

**Hydrogen isotope composition
of algal chemical fossils:
a tool for reconstructing
paleo sea surface salinity**

Gabriella M. Weiss

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Hydrogen isotope composition of algal chemical fossils: a tool for reconstructing paleo sea surface salinity

De waterstofisotoopsamenstelling van
chemische algen fossielen: gereedschap voor
het reconstrueren van het zoutgehalte van
vroegere oceanen
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Summary	1
Samenvatting in het Nederlands	3
1 Introduction	7
2 Effects of alkalinity and salinity at low and high light intensity on hydrogen isotope fractionation of long-chain alkenones produced by <i>Emiliana huxleyi</i>	17
3 Hydrogen isotope fractionation response to salinity and alkalinity in a calcifying strain of <i>Emiliana huxleyi</i>	34
4 Impact of metabolic pathways and salinity on the hydrogen isotope ratios of haptophyte lipids	42
5 Constraining the application of hydrogen isotopic composition of long-chain alkenones as a salinity proxy using marine surface sediments	62
6 Alkenone distributions and hydrogen isotope ratios show changes in haptophyte species and source water in the Holocene Baltic Sea	92
7 Paleosensitivity of hydrogen isotope ratios of long-chain alkenones to salinity changes at the Chile Margin	112
References	128
Acknowledgements	148
Curriculum Vitae	150
Publications	151



The RV Pelagia waiting in the harbor at Cyprus prior to setting out to collect samples from the Mediterranean Sea.

Summary

Continental climate is strongly influenced by changes in ocean currents; a well-known example is thermohaline circulation which is driven in part by the temperature and salinity of sea water. Thermohaline circulation in the Atlantic Ocean brings warm, saline waters to high northern latitudes, where heat is then lost to the atmosphere, causing the water to cool. This cold, dense water sinks moves southward, and regains heat in the high southern latitudes to restart the process. The amount of rainfall relative to the amount of evaporation, the volume of water stored in ice sheets and glaciers, as well as atmospheric temperature, can cause perturbations in ocean circulation and how water is transported around the planet, resulting in a shift in continental climate regimes. To constrain these climate effects we can look at past time periods to see how changes in temperature and salinity affected global climate. Paleoceanographers have a number of tools for reconstructing temperature of the ocean, but reconstructing salinity remains an arduous task.

Reconstructions of sea surface salinity from environmental archives have relied heavily on oxygen isotope ratios, specifically measured on calcium carbonate, as these correlated strongly with salinity. However, oxygen isotope ratios of calcium carbonate must be corrected for temperature, making the errors on these estimates substantial. Recently, well-preserved organic compounds containing hydrogen that is not easily exchanged with the environment, have been used to reconstruct salinity. In particular, the hydrogen isotopic composition of long-chain alkenones, compounds synthesized by the Isochrysidales order of Haptophytes, have been tested for this purpose. Importantly, alkenones show a strong correlation to water hydrogen isotope ratios and salinity in batch culture experiments. However, growth phase, growth rate, nutrient concentration, light intensity, and different species have also been demonstrated to impact the offset between water isotope ratios and alkenone hydrogen isotopes, known as hydrogen isotope fractionation. Furthermore, a lack of correlation with fractionation has been noted in hypersaline lakes in North America and in the Chesapeake Bay, likely due to mixing of different species. Down-core reconstructions of salinity from hydrogen isotope ratios of alkenones around the African continent, Mediterranean Sea, Black Sea, off-shore Colombia, show promise but sensitivities differ regionally. This thesis focuses on understanding the relationship between $\delta^2\text{H}_{\text{C}37}$ and salinity in controlled culture settings, but also aims to characterize the environmental signal and apply the proxy in down-core reconstructions to obtain better constraints on the paleosensitivity of $\delta^2\text{H}_{\text{C}37}$ to salinity.

