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Effects of alkalinity and salinity at low and high light intensity on hydrogen isotope fractionation of long-chain alkenones produced by *Emiliania huxleyi*

Gabriella M. Weiss[1], Eva Y. Pfannerstill[1], Stefan Schouten[1,2], Jaap S. Sinninghe Damsté[1,2], Marcel T.J. van der Meer[1]

[1]Department of Marine Microbiology and Biogeochemistry, NIOZ, Royal Netherlands Institute for Sea Research and Utrecht University, Den Burg, 1790 AB, The Netherlands
[2]Department of Earth Sciences, Faculty of Geosciences, Utrecht University, Utrecht, 3508 TA, The Netherlands

Correspondence to: Gabriella M. Weiss (gabriella.weiss@nioz.nl)

Abstract Over the last decade, hydrogen isotope fractionation of long-chain alkenones have been shown to be a promising proxy for reconstructing paleo sea surface salinity due to a strong hydrogen isotope fractionation response to salinity across different environmental conditions. However, to date, the decoupling of the effects of alkalinity and salinity, parameters that co-vary in the surface ocean, on hydrogen isotope fractionation of alkenones has not been assessed. Furthermore, as the alkenone-producing haptophyte, *Emiliania huxleyi*, is known to grow in large blooms under high light intensities, the effect of salinity on hydrogen isotope fractionation under these high irradiances is important to constrain before using hydrogen isotope fractionation to reconstruct paleosalinity. Batch cultures of the marine haptophyte *E. huxleyi* strain CCMP 1516 were grown to investigate the hydrogen isotope fractionation response to salinity at high light intensity and independently assess the effects of salinity and alkalinity. Our results suggest that alkalinity does not significantly influence hydrogen isotope fractionation of alkenones, but salinity does have a strong effect. Additionally, no significant difference was observed between the fractionation responses to salinity recorded in alkenones grown under both high and low light conditions. Comparison with previous studies suggests that the fractionation response to salinity in culture is similar under different environmental conditions, strengthening the applicability of hydrogen isotope fractionation as a paleosalinity proxy.

1 Introduction

Ocean circulation plays a strong role in global heat and moisture transport (Rahmstorf, 2002) and is controlled in part by differences in temperature and salinity, known as thermohaline circulation. Therefore, knowing these parameters is important to reconstruct ocean circulation in the geological past, which leads to a more robust understanding of our climate system. A number of valuable proxies exist to reconstruct sea surface temperature, for example, $\delta^{18}O_{foram}$ (Emiliani, 1955), Mg/Ca (Elderfield and Ganssen, 2000), TEX_{86} (Schouten et al., 2002), $U_{37}^{K'}$ (Brassell et al., 1986), LDI (Rampen et al., 2012). However, there are currently very few proxies for reconstructing sea surface salinity (SSS).

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Evaporation, precipitation, continental runoff, and ice melt cause changes in seawater salinity, thereby influencing ocean circulation. The isotopic ratios of oxygen ($\delta^{18}O$) and hydrogen (δD) of water are strongly tied to these environmental parameters (Craig and Gordon, 1965). Increasing evaporation causes both enrichment in heavy isotopes (Clark and Fritz, 1997) and increase in surface water salinity. The resulting water vapor has a depleted isotopic signature (Clark and Fritz, 1997) and the longer the water stays in vapor form, the more depleted the isotopic signature of the vapor becomes as relatively enriched water precipitates first. Therefore, continental bodies of water (i.e. meteoric waters) fed by precipitation are characterized by a depleted isotopic signature and low salinity. This leads to a strong linear correlation between $\delta^{18}O_{water}$ and salinity in ocean water and therefore the $\delta^{18}O_{water}$ is a suitable proxy for sea surface salinity. However, the slope of the correlation varies in space (ocean region) and time (Duplessy et al., 1993; Mashiotta et al., 1999), severely complicating reconstructions of ancient $\delta^{18}O_{water}$-S relationships and, thus, paleosalinity reconstructions. Therefore, constraining the correlation between $\delta^{18}O_{water}$ and S currently poses a challenge in attempts to extract reliable palaeosalinity estimates from inferred $\delta^{18}O_{water}$.

Over the last decade, culture studies have shown that the hydrogen isotopic ratios of long chain alkenones (δD_{C37}), biomarkers of Haptophyte algae from the order Isochrysidales (Volkman et al., 1980), correlate with the hydrogen isotopic ratios of the water in which the algae grow (δD_{H2O}) (Englebrecht and Sachs, 2005; Paul, 2002), which in turn is correlated with salinity (Craig and Gordon, 1965). In addition to the observed relationship between δD_{C37} and δD_{H2O}, biological hydrogen isotope fractionation (expressed as the isotope fractionation factor α) has been shown to decrease with increasing salinity, thereby amplifying the salinity to seawater δD relationship of alkenones grown in culture (Schouten et al., 2006; Wolhowe et al., 2009; M'Boule et al., 2014; Chivall et al., 2014; Sachs et al., 2016). Therefore δD_{C37} has been proposed as an appropriate proxy for reconstructing sea surface salinity (SSS) (Englebrecht and Sachs, 2005; Schouten et al., 2006). For example, δD_{C37} measured on alkenones extracted from Mediterranean sapropel S5 shows similar trends to $\delta^{18}O$ measured on planktonic foraminifera and suggests a salinity decrease of 6 in the Eastern Mediterranean at the onset of sapropel formation (van der Meer et al., 2007). δD_{C37} from Panama Basin sediments show changes in amount of runoff from the San Juan River, aligning well with instrumental data and even tracking glacial to interglacial changes in salinity (Pahnke et al., 2007). Salinity changes in the Agulhas Current system were also recorded by changes in δD_{C37} during glacial termination I and II and from the LGM into the Holocene, which align with $\delta^{18}O_{foram}$ from the same region (Simon et al., 2015; Petrick et al., 2015; Kasper et al., 2014).

