Biogeosciences

The effect of temperature, salinity and growth rate on the stable hydrogen isotopic composition of long chain alkenones produced by *Emiliania huxleyi* and *Gephyrocapsa oceanica*

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Abstract. Two haptophyte algae, *Emiliania huxleyi* and *Gephyrocapsa oceanica*, were cultured at different temperatures and salinities to investigate the impact of these factors on the hydrogen isotopic composition of long chain alkenones synthesized by these algae. Results showed that alkenones synthesized by *G. oceanica* were on average depleted in D by 30‰ compared to those of *E. huxleyi* when grown under similar temperature and salinity conditions. The fractionation factor, $\alpha_{\text{alkenones}-\text{H}_2\text{O}}$, ranged from 0.760 to 0.815 for *E. huxleyi* and from 0.741 to 0.788 for *G. oceanica*. There was no significant correlation of $\alpha_{\text{alkenones}-\text{H}_2\text{O}}$ with temperature but a positive linear correlation was observed between $\alpha_{\text{alkenones}-\text{H}_2\text{O}}$ and salinity with ∼3‰ change in fractionation per salinity unit and a negative correlation between $\alpha_{\text{alkenones}-\text{H}_2\text{O}}$ and growth rate. This suggests that both salinity and growth rate can have a substantial impact on the stable hydrogen isotopic composition of long chain alkenones in natural environments.

1 Introduction

The oxygen and hydrogen isotopic composition of sea water mainly depends on the degree of evaporation and influx of freshwater. Hence, ancient records of δD and $\delta^{18}O$ of marine waters can, for example, be used to estimate the salinity or to trace the relative influx of rivers. The $\delta^{18}O$ of waters is recorded in the carbonate shells of foraminifera. Hydrogen isotopic compositions may be recorded in the non-exchangeable hydrogen in organic matter although with a considerable "vital effect" (e.g. isotope fractionation related to the biosynthetic pathways). This is due to the production of NADPH from NADP$^+$, leading to an initial deple-

tion of ca. 171‰ in the primary photosynthate (Yakir and DeNiro, 1990). The δD value of fossil bulk organic matter has sometimes been used to reconstruct changes in salinities. For example, Krishnamurthy et al. (2000) suggested that Mediterranean sapropels were deposited under different surface salinities based on the different δD of bulk organic matter in the sapropel. However, analysis of hydrogen isotopic compositions of fossil organic matter is complicated by the large potential for exchange of hydrogen after deposition of the initial organic matter (e.g. Schimmelman et al., 1999). Thus, the δD of bulk organic matter has rarely been used for paleoenvironmental reconstructions.

A better method for reconstructing ancient δD values of organic matter is now available through some recent technical innovations, i.e. compound-specific hydrogen isotope analysis. Through this technique δD values of individual compounds may be determined with an accuracy of 3–5‰. Recent investigations by Sessions et al. (2004) indicate that exchange of hydrogen of alkanes and sterols after deposition of the compounds in the sediments will only have a relatively small effect on short geological time scales. Thus, compound-specific hydrogen seems to be a unique tool to reconstruct ancient deuterium contents of organic matter and, if the isotopic fractionation factor is known, of water in which the organisms grew.

Similar to stable carbon isotopes, the δD values of individual compounds cannot be straightforwardly interpreted without knowledge of the effects of biosynthetic pathways and environmental parameters. Preliminary results using cultures of a diverse set of microorganisms (Sessions et al., 1999) and re-evaluation of earlier work led Hayes (2001) to suggest that the stable hydrogen isotopic composition of lipids in organisms are primarily a function of their biosynthetic pathways and not so much of the carbon acquisition mechanisms (in strong contrast to ^{13}C-contents of lipids). Acetogenic lipids such as fatty acids are depleted by ca. 150 to 250‰ compared

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to culture water whilst isoprenoidal lipids are additionally depleted in D by 10–130‰ depending on whether they are synthesized in the cytosol (e.g. sterols) or in the plastid (e.g. phytol). The impact of growth conditions on the isotopic difference between lipids and culture medium has not yet been established in any great detail. However, Sauer et al. (2002) found a consistent difference of ∼200‰ between δD values of sterols and that of surface waters in a diverse number of marine and lacustrine sediments. Huang et al. (2004) found a strong relation between the δD values of palmitic acid and waters for several lakes. Sachse et al. (2004) analysed lake surface sediments and found a consistent fractionation of ∼157‰ between the C_{17} n-alkane and meteoric waters. This suggests that certain lipids can be used to track the original isotopic composition of the water in which their parent organisms grew.

