ‰) for δD analyses of each compound in all 38 analyzed samples.
[c] A single leaf was sectioned into lower, middle, and upper thirds, each of which was then homogenized for analysis.

D-depleted values were recorded for plants collected during late July, 2003. A similar, though smaller, depletion of D was observed during July of 2002. Although β-sitosterol and lupenol appear to have a slight seasonal pattern of opposite sign (i.e., D-enrichment during the summer), this pattern is strongly influenced by a single sample collected in December 2002. That same sample possessed anomalously high abundances of lipids and is likely an outlier. Hence seasonal changes in δD values of the triterpenoids are not considered statistically significant.

3.3. Variations in biosynthetic fractionation

Given a virtually constant isotopic composition of environmental water, seasonal changes in lipid δD values from *S. alterniflora* will be controlled by three types of effects. First, evapotranspiration can alter the D/H ratio of leaf water due to preferential evaporation of the light isotope. Second, isotope effects accompanying specific biosynthetic reactions, for example the hydrogenation of carbon skeletons by NADPH, could vary. Third, the isotopic composition of substrates feeding into the biosynthetic reaction network can change as substrates are produced and consumed by varying metabolic pathways (Hayes, 2002).

The pattern of D-depletion in lipids during summer months is opposite that expected for evaporative enrichment of D in leaf water (effect 1 above). Leaf waters should become more D-enriched during the hotter, drier summer months (Roden et al., 2000), leading to a D-enrichment in lipids synthesized during that time. Although such effects must surely exist, they are apparently overwhelmed by competing biosynthetic fractionations. The summertime depletions in D of lipids must therefore represent minimum values for changes in fractionation associated with biosynthesis. Isotopic analysis of leafwater δD values would provide a means for parsing the net biological fractionation into its biophysical and biochemical components.

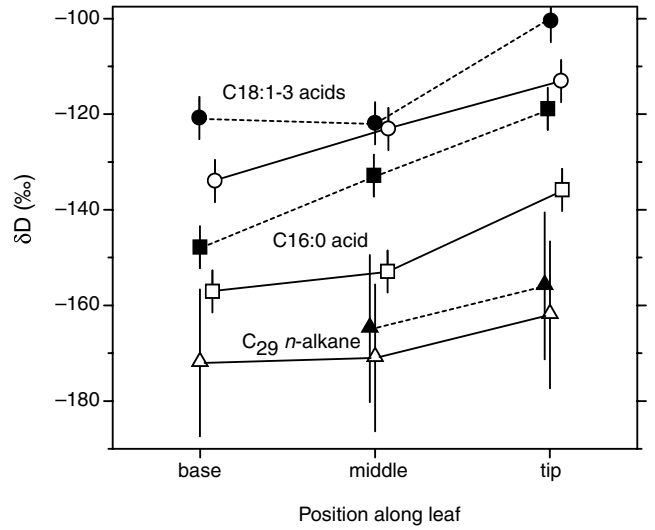

Fig. 3. Values of lipid δD (‰) as a function of position along a single leaf. Error bars represent the standard deviation (1σ) for each compound obtained for replicate extractions of a single homogenized sample (Table 3). Symbols have been slightly offset horizontally to improve visual clarity. Solid symbols represent samples collected in July, 2002, open symbols represent those collected in October, 2003.

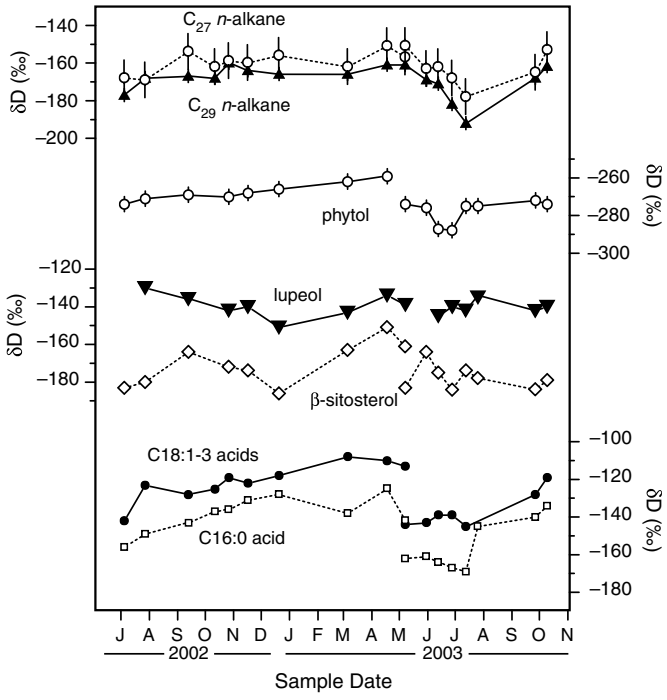

Fig. 4. Seasonal changes in lipid δD values (‰). Where multiple samples were collected on a single day, average values are plotted. Error bars represent the standard deviation (1σ) for each compound obtained for replicate extractions of a single homogenized sample (Table 3). Where error bars are not visible, they are smaller than the symbol size. The break in lines connecting symbols at May, 2003 demarks the emergence of a new year's growth.

