SALINITY DEPENDENT HYDROGEN ISOTOPE FRACTIONATION IN ALKENONES PRODUCED BY COASTAL AND OPEN OCEAN HAPTOPHYTE ALGAE

Daniela M'BOULE, David CHIVALL, Danielle SINKE-SCHOEN, Jaap S. SINNINGHE DAMSTÉ, Stefan SCHOUTEN and Marcel T.J. VAN DER MEER*

NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry,1790 AB Den Burg (Texel), Netherlands
*(*corresponding author: Marcel.van.der.Meer@nioz.nl, Tel. +31222369568, Fax: +31222319674)*

Abstract.

The hydrogen isotope fractionation in alkenones produced by haptophyte algae is a promising new proxy for paleosalinity reconstructions. To constrain and further develop this proxy the coastal haptophyte *Isochrysis galbana* and the open ocean haptophyte alga *Emiliania huxleyi* were cultured at different salinities. The fractionation factor, $\alpha_{alkenones\text{-}water}$, ranged between 0.853 and 0.902 for *I. galbana* and 0.789 and 0.822 for *E. huxleyi*. The results show a strong linear correlation between the fractionation factor α and salinity for *E. huxleyi*, in agreement with earlier studies, but also for *I. galbana*. Both haptophytes show the same response to changes in salinity, represented by the slopes of the α-salinity relationship (~0.002 per salinity unit). This suggests that the same process, in both coastal as well as open ocean haptophytes, is responsible for reducing fractionation with increasing salinity. However, there is a significant difference in absolute isotope fractionation between *E. huxleyi* and *I. galbana*, i.e. *E. huxleyi* produces alkenones which are 90‰ more depleted in D under the same culturing conditions than *I. galbana*. Our data suggest that the δD of alkenones can be used to reconstruct relative shifts in paleosalinity in coastal as well as open ocean environments with careful consideration of species composition and other complicating factors especially in coastal regions.

1 Introduction

Ocean currents transport heat and moisture around the globe and are therefore a critical factor in the Earth's climate. Salinity, along with temperature, determines the density of seawater and thereby the sinking of water masses, affecting the overturning circulation. Being able to reconstruct paleosalinity could, therefore, yield important information about past ocean circulation and the global water cycle. Epstein and Mayeda (1953) and Craig and Gordon (1965) showed that the isotopic composition of seawater is a good tracer for salinity as they are strongly correlated with each other because both are being altered by evaporation of surface waters. However, this strong relationship between $\delta^{18}O$ and salinity varies regionally (Craig and Gordon, 1965) and seasonally (Fairbanks, 1982; Strain & Tan, 1993) and probably on longer timescales (Rohling and Bigg, 1998). Moreover, the $\delta^{18}O$-salinity relationship is greatly complicated by mixing and evaporation/precipitation effects (Craig and Gordon, 1965).

Nevertheless, several attempts have been made to reconstruct sea surface paleosalinity making use of this $\delta^{18}O$-salinity relationship. For this, the $\delta^{18}O$ of foraminiferal carbonate shells is used to estimate the seawater $\delta^{18}O$ (Fillon and Williams, 1984; Broecker, 1989; Broecker, 1990; Spero and Williams, 1990; Duplessy et al., 1991; Sikes and Keigwin, 1996). The $\delta^{18}O$ of foraminiferal carbonate shells depends on both the $\delta^{18}O$ of the seawater and the calcifying temperature (Erez and Luz, 1983). Therefore, for calculating the $\delta^{18}O$ of the seawater, the $\delta^{18}O$ of foraminiferal carbonate needs to be corrected for temperature using e.g. Mg/Ca ratios (Nurnberg et al., 1996; Elderfield & Ganssen, 2000) from the same species of foraminifera. However, this results in relatively large uncertainties in the reconstructed absolute salinity values (Rohling, 2007). Therefore, at this moment paleosalinity cannot be determined with enough accuracy to give reliable salinity estimates (Rohling, 2007).

Besides the oxygen isotopic composition, the hydrogen isotopic composition (δD) of meteoric waters is also strongly correlated with salinity as there is a close relationship between $\delta^{18}O$ and δD values of precipitation and seawater that has not undergone evaporation (Craig, 1961). Thus, targeting the δD of seawater provides an alternative approach to estimate paleosalinity. The δD of seawater is recorded in the hydrogen isotopic composition of non-exchangeable hydrogen in organic matter and organic compounds and therefore can potentially be used for paleo-oceanographic and paleo-climate applications (Sessions et al., 1999; Sauer et al., 2001; Sachse et al., 2004; Englebrecht & Sachs, 2005). Long chain alkenones are attractive compounds for this purpose as they do not contain exchangeable hydrogen and are derived from specific haptophyte algae (Volkman et al., 1998). Initial studies of the hydrogen isotope fractionation between the culture medium and the C_{37} alkenones produced by the marine haptophyte *Emiliania huxleyi* showed a constant offset of ~225-232‰ between the medium and the alkenones when varying the δD of the culture medium (Paul, 2002; Englebrecht & Sachs, 2005). However, Schouten et al., (2006) showed that the isotopic fractionation during synthesis of C_{37} alkenones also strongly depends on salinity in the marine haptophyte algae *E. huxleyi* and *Gephyrocapsa oceanica* with a positive linear correlation of the fractionation factor α, between the growth water and alkenones ($\alpha_{alkenone\text{-}water}$), and salinity. The fractionation factor α changed by 0.003 per salinity unit over a salinity range from 25 to 35 psu for both species but with an offset of ca. 30‰ between *G. oceanica* and *E. huxleyi*. Additionally, the hydrogen isotope fractionation by these marine algae was negatively correlated with growth rate (Schouten et al., 2006). Growth phase in batch culture was also shown to affect the hydrogen isotopic fractionation for C_{37} alkenones (Wolhowe et al., 2009). The first attempts to use these fractionation factor α-salinity relationships determined in culture studies to reconstruct sea surface paleosalinities (SSS) are promising. For example, the application of the δD of C_{37} alkenones – salinity- relationship led to the reasonable

reconstruction of the SSS in the Eastern Mediterranean and the Black Sea, respectively (van der Meer et al., 2007, 2008; Giosan et al., 2012). Recently, however, Schwab and Sachs (2011) found no relationship between $\alpha_{alkenone\text{-}water}$ and salinity in a natural salinity gradient in the Chesapeake Bay estuary. The authors suggested that there might be differences in the sensitivity of D/H fractionation to salinity between different haptophytes, i.e. coastal versus open ocean species.