Potentially valuable biomarkers to reconstruct ancient δD values of water are long chain alkenones produced by haptophyte algae. Paul (2002) performed an initial investigation of the hydrogen isotopic composition of C_{37} alkenones using a culture of *Emiliania huxleyi* grown in culture media of different stable hydrogen isotopic compositions. Fractionation between δD of C_{37} alkenones and culture media was relatively consistent at ∼232‰. Recently, Englebrecht and Sachs (2005) found a similar fractionation of ∼225‰. Using this, Paul (2002) was able to reconstruct ancient δD values of Mediterranean seawater during sapropel formation using the δD values of C_{37} alkenones. These values were 30–35‰ depleted in D compared to today's seawater suggesting substantial freshening of the surface waters during sapropel deposition, consistent with most depositional models of sapropel formation. However, many factors affecting the relation between paleohydrology and stable hydrogen isotopic compositions of C_{37} alkenones still remain unknown. Here we investigated some of these factors by analysing the stable isotopic composition of C_{37} alkenones in cultures of *E. huxleyi* and *Gephyrocapsa oceanica* grown at different temperatures and salinities.

## 2	Material and methods

### 2.1	Culturing

Monospecific cultures of the haptophyte algae *Emiliania huxleyi* (strain PML B92/11) and *Gephyrocapsa oceanica* (strain JSI) were acclimatized to different salinities for 10 days prior to the start of the experiments. Acclimatization was carried out at a constant temperature of 15°C. The incident photon flux density was 300 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 16:8 h light:dark cycle. In order to achieve salinities above 29, natural seawater was evaporated at 60°C. Salinities below 29 were realized by adding ultrapure water to the medium. Subsequently, the seawater was enriched with nutrients, trace

metals and vitamins according to F/2 (Guillard, 1975) and sterile filtered using a 0.45 μm and 0.2 μm filter cartridge. At each salinity/temperature combination (Table 1), 1 l cultures were inoculated from pre-adapted stock cultures to provide an initial cell density between 500 and 7000 cells ml^{-1}. Bottles were closed and incubated for 4–12 days depending on algal growth rate. Cell counts were measured daily from 20 ml subsamples using a Beckman Coulter Multisizer 3 particle counter. Salinity measurements were performed by using a conductivity meter (WTW Multi 340i) combined with a TetraCon® 325 sensor. Prior to the measurements, the cell constants were calibrated using a control standard solution (0.01 mol/l KCl). The internal reference temperature (Tref) was set to 25°C. Precision of the conductivity measurements was ≤1% of the measured conductivity value. The meter translates conductivity into salinity based on the international standard KCl solution (S=35.000, Cl=19.374‰) at 15°C. The resulting accuracy is ±0.1 salinity units (http://www.wtw.com).

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

$$\mu = \frac{\ln\left(N_f\right) - \ln\left(N_0\right)}{t}$$

where N_0 and N_f are the cell densities at the beginning and end of the experiment and t is the duration of incubation in days. The cultures were harvested when they were in exponential growth phase and cell densities of $\geq 1 \times 10^5$ cells ml^{-1} by filtration over a precombusted 0.7 μm GF/F filter (Whatman). Filters were frozen immediately and stored at −80°C until analysis. Filtered waters were analysed by Elemental Analysis/Thermal Conversion/ isotope ratio monitoring mass spectrometry (EA/TC/irmMS).

Filters were extracted ultrasonically using methanol, methanol:dichloromethane 1:1 v:v mixture and dichloromethane. The total extract was separated using column chromatography with Al_2O_3 as stationary phase and a mixture of hexane and dichloromethane (9:1, v:v) to elute the apolar fraction, a mixture of hexane and dichloromethane (1:1, v:v) to elute the alkenone fraction and a mixture of methanol and dichloromethane (1:1, v/v) to elute the residual polar fraction. The alkenone fraction was analysed by gas chromatography (GC), GC/mass spectrometry (GC/MS) and GC/TC/irmMS.