The effects of alkalinity, salinity, high light intensity and calcification on hydrogen isotope fractionation were tested in batch culture experiments of the common marine haptophyte *Emiliania huxleyi*. In one set of batch cultures, a non-calcifying strain of *E. huxleyi* was used to understand the individual effects of alkalinity and salinity and test the correlation between $\delta^2\text{H}_{\text{C}37}$ and salinity under high light conditions. No effect of alkalinity on isotopic fractionation was observed, but a strong effect of salinity was, confirming the effect of salinity noted in previous studies. Under high irradiance of $600 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, there was no

Summary

decrease in sensitivity of the $\delta^2\text{H}_{C37}$ – salinity relationship. The individual effects of alkalinity and salinity were also tested in a calcifying strain of *E. huxleyi* in a second set of batch culture experiments. Long-chain alkenones from this calcifying strain showed more fractionation and had more depleted $\delta^2\text{H}_{C37}$ values relative to non-calcifying strains, but the sensitivity to salinity was similar. These culture experiments confirmed that while alkalinity and salinity co-vary in natural waters, salinity causes the variation in $\delta^2\text{H}_{C37}$ ratios. Furthermore, the salinity effect is not altered by calcification and is still present under high light intensity, factors which are important to paleo application of this proxy for two main reasons. First, haptophytes generally bloom under high light conditions, and these blooms are thought to transport a large portion of alkenones into the sedimentary record. Second, a majority of *E. huxleyi* cells found in the natural environment are calcifying.

While it is relatively well-understood that $\delta^2\text{H}_{C37}$ ratios become more enriched with increasing salinity in a variety of laboratory culture experiments, it is not known what governs this relationship. To improve understanding of the salinity mechanisms reflected in hydrogen isotope ratios of certain lipids, we compared $\delta^2\text{H}$ values of fatty acids, long-chain alkenones, brassicasterol and phytol from four different alkenone-producing haptophytes. Analysis of these different compounds highlighted separate cellular compartments as important controls of the correlation between $\delta^2\text{H}$ ratios and salinity. We also provide the first hydrogen isotope values of alkenones from *Tisochrysis lutea*, a marginal marine alkenone-producing haptophyte. Values from *T. lutea* align well with other marginal marine haptophyte species, confirming a species offset noted in some cultures and environments.

To characterize the natural $\delta^2\text{H}_{C37}$ – salinity relationship, alkenones were analyzed from marine surface sediments covering a salinity range of 7 to 39. No correlation with fractionation was noted, similar to Chesapeake Bay results, likely because of different species present along the salinity gradient. As a result, identifying alkenone producers is important for application of the $\delta^2\text{H}_{C37}$ salinity proxy. The Baltic Sea is the ideal location to test this species effect in a down-core record to track changes over the Holocene. Changes in distribution and hydrogen isotope ratios provided a more robust understanding of species shifts and hydrological regimes in the Baltic Sea. Distribution changes showed that species shifts occurred at the time of Baltic Sea phase shifts which were driven by changes in salinity. Hydrogen isotope ratios followed these species shifts in one case, and in another showed a shift in source water as a result of the melting of the Scandinavian Ice Sheet.

Finally, the $\delta^2\text{H}_{C37}$ – salinity relationship was also analyzed in a longer down-core record from the Chile Margin covering the last 150 kyr. More enriched values were noted for colder time periods and more depleted values for warmer periods. We compared different calibrations for reconstructing salinity and determined that paleosensitivity is generally much larger than noted in laboratory settings or surface sediments. The larger paleosensitivity might stem from our lack of concrete understanding about what governs the salinity mechanism, or it might be that changes in salinity of the surface ocean were larger in the past.

The results of the thesis show that salinity and species are the two strongest controls on $\delta^2\text{H}_{C37}$ ratios of alkenones in both laboratory and environmental settings. Mixing of coastal and marine species can cause a significant decrease in the sensitivity of $\delta^2\text{H}_{C37}$ to salinity. It is vital to determine which species produced alkenones preserved in the sedimentary record. Future attempts to reconstruct salinity should employ a multiproxy approach in order to more accurately understand paleosalinity changes in the past.