To address the possible effect of different species on the δD of C_{37} alkenones synthesized by haptophytes living in different salinity regimes, we have grown the open ocean haptophyte *E. huxleyi*, a different strain than used in Schouten et al. (2006), and the coastal haptophyte *I. galbana* at salinities ranging from 10 to 35, substantially extending the range of Schouten et al. (2006). Our results provide further insights into the use of δD of alkenones for paleosalinity reconstructions, in particular in coastal regions.

2 Material and methods

2.1 Culturing

The haptophyte algae *Emiliania huxleyi* (no longer producing coccoliths, strain CCMP 1516) and *Isochrysis galbana* (CCMP 1323) were grown in batch cultures at salinities ranging from 25 to 35 and 10 to 35, respectively. The mono-specific cultures were grown in closed Erlenmeyer flasks at different salinities, constant temperature (15°C) and under constant, cool-white light (ca. 50 μE m^{-2} s^{-1}, 16:8 h light:dark cycle). Each culture was inoculated from a starter culture (50 ml) grown at the same salinity as the main experiment. The starter cultures were generated from a single stock culture grown at a salinity of approximately 34 using a 10% inoculum and transferred 5 times in fresh medium with the

final salinity, each time inoculating with 10% inoculum, and grown under the same conditions as the main experimental cultures.

In order to achieve salinities under 35, ultrapure water was added to filtered North Sea water with a salinity of approximately 34. For salinities over 35 filtered natural seawater was evaporated in an open bottle at 60°C in the oven. After achieving the intended salinities the filtered seawater was autoclaved and enriched with sterile nutrients, trace metals and vitamins according to F/2 medium of Guillard (1975).

E. huxleyi cultures were grown in triplicate at the salinities 27, 29, 32, 35 and 37. Initial cell densities varied between $5 \times 10^4 - 2.5 \times 10^5$ cells/ml. A second experiment with *E. huxleyi* at these 5 different salinities were inoculated with less material and started with cell densities between 2×10^3 and 5×10^3 cells/ml to test for possible memory effects from the starter cultures. Cells were harvested during the exponential growth phase after 7 or 8 days. *I. galbana* cultures were grown in triplicate at salinities 10, 15, 20, 25, 30, 35. Initial cell densities were between 2.3×10^3 and 4.5×10^3 cells/ml. Cells were harvested during exponential growth phase after 11 days.

Growth rate was measured by counting cells daily in 2 ml subsamples using a Accuri Flow Cytometer. Salinities were measured at the point of harvesting with a handheld conductivity meter VWR EC 300. The conductivity meter was calibrated against 4 reference waters with salinities of 10, 30, 35, 37. The resulting linear equation was applied to calculate the salinities of the culture media. The accuracy of the instrument after calibration is 0.1 practical salinity units (psu).

2.2 Harvesting and extraction

The cultures were harvested in exponential growth phase and cell densities were over or close to 1 x 10^6 cells per ml. Cells were harvested by filtration over a precombusted 0.7 µm GF/F filter (Whatman) and stored at -80°C until they were freeze dried. Samples for hydrogen isotope analysis of the medium water were taken at the start of the experiment and at the point of harvesting. Salinity changed negligibly (0.1 – 0.2 psu) in the cultures during the course of the experiment due to evaporation.

Filters were extracted ultrasonically using dichloromethane (DCM)/methanol (MeOH) 2/1 (v:v) mixture. The total lipid extract was separated over Al_2O_3 using hexane/DCM 9/1 to elute the apolar fraction, hexane/DCM 1/1 to elute the alkenone fraction and DCM/MeOH 1/1 to elute the polar fraction. The alkenones were identified using gas chromatography (GC) and GC-mass spectrometry (MS) using methods described by Van der Meer et al. (2007).

2.3 Hydrogen isotope analysis

The hydrogen isotopic compositions (δD values) of the culture media were determined by Thermal Conversion (TC)/ Elemental Analyzer (EA)/ isotope ratio monitoring mass spectrometry (irMS) using a Thermo Electron TC/EA coupled to a Thermo Electron DELTA^{PLUS} XL mass spectrometer. Approximately 1 µl of water was injected into a ceramic tube filled with glassy carbon at a temperature of 1450°C. Waters were analyzed with 10 or 11 replicate analyses per analytical run. H_3^+-factors were determined daily and varied between 7.1 and 7.4 ppm mV^{-1} and between 7.4 and 7.6 ppm mV^{-1} for the measurements of the culture media of *I. galbana* and *E. huxleyi*, respectively. H_2 gas with known isotopic composition was used as monitoring gas during each analysis and isotope values were corrected against in-house lab standards (North Sea water: 5‰ and bidistilled water: -76‰) calibrated against VSMOW and VSLAP.

Hydrogen isotope analysis of alkenones from *I. galbana* cultures were performed by GC/TC/irMS with a Thermo Electron DELTA V mass spectrometer, while the $\delta D_{alkenones}$ of the *E. huxleyi* cultures were determined using a Thermo Electron DELTA^{Plus} XL mass spectrometer. The δD values of the C_{37} alkenones represent the combined hydrogen isotopic composition of the $C_{37:4}$ (for *I. galbana*), $C_{37:3}$ and $C_{37:2}$ alkenones (van der Meer et al., 2013). The GC was equipped with a 100% dimethylpolysiloxane coated fused-silica capillary column (Agilent CP-Sil 5 CB; 25m × 0.32 mm ID; film thickness = 0.4 µm). A constant flow of the carrier gas He was used at 1 ml/min. For the DELTA V samples were injected on column at 70 °C and the oven was then heated up to 145 °C at 20°C/min then at 8°C/min to 200°C and further at 4°C/min to 320°C where it was held isothermal for 20 min. For the DELTA^{PLUS} XL samples were injected on column at 70 °C and the oven was then heated up to 145 °C at 20°C/min and then further at 4°C/min to 320°C where it was held isothermal for 15 min. H_3^+-factors were determined daily and varied between 6.2 and 6.4 ppm mV^{-1} for the DELTA V and between 7.0 and 7.3 ppm mV^{-1} for the DELTA^{PLUS} XL. H_2 gas with known isotopic composition was used as calibration gas and a mixture of C_{16}-C_{32} n-alkanes of known isotopic composition (Schimmelmann Mix B2 and B3) was used to monitor the performance of the system. The average offsets between δD of the n-alkane mixture and their known values were generally 5‰ or less on both instruments. Analyses were done in duplicate, occasionally in triplicate or quadruplicate (indicated in Table 1 and 2), and the reproducibility was better than 3‰ for the DELTA V and 8 ‰ for the DELTA^{Plus} XL mass spectrometer. Squalane with a known δD value of -170‰ ± 4.0‰ was co-injected with every sample. The average value of all $\delta D_{squalane}$ measurements was -171.5 ± 5 ‰ for the DELTA V, and -164.4 ± 3‰ for the DELTA^{Plus} XL.