Although a relationship between δD_{C37}, δD_{H2O} and fractionation with salinity has been observed in culture and some paleostudies show promising results, this relationship is not always found in nature. Häggi et al. (2015) did not find a significant relationship between δD_{C37} and salinity in SPM from the Amazon Plume. In the Chesapeake Bay Estuary (USA), δD_{C37} in sediments relate to δD_{C37} from SPM filters and δD_{H2O} values, but fractionation does not show a relationship with salinity (Schwab and Sachs, 2011). These environmental datasets suggest that there are other factors affecting hydrogen isotope fractionation, which complicate the use of δD_{C37} as a salinity proxy. Indeed, culture studies have indicated that

hydrogen isotope fractionation can be influenced by a number of parameters, i.e., growth rate (Schouten et al., 2006), growth phase (Chivall et al., 2014), species composition (M'Boule et al., 2014; Chivall et al., 2014), and irradiance (van der Meer et al., 2015). When the hydrogen isotope ratios of both the C37:3 and C37:2 alkenones are integrated (van der Meer et al., 2013), the effect of temperature on α has been shown to be negligible on the δD_{C37} SSS proxy, eliminating one impeding factor (Schouten et al., 2006).

The effect of alkalinity on hydrogen isotope ratios and fractionation has not yet been tested. This factor may be important as the culture experiments investigating hydrogen isotopes from alkenones created media of different salinities by evaporation, which changed alkalinity together with salinity in the culture media. Similarly, in the natural environment, precipitation and evaporation are not only influential on salinity, but also affect the total alkalinity (A_T) of the surface ocean. In fact, a strong positive linear correlation between A_T and salinity is observed in surface ocean waters (Millero et al., 1998; Lee et al., 2006), and, on top of that, large coccolithophore blooms can bring about a significant decline in surface water A_T (Anning et al., 1996). Since alkalinity is essentially the capacity to take up H+, it could potentially affect the hydrogen isotope composition of internal cell water as well as hydrogen isotope fractionation during the synthesis of organic compounds. It is, therefore, crucial to decouple the effects of salinity and alkalinity and assess how each effect hydrogen isotope fractionation independently.

Furthermore, culture work has shown light intensity to have a strong effect on α_{C37} at light intensities below 200 µmol photons $m^{-2}s^{-1}$, but not above (van der Meer et al., 2015). However, most of the culture studies that reported a strong correlation between the fractionation factor α_{C37} and salinity were performed at relatively low light intensities. Since algal blooms occur under high light conditions in surface waters across the globe, and α is more stable at high light conditions (van der Meer et al., 2015), the effect of salinity on α at high light intensity needs to be studied to better understand the potential effect of salinity on alkenones synthesized in nature.

Here we addressed these two issues by using batch cultures of the haptophyte algae *Emiliania huxleyi* in experiments where alkalinity was varied independently of salinity and where salinity was varied under high light conditions.

2 Materials and Methods

2.1 Media and Culture Conditions

Two separate batch culture experiments were conducted: 1) to assess whether alkalinity affects hydrogen isotope fractionation between alkenones and growth water ('alkalinity/salinity' experiment) and 2) to examine if the fractionation-salinity relationship seen in previous culture experiments still holds under high light conditions ('high light' experiment). A no longer calcifying strain of *E. huxleyi*, CCMP 1516, was used in these batch cultures. Media for all experiments was made using filtered North Sea water with added nutrients, trace metals and vitamins following the method for F/2 medium (Guillard and Ryther, 1962). Medium was diluted with ultrapure water to a salinity of approximately 25 and NaCl was added to achieve higher salinities.

The alkalinity/salinity experiments consisted of batch cultures with a salinity range of 26-42 and A_T constant at 2.44 mM and batch cultures of salinity 34 and A_T between 1.44 and 4.6 mM. For batches where alkalinity was changed, $NaHCO_3$ and Na_2CO_3 were added to bring the medium to an A_T of 2.44 mM, an average value for open ocean waters, which typically fall between 2.1 and 2.5 mM in the modern day ocean (Ilyina et al., 2009; Takahashi et al., 1981). Concentrated HCl was added to reduce alkalinity of the medium to 1.44 mM, and bubbling with air for 48 h allowed for equilibration of CO_2 with the atmosphere following the method of Keul et al. (2013). To increase alkalinity of the medium, $NaHCO_3$ and Na_2CO_3 were added to achieve A_T of 3.3 and 4.6 mM, respectively. Temperature was a constant 15˚C and light intensity was consistently kept at 75 µmol photons $m^{-2}s^{-1}$ using cool white fluorescent light, with a light:dark cycle of 16:8 h.

The high light experiment was performed at five different salinities, from 25-35, under a light intensity of 600 µmol photons $m^{-2}s^{-1}$ using cool white fluorescent light, with a light:dark cycle of 16:8 h, and constant temperature of 18.5˚C.

All batch culture experiments were performed in triplicate. Cultures were transferred to new medium five times prior to starting the experiment to remove any possible memory effects from the original stock culture and adapt the algae to the desired experimental conditions. An Accuri C6 flow cytometer was used to count cell concentrations daily to calculate growth rate over the length of the experiment. Cells were harvested during exponential growth when cell abundance reached $>10^6$ cells mL^{-1} to prevent effects of shading or reduced nutrient content of the medium by the haptophytes. Cultures were filtered over 0.7 µm GF/F filters to collect organic material and medium was subsequently collected following filtration to determine δD of the growth water.

