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The effect of temperature and salinity 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 per mil compared to those of *E. huxleyi* when grown under similar conditions. The fractionation factor, $\alpha_{\text{alkenones}-H_2O}$, 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 $\alpha_{\text{alkenones}-H_2O}$ with temperature but a positive linear correlation was observed between $\alpha_{\text{alkenones}-H_2O}$ and salinity with ~3 per mil change in fractionation per salinity unit. This suggests that salinity can have a substantial impact on the stable hydrogen isotopic composition of long chain alkenones in natural environments and, vice versa, that δD can possibly be used as a proxy to estimate paleosalinity.

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 δ^{18}O of marine waters can, for example, be used to estimate the salinity or to trace the relative influx of rivers. The δ^{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 depletion of ca 170‰ in the primary photosynthate (reviewed by Hayes, 2001). 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 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 water 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. Saches 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 culture water of their parent organisms.

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 waters of different stable hydrogen isotopic compositions. Fractionation between δD of C_{37} alkenones and culture water was relatively consistent at ∼232‰. Recently, Englebracht 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

Emiliania huxleyi (strain PMLB92/11) and *Gephyrocapsa oceanica* (strain JSI) were grown in F/2 medium at different salinities and temperatures. F/2 medium was prepared by adding the usual nutrient-, trace-element-, and vitamin supplements to natural seawater with a salinity of 32.5 PSU (Guillard, 1975). After filtration, part of the medium was evaporated at 50°C until the target salinity above 32.5 PSU was reached. Salinities below ambient seawater were realized by adding ultrapure water. Cultures were grown

above saturation light intensities (ca. 300 μE,m^{-2}s^{-1}). Cell counts were carried out every day or every other day, depending on the calculated growth rates. Once the cultures reached a minimum of 10^5 cells ml^{-1}, the experiment was terminated by filtration over a precombusted 0.7 mm GFF filter. 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-thermal conversion-isotope ratio mass spectrometry (GC/TC/irmMS).

2.2. Hydrogen isotope analysis

The hydrogen isotopic compositions of waters in which the algae were grown were determined by EA/TC/irmMS with a Thermo Electron EA/TC coupled to a Thermo Electron DELTA Plus XL mass spectrometer. Ca. 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 were analysed at least 6 times. 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 VSMOW and 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. 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 off sets between the measured hydrogen isotopic composition of the C_{16}–C_{32} n-alkanes and their off-line determined values were generally 5‰ or less. Analyses were done at least in duplicate with reproducibility 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). Stable hydrogen isotopic analysis 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 toward more saline media and the mixing line between the stock F/2 medium (32.5 PSU, δD=9‰, Table 1) and the ultrapure water δD=−45±1.9‰ (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):

$$\delta D_{\text{alkenones}} = \alpha \times \delta D_{\text{water}} + (\alpha - 1) \times 1000 \quad (1)$$

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 a similar slope in the regression lines (2.9 vs. 2.6) but with a different intersect (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 is only depending on the hydrogen isotopic composition of culture water then the fractionation factor α can be calculated from either the slope or the intersect 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 data of Paul (2002) and Englebracht and Sachs (2005) for the C_{37} di-unsaturated 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 intersect (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 intersects (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 water 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. Two environmental factors can be examined as they have varied in our experimental set up, i.e. temperature and salinity. 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 obvious 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 correlation is observed between α and salinity for both *E. huxleyi* and *G. oceanica* (Fig. 2b); with increasing salinity there is a linear decrease in isotopic fractionation 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 $\delta D_{alkenones}$ with salinity with a slope (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 $\delta D_{alkenones}$ and δD_{water} has slopes much larger than 1 (Fig. 1b).

The reasons for the different hydrogen isotopic fractionation of *E. huxleyi* and *G. oceanica* and the dependence of the fractionation on salinity are unclear. 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. However, substantial exchange can occur between organic-bound hydrogen and cellular water (reviewed by Hayes, 2001). The fractionation from water via NADPH and H+ is thought to lead to an initial depletion in D of the primary photosynthate of ~171‰ (Yakir and

DeNiro, 1990). Fractionation will likely also 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. As there is a consistent difference between the isotopic composition of alkenones derived from *E. huxleyi* and *G. oceanica* some unknown species specific factor must substantially affect the hydrogen isotopic composition of long chain alkenones. Further research should point out which step in the sequence of fractionation, e.g. NADPH production or lipid biosynthesis, is responsible for this species specific difference and which step is sensitive to salt concentrations.

Our results suggest that, assuming that the culture experiments can be extrapolated to the natural environment, besides the stable hydrogen isotopic composition of water, salinity can 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 deceasing 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 per mill in salinity 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 an excellent tool to reconstruct large scale variations in paleosalinity.