2.4 Statistical analyses

A comparison of the α – salinity relationships of the *E. huxleyi* CCMP 1516, *I. galbana* and the relationships from earlier studies of *E. huxleyi* PML B92/11 and *G. oceanica* cultures (Schouten et al., 2006) was done by using analysis of covariance (ANCOVA) to compare slopes (homogeneity of the slopes test) and intercepts of the relationships. The significance level (p value) of 0.005 (Johnson, 2013) is used to characterize the data sets. Additional values provided together with the p value are df = degree of freedom and F = test statistic which gives variance of the group means / mean of the within group variances. All statistical analyses were performed in the R software package.

3 Results

Two haptophyte algae, *E. huxleyi* and *I. galbana* were cultured at 15°C at salinities ranging from 27 to 37 and 10 to 35, respectively (Table 1 and 2). Growth rates were 0.25-0.45 day^{-1} for *E. huxleyi*, covering a smaller range then the cultures of Schouten et al. (2006), and relatively constant with 0.60-0.65 day^{-1} for *I. galbana*, possibly due to the relatively low light intensity of about 50 $\mu E/m^2/s$. The stable hydrogen isotopic composition of the culturing water varied from -12‰ at a salinity of 27 to +7‰ at a salinity of 37 for *E. huxleyi* (Table 1) and from -32 to +4‰ for *I. galbana* at salinities of 10 and 35, respectively (Table 2). The C_{37} alkenones were depleted in D compared to the δD_{water} and varied between -173 to -218‰ for *E. huxleyi* (Table 1) and -97 to -170‰ for *I. galbana* (Table 2). Thus, the D/H fractionation between C_{37} alkenones and water ($\alpha_{alkenones - water} = (D/H)_{alkenones} / (D/H)_{water}$) varied between 0.789 and 0.822 for *E. huxleyi* and 0.853 and 0.902 for *I. galbana*.

The relative abundance of the tetra-unsaturated C_{37} alkenone, calculated as $\%C_{37:4} = C_{37:4}/(C_{37:4}+C_{37:3}+C_{37:2})$ (Marlowe et al., 1984) was between 4.4 and 12.2 % in the *I. galbana* culture (Table 2). The ratio of C_{37} to C_{38} alkenones, calculated as $C_{37}/C_{38} = (C_{37:3}+C_{37:2})$ /

197 ($C_{38:3}$+$C_{38:2}$) (Marlowe et al., 1984) was between 2.1 and 3.4 in the *I. galbana* culture (Table
198 2).

199

200 **4 Discussion**

201 To avoid any possible carry over from the stock culture grown at a salinity of approximately
202 34, all cultures were transferred to their final salinities and transferred to fresh medium 5
203 times in 50 ml flasks before inoculating the final culture in 600 ml flasks. However, the initial
204 batches of the *E. huxleyi* experiment started with relatively high cell numbers (see Methods
205 section). To be absolutely sure that these relatively high cell numbers from the starter culture
206 did not affect the outcome of the experiment an additional batch experiment was set up in
207 which the initial cell densities were kept very low. Although there is some variability in the α-
208 salinity relationships of the two experiments, they do not differ significantly from the overall
209 α-salinity based on all data points (Fig. 1, homogeneity of the slopes test: df=2, F=0.150,
210 p=0.862, ANCOVA: df=2, F=3.278, p=0.0503). Therefore, we use the α-salinity relationship
211 based on all data points in the following discussion.

212 **4.1 Impact of salinity and growth rate on hydrogen isotopic fractionation**

213 The two haptophyte algae investigated show a strong linear correlation between the
214 fractionation factor α and salinity (Fig. 2). With increasing salinity the fractionation factor α
215 increases, representing less D/H fractionation at higher salinities. The response to changes in
216 salinity, represented by the slopes of the α-salinity relationship, is indistinguishable for *E.*
217 *huxleyi* and *I. galbana* cultures (~0.002 per salinity unit) grown under identical temperature
218 and light conditions (homogeneity of the slopes test: df=1, F=0.866, p=0.359). This suggests
219 that the same process, in both coastal as well as open ocean haptophytes, is responsible for
220 reducing fractionation with increasing salinity. Previous culture experiment results for *E.*

huxleyi (strain PML B92/11) and *G. oceanica* of Schouten et al. (2006) show a slightly stronger response to salinity, i.e. a change in α of ~0.003 per salinity unit with no significant difference between the *E. huxleyi* PML B 92/11 and *G. oceanica* relationships (df=1, F=0.06, p=0.809) [Fig. 2]. However, the difference between the slopes of the current *E. huxleyi* experiment and that of *E. huxleyi* (strain PML B92/11) of 0.1 ‰ S^{-1} is not statistically significant especially when using the p>0.005 criterion of Johnson (2013) [homogeneity of the slopes test: df=1, F=4.2, p=0.051 with p-values >0.005 considered not significant (Johnson, 2013)].

In addition, Schouten et al., (2006) observed a negative correlation between growth rate and α for *E. huxleyi* and *G. oceanica*. When we plot α against growth rate no clear correlation is visible for *I. galbana* since growth rates were relatively constant for these cultures compared to those of Schouten et al. (2006) (Fig. 3). These relatively constant growth rates might also explain the high correlation coefficient (R^2 of 0.97) for the α - salinity relationship of this species (Fig. 2). We also observed no correlation of growth rate with α for *E. huxleyi* CCMP 1516 in our study. Interestingly, however, the α – growth rate data of *E. huxleyi* CCMP 1516 falls on the α – growth rate correlation for *E. huxleyi* PML B92/11, suggesting there might be a growth rate effect (Fig. 3). These results thus show that hydrogen isotope fractionation of all alkenone-producing species is strongly related to salinity and that there might be also a growth rate effect.