Samenvatting in het Nederlands

Het continentale klimaat wordt sterk beïnvloed door veranderingen in zeestromingen; een bekend voorbeeld is de thermohaliene circulatie die deels wordt aangedreven door verschillen in temperatuur en zoutgehalte van zeewater. De thermohaliene circulatie in de Atlantische Oceaan brengt warm en zout water naar hoge noordelijke breedtegraden, waar de warmte dan verloren gaat aan de atmosfeer, waardoor het water afkoelt. Dit koude water met hoge dichtheid zinkt en stroomt naar zuiden en herwint warmte op de zuidelijke breedtegraden om het proces opnieuw te starten. De hoeveelheid neerslag in verhouding tot de hoeveelheid verdamping, het volume van het water opgeslagen in ijskappen en gletsjers, evenals de atmosferische temperatuur, kan verstoringen veroorzaken in de oceaancirculatie en de manier waarop water wordt getransporteerd over de planeet, wat resulteert in een verandering van continentale klimaat regimes. Om deze klimaateffecten te begrijpen, kunnen we kijken naar het verleden om te zien hoe veranderingen in temperatuur en zoutgehalte het klimaat op aarde beïnvloedden. Paleooceanografen hebben meerdere hulpmiddelen voor het reconstrueren van de temperatuur van de oceaan, maar het reconstrueren van het zoutgehalte blijft een moeilijke taak.

Reconstructies van het zoutgehalte van het zeeoppervlak uit natuurlijke archieven, zoals sedimenten, leunen sterk op zuurstofisotoopverhoudingen, specifiek gemeten op calciumcarbonaat, omdat deze sterk correleerden met het zoutgehalte. De zuurstofisotoopverhoudingen van calciumcarbonaat moeten echter worden gecorrigeerd voor de temperatuur, waardoor de fouten op deze schattingen aanzienlijk zijn. De laatste jaren worden goed geconserveerde organische verbindingen die niet makkelijk met de omgeving uitwisselbaar waterstof bevatten, gebruikt om het zoutgehalte te reconstrueren. Met name de waterstofisotoopsamenstelling van langeketenalkenonen, verbindingen gesynthetiseerd door de orde Haptopyten van de Isochrysidales, zijn voor dit doel getest. Belangrijk is dat alkenonen een sterke correlatie vertonen met de waterstofisotoopverhouding van het water en zoutgehalte in batch kweek experimenten. Er is echter ook aangetoond dat de groeifase, groeisnelheid, voedingsstofconcentratie, lichtintensiteit en verschil in soorten van invloed zijn op het verschil tussen de waterstofisotoopverhouding van het water en de alkenonen, beter bekend als waterstofisotoopfractionering. Bovendien is er geen correlatie van fractionering met zoutgehalte gevonden in hypersaliene meren in Noord-Amerika en in de Chesapeake Bay, waarschijnlijk als gevolg van alkenonen afkomstig van meerdere soorten Haptophyten. Reconstructies van het zoutgehalte in het verleden doormiddel van de waterstofisotoopverhoudingen van alkenonen rond het Afrikaanse continent, de Middellandse Zee, de Zwarte Zee en net uit de kust van Colombia zijn veelbelovend, maar de gevoeligheid van deze methode verschilt regionaal. Dit proefschrift richt zich op het begrijpen van de relatie tussen de waterstofisotoopverhouding in alkenonen ($\delta^2\text{H}_{\text{C}37}$) en zoutgehalte onder gecontroleerde kweek omstandigheden, maar heeft ook tot doel natuurlijke isotopenverhoudingen te karakteriseren en de proxy toe te passen in reconstructies van het

verleden om een beter idee van de beperkingen te krijgen, onder andere de verschillende paleosensitiviteit van $\delta^2\text{H}_{\text{C}37}$ ten opzichte van het zoutgehalte.