Fractionations accompanying biosynthetic reactions (effect 2) are potentially large. However, they are also intrinsic to specific reaction mechanisms and so are thought to be nearly independent of variables such as temperature and reaction rate (Cleland, 2003). Large seasonal variations in these specific isotope effects are thus unlikely. Seasonal expression of different biosynthetic pathways and/or enzymes could lead to variations in observed fractionation, but seasonal changes in biosynthetic pathways have not been previously reported.

Changes in the isotopic composition of biosynthetic feedstocks (effect 3) provide the most plausible explanation for the observed seasonal changes in δD values. *n*-Alkyl lipids such as fatty acids and *n*-alkanes are biosynthesized via the successive polymerization and reduction of 2-carbon acetate monomers. Analysis of the details of this pathway suggests that ~25% of hydrogen atoms in the resulting lipid are derived from the precursor acetate molecules, ~50% derive from the reductant NADPH, and ~25% derive from cellular water during hydrogenation of double bonds (Sessions et al., 1999). Acetate is derived from simple sugars via glycolysis. Those sugars can, in turn, derive either from the immediate products of photosynthesis or from carbohydrates involved in other metabolic processes, including energy storage. NADPH is generated both during photosynthesis and from the heterotrophic recycling of sugars in the pentose-phosphate pathway. In the former case, the source of H used to reduce $NADP^+$ to NADPH is certainly water. In the latter case, the source of H is unknown and could be either cellular water or the oxidized sugar (Sessions et al., 1999).

Yakir (1992) reviewed experimental evidence that the δD values of plant carbohydrates are influenced by two competing fractionations. First, the immediate products of photosynthesis are strongly depleted in D relative to water, with an estimated fractionation of −171‰ (Yakir and DeNiro, 1990). This large depletion may be the result of kinetic isotope effects during the reduction of $NADP^+$ to NADPH, or may reflect the greater activity of H^+ relative to D^+ in water (Luo et al., 1991). Second, carbohydrates become progressively enriched in D as a result of heterotrophic reprocessing. For example, Luo and Sternberg (1991) showed that starch produced in chloroplasts (a direct product of photosynthesis) was depleted in D by up to 165‰ relative to available water while cytoplasmic cellulose was simultaneously enriched in D by as much as 25‰ relative to water. Up to one half of the carbon-bound hydrogen in carbohydrates is subject to isotopic exchange with water during heterotrophic carbohydrate metabolism as a result of rearrangement and isomerization reactions involving the abstraction of carbon-bound H (Yakir, 1992). Whether the enrichment of D in carbohydrates during such exchange represents a true equilibrium isotope effect, or preferential abstraction of H relative to D during isomerization, remains uncertain. Regardless, the data suggest that the δD values of plant carbohydrates are a function of the plant's net metabolic status, becoming more (or less) enriched in D with increasing (or decreasing) levels of respiration relative to photosynthesis. This principal has been confirmed in several subsequent studies of plant cellulose both in the laboratory (Terwilliger and DeNiro, 1995)

and in the field (Roden and Ehleringer, 1999, 2000; Terwilliger et al., 2001). Roden et al. (2002) further suggest that "when discussing inputs from different carbon sources for cellulose synthesis the terms *current photosynthate* and *stored reserves* should be adopted to reduce confusion," a convention that is followed below.

The seasonal changes observed in lipid δD values for *S. alterniflora* are in good qualitative agreement with the hypothesis of changing substrate utilization for biosynthesis. Photosynthetic rates are highest in mid-summer, when leaf surface area is at a maximum, incident sunlight is strongest, and temperatures are highest. Lipids synthesized during this period exhibit maximal depletion of D, consistent with utilization of acetate and NADPH derived directly from current photosynthate for lipid biosynthesis. In early spring, biomass is synthesized from energy reserves stored as carbohydrates in roots. In fall, heterotrophy again increases as photosynthesis diminishes and carbohydrates are stored for the following winter. Lipids produced during the fall and spring are more enriched in D, consistent with an increase in the utilization of stored reserves for biosynthesis.

If correct, this interpretation indicates a significant hurdle for efforts to use lipid δD values as proxies for paleoclimate. In particular, a large part of the lipid δD 'signal' previously attributed to variations in precipitation δD and humidity may derive from alterations in growth rate, seasonality, and metabolic status of plants. Differences between δD values measured in the summers of 2002 versus 2003 also suggest the possibility of annual variations in biosynthetic fractionation, though this will need to be confirmed over longer timescales. If lipid δD values are to be quantitative rather than qualitative proxies, such factors will need to be accounted for in more detail. The measurement of lipids from lake sediments does have the ability to capture average annual isotopic compositions of diverse ecosystems. However, such an approach will confound variability due to environmental and biological factors. Indeed, latitudinal changes in plant physiology and the length of growing season may help explain the fact that empirical calibrations do not measure a constant isotopic fractionation between environmental water and lipids over geographic transects (Sessions and Hayes, 2005).