4.2 Different isotopic fractionation between coastal and open ocean haptophytes

There is a significant difference in isotopic fractionation between *E. huxleyi* and *I. galbana,* i.e. *E. huxleyi* produces alkenones which are 90‰ more depleted in D under the same culturing conditions than *I. galbana* (Table 1 and 2) (ANCOVA: df=1, F=3264.6, p<0.0001). This confirms the observation from Schouten et al. (2006) that different species fractionate

246 differently. All haptophytes investigated in this and the Schouten et al. (2006) study are
247 significantly different in the intercepts of the α-salinity relationships (Fig. 2). In the group of
248 the open ocean haptophytes there are also differences in D/H fractionation between *E. huxleyi*
249 CCMP 1516, *E. huxleyi* PML B92/11 and *G. oceanica*. Of these three, *G. oceanica*
250 fractionates the most, and synthesizes alkenones that are 27‰ and 52‰ more depleted in D
251 compared to *E. huxleyi* PML B92/11 and *E. huxleyi* CCMP 1516, respectively. The two
252 strains of *E. huxleyi* also fractionate significantly differently against D under similar culture
253 conditions by approximately 25‰ (ANCOVA: df=1, F=19.67, p<0.001).
254 One reason for the different fractionation between *I. galbana* and the open ocean haptophytes
255 might be the presence of the $C_{37:4}$ alkenones in *I. galbana* since we measured the hydrogen
256 isotopic composition of the combined C_{37} alkenones. Indeed, there is a correlation between
257 $\alpha_{alkenones\text{-}water}$ and $\%C_{37:4}$ (R^2=0.54, p<0.001) for the *I. galbana* cultures, suggesting that $\%C_{37:4}$
258 might affect $\alpha_{alkenones\text{-}water}$. However, this correlation is likely due to the fact that $\%C_{37:4}$ is
259 itself correlated to salinity (R^2=0.62, p=0.0001; Fig. 4). A multiple regression of $\alpha_{alkenones\text{-}water}$
260 against S and $\%C_{37:4}$ was performed resulting in: R^2=0.97, with a p value for salinity of
261 <0.001 and for $\%C_{37:4}$ = 0.56, which would suggest that $\%C_{37:4}$ does not affect $\alpha_{alkenones\text{-}water}$.
262 Also, in *I. galbana* the relative abundance of $C_{37:4}$ is relatively small (4-12%) and therefore
263 unlikely to significantly affect $\alpha_{alkenones\text{-}water}$.
264
265 The observed differences in fractionation between the different species might be due to the
266 culturing conditions and, in particularly, growth rate (Schouten et al., 2006). To account for
267 changes in growth rate, α was also plotted against growth rate divided by salinity for all
268 experiments (Fig. 5; cf. Schouten et al., 2006). Both *E. huxleyi* strains fall on the same
269 regression line, suggesting that the differences in α versus salinity of these two strains of the
270 same species (Fig. 2) are due to the differences in growth rate. However, there are large

difference in the α versus growth rate divided by salinity between the haptophyte species, *I galbana*, *E. huxleyi* and *G. oceanica*, (Fig. 5) illustrating the species-specific fractionation of different haptophyte species.

All the investigated species belong to the same class of algae (Prymnesiophyceae) and they most likely share the same biosynthetic pathway for alkenone synthesis. Thus, there have to be other reasons for the relatively large difference in hydrogen isotope fractionation between the different haptophytes. The production of initial photosynthate in photoautotrophs is associated with a large hydrogen isotope fractionation effect of approximately 171‰ (Yakir & Deniro, 1990). As discussed by Hayes (2001) there are basically three main processes that together could explain this hydrogen isotopic fractionation in photoautotrophic organisms: the splitting of water by the oxygen evolving complex of Photosystem II, the reduction of NADP to NADPH by ferrodoxin-NADP reductase linked to Photosystem I and the transfer of H from NADPH to 3-phosphoglycerate in the Calvin cycle. Hence, differences in photosynthetic activity could cause differences in fractionation and thus differences between species and culture experiments. Different light intensities for example are believed to have a strong impact on the $\delta D_{alkenone}$, with higher light intensities resulting in less fractionation (Sachse et al., 2012). The light intensity in the current study was lower (50 $\mu E/m^2/s$) than in the experiments performed by Schouten et al. (2006) (80 $\mu E/m^2/s$), but none the less hydrogen isotopic fractionation in the current study is slightly lower, suggesting that different light conditions alone cannot explain the difference in fractionation between the different species in these experiments.

Another possible explanation for the different hydrogen isotopic fractionation could be differences in the hydrogen isotopic composition of the internal water of the cell in different haptophyte species. Previous results for *Escherichia coli* have shown large differences between the isotopic composition of external and internal water pools due to fluxes of D-

296 depleted water derived from metabolic processes (Kreuzer-Martin et al., 2005). Therefore, a
297 possible explanation for internal water pools with different hydrogen isotopic compositions
298 could be the differences in water exchange between the cell and the environment between
299 different haptophyte species (Schwab and Sachs, 2011). *G. oceanica* grows optimally at
300 salinities around 33 and is therefore the most adapted to high salinities and possibly the least
301 tolerant to salinity change (Brand, 1984). *I. galbana* grows optimally at salinities around 15
302 and thrives in coastal areas with fluctuating salinities and therefore possibly has the highest
303 tolerance to salinity changes (Brand, 1984). Finally, *E. huxleyi* is the intermediate with
304 optimal growth at salinities from 20-25 (Brand, 1984). These different species seem to be
305 adapted to different salinities and salinity variability, based on their different growth optima
306 and natural niches. In order to survive at these different salinities these organisms will have to
307 have developed different strategies to deal with the osmotic challenges of their natural habitat
308 (Brand, 1984). We hypothesize that these different strategies could possibly lead to different
309 water exchange rates over the cell membrane resulting in isotopically different internal water
310 pools between the species. Hereby, *G. oceanica*, adapted to stable high salinities, could
311 potentially reduce water exchange to a minimum to avoid losing water to the environment
312 resulting in an increasing percentage D-depleted metabolic water and shows therefore the
313 largest fractionation. On the other hand, *I. galbana* as very flexible low salinity coastal
314 species might have a different strategy involving osmolytes to cope with higher salinities and
315 salinity changes, but keeping the ability for water exchange and shows therefore the least D/H
316 fractionation compared to the medium. *E. huxleyi*, although similar to *G. oceanica*, seems to
317 thrive at slightly lower salinities allowing perhaps for slightly more water exchange and
318 slightly decreased isotope fractionation compared to *G. oceanica.*
319
320 **4.3 Implications for paleosalinity reconstructions**

Our results suggest that the δD value of alkenones produced by *I. galbana* can in principle be used to reconstruct changes in salinity not only in open marine environments but also in coastal environments. However, Schwab and Sachs (2011) found no relationship between $\alpha_{alkenone\text{-}water}$ and salinity along a natural salinity gradient in the Chesapeake Bay (CB) estuary. The authors suggested, among others, that this might be caused by differences in the sensitivity of D/H fractionation to salinity between different haptophytes, i.e. coastal versus open ocean species. However, our experiments shows that *I. galbana* and *E. huxleyi* respond in the same way to salinity change, although the extent of fractionation is significantly different. Another possible explanation put forward by the authors is a changing community composition of the alkenone-producing haptophytes, from a more low salinity adapted coastal species dominated community in the low salinity areas to a more open ocean species dominated community in the high salinity areas. Based on our culture data we can hypothesize how this might work. A change from a 100% *I. galbana* population at low salinities, i.e. a salinity of 10, to a mixed population of approximately 60% *E. huxleyi* and 40% *I. galbana* at high salinities, salinity of 30, would result in a similar α value of ~ 0.85, assuming that both species produce the same amount of alkenones. However, it is unknown if such a community composition change took place in this gradient.