2.2 Water Isotope Analysis

Hydrogen isotopic ratios of the medium (δD_{H2O}) were measured on water collected prior to starting the experiment and after the experiment concluded. δD_{H2O} was measured using elemental analysis-thermal conversion-isotope ratio monitoring mass spectrometry (EA/TC/irMS) (see Schouten et al., 2006). 1 µL of sample water was injected at least 10 times during a single analytical run. δD_{H2O} values were corrected to an in-house North Sea (5‰) standard, which was calibrated against VSMOW and VSLAP.

2.3 Alkenone Analysis

Following filtration, filters were freeze-dried and extracted ultrasonically five times for 10 min each time using DCM/MeOH (2:1) to obtain total lipid extracts (TLE). TLEs were then separated into three fractions over Al_2O_3 column using hexane/DCM 9:1 (v:v) to elute the apolar fraction, hexane/DCM 1:1 (v:v) to elute the ketone (alkenone) fraction, and DCM/MeOH 1:1 (v:v) to elute the polar fraction. Ketone fractions were run on a gas chromatograph coupled to a flame ionisation detector (GC-FID) to determine alkenone concentrations prior to running on GC/TC/irMS to measure compound specific hydrogen isotope ratios (δD_{C37}). The H_3^+ factor was measured daily on the GC/TC/irMS prior to running samples; values ranged between 2.8 and 2.9 ppm mV^{-1} for the alkalinity/salinity experiments and 5.4 and 5.5 ppm mV^{-1} for the high light experiments. A Mix B standard (supplied by A. Schimmelmann, Indiana University) was run to assess machine

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accuracy on a daily basis and samples were only run when standard deviation and error of the Mix B standard were less than 5‰. Samples were measured in duplicate and squalane was co-injected with each analytical run to monitor quality of runs; average value for squalane co-injected with high light experiment samples was -164.8‰ with standard deviation of 2.2 and -163.4‰ with standard deviation of 2.7 when co-injected with the alkalinity/salinity experiment samples. All C_{37} alkenone peaks were integrated as a single peak and values are thus reported as the combined values of the C37:2 and C37:3 alkenones (van der Meer et al., 2013). The isotopic fractionation of alkenones compared to media is expressed as α_{C37} and calculated using the equation:

Eq. (1) : $\alpha_{C37} = \frac{\delta D_{C37} + 1000}{\delta D_{H2O} + 1000}$

2.4 Statistics

Analysis of covariance (ANCOVA) was applied to test if a significant different exists between equations of the linear regression models representative of the α_{C37}-salinity relationship between this study and previous culture studies of *E. huxleyi*. All statistical analyses were run in R using the R stats package.

3 Results

A no longer calcifying strain of *E. huxleyi* was grown under high light conditions over a salinity range from 25 – 35 with constant alkalinity in our high light experiment, and low light conditions over a salinity range from 26-42 with an alkalinity range of 1.4 – 4.6 mM in our alkalinity/salinity experiment. Changes in salinity, alkalinity and pH were negligible over the course of the batch culture experiments (Table 1). Changes in δD_{H2O} of the culture media sampled prior to beginning the experiments and at the end of the experiments were minimal, generally <0.4‰ (Table 1). Slightly larger changes in δD_{H2O} occurred for media of the alkalinity/salinity experiments, but still <1.3‰ (Table 1) and therefore neglected. Since the salinity of the media of was not altered by evaporation but by addition of NaCl, in contrast to previous culture studies, the δD_{H2O} was not correlated with salinity (Figure 1). δD_{C37} values ranged from -230.9‰ to -197‰ across all experiments, with more depleted values at lower salinities (Table 1). Growth rates (μ) ranged from 0.65 – 0.93 d^{-1} for the alkalinity/salinity experiment and 0.54 – 0.67 d^{-1} for the high light experiment, with lower growth rates at higher salinities (Table 1). Higher α_{C37} occurs at higher salinities for both the alkalinity/salinity and high light experiments. A strong linear relationship between α_{C37} and salinity is observed in both experiments (Figure 2a,b): for the high light experiment, α_{C37}=0.002S + 0.7408 (R^2 0.92, n=14; p<0.001), and for the alkalinity/salinity experiment, α_{C37}=0.0026S +0.7098 (R^2 0.86, n=24; p<0.001).

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4 Discussion

4.1 Impact of alkalinity and light

In the alkalinity/salinity experiment, α_{C37} changed from 0.776 to 0.824 over a salinity range of 26 to 42 and constant alkalinity, and remained constant at 0.799±0.003 at alkalinities ranging from 1.42 to 4.61 (Figure 2c). This shows that alkalinity, in contrast to salinity, does not affect hydrogen isotope fractionation of non-calcifying *E. huxleyi.* We note, however, that this experiment was performed with a no longer calcifying strain of *E. huxleyi*, and results might be different when haptophytes are calcifying since calcification may be impacted by alkalinity, which in turn could have consequences for other intracellular processes. At constant alkalinity over a range of salinity, we see a 2.6‰ change per salinity unit, confirming that salinity does indeed have an effect on hydrogen isotope fractionation between alkenones and growth water (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014; Sachs et al., 2016).

Alkenones synthesized by haptophytes growing at different salinities under high light (600 µmol photos m^{-2} s^{-1}) show a strong correlation between α_{C37} and salinity. This unambiguously shows that there is also a strong correlation between salinity and hydrogen isotopic fractionation in alkenones at high light intensities, as encountered in the surface layers of the ocean.