The δD of the alkenones may be used directly to estimate paleosalinity by applying the relationship of e.g. *E. huxleyi*: S (PSU)=($\delta D_{alkenones}$+350)/4.8 (see Fig. 1d). However, the $\delta D_{alkenones}$ is likely to be a mixture of *E. huxleyi* and *G. oceanica*. To circumvent this problem the contributions of *E. huxleyi* and *G. oceanica* to the alkenone pool may be estimated from the relative ratio of C_{37-38} alkenoates to alkenones (Sawada et al., 1996). A mass balance of the experimentally determined equations in this study

can then subsequently be used to estimate paleosalinity. A second problem with the direct approach is that it implicitly assumes that the salinity-δD_{H_2O} relationship in our culture medium (see Fig. 1a) is universally valid, which is not the case. Salinity-$\delta^{18}O_{H_2O}$ relationships are regionally quite variable (Fairbanks et al., 1992). An alternative approach is to use the relationship of $\alpha_{alkenones-H_2O}$ with salinity (Fig. 2b). For this we need to know the δD_{H_2O} of the paleo-waters. Since δD_{H_2O} and $\delta^{18}O_{H_2O}$ are directly related via the meteoric water line ($\delta D_{H_2O}=8\times\delta^{18}O_{H_2O}+10$; Dansgaard, 1964) we can calculate δD_{H_2O} if we can determine $\delta^{18}O_{H_2O}$. The oxygen isotopic composition of paleo-water can be estimated by solving the paleotemperature equation for $\delta^{18}O_{H_2O}$ after inserting $\delta^{18}O_{calcite}$ determined on foraminifera and another, independent temperature proxy such as Mg/Ca or $U^{K}_{37'}$. However, it is obvious that the errors associated with each of these proxies will propagate thereby deteriorating the estimate of δD_{H_2O} and, subsequently, $\alpha_{alkenones-H_2O}$ and hence decrease the accuracy and precision of the salinity reconstruction. In addition, deuterium excess as well as the regional salinity-δD relationships may have been significantly different in the past. Therefore we propose to follow an alternative approach. It seems a reasonable assumption that the fractionation characteristics (offsets and slopes) for different compounds and different phytoplankton will be different. If this is the case, we can deconvolve salinity from the absolute difference between two compounds from the same or from different species, without involving δD_{H_2O}. Therefore, future studies will be directed at obtaining the salinity-δD relationships for different compounds from other marine phytoplankton.

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

Species	S (PSU)	T (°C)	δD H_2O (‰ vs. VSMOW)	δD C_{37} (‰ vs. VSMOW)	δD C_{38} (‰ vs. VSMOW)	α_{37}-H_2O
E. huxleyi	24.9	10	−5.2±1.6	−213.3±0.6	−223.0±0.9	0.791
	24.9	15	−5.4±3.1	−245.3±5.8	−250.5±2.7	0.760
	25.0	21	−2.5±1.9	−229.9±1.3	−229.0±4.2	0.776
	27.2	15	−0.7±1.9	−212.8±4.9	−216.0±6.1	0.788
	29.0	10	0.8±1.3	−207.0±2.1	−214.3±0.7	0.792
	29.0	15	1.4±1.9	−212.9±0.2	−219.8±0.8	0.786
	29.0	21	2.5±1.8	−215.3±7.0	−209.4±5.0	0.783
	32.4	15	9.0±2.1	−199.4±4.6	−209.4±3.8	0.793
	35.1	10	12.8±2.1	−174.5±6.1	−188.3±4.8	0.815
	35.1	15	12.2±1.1	−182.5±2.9	−189.8±1.6	0.808
	35.1	21	16.4±2.2	−176.2±4.1	187.3±6.1	0.810
G. oceanica	24.8	15	−2.8±1.8	−244.5±2.6	−254.8±3.3	0.758
	24.7	21	−3.6±0.9	−261.3±0.7	−269.7±6.1	0.741
	27.3	15	−1.1±2.2	−240.2±1.1	−257.7±3.0	0.761
	29.0	15	3.6±1.6	−220.1±4.1	−229.5±5.7	0.777
	29.0	21	4.0±1.4	−246.6±6.2	−257.4±2.4	0.751
	32.5	15	9.4±1.7	−210.5±1.4	−219.7±0.4	0.782
	35.1	15	11.3±1.4	−203.0±0.1	−205.6±0.4	0.788
	35.1	21	10.9±1.8	−220.7±1.1	−221.3±6.2	0.771