Another difference is that Schwab and Sachs (2011) measured the δD of the $C_{37:2}$ and $C_{37:3}$ alkenones separately whereas in our study we measured the combined alkenones (cf. van der Meer et al., 2013). However, the weighted average δD of the di- and tri-unsaturated C_{37} alkenones from Chesapeake Bay (calculated from Schwab and Sachs, 2011) showed also no correlation between $\alpha_{alkenone\text{-}water}$ and salinity and thus cannot explain the difference with our culture results.

One other possibility to explain the absence of an α-salinity relationship in the CB could be the potential mixing of alkenones from different sources, i.e. through lateral transport and re-

suspension of particles, due to the constantly moving water masses in the estuary. This strong mixing would dampen the original salinity signal in the alkenones. This explanation is supported by the absence of a correlation between the $U^{k'}_{37}$ and in-situ water temperature in CB (Schwab and Sachs, 2011), a correlation that would be expected if the alkenones were produced in the same water mass they were sampled from. Clearly future investigations, particularly on species compositions in estuarine settings, are needed to examine the effect of different factors on the hydrogen isotopic composition of alkenones in coastal systems. Since *I. galbana* has no coccoliths, haptophyte DNA analysis would be required to identify changes in species compositions. Alternatively, the ratio of C_{37}/C_{38} alkenones may be used to distinguish between haptophyte species since different C_{37}/C_{38} values were observed for different haptophytes; coastal haptophytes tend to have higher ratios compared to open ocean species (Prahl et al., 1988; Marlowe et al., 1984; Schulz et al., 2000). However, environmental conditions, e.g. temperature, can also affect C_{37}/C_{38} alkenone ratio (Conte et al., 1998; Sun et al., 2007). Recently, Liu et al. (2011) found distinct C_{37}/C_{38} ratios in different geographical regions, ranging from 0.6-2.0 (surface water) for the arid Qaidam Basin with higher salinities and 3.1-4.5 (surface water) for the semi-arid region of Lake Qinghai with lower salinities, suggesting contributions from different alkenone producers. In our study we do not observe a relationship between C_{37}/C_{38} alkenones and salinity (Table 2) which would support these earlier findings.

The relative abundance of $C_{37:4}$ alkenones has also been reported as a potential salinity indicator or even a paleosalinity proxy (Rosell-Mele, 1998; Schulz et al., 2000; Blanz et al., 2005; Liu et al., 2008 and 2011), with coastal haptophytes as their potential source organisms producing higher concentration of the $C_{37:4}$ and therefore indicating low salinities. However, sometimes none or a positive relationship of $\%C_{37:4}$ with salinity has been observed (Sikes and Sicre, 2002; Theroux et al., 2010; Toney et al., 2010; Schwab and Sachs, 2011), possibly

because $\%C_{37:4}$ varies in different alkenone-producing species and strains (Marlowe et al., 1984; Conte et al., 1995). Our *I. galbana* culture results also show a positive $\%C_{37:4}$ – salinity relationship (Table 2; Fig. 4) indicating that higher concentrations of $C_{37:4}$ are produced at higher salinities, similar to the observations in Chesapeake Bay (Schwab and Sachs; 2011).

5. Summary

The coastal haptophyte *I. galbana* and the open ocean haptophyte algae *E. huxleyi* were cultured at 15°C at salinities ranging from 27 to 37 and 10 to 35, respectively. The two haptophytes show a strong linear correlation between the fractionation factor α and salinity. With increasing salinity the fractionation factor α increases, representing less D/H fractionation at higher salinities confirming earlier results. The response to changes in salinity, represented by the slope of the α-salinity relationship, is the same for *E. huxleyi* and *I. galbana* cultures (~0.002 per salinity unit). However, there is a significant difference in absolute isotope fractionation between *E. huxleyi* and *I. galbana, E. huxleyi* produces alkenones that are 90‰ more depleted in D under the same culturing conditions than *I. galbana*. An explanation for this could be that the different species are adapted to different salinities and salinity variability and have developed different strategies to deal with the osmotic challenges of their natural habitat leading to different fractionations.

Our results suggest that the δD of alkenones can potentially be used to reconstruct relative shifts in paleosalinity in coastal as well as open ocean environments. However, changes in species composition and growth rate may potentially complicate its application in coastal environments.

Acknowledgements

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538 Table 1 Cell density, growth rate, $\delta D_{alkenones}$, δD_{water}, and $\alpha_{alkenones\text{-}water}$ for batch cultures of
539 *Emiliania huxleyi* grown at salinity 26 to 37 at 15°C.

Salinity	Batch	cell density (ml^{-1})	Growth rate (d^{-1})	$\delta D_{C37alkenones}$ (‰)	δD_{water} (‰)	$\alpha_{alkenones\text{-}water}$
26.6	a	$7.4x10^5$	0.37	-208 ± 5 [b]	-11 ± 3	0.802 ± 0.006
26.6	b	$7.6x10^5$	0.36	-210 ± 3	-10 ± 2	0.798 ± 0.003
26.6	c	$1.3x10^6$	0.38	-218 ± 1 [a]	-9 ± 4	0.789 ± 0.003
26.6	d	$4.4x10^5$	0.38	-207 ± 1	-12 ± 4	0.803 ± 0.003
29.1	a	$8.6x10^5$	0.36	-203 ± 1	-6 ± 4	0.802 ± 0.003
29.1	b	$1.4x10^6$	0.30	-202 ± 1	-6 ± 3	0.803 ± 0.002
29.1	c	$1.2x10^6$	0.31	-211 ± 6 [a]	-5 ± 3	0.792 ± 0.006
29.1	d	$5.8x10^5$	0.37	-200 ± 2	-5 ± 2	0.805 ± 0.002
32.0	a	$1.3x10^6$	0.42	-195 ± 3 [a]	0 ± 2	0.805 ± 0.004
32.1	b	$1.2x10^6$	0.33	-194 ± 2 [a]	-3 ± 3	0.809 ± 0.004
32.0	c	$1.5x10^6$	0.30	-197 ± 3	0 ± 3	0.803 ± 0.003
32.1	d	$1.0x10^6$	0.40	-187 ± 2	-1 ± 4	0.814 ± 0.004
35.2	a	$1.0x10^6$	0.30	-185 ± 2	+2 ± 1	0.813 ± 0.002
35.2	b	$7.3x10^5$	0.36	-180 ± 1	+3 ± 3	0.818 ± 0.002
35.2	c	$1.3x10^6$	0.25	-182 ± 7 [a]	+3 ± 2	0.811 ± 0.006
35.3	d	$4.9x10^5$	0.45	-180 ± 4	+2 ± 3	0.818 ± 0.004
37.3	a	$9.5x10^5$	0.29	-176 ± 1	+7 ± 2	0.818 ± 0.002
37.4	b	$7.6x10^5$	0.34	-173± 2	+7 ± 3	0.821 ± 0.003
37.3	c	$1.3x10^6$	0.26	-176 ± 3	+7 ± 2	0.819 ± 0.003
37.4	d	$4.4x10^5$	0.43	-174 ± 3 [a]	+6 ± 3	0.822 ± 0.004