### 2.2	Hydrogen isotope analysis

The hydrogen isotopic compositions of the culture media in which the algae were grown were determined by Elemental Analysis (EA)/Thermal Conversion (TC)/irmMS with a Thermo Electron EA/TC coupled to a Thermo Electron DELTA^{Plus} XL mass spectrometer. About 2 μl of water was injected into a ceramic tube coated with graphite at a temperature of 1450°C. H_3^+-factors were determined daily on the isotope mass spectrometer and varied between 3–4. Waters

Table 1. Growth conditions and stable hydrogen isotopic composition of culture water and C_{37} and C_{38} alkenones in cultures of *E. huxleyi* and *G. oceanica*.

| Species	S	T (°C)	Growth rate (d^{-1})	δD H$_2$O (‰ vs. VSMOW)	δD C$_{37}$ (‰ vs. VSMOW)	δD C$_{38}$ (‰ vs. VSMOW)	αC$_{37}$-H$_2$O
E. huxleyi	24.9	10	0.51	−5.2±1.6	−213.3±0.6	−223.0±0.9	0.791
	24.9	15	1.42	−5.4±3.1	−245.3±5.8	−250.5±2.7	0.760
	25.0	21	1.20	−2.5±1.9	−229.9±1.3	−229.0±4.2	0.776
	27.2	15	1.44	−0.7±1.9	−212.8±4.9	−216.0±6.1	0.788
	29.0	10	0.58	0.8±1.3	−207.0±2.1	−214.3±0.7	0.792
	29.0	15	1.31	1.4±1.9	−212.9±0.2	−219.8±0.8	0.786
	29.0	21	1.14	2.5±1.8	−215.3±7.0	−209.4±5.0	0.783
	32.4	15	1.29	9.0±2.1	−199.4±4.6	−209.4±3.8	0.793
	35.1	10	0.40	12.8±2.1	−174.5±6.1	−188.3±4.8	0.815
	35.1	15	0.68	12.2±1.1	−182.5±2.9	−189.8±1.6	0.808
	35.1	21	0.56	16.4±2.2	−176.2±4.1	−187.3±6.1	0.810
G. oceanica	24.8	15	0.63	−2.8±1.8	−244.5±2.6	−254.8±3.3	0.758
	24.7	21	1.02	−3.6±0.9	−261.3±0.7	−269.7±6.1	0.741
	27.3	15	0.66	−1.1±2.2	−240.2±1.1	−257.7±3.0	0.761
	29.0	15	0.64	3.6±1.6	−220.1±4.1	−229.5±5.7	0.777
	29.0	21	1.03	4.0±1.4	−246.6±6.2	−257.4±2.4	0.751
	32.5	15	0.59	9.4±1.7	−210.5±1.4	−219.7±0.4	0.782
	35.1	15	0.58	11.3±1.4	−203.0±0.1	−205.6±0.4	0.788
	35.1	21	0.79	10.9±1.8	−220.7±1.1	−221.3±6.2	0.771

were analysed with at least 6 replicate analyses. H$_2$ gas with known isotopic composition was used as reference and the isotope values were calibrated against in-house lab standards (North Sea water: +5‰ and bidistilled water: −76‰, calibrated using Vienna Standard Mean Ocean Water (VSMOW) and Greenland Ice Sheet Precipitation (GISP) standards)

Compound-specific hydrogen isotopic compositions of the n-alkanes were determined by GC/TC/irmMS with a Thermo Electron DELTA^{Plus} XL mass spectrometer using high temperature conversion. GC conditions were similar to conditions for GC and GC/MS analysis except that the film thickness of the CPSil 5 column was 0.4 μm and that a constant flow of He was used at 2 ml min^{-1}. Compounds were pyrolyzed in an empty ceramic tube heated at 1450°C which was pre-activated by injecting 1 μl of n-hexane. H$_2$ gas with known isotopic composition was used as reference and a mixture of C$_{16}$-C$_{32}$ n-alkanes with known isotopic composition (ranging from −42‰ to −256‰ vs. VSMOW) was co-injected and monitored during analysis. The average offsets between the measured hydrogen isotopic composition of the C$_{16}$-C$_{32}$ n-alkanes and their values determined off-line were generally 5‰ or less. Analyses were done at least in duplicate and the reproducibility was always better than 7‰ (Table 1).