De effecten van alkaliniteit, zoutgehalte, hoge lichtintensiteit en calcificatie op waterstofisotoopfractionering werden getest in batchcultuurexperimenten van de open oceaan haptophyte *Emiliana huxleyi*. In één batchcultuur experiment werd een niet-calcifiërende stam van *E. huxleyi* gebruikt om de individuele effecten van alkaliniteit en saliniteit te begrijpen en de correlatie tussen $\delta^2\text{H}_{\text{C}37}$ en zoutgehalte onder omstandigheden met veel licht te testen. Alkaliniteit had geen effect op de isotoopfractionering, maar er was wel een sterk effect van saliniteit, wat het effect van zoutgehalte uit eerdere studies bevestigde. Onder hoge lichtintensiteit van $600 \mu\text{mol fotonen m}^{-2} \text{ s}^{-1}$ was er geen afname in gevoeligheid van de $\delta^2\text{H}_{\text{C}37}$ - zoutgehalte relatie. De individuele effecten van alkaliniteit en zoutgehalte werden ook getest in een calcifiërende stam van *E. huxleyi* in een tweede reeks batchcultuurexperimenten. Langeketenalkenonen van deze calcifiërende stam vertoonden meer fractionering en hadden meer negatieve $\delta^2\text{H}_{\text{C}37}$ - waarden ten opzichte van niet-calcifiërende stammen, maar de gevoeligheid voor zoutgehalte was vergelijkbaar. Deze kweekexperimenten bevestigden dat hoewel alkaliniteit en saliniteit co-variëren in natuurlijke wateren, saliniteit de variatie in $\delta^2\text{H}_{\text{C}37}$ -waardes veroorzaakt. Verder wordt het zoutgehalte-effect niet veranderd door kalkvorming en is het nog steeds aanwezig onder hoge lichtintensiteit, factoren die belangrijk zijn voor het gebruik van deze proxy. Dit is belangrijk om twee hoofdredenen: Ten eerste bloeien haptophytes meestal onder omstandigheden met veel licht, en van deze bloeien wordt gedacht dat ze een groot deel van de alkenonen in het sediment produceren. Ten tweede maken de meeste *E. huxleyi*-cellen in de natuur kalkplaatjes.

Hoewel uit een verscheidenheid aan laboratoriumcultuurexperimenten het duidelijk geworden is dat $\delta^2\text{H}_{\text{C}37}$ -waardes hoger worden met toenemende saliniteit, is het niet bekend wat deze relatie regelt. Om het begrip van het effect van zoutgehalte, weerspiegeld in waterstofisotopenverhoudingen van bepaalde lipiden, te verbeteren, vergeleken we $\delta^2\text{H}$ -waardes van vetzuren, langeketenalkenonen, brassicasterol en fytol van vier verschillende alkenonenproducerende haptophyten. Analyse van deze verschillende verbindingen benadrukte afzonderlijke cellulaire compartimenten als belangrijke factor in de correlatie tussen $\delta^2\text{H}$ -waardes en zoutgehalte. We rapporteren ook de eerste waterstofisotoopwaarden van alkenonen van *Tisochrysis lutea*, een marginale, meer kust gebonden, mariene alkenonenproducerende haptophyte. Waarden van *T. lutea* komen goed overeen met andere marginale mariene haptophytesoorten, wat een offset tussen soorten bevestigt die in sommige culturen en natuurlijke omgevingen wordt opgemerkt.

Om de natuurlijke $\delta^2\text{H}_{\text{C}37}$ -zoutgehalte relatie te karakteriseren, werden alkenonen geanalyseerd uit mariene oppervlakesedimenten met een zoutgehalte van 7 tot 39. Er werd geen correlatie met fractionering vastgesteld, vergelijkbaar met de Chesapeake Bay-resultaten, waarschijnlijk vanwege de verschillende soorten aanwezig langs deze zoutgradiënt. Daarom is het identificeren van alkenonenproducenten belangrijk voor de toepassing van de $\delta^2\text{H}_{\text{C}37}$ saliniteitsproxy. De Oostzee is de ideale locatie om dit soort effecten te testen in een sedimentkern die de veranderingen over het Holoceen omvat. Veranderingen in distributie en waterstofisotoopverhoudingen van langeketenalkenonen zorgden voor een meer gedegen inzicht in soortverschuivingen en hydrologische omstandigheden in de Oostzee gedurende het Holoceen. Veranderingen in de alkenonendistributie lieten zien dat verschuivingen van soorten plaatsvonden ten tijde van vernadering in fases van de Baltische Zee, soortenverschuivingen die werden aangedreven door veranderingen in het zoutgehalte. Waterstofisotoopverhoudingen volgden deze soortverschuivingen in één geval en in een ander toonde het een verschuiving in de oorsprong van het zoete water als gevolg van het smelten van de Scandinavische ijskap.