Four details of the seasonal pattern of δD variation warrant further consideration. First, the timing of D-depletion varied significantly between *n*-alkanes and fatty acids. Fatty acids maintained relatively low δD values beginning with new growth in May and continuing through late summer, followed by a gradual increase in δD values throughout the remainder of the year (Fig. 4). In contrast, *n*-alkanes from new growth possessed δD values that were identical to the previous year's growth in May, but then declined rapidly in June–July and recovered rapidly to ~constant values for the remainder of the year. These data are consistent with numerous studies showing that fatty acid and leaf-wax biosynthesis are compartmentalized (summarized in Eglinton and Hamilton, 1967; Kolattuk, 1968). Fatty acids (excluding long-chain, leaf wax acids) are synthesized in the chloroplast of plant cells (Ohlrogge and Jaworski, 1997), and thus are able to access the immediate products of photosynthesis. In contrast, *n*-alkanes are thought to be produced only in epidermal cells, and their synthesis is independent of light availability and hence chloroplast activity (Kolattuk, 1970). A likely explanation for the differing seasonal patterns is that *n*-alkane biosynthesis draws on stored carbohydrates as biochemical feedstock early in the growing season, while fatty acid biosynthesis does not.

Second, the ~30‰ drop in midsummer δD values of *n*-alkanes and fatty acids is only a fraction of the potential difference (up to 190‰) between current photosynthate and stored reserves. One interpretation of this diminution is that 25% or less of lipid hydrogen is derived directly from acetate precursors, and that the ~50% of lipid hydrogen derived from NADPH mirrors the isotopic composition of leaf water rather than organic substrates. If correct, this would imply that substrate D/H ratios are not faithfully transmitted to lipids through the hydride carrier NADPH, a result with important implications for heterotrophic metabolism. Alternatively, storage carbohydrates in *S. alterniflora* may not be as D-enriched as those described by Yakir and DeNiro (1990).

Third, phytol exhibits a moderate seasonal cycle in δD (Fig. 4) while the triterpenoids β-sitosterol and lupenol do not. The strong depletion of D in phytol relative to other isoprenoid products has been widely observed (Sessions et al., 1999; Yang and Huang, 2003; Chikaraishi et al., 2004a), but the reasons for this difference have remained elusive. One possibility suggested by Chikaraishi et al. (2004a) is that there is significant isotopic fractionation during the hydrogenation of phytol precursors but not that of sterol precursors. The current results suggest an additional possibility, namely that biosynthesis of the two types of isoprenoid lipids draws on different pools of organic substrates. Thus the depletion of D in phytol relative to sterols may in part be explained by a greater utilization of current photosynthate for its biosynthesis.

Fourth, δD values of *n*-alkanes drop rapidly during late June and recover rapidly during the late summer, despite negligible changes in their abundance during this time (Fig. 2). Several previous studies have recognized rapid turnover of leaf wax compounds based on seasonal changes in $\delta^{13}C$ value (Lockheart et al., 1997; Conte and Weber, 2002), but were unable to quantify the extent of turnover based on those data. The observations reported here that (i) *n*-alkanes show the same magnitude of seasonal depletion in D as fatty acids, and (ii) changes in *n*-alkane δD values are as rapid as for fatty acids, suggest that leaf-wax *n*-alkanes likely turn over entirely on timescales of less than a month. This result is significant in that windborn *n*-alkanes have been proffered as an ideal integrator of seasonal ecosystem processes over large spatial scales (Conte and Weber, 2002). The current D/H data will help to constrain the temporal scales of integration for such a proxy. This result also indicates that the gradual accumulation of *n*-alkanes in *S. alterniflora* throughout the year likely

represents their greater resistance to microbial degradation rather than a slower rate of metabolic turnover per se.

4. Conclusions

Hydrogen-isotopic fractionations between environmental water and lipids of *S. alterniflora* exhibit substantial seasonal changes in magnitude. Fatty acids, *n*-alkanes, and phytol all become depleted in D by up to 40‰ during summer months, whereas environmental water remains at an approximately constant δD value. This pattern was reproduced across portions of two growing seasons, and is statistically robust. Because the summertime depletion of D is in the opposite direction of that expected due to leafwater evaporation, the pattern can likely be attributed to changes in biosynthetic fractionations. The varying fractionation between lipids and water can readily be explained as reflecting the variable utilization of current photosynthate versus stored reserves for lipid biosynthesis. If confirmed in other species, this observation will have two important implications for the use of lipid D/H ratios by geochemists. First, efforts to establish lipid δD values as a paleoclimatic proxy will need to consider biological as well as environmental variables in the calibration of lipid/water fractionation factors. Second, differences in lipid δD values between plant species may not be characteristic of C3 versus C4 metabolism, as has been previously suggested (Chikaraishi et al., 2004b). Instead, they may reflect varying growth strategies involving the storage of carbohydrate reserves during winter. On the other hand, such biological subtleties suggest that measurements of lipid δD could provide an important new tool in studies of plant metabolism and ecosystem function. In this role, they would serve as a useful constraint on existing models for the isotopic composition of tree-ring cellulose, another paleoclimate proxy.

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Appendix A. Supplementary data

Microsoft Excel workbook containing sample descriptions, lipid abundance, and D/H ratio data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gca.2006.02.003.

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