3 Results and discussion

Two haptophyte algae, *E. huxleyi* and *G. oceanica*, were cultured at salinities ranging from 25 to 35 and temperatures ranging from 10 to 21°C (Table 1). Due to these different conditions average growth rates also varied between 0.4 and 1.4 d^{-1} (Table 1). Analysis of the stable hydrogen isotopic composition showed that the δD values of the water of the algal growth medium varied from −5 to +16‰. The δD value of the water is linearly correlated with salinity reflecting the fractionation due to evaporation towards more saline media and the mixing line between the stock F/2 medium (salinity of 29, $\delta D=\sim$2‰, Table 1) and the ultrapure water ($\delta D=-45\pm1.9$‰) (Fig. 1a). The δD values of the alkenones, i.e. the combined hydrogen isotopic composition of the C$_{37:2}$ and the C$_{37:3}$ alkenones, were considerably depleted in D compared to the δD values of the water in which they were synthesized and varied from −175 to −261‰ (Table 1). As the hydrogen for the long chain alkenones was ultimately derived from the water in which they grew, isotopic mass balance dictates that the stable hydrogen isotopic composition of alkenones should be correlated to the stable hydrogen isotopic composition of water. If a single constant fractionation step would be responsible for the isotopic depletion in D of long chain alkenones compared to water then the following equation applies (cf. Sessions and Hayes, 2005):