Ten slotte werd de $\delta^2\text{H}_{\text{C}_{37}}$ -zoutgehalte relatie ook geanalyseerd in een langer sedimentkern genomen voor de kust van Chili, deze kern omvat de laatste 150,000 jaar. Hogere waarden werden gemeten in koudere perioden en meer negatieve, of lagere, waarden in warmere periodes. We vergeleken verschillende kalibraties voor het reconstrueren van het zoutgehalte en stelde vast dat de paleosensitiviteit van de $\delta^2\text{H}$ proxy over het algemeen veel groter is dan in laboratoriumexperimenten of oppervlaktensedimenten. De grotere paleosensitiviteit kan het gevolg zijn van ons gebrek aan begrip van hoe het zoutgehalte de watersofisotoopverhouding van langeketenalkenonen precies beïnvloed, of het kan zijn dat veranderingen in het zoutgehalte van de oceaanoppervlakte in het verleden groter waren dan we verwachten.

De resultaten van het proefschrift laten zien dat zoutgehalte en de soort haptophyte de twee sterkste controles zijn op $\delta^2\text{H}_{\text{C}_{37}}$ -waardes van alkenonen in zowel het laboratorium- als de natuurlijke omgeving. Het mengen van (alkenonen van) kust- en mariene soorten kan een significante afname van de gevoeligheid van $\delta^2\text{H}_{\text{C}_{37}}$ tot zoutgehalte veroorzaken. Het is van vitaal belang om te bepalen welke soort haptophyte de alkenonen heeft geproduceerd die in het sedimentaire archief zijn bewaard. Toekomstige pogingen om het zoutgehalte te reconstrueren zouden een multiproxy-benadering moeten gebruiken om veranderingen in saliniteit in het verleden beter te kunnen begrijpen.



Passing through the Corinth Channel, Greece aboard the RV Pelagia.

Chapter 1

Introduction

1 The role of sea surface salinity in global climate

Surface ocean dynamics play an important role in governing the magnitude of changes in the Earth's climate (Chahine, 1992). In fact, the mixing of waters with different salt concentrations is a vital part of ocean currents and ocean circulation, and has strong implications for global climate change (Rahmstorf, 2002). Much attention has been directed toward what the future holds for our planet under increased warming (IPCC, 2014). In order to better understand what scenarios exist for the future, it is essential to obtain a concrete understanding of how the climate system has reacted in the past. A lot of effort has been made to reconstruct surface ocean temperature and atmospheric CO₂ on geologic timescales (e.g., Emiliani, 1955; Brassell et al., 1986; Zachos et al., 2001; Seki et al., 2010; Plancq et al., 2014; Inglis et al., 2015), providing a window into significantly warmer and colder worlds than our present climate, for which no instrumental records exist. A wealth of knowledge has been gained from reconstructions of these aforementioned parameters, but very little is known about the salinity of the ocean surface in the past.

The movement of ocean currents around the globe driven in part by temperature and salinity has been termed thermohaline circulation (Wunsch, 2002). The thermohaline circulation in our present climate (Fig. 1) transports warm, saline surface water northward in the Atlantic, where it releases heat to the atmosphere (Broecker, 1997; Clark et al., 2002). As these warm, saline waters travel north, heat is lost to the atmosphere creating cooler, denser water which sinks to deeper depths (known as North Atlantic Deep Water). The colder current then moves southward and picks up heat in the high southern latitudes to start the process again (Broecker, 1997; Clark et al., 2002). Knowledge about past fluctuations in salinity and temperature from the geologic record can help better inform our understanding of ocean circulation processes, and illustrate about how perturbations to it, like the shutdown or alteration of thermohaline circulation, may have driven abrupt global climate change (Clark et al., 2002).