[a] Mean of 3 measurements ± 1 standard deviation, [b] Mean of 4 measurements ± 1 standard deviation

δD_{water} and salinity values are the mean of measurements taken at the start and end of the culture. Errors in $\delta D_{alkenones}$ are the range between duplicate measurements unless stated. Errors in δD_{water} are propagated from the standard deviation of at least ten replicate injections. $\alpha_{alkenones\text{-}water}$ is defined as $(D/H)_{alkenones}/(D/H)_{water} = (\delta D_{alkenones} + 1000)/(\delta D_{water} + 1000)$, Errors in $\alpha_{alkenones\text{-}water}$ are propagated from the errors of $\delta D_{alkenones}$ and δD_{water}.

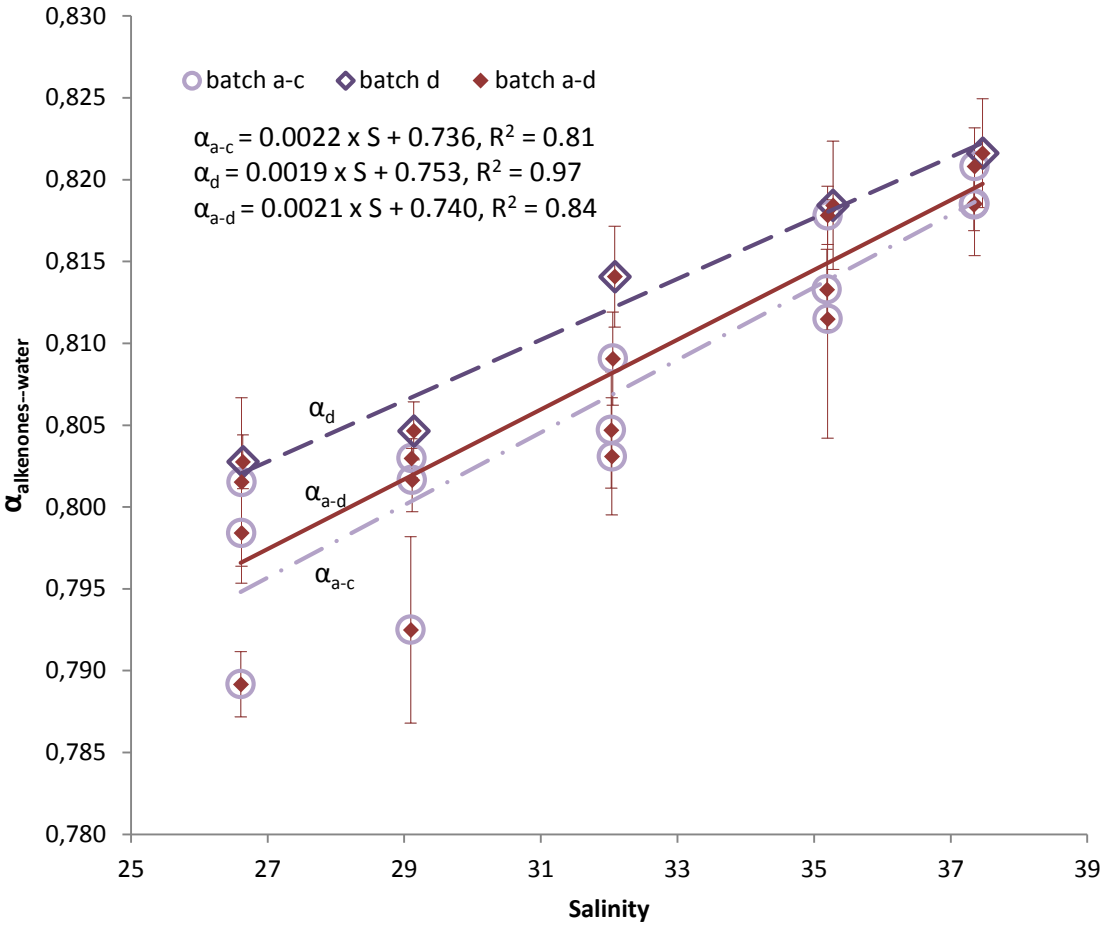
540

Table 2 Cell density, growth rate, $\delta D_{alkenones}$, δD_{water}, and $\alpha_{alkenones\text{-}water}$, $\%C_{37:4}$ and C_{37}/C_{38} for batch cultures of *Isochrysis galbana* grown at salinity 10 to 35 at 15°C.