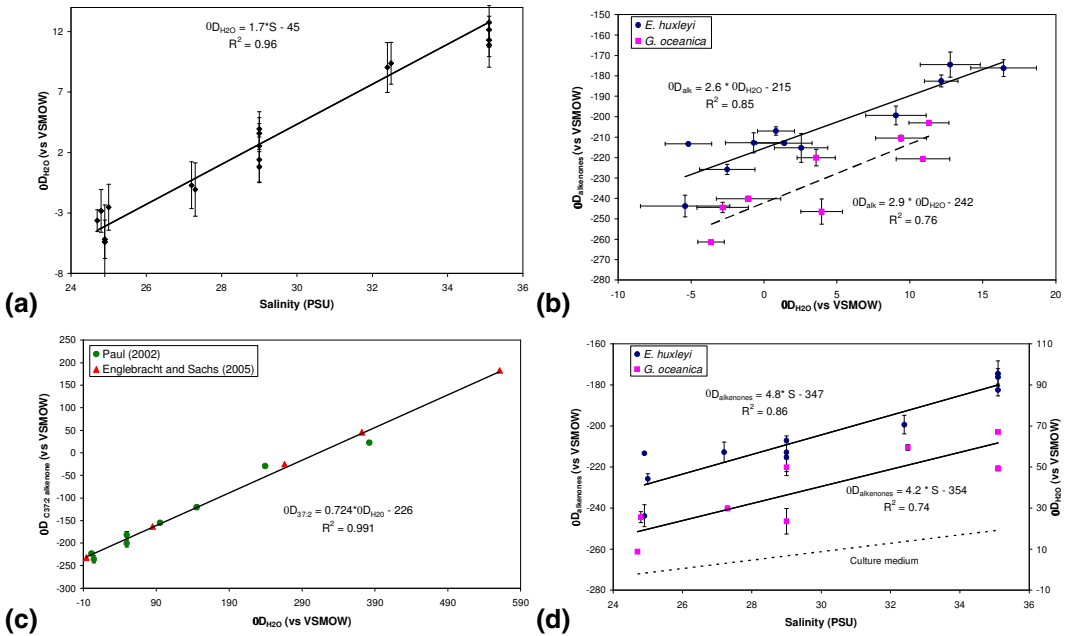

Fig. 1. Results of δD measurements of water and alkenones for cultures of *E. huxleyi* and *G. oceanica* plotted against experimental variables. **(a)** δD of culture waters plotted against salinity, **(b)** δD of C_{37} alkenones plotted against δD of culture water, **(c)** δD of $C_{37:2}$ alkenone plotted against δD of culture water from Paul (2000; filled circles) and Engelbracht and Sachs (2005, filled triangles) (cf. Sessions and Hayes, 1995) and **(d)** δD of C_{37} alkenones and δD of culture waters (dotted line) plotted against salinity.

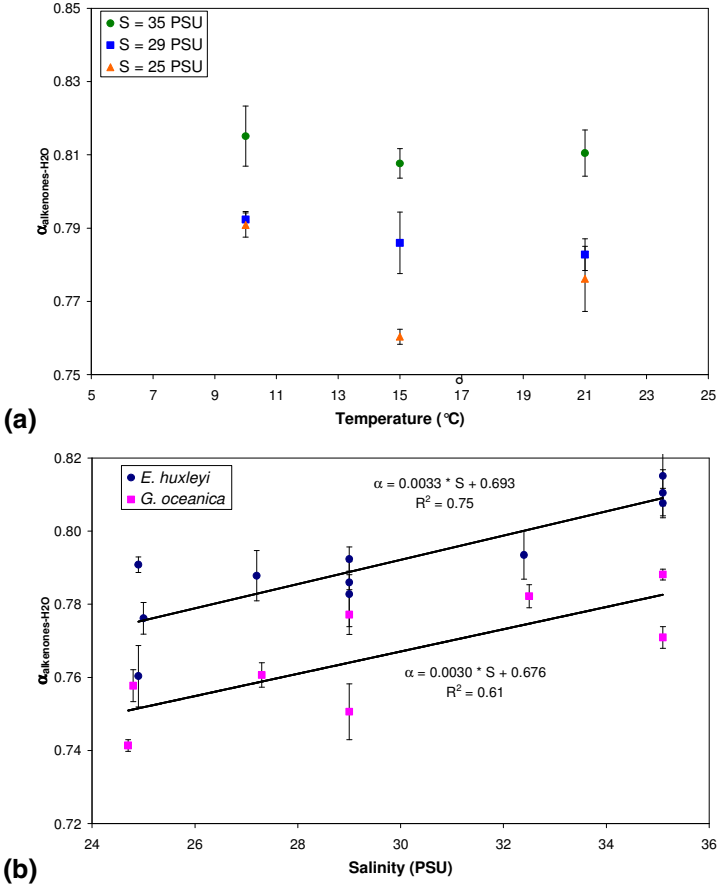

Fig. 2. Fractionation factor α of C_{37} alkenones versus water for *E. huxleyi* and *G. oceanica* plotted against **(a)** culture temperature and **(b)** against salinity.