The slope of the α-salinity correlation, or fractionation response per unit salinity, is statistically similar for both the alkalinity/salinity and the high light experiments. There is a weak negative correlation of growth rate (µ) with fractionation for both the alkalinity/salinity and high light experiments, $\alpha = -0.0692\mu + 0.8557$ (R^2=0.25, n=24, $p<0.05$) and $\alpha = -0.1257\mu + 0.8776$ (R^2=0.35, n=14, $p<0.05$), respectively (Figure 2). Growth rate is also negatively correlated with salinity in both experiments (Table 1; Figure 2), which is consistent with earlier work of Schouten et al. (2006) and Sachs and Kawka (2015). However, our results show a direct effect of salinity on both growth rate and fractionation, suggesting the correlation between growth rate and fractionation might be largely indirect.

4.2 Comparison with previous studies

We performed a statistical comparison using ANCOVA between the different α_{C37}-salinity relationships for previous *E. huxleyi* cultivation experiments (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014; Sachs et al., 2016; Table 2) and our experiments. This showed a strong similarity between slopes of the α_{C37}-salinity response seen in all *E. huxleyi* datasets. Furthermore, the strong correlation between α_{C37} and salinity is present under both low and high light conditions. The fact that the strong α_{C37}-salinity response is also identified at high light conditions is important for understanding the influence of light and depth effects (i.e., van der Meer et al., 2015; Wolhowe et al., 2015) on the α_{C37} of alkenones preserved in the sedimentary record. In fact, because the most dominant source of alkenones that end up being preserved in marine sediments are likely to be high-light summer blooms occurring in the surface ocean, these light and depth effects (i.e., van der Meer et al., 2015; Wolhowe et al., 2015) might not have such a large effect on the α_{C37}-salinity response.

In contrast, with the exception of the α_{C37}-salinity relationship reported by M'Boule and our high light experiment, the intercepts of the regression models applied to the α_{C37}-salinity relationships for the *E. huxleyi* culture data are all significantly different, i.e. the absolute fractionation differs between the different studies. These differences in intercept may be explained by a number of potential factors. One explanation could be due to the different strains of *E. huxleyi* used in the cultivations, as each strain would respond in a similar fashion to salinity changes but fractionate to a different extent. This could be due differences in fractionation and sources of hydrogen or differences in lipid synthesis rates. Another explanation could be analytical differences between laboratories, i.e. small offsets in measured absolute values of C_{37} alkenones. Inter-laboratory comparison of measured hydrogen isotope values of an alkenone standard could help to eliminate this uncertainty.

4.3 Potential mechanisms for a response to salinity

Our results show that the response in hydrogen isotopic fractionation of alkenones to salinity is similar across different *E. huxleyi* strains and under different growth conditions, including low and high light conditions. How salinity affects hydrogen isotope fractionation is still unknown, although several possible mechanisms have been proposed (e.g., Maloney et al., 2016). The effect of salinity on hydrogen isotope fractionation seems to be a general feature recorded in alkenones, fatty acids and sterols produced by photoautotrophic organisms (Heinzelmann et al., 2015; Schouten et al., 2006; Sachse and Sachs, 2008; Sachs and Schwab, 2011). Nicotinamide adenine dinucleotide phosphate (NADPH) and intracellular water are the two sources of H available for use in organic compound synthesis, the latter of which is less affected by fractionation, evidenced by a comparably smaller depletion in deuterium (D) compared to that of extracellular water (Schmidt et al., 2003). NADPH has been proposed to supply around 50% of the H eventually used for lipid production in the bacterium *Escherichia coli* (Kazuki et al., 1980), and it is presumed to be roughly the same for photosynthetic algae (Zhang et al., 2009). The cell generates NADPH either photosynthetically or via the oxidative portion of the pentose phosphate pathway (OPP pathway) (Schmidt et al., 2003; Wamelink et al., 2008). Photosynthetic production causes NADPH to be depleted by ~600‰ in D when compared to intracellular water, whereas NADPH produced via the OPP pathway is also depleted compared to intracellular water, but much less than photosynthetically produced NADPH (Schmidt et al., 2003; Maloney et al., 2016). Schmidt et al. (2003) suggested that a transfer of H from NADPH generated as part of the OPP pathway cause depletion in D which is further enhanced during continued biosynthesis, meaning organic compounds containing H largely derived from metabolically reduced NADPH are characterized by depletion in D. However, this depletion is still less than what is observed for photosynthetically-derived NADPH. Up or down regulation of the OPP pathway relative to other NADPH generating pathways (photosynthetically-derived, for instance) could, therefore, cause differences in the amount of D depletion of organic compounds. Up regulation of the pentose phosphate pathway observed in the bacterium *Vibrio sp* at high salinities led to an increase in NADPH generated by the pathway for use in biosynthesis (Danevčič and Stopar, 2011). A similar mechanism could be present in *E. huxleyi*, causing the metabolically reduced NADPH pool to increase relative to other pools, and possibly become a more important source of NADPH for biosynthesis. However, in photoautotrophic organisms, the reduction of $NADP^+$ to NADPH is also directly linked to photosystem activity and, therefore, light intensity