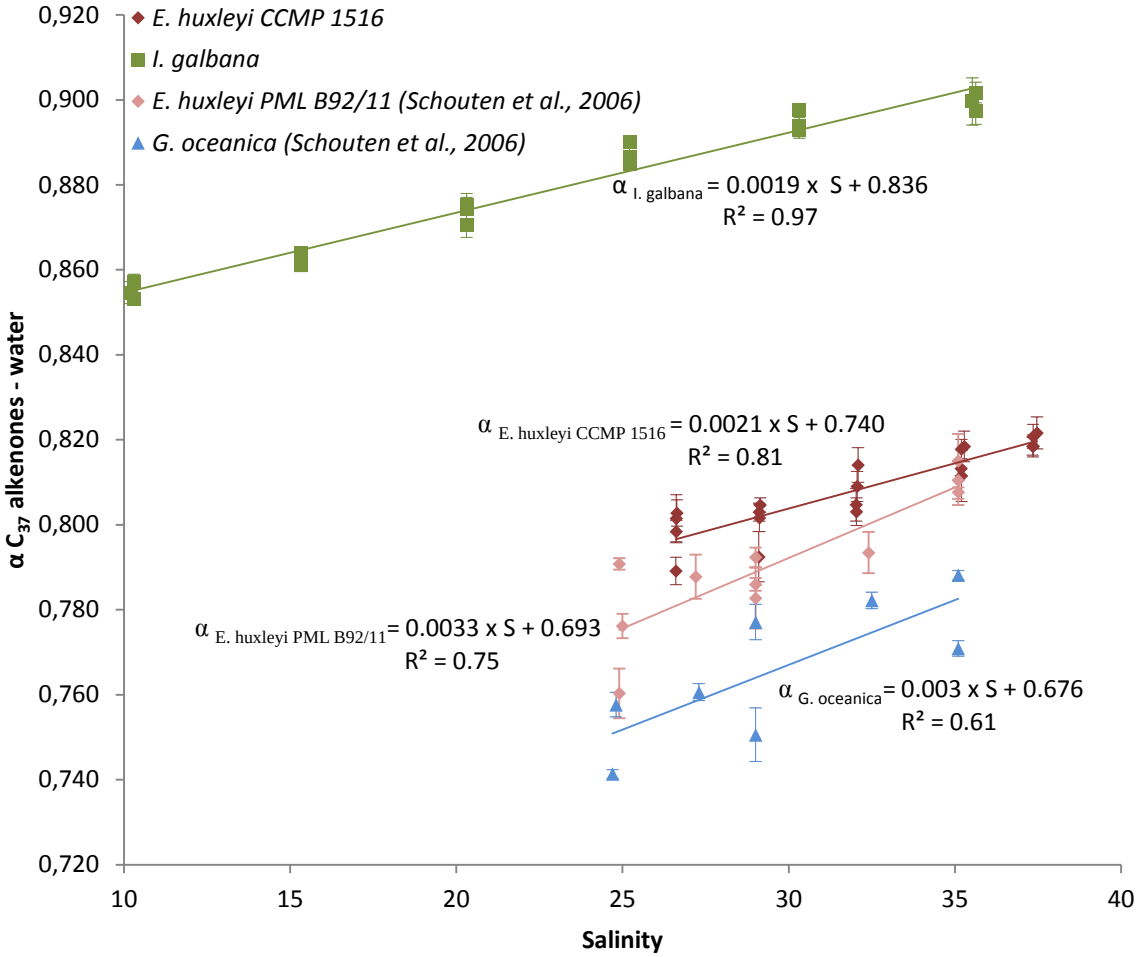
Salinity	cell density (ml^{-1})	Growth rate (d^{-1})	$\delta D_{C37alkenones}$ (‰)	δD_{water} (‰)	$\alpha_{alkenones\text{-}water}$	$\%C_{37:4}$	C_{37}/C_{38}
10.3	$1.4x10^6$	0.63	-172 ± 1	-29 ± 1	0.853 ± 0.001	6.3	2.4
10.2	$1.3x10^6$	0.63	-172 ± 2[a]	-30 ± 1	0.853 ± 0.003	6.7	3.4
10.3	$1.6x10^6$	0.62	-170 ± 1	-30 ± 2	0.856 ± 0.002	7.2	2.2
15.3	$1.6x10^6$	0.58	-158 ± 1[a]	-22 ± 1	0.861 ± 0.001	6.0	2.3
15.3	$1.8x10^6$	0.61	-157 ± 1	-22 ± 1	0.863 ± 0.001	7.3	2.4
15.3	$1.8x10^6$	0.61	-158 ± 1	-22 ± 1	0.860 ± 0.002	4.4	2.6
20.3	$1.8x10^6$	0.63	-142 ± 3	-17 ± 2	0.873 ± 0.004	5.7	2.2
20.3	$2.1x10^6$	0.65	-140 ± 1	-17 ± 2	0.875 ± 0.002	6.1	2.5
20.3	$1.6x10^6$	0.65	-144 ± 3	-16 ± 2	0.871 ± 0.003	5.5	2.4
25.2	$1.6x10^6$	0.61	-122 ± 1	-9 ± 2	0.886 ± 0.002	6.1	2.5
25.2	$1.5x10^6$	0.63	-126 ± 1	-11 ± 1	0.883 ± 0.001	7.4	2.5
25.2	$1.7x10^6$	0.64	-121 ± 1	-10 ± 1	0.889 ± 0.001	7.9	2.5
30.3	$1.3x10^6$	0.63	-111 ± 1	-3 ± 2	0.892 ± 0.002	8.4	2.6
30.3	$1.4x10^6$	0.63	-106 ± 1	-3 ± 1	0.896 ± 0.001	8.6	2.3
30.3	$1.3x10^6$	0.63	-109 ± 2	-2 ± 1	0.893 ± 0.002	9.2	2.5
35.6	$8.0x10^5$	0.62	-97 ± 2	+4 ± 2	0.900 ± 0.002	11.8	2.2
35.6	$7.3x10^5$	0.60	-99 ± 3[a]	+5 ± 1	0.896 ± 0.003	10.5	2.1
35.5	$7.3x10^5$	0.63	-98 ± 5[a]	+5 ± 1	0.898 ± 0.006	12.2	2.2

[a] Mean of triplicate measurement ± 1 standard deviation

δD_{water} and salinity values are the mean of measurements taken at the start of the culture and at the time of sampling. Errors in $\delta D_{alkenones}$ are the range between duplicate measurements unless stated. Errors in δD_{water} are propagated from the standard deviation of at least ten replicate injections. α is defined as $(D/H)_{alkenones}/(D/H)_{water} = (\delta D_{alkenones} + 1000)/(\delta D_{water} + 1000)$, errors in $\alpha_{alkenones\text{-}water}$ are propagated from the error of $\delta D_{alkenones}$ and δD_{water}