2 The hydrological cycle and isotopic composition of water

Stable isotope ratios of oxygen ($\delta^{18}\text{O}$) and hydrogen ($\delta^2\text{H}$) are widely applied tools for reconstructing past hydrological conditions due to the fact that these isotope ratios change throughout the hydrological cycle (Fig. 2). In the oceans, as water evaporates, the heavier isotope and salts remain in the liquid phase, causing enrichment of the $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios

Introduction

of the water and increased salinity (Craig and Gordon, 1965). Meanwhile, the vapor phase contains more of the light isotope, resulting in relatively depleted $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios (Craig and Gordon, 1965, Clark and Fritz, 1997; Fig. 2). Increased residence time of vapor in the atmosphere and increased distance from the source of evaporation prior to precipitation also causes isotopic depletion (Dansgaard, 1964; Craig and Gordon, 1965; Fig. 2). Precipitation follows a Rayleigh fractionation pattern: as the precipitation reservoir decreases in size, there is continued removal of the heavier isotope, leading to overall depletion of precipitation over time and distance. In equatorial latitudes, precipitation exceeds evaporation and falls relatively close to the evaporation source, which leads to precipitation with a similar isotopic value as the sea water, causing lower sea water salinity but little isotopic change (Craig and Gordon, 1965). In the mid-latitudes, evaporation exceeds precipitation, which increases ocean salinity. Polar regions receive precipitation that originates in the tropical latitudes and therefore have the most depleted precipitation due to the increased distance from the evaporation source. High latitude oceans receiving freshwater input (via ice melt and continental runoff) are characterized by both relatively low $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios and lower salinities. A culmination of the aforementioned processes results in a strong, positive correlation between both $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios as well as salinity in meteoric and surface ocean waters (Fig. 3), and this relationship can be implemented as a tool to gain insight about sea surface salinity in the past.

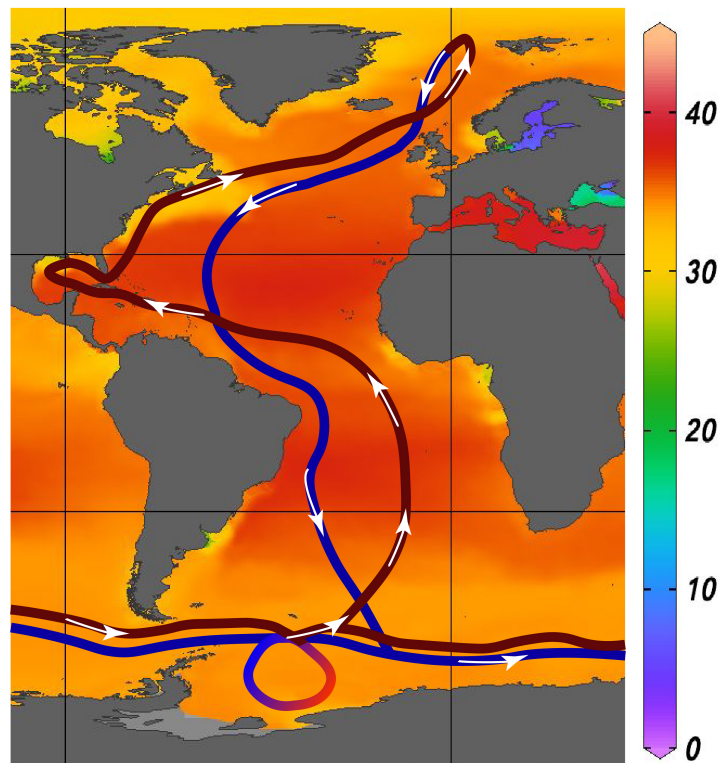


Figure 1: Thermohaline circulation in the Atlantic Ocean. The red lines represent warm surface currents and the blue lines represent cold, deeper currents. Figure adapted from Rahmstorf (2002).