(Allen, 2002), with this initial reduction being characterized by a fractionation step of approximately -600‰ (Schmidt et al., 2003; Maloney et al., 2016). Because of this, we would expect isotope ratios to change with different light intensities. Indeed, van der Meer et al. (2015) showed this to be the case for irradiances between 15 and 200 µmol photons m^{-2} s^{-1}, but the effect of changing light intensity on hydrogen isotope fractionation is much lower at light intensities >200 µmol photons m^{-2} s^{-1}. Furthermore, Sachs et al. (2017) showed a light effect on hydrogen isotope fractionation of C14:0 fatty acid, but no effect of light was observed on hydrogen fractionation of C16:0 and C16:1 fatty acids from the diatom *Thalassiosira pseudonana* grown over a low light range from 6 – 47 µmol m^{-2} s^{-1}. The lack of correlation with light intensity for the longer fatty acids is explained by enzymatic reprocessing that causes further hydrogen fractionation and overwrites the light effect seen for the C14:0 (Sachs et al., 2017). This effect could also apply to alkenones since alkenone synthesis has been linked to fatty acid biosynthesis (Volkman et al., 1980; Marlowe et al., 1984; Rontani et al., 2006). However, at high light conditions, where more photosynthetically derived NADPH is expected to be available (e.g. our high light experiment), the same fractionation response to salinity is observed as at low light conditions (e.g. M'Boule et al., 2014), where less photosynthetically derived NADPH is expected. This suggests that light intensity does not directly have an effect on the predominance of photosynthetically derived versus metabolically derived or enzymatically reprocessed NADPH used in biosynthesis, or that the up-regulation of metabolically derived NADPH with increasing salinity exerts a stronger control on hydrogen isotope fractionation than irradiance.

Another explanation for the observed significant correlation with salinity at both high and low light intensity could be that the cell synthesizes alkenones in a closed cell compartment, similar to the 'coccolith vesicle-reticular body' in which coccoliths are formed (Wilbur et al., 1963; Sviben et al., 2016), where the amount of NADPH used for biosynthesis is regulated and the fraction NADPH derived from the OPP pathway into the closed compartment increases with increasing salinity.

In addition to higher abundance of NADPH generated by OPP pathway at higher salinities, cells also could produce more D-depleted compounds, osmolytes for instance (Dickson et al., 1982), which would leave the intracellular NADPH pool more enriched, which would result in D enrichment of other biosynthetic products such as alkenones. The production of DMSP, an osmolyte produced by marine microalgae, is not coupled to light intensity (van Rijssel and Gieskes, 2002), therefore, osmolyte production could be a major factor responsible for the salinity response observed over a range of light intensities. An added complication could be that cells excrete more isotopically depleted osmolytes at high salinities than at low salinities (Demidchik et al., 2014), which could leave the fraction of NADPH used for other organic compounds more isotopically enriched at high salinities. These processes are correlated with salinity, however, if NADPH plays a central role in hydrogen isotope fractionation and the reduction of $NADP^+$ to NADPH is directly coupled to photosystem activity and, therefore, light intensity, different slopes for α_{C37}-salinity are expected for cells grown at high light and low light conditions, which is in contrast to what our results show.

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4.4 Potential mechanisms for a lack of light effect

Based on the compilation of *E. huxleyi* culture data, a significant relationship between hydrogen isotope fractionation and salinity is observed. However, we do not see a clear relationship between hydrogen isotope fractionation and light intensity. At higher light intensities, we expect a larger pool of photosynthetically derived NADPH inside the cell, which could cause differences in hydrogen isotope fractionation during the synthesis of alkenones. Transhydrogenase enzyme activity can remove NADPH when in excess by reducing NAD+ to NADH (Kim and Gadd, 2008; Zhang et al., 2009), which is associated with a large isotope fractionation effect of between 800-3500‰ (Zhang et al., 2009) leaving a relatively D enriched pool of NADPH behind. At high light conditions, an excess of NADPH is expected, and therefore, increased transhydrogenase activity. However, if transhydrogenase enzyme activity is responsible for reducing the excess NADPH, we might expect to see a difference in isotope values and a different fractionation response to salinity at high and low light conditions, which is not the case.

There is, of course, the possibility that the cell could use excess NADPH for other intracellular processes or synthesis of compounds that are not being investigated or measured in our experiments, which could be light intensity dependent as well. This would explain why having an abundance of NADPH at high light intensities does not seem to affect hydrogen isotope fractionation of alkenones since this abundance of photosynthetically derived NADPH is being used for other processes. A better understanding of the hydrogen isotopic composition of different relevant hydrogen pools is required for more accurate determinations of the mechanism responsible for the strong salinity response to hydrogen isotope fractionation of alkenones.

5 Conclusions

Our results show that salinity has a strong effect on hydrogen isotope fractionation of alkenones in cultivated *E. huxleyi* at high light intensities. In contrast, alkalinity, although co-varying with salinity in environmental waters, does not affect hydrogen isotope fractionation between alkenones and growth water. Interestingly, we see a similar effect of salinity on hydrogen isotope fractionation at both high and low light conditions. Our present knowledge of biosynthetic mechanisms does not allow us to explain the similarity of the fractionation response to salinity at high and low irradiance in absolute terms. However, further investigation of intracellular sources and partitioning of intracellular hydrogen could allow us to explain this mechanism more accurately. The fact that light intensity is a function of depth in the water column, and the abundance of alkenones decreases with depth, the effect of lower light intensity on hydrogen isotope fractionation observed in previous studies is likely minor for sedimentary alkenones. Our results show the consistency of the hydrogen isotope fractionation response to salinity for multiple E. huxleyi strains grown under different conditions further supports the use of α_{C37} for reconstructing paleo sea surface salinity.

Author Contribution

Gabriella Weiss, Marcel T.J. van der Meer and Eva Pfannerstill designed the experiments and Gabriella Weiss and Eva Pfannerstill carried them out. Gabriella Weiss prepared the manuscript with contributions for all co-authors.

Competing Interests

Marcel T.J. van der Meer is an Associate Editor of Biogeosciences.