$$\delta D_{alkenones} = \alpha \times \delta D_{water} + (\alpha - 1) \times 1000 \qquad (1)$$
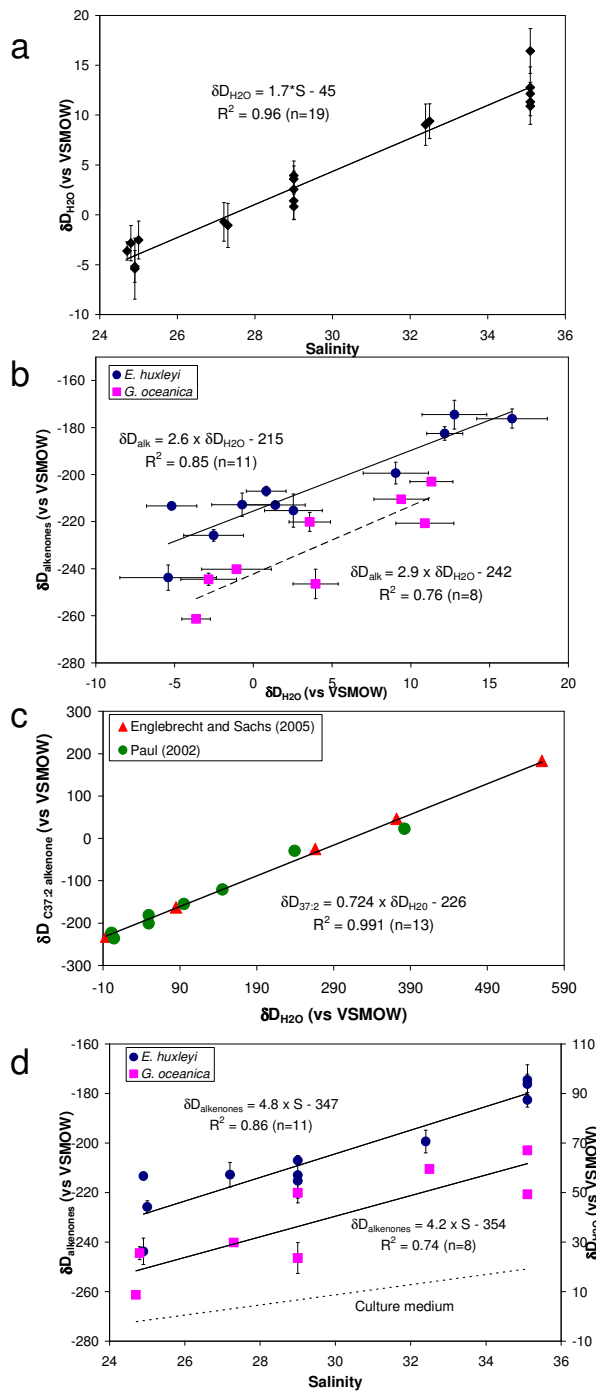
Fig. 1. Results of δD measurements of water and alkenones for cultures of *E. huxleyi* and *G. oceanica* plotted against experimental variables. **(a)** δD of culture waters plotted against salinity, **(b)** δD of C_{37} alkenones plotted against δD of culture water, **(c)** δD of $C_{37:2}$ alkenone plotted against δD of culture water from Paul (2000, filled circles) and Englebrecht and Sachs (2005, filled triangles) (cf. Sessions and Hayes, 2005) and **(d)** δD of C_{37} alkenones and δD of culture waters (dotted line) plotted against salinity. Error bars represent standard deviations of replicate measurements.
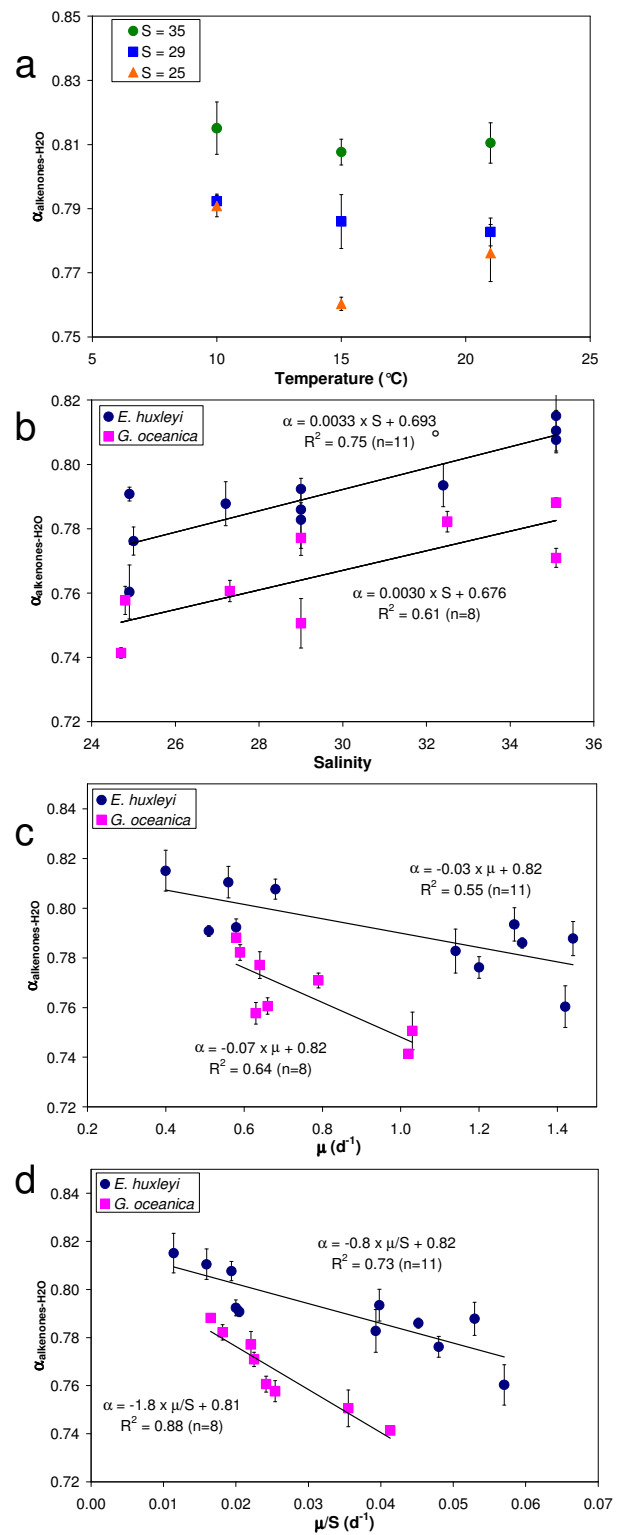
Fig. 2. Fractionation factor α of C_{37} alkenones versus water for *E. huxleyi* and *G. oceanica* plotted against **(a)** culture temperature, **(b)** against salinity, **(c)** against growth rate and **(d)** against growth rate divided by salinity.

where α = the isotopic fractionation factor:

$$\alpha = (D/H)_{\text{alkenones}}/(D/H)_{\text{water}} \quad (2)$$

Plotting the δD values of the alkenones against the δD values of the water should, thus, according to Eq. (1), yield a linear correlation. Indeed, this is observed for both *E. huxleyi* and *G. oceanica* with different slopes in the regression lines (2.9 vs. 2.6) and different intercepts (215 vs. 242‰) (Fig. 1b). The fact that two different regression lines are obtained shows that the two haptophyte algae fractionate differently compared to each other, i.e. *G. oceanica* synthesizes alkenones which are ∼30‰ more depleted in D compared to *E. huxleyi* under similar growing conditions (Table 1).