3 Methods for reconstructing past salinity

Based on the tight coupling between salinity and isotope ratios of water, past salinities can be reconstructed if the isotope ratio of the original water is known. Water from past ocean settings is not readily preserved in geological archives and available for analysis, thus other sources must be utilized to inform about ancient water conditions. The principal tool for reconstructing the salinity of paleo water has been the $\delta^{18}\text{O}$ ratios of calcium carbonate, in particular that of foraminifera. However, $\delta^{18}\text{O}$ ratios of carbonate are not only impacted by the $\delta^{18}\text{O}$ composition of the sea water, but are also dependent upon temperature (Shackleton, 1967, 1974; Erez and Luz, 1982; Ravelo and Hillaire-Marcel, 2007). Therefore, to reconstruct sea water $\delta^{18}\text{O}$ ratios, one must also know calcification temperature. Temperature can be inferred using Mg/Ca ratios of foraminiferal calcite, preferably measured on the same calcite as $\delta^{18}\text{O}$ ratios (Lear et al., 2000). Indeed, paired analyses of $\delta^{18}\text{O}$ and Mg/Ca ratios on calcite, mainly from foraminifera, have shown promise for reconstructing paleosalinities (Elderfield and Ganssen, 2000). The calibrations established for the relationship between $\delta^{18}\text{O}_{\text{foram}}$ and $\delta^{18}\text{O}_{\text{seawater}}$ might vary on spatial and temporal scales, due in part to freshwater runoff into oceans, which does not have a fixed isotopic signature (Rohling, 2007), posing an additional challenge. Besides temperature, Mg/Ca is also affected by variations in salinity, complicating the situation further (Nurnberg et al., 1996). Clumped isotope geochemistry, utilizing the natural “clumping” of oxygen and carbon isotopes in carbonates, is a relatively new tool used for reconstructing temperature (Eiler, 2011), and in principle the $\delta^{18}\text{O}$ of sea water, which can combat some of the issues encountered with combined $\delta^{18}\text{O}$ and Mg/Ca salinity reconstructions.

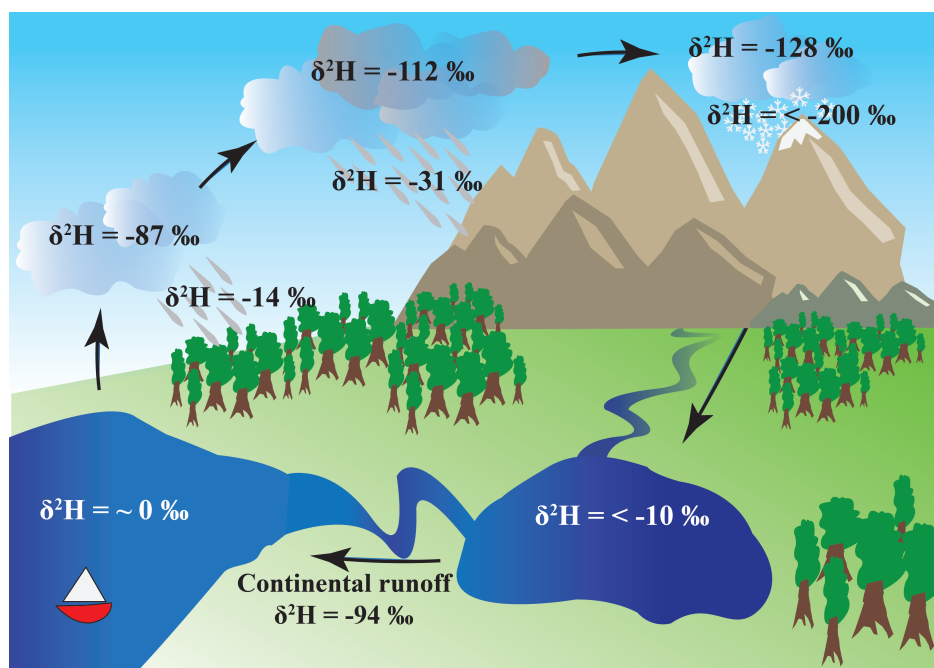


Figure 2: Relative changes in hydrogen isotope ratios ($\delta^2\text{H}$) throughout the hydrological cycle. Oceans are characterized by relatively enriched $\delta^2\text{H}$ ratios compared to freshwater sources which tend to have relatively depleted $\delta^2\text{H}$ ratios. Vapor and precipitation become more depleted the further away from the source they travel. Figure adapted from Craig and Gordon (1965) and Gat (2010).