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Figures

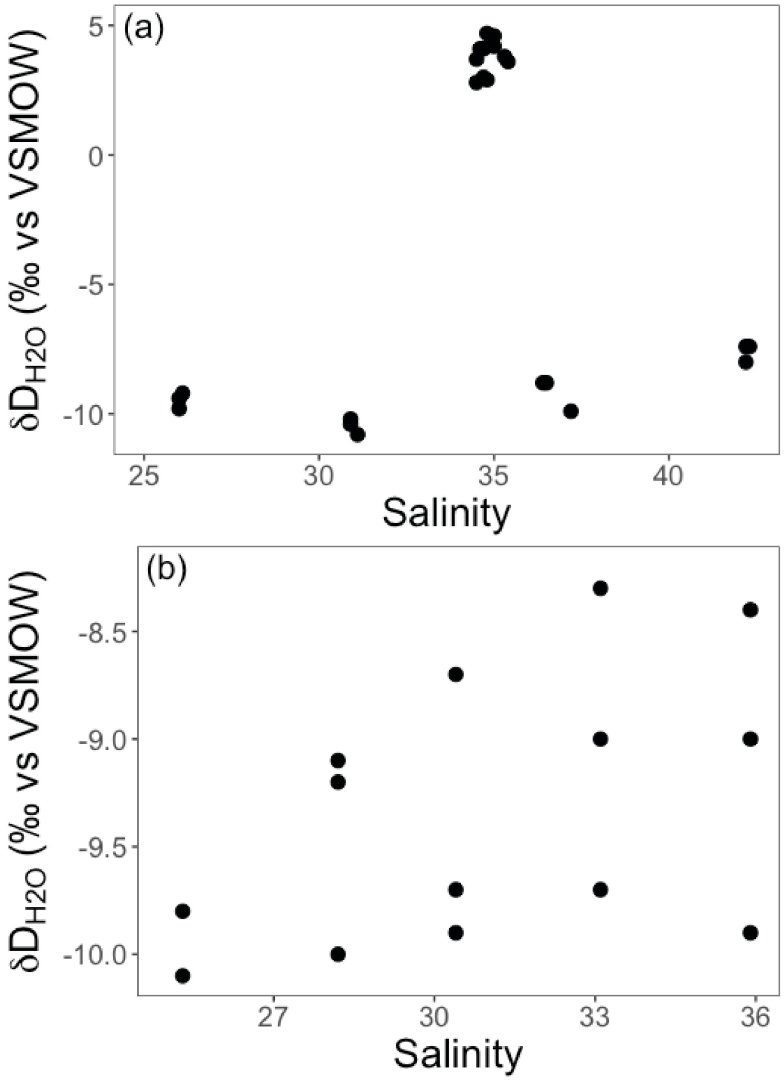

Figure 1: δD_{H2O} of the culture media plotted against salinity of the culture media for (a) the Alkalinity/Salinity experiment and (b) the High Light experiment.

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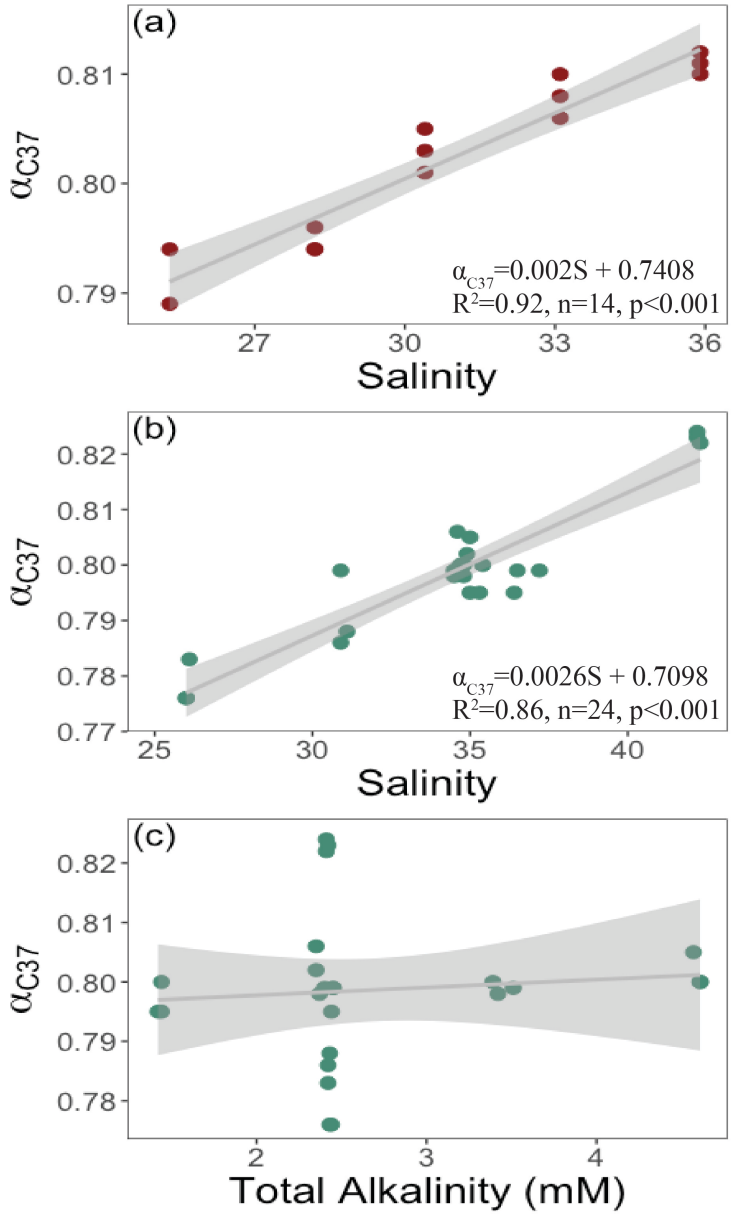

Figure 2: Hydrogen isotope fractionation factor (α_{C37}) plotted against salinity for (a) the High Light experiment and (b) the Alkalinity/Salinity experiment. 2c shows α_{C37} plotted against Total Alkalinity for the Alkalinity/Salinity experiment.