If the hydrogen isotopic composition of alkenones only depends on the hydrogen isotopic composition of culture medium then the fractionation factor α can be calculated from either the slope or the intercept of the equations in Fig. 1a (cf. Sessions and Hayes, 2005, Eq. 8: $\delta_{\text{product}} = \alpha \times \delta_{\text{source}} + \varepsilon$). For instance, when the data of Paul (2002) and Englebrecht and Sachs (2005) for the C_{37} diunsaturated alkenone of *E. huxleyi* are plotted (Fig. 1c), the fractionation factor α calculated using Eq. (1) from the slope (0.724) is relatively similar to that calculated from the intercept (0.774). However, the fractionation factors α calculated from the slopes of the regression lines (2.9 and 2.6 for *E. huxleyi* and *G. oceanica*, respectively) are much larger than those derived from the intercepts (0.785 and 0.758 for *E. huxleyi* and *G. oceanica*, respectively) suggesting that more than one process is responsible for the fractionation between hydrogen in the culture medium and that in the alkenones. In fact, in our experiments the α values derived from the slope of the regression lines are >1 suggesting fractionation of H rather than D which is highly unlikely. Hence, the fractionation of hydrogen during the synthesis of alkenones must have varied during our experiments and, therefore, the method for analysis of hydrogen isotopic fractionation described by Sessions and Hayes (2005) does not apply here as this assumes a constant fractionation factor.

Three factors must be considered as they have varied in our experimental set up, i.e. temperature, salinity and growth rate. To correct for the change in the isotopic composition of the culture water we calculated α for every experiment:

$$\alpha_{\text{alkenones}-H_2O} = (1000 + \delta D_{\text{alkenones}})/(1000 + \delta D_{\text{water}}) \quad (3)$$

When α is plotted against temperature for experiments where *E. huxleyi* was grown at constant salinity no clear correlation is visible (Fig. 2a). For example, alkenones synthesized by *E. huxleyi* grown at a constant salinity of 35 and at temperatures of 10, 15 and 21°C have similar α values of 0.815, 0.808 and 0.810, respectively (Table 1). The same is observed for the culture experiments of *G. oceanica* (Table 1). This suggests that temperature is not substantially affecting isotopic fractionation of hydrogen during the synthesis of alkenones. In contrast, a strong positive correlation
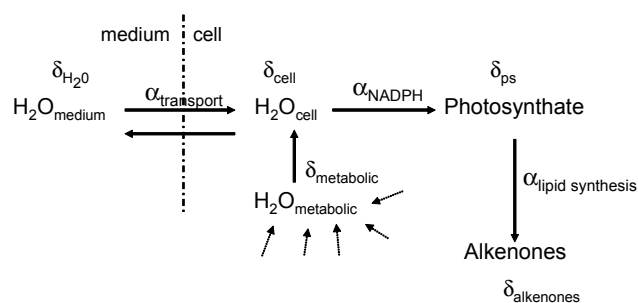
Fig. 3. Conceptual scheme of hydrogen fluxes during the biosynthesis of alkenones.

is observed between α and salinity for both *E. huxleyi* and *G. oceanica* (Fig. 2b); with increasing salinity there is a linear increase in the isotopic fractionation factor α of ∼0.003 or ∼3‰ per salinity unit during the synthesis of alkenones. As mentioned above, *G. oceanica* fractionates more strongly than *E. huxleyi* but the slope of the correlation line between α and salinity is similar for *E. huxleyi* and *G. oceanica* suggesting that the same process in both species is responsible for reducing fractionation with increasing salinity. Thus, our results suggest that salinity has a substantial impact on the isotopic fractionation factor α during synthesis of alkenones. The strong correlation between salinity and δD of the water ("meteoric water line") results in a strong correlation between the $\delta D_{\text{alkenones}}$ with salinity with a slope of 4–5‰ per salinity unit (Fig. 1d). This is much larger than expected if the fractionation factor α would have remained constant and the slope would be primarily determined by the change in δD_{water} with salinity (1.7‰ per salinity unit in our experiments; dashed line in Fig. 1d) and explains why the linear correlation between $\delta D_{\text{alkenones}}$ and δD_{water} has slopes much larger than 1 (Fig. 1b).