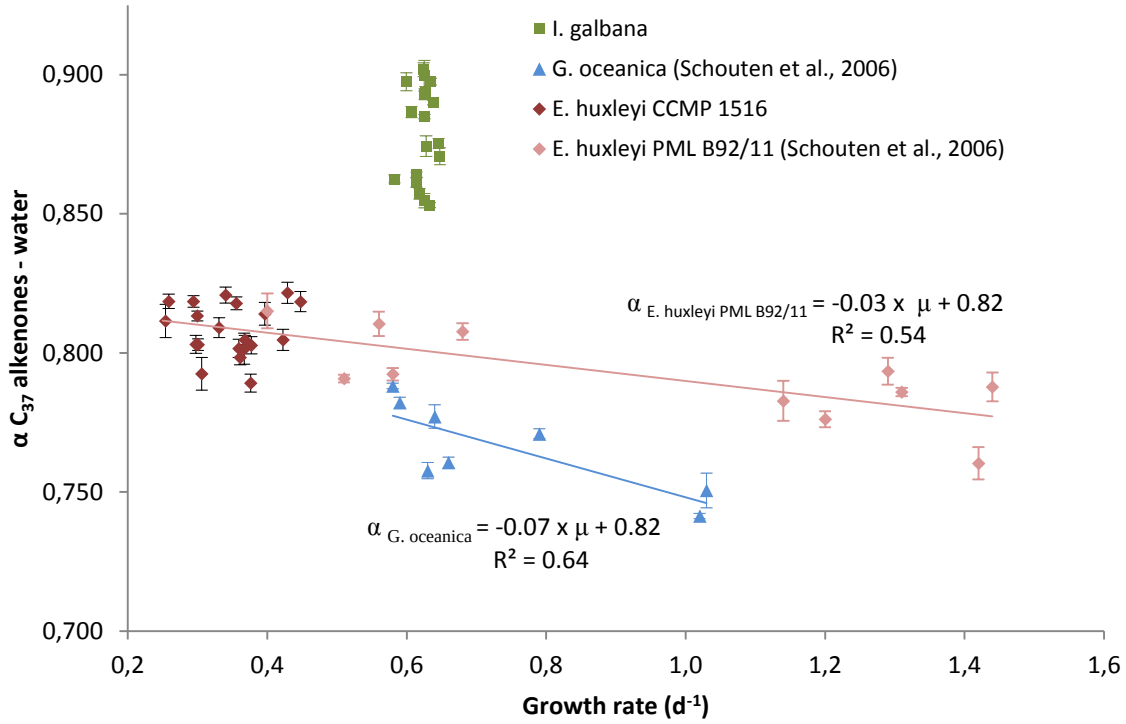


547
548 Fig. 1 α-salinity relationships of the different batches of the *E. huxleyi* culture experiment.



549

550 Fig. 2 Fractionation factor α of C_{37} alkenones plotted against salinity.



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552 Fig. 3 Fractionation factor α of C_{37} alkenones plotted against growth rate (μ).
553

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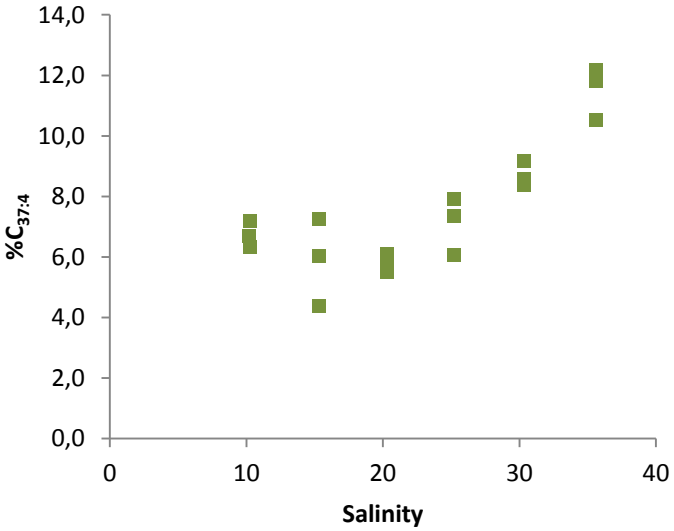
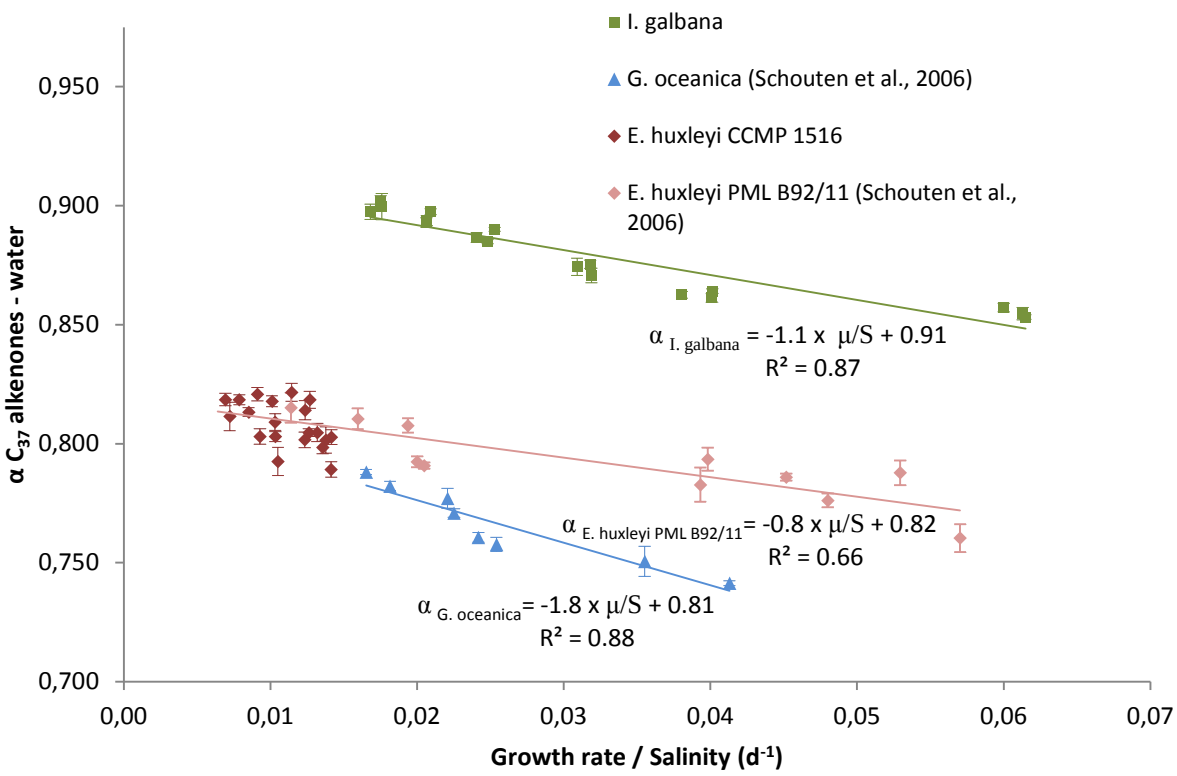


556

557 Fig. 4 Variations of $\%C_{37:4}$ with salinity from
558 the *I. galbana* cultures.
559



560

561 Fig. 5 Fractionation factor α of C_{37} alkenones plotted against growth rate divided by salinity
562 (μ/S).