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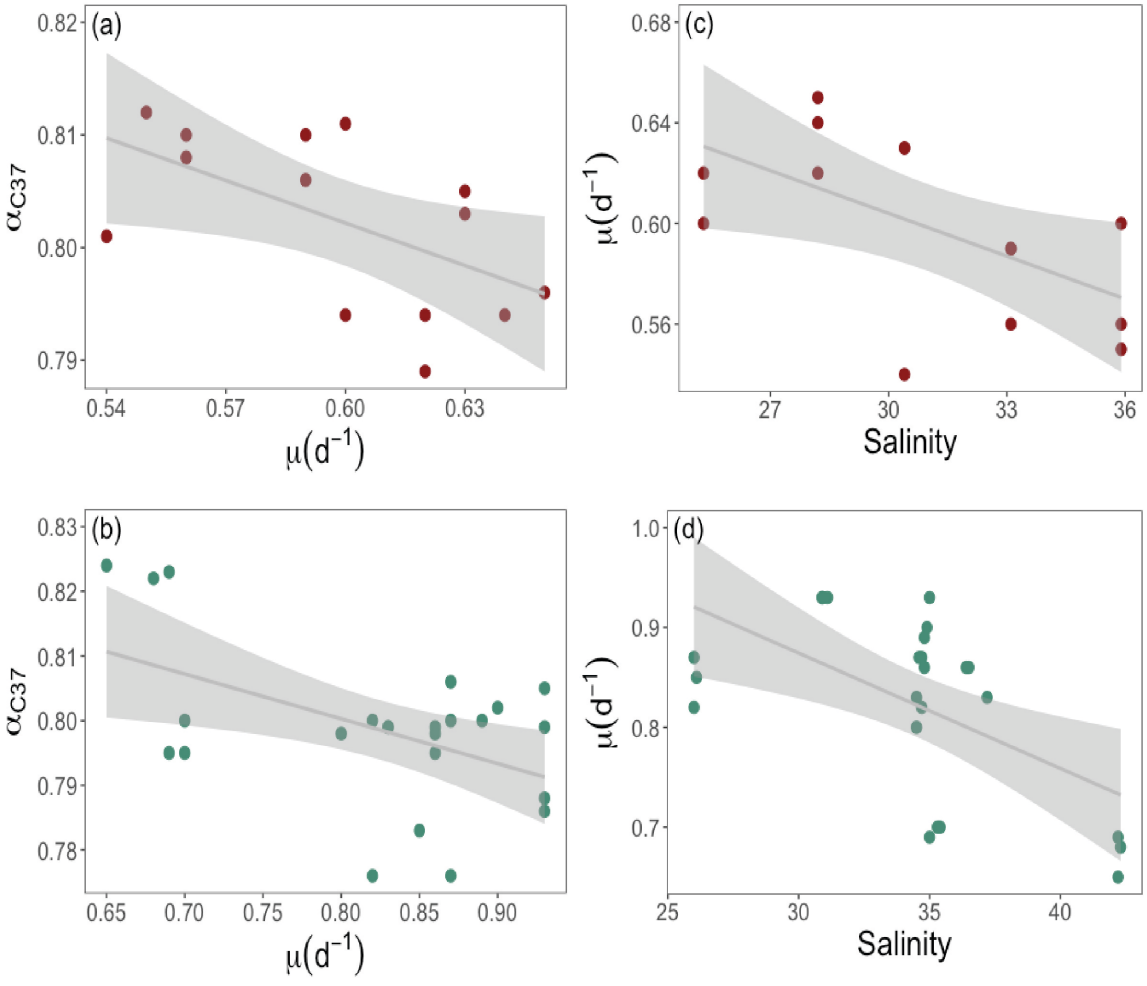

Figure 3: Hydrogen isotope fractionation factor (α_{C37}) plotted against growth rate (μ d^{-1}) for (a) the High Light experiment and (b) the Alkalinity/Salinity experiment. Growth rate (μ d^{-1}) is plotted against salinity for (c) the High Light experiment and (d) the Alkalinity/Salinity experiment.