The final factor which varied in our experiments is the average growth rate which varied from 0.4 to 1.4 d^{-1} (Table 1). Interestingly, a negative correlation is observed between growth rate and the isotopic fractionation factor α, especially for *G. oceanica* (Fig. 2c). This correlation is only partly caused by a weak negative correlation between growth rate and salinity (R^2=0.24). In fact, when α is plotted against growth rate divided by salinity excellent correlations are obtained for both *E. huxleyi* and *G. oceanica* (Fig. 2d). This suggests that salinity and growth rate have opposite effects on the isotopic fractionation of hydrogen. The magnitude of these effects is different for the two species, i.e. the slope of the correlation lines is much steeper with *G. oceanica* (−1.8 d per salinity unit) then with *E. huxleyi* (−0.8 d per salinity unit). Notably, however, the intercepts for the correlation lines are nearly identical at 0.81 and 0.82, respectively.

The reasons for the different hydrogen isotopic fractionation of *E. huxleyi* and *G. oceanica* and the dependence of the fractionation on salinity and growth rate must lie in

the isotopic fractionations which occur during the synthesis of alkenones. Figure 3 shows the major fluxes and fractionations of hydrogen from water to the covalently-bound hydrogen of alkenones. Water enters the cell and is then used to transfer $NADP^+$ to NADPH and H^+ which is subsequently fed into the Calvin cycle and used to reduce 3-phosphoglyceric acid and synthesize hexoses. These hexoses are subsequently transformed into acetate and, finally, into long chain alkenones. One of the main factors determining the hydrogen isotopic fractionation during biosynthesis of lipids, and thus of long chain alkenones, is the reduction of cellular metabolites by NADPH and H^+ as this is the original main source of hydrogen in lipids. The fractionation from water via NADPH and H^+ is thought to lead to an initial depletion in deuterium of the primary photosynthate of ∼171‰ (Yakir and DeNiro, 1990). Fractionation will also likely occur during the biosynthesis of alkenones from the original photosynthate and other source of hydrogen may be used in this process although ultimately they will all derive from the water of the growth medium. However, it has also recently been shown that the isotopic composition of water inside a cell can be considerably different from that outside the cell due to fluxes of water derived from metabolic processes inside the cell (Kreuzer-Martin et al., 2005). In fact, Kreuzer-Martin et al. (2005) showed that at high growth rates up to 70% of the water inside the cells is derived from metabolic water. Considering this complex scheme of hydrogen fluxes, isotopic fractionations and (isotopically) different pools of water it is remarkable that the fractionation of hydrogen during synthesis of alkenones is relatively simply linearly related to μ/S. Another remarkable observation is that the slope is different for the different species and that the intercept is ∼0.82 for both *E. huxleyi* and *G. oceanica*. This latter value is very close to that of α_{NADPH}(∼0.83, Yakir and DeNiro, 1990) possibly suggesting that at very low growth rates and high salinities the formation of NADPH may be the main fractionation step. Clearly more work culturing other species and analyzing different compounds and rigorous modeling of isotopic fluxes are needed to ascertain if the observed relationship between isotopic fractionation of lipids and growth and salinity reported here is a general feature in unicellular organisms.

Assuming that these culture experiments can be extrapolated to the natural environment, our results thus suggest that in addition to the stable hydrogen isotopic composition of water, salinity and growth rate have a substantial impact on the stable hydrogen isotopic composition of alkenones and possibly of other lipids as well. There is a potential for the use of δD of long chain alkenones as a paleosalinity proxy but not in the sense of Paul (2002). In natural environments a decreasing salinity is commonly associated with a decrease in δD of the water ("meteoric water line"). This decrease depends on the relative depletion in D of the freshwater influx and the rate of evaporation and varies from 1–3% per salinity unit. Our results suggest that with a decreasing salinity

there is also an increasing fractionation by ∼3‰ per salinity unit which amplifies the change in δD of alkenones due to changing salinity. For example, in our experiments a change of 1 salinity unit led to a 4–5‰ change in δD of long chain alkenones for both haptophytes whilst the δD of the water only changed by 1.7‰. Hence, δD of long chain alkenones may potentially be a good tool to reconstruct large scale variations in paleosalinity provided that the effect of growth rates can be constrained and the source organisms are known. In practice, the latter two variables will be difficult to constrain in paleostudies. In addition, there is a considerable diversity in alkenone-producing organisms which may all have their specific fractionation patterns. Further culture experiments using different conditions and especially field experiments are needed to verify the relation between the δD of alkenones and environmental parameters such as salinity and growth rate.

Edited by: J.-P. Gattuso

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