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Salinity	Temperature (°C)	Irradiance (µmol photons $m^{-2}s^{-1}$)	A_T mM initial	A_T mM end	pH initial	pH end	Growth rate (d^{-1})	δD_{H2O} (‰ vs. VSMOW) initial	St. dev.	δD_{H2O} (‰ vs. VSMOW) end	St. dev.	δD_{C37} (‰ vs. VSMOW)	St. dev.	α	Error
Alkalinity and Salinity Experiment Strain CCMP1516															
26.0	15	75	2.39	2.49	8	8.8	0.82	-10.0	1.1	-8.8	1.8	-230.9	1.8	0.776	0.002
26.0	15	75	2.39	2.46	8	8.7	0.87	-10.0	1.1	-9.6	1.6	-231.1	1.4	0.776	0.001
26.1	15	75	2.39	2.46	8	8.7	0.85	-10.0	1.1	-8.5	2.0	-224.3	2.1	0.783	0.002
30.9	15	75	2.38	2.42	7.9	7.8	0.93	-10.8	1.7	-10.1	2.6	-209.1	0.7	0.799	0.001
31.1	15	75	2.38	2.47	7.9	8.7	0.93	-10.8	1.7	-10.7	0.9	-220.3	0.2	0.788	0.000
30.9	15	75	2.38	2.46	7.9	8.6	0.93	-10.8	1.7	-9.6	1.1	-221.6	1.8	0.786	0.002
36.5	15	75	2.42	2.48	7.8	8.7	0.86	-8.7	1.1	-8.9	1.8	-207.8	0.5	0.799	0.001
37.2	15	75	2.42	2.49	7.8	8.8	0.83	-8.7	1.1	-11.1	1.5	-209.3	1.6	0.799	0.002
36.4	15	75	2.42	2.45	7.8	8.6	0.86	-8.7	1.1	-8.9	1.4	-212.5	1.1	0.795	0.001
42.2	15	75	2.38	2.46	7.9	8.6	0.69	-7.4	1.6	-7.4	1.6	-183.1	2.9	0.823	0.003
42.2	15	75	2.38	2.45	7.9	8.6	0.65	-7.4	1.6	-8.6	0.9	-182.8	2.3	0.824	0.002
42.3	15	75	2.38	2.45	7.9	8.7	0.68	-7.4	1.6	-7.3	1.0	-184.4	0.2	0.822	0.000
35.4	15	75	1.39	1.5	7.8	8	0.7	3.6	1.2	3.5	1.6	-197.0	1.4	0.800	0.001
35.3	15	75	1.39	1.45	7.8	8.5	0.7	3.6	1.2	4.0	1.7	-201.8	0.2	0.795	0.000
35.0	15	75	1.39	1.48	7.8	8.5	0.69	3.6	1.2	5.6	2.1	-201.5	0.3	0.795	0.000
34.8	15	75	2.32	2.42	7.9	8.6	0.86	4.4	1.2	5.0	1.1	-198.7	2.2	0.798	0.002
34.6	15	75	2.32	2.39	7.9	8.5	0.87	4.4	1.2	3.8	1.0	-190.2	3.0	0.806	0.003
34.9	15	75	2.32	2.39	7.9	8.6	0.9	4.4	1.2	4.8	1.7	-194.5	2.1	0.802	0.002
34.7	15	75	3.32	3.45	8	8.6	0.82	2.3	1.1	3.8	1.2	-197.1	1.0	0.800	0.001
34.5	15	75	3.32	3.52	8	8.8	0.8	2.3	1.1	3.3	1.0	-199.8	0.7	0.798	0.001
34.5	15	75	3.32	3.69	8	8.7	0.83	2.3	1.1	5.1	1.1	-198.1	1.4	0.799	0.001
35.0	15	75	4.58	4.56	7.9	8.5	0.93	4.1	1.5	4.2	1.3	-191.6	0.7	0.805	0.001
34.8	15	75	4.58	4.63	7.9	8.6	0.89	4.1	1.5	1.7	1.5	-197.5	0.6	0.800	0.001
34.7	15	75	4.58	4.63	7.9	8.5	0.87	4.1	1.5	4.0	1.4	-197.0	0.8	0.800	0.001
High Light Experiment Strain CCMP1516															
25.3	18.5	600					0.60	-9.9	1.0	-10.1	0.9	-214.3	3.4	0.794	0.003
25.3	18.5	600					0.62	-9.9	1.0	-9.8	1.0	-218.3	0.4	0.789	0.000
28.2	18.5	600					0.65	-9.3	1.2	-10.0	0.8	-212.1	1.3	0.796	0.000
28.2	18.5	600					0.62	-9.3	1.2	-9.2	1.0	-213.1	0.0	0.794	0.001
28.2	18.5	600					0.64	-9.3	1.2	-9.1	1.1	-213.2	0.6	0.794	0.001
30.4	18.5	600					0.63	-10.7	1.2	-9.9	0.7	-204.9	0.5	0.803	0.001
30.4	18.5	600					0.54	-10.7	1.2	-8.7	1.2	-205.6	0.8	0.801	0.001
30.4	18.5	600					0.63	-1.7	1.2	-9.7	1.0	-203.1	0.8	0.805	0.001
33.1	18.5	600					0.56	-9.0	1.5	-9.7	1.3	-199.6	0.7	0.808	0.003
33.1	18.5	600					0.59	-9.0	1.5	-8.3	0.9	-196.8	2.9	0.810	0.000
33.1	18.5	600					0.59	-9.0	1.5	-9.0	1.5	-201.1	0.0	0.806	0.000
35.9	18.5	600					0.60	-9.5	0.9	-9.9	0.7	-197.2	0.0	0.811	0.001
35.9	18.5	600					0.55	-9.5	0.9	-9.0	1.3	-195.1	0.7	0.812	0.002
35.9	18.5	600					0.56	-9.5	0.9	-8.4	1.3	-197.2	1.9	0.810	0.000

Table 1: Growth parameters and hydrogen isotope measurements for Alkalinity/Salinity and High Light batch cultures of *Emiliania huxleyi* CCMP 1516.

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Reference	Strain	α-Salinity relationship	R^2	Number of points
Schouten et al., 2006	*E. huxleyi* PML B92/11	α=0.0033S + 0.6928	0.74	11
M'Boule et al., 2014	*E. huxleyi* CCMP 1516	α=0.0021S + 0.7401	0.80	20
Sachs et al., 2016	*E. huxleyi* CCMP 374	α=0.0015S + 0.777	0.88	9
Alkalinity/Salinity	*E. huxleyi* CCMP 1516	α=0.0026S + 0.7098	0.86	24
High Light	*E. huxleyi* CCMP 1516	α=0.002S + 0.7408	0.92	14

Table 2: Linear regression equations for hydrogen isotope fractionation - salinity (α-salinity) relationships for a compilation of culture experiments growing different strains of *Emiliania huxleyi.*