Likewise, elemental ratios measured on foraminiferal calcite, for example Na/Ca ratios

(Wit et al., 2013), have been explored as potential salinity proxies which might be more directly related to salinity than water isotope ratios. Nonetheless, there have been no environmental calibrations or wide applications of these proxies to date, leaving salinity a difficult parameter to reconstruct. As discussed briefly above, existing methods require corrections for temperature (or other factors) in order to obtain estimates of water isotope ratios which in turn allow for an evaluation of salinity changes. Water isotope ratios are also affected by parameters like the volume of ice stored in ice sheets and glaciers globally (Shackleton, 1967), adding additional uncertainty. Furthermore, large errors are associated with these currently established methods, making reconstructions of absolute salinity harder to achieve (e.g. Rohling et al., 2007). As a consequence, there is still a need for complimentary methods to strengthen understanding of how salinity changed in the past.

4 A salinity proxy based on hydrogen isotope ratios of alkenones

4.1 Culture studies

Haptophyte algae are ubiquitous phytoplankton in modern and past oceans, familiar to some because their calcium carbonate skeletons comprise the White Cliffs of Dover and they form extensive blooms which are visible from space. Haptophytes exist in both calcified (coccolithophores) and non-calcified forms, and calcite-producing cells are thought to be one of the largest producers of calcium carbonate globally (Laguna et al., 2001; Paasche, 2002). A select group of haptophytes from the order Isochrysidales synthesize organic compounds known as long-chain alkenones: methyl and ethyl ketones with carbon chain lengths from 35-40 (de Leeuw et al., 1980; Volkman et al., 1980; Longo et al., 2013). Found in the geologic record as far back as 120 Ma (Brassell et al., 2004 and references cited therein), long-chain alkenones are present from the Late Cretaceous to the modern day. Most commonly, long-chain alkenones have been used to reconstruct temperatures of the surface ocean globally using the U_{37}^K and $U_{37}^{K'}$ indices (Brassell et al., 1986; Prahl and Wakeham, 1987), and stable carbon isotope ratios of long-chain alkenones have been explored as a tool for reconstructing atmospheric CO_2 (Jasper and Hayes, 1990; Pagani, 2002, 2014). Long-chain alkenones have mostly carbon-bound hydrogen, which is thought to experience relatively little exchange with the environment on short geologic timescales (Sessions et al., 2004), and they are not easily degraded due to the five methylene spacing between double bonds of the uncommon E configuration (Rechka and Maxwell, 1988; Rontani et al., 2006). Thus, their hydrogen isotopic composition has also been explored for proxy potential.

The first studies showed that, as expected, the hydrogen isotope ratios of long-chain alkenones (δ^2H_{C37}) correlated strongly with growth water isotope ratios ($\delta^2H_{H_2O}$) in laboratory cultures (Paul, 2002; Englebrecht and Sachs, 2005). The offset between $\delta^2H_{H_2O}$ and δ^2H_{C37} ratios was approximately -171 ‰ in these initial studies. This offset was first thought to be constant, thus it was believed that this value could be utilized to reconstruct δ^2H of sea water, and therefore salinity. However, it was subsequently realized that this hydrogen isotope fractionation, expressed as the hydrogen isotope fractionation factor α , or the difference between $\delta^2H_{H_2O}$ and δ^2H_{C37} ratios, is dependent on many variables. In particular, there is a strong effect of salinity on α values, which appears to be a robust observation in laboratory cultures (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014). An increase in fractionation factor α (meaning a decrease in fractionation) is noted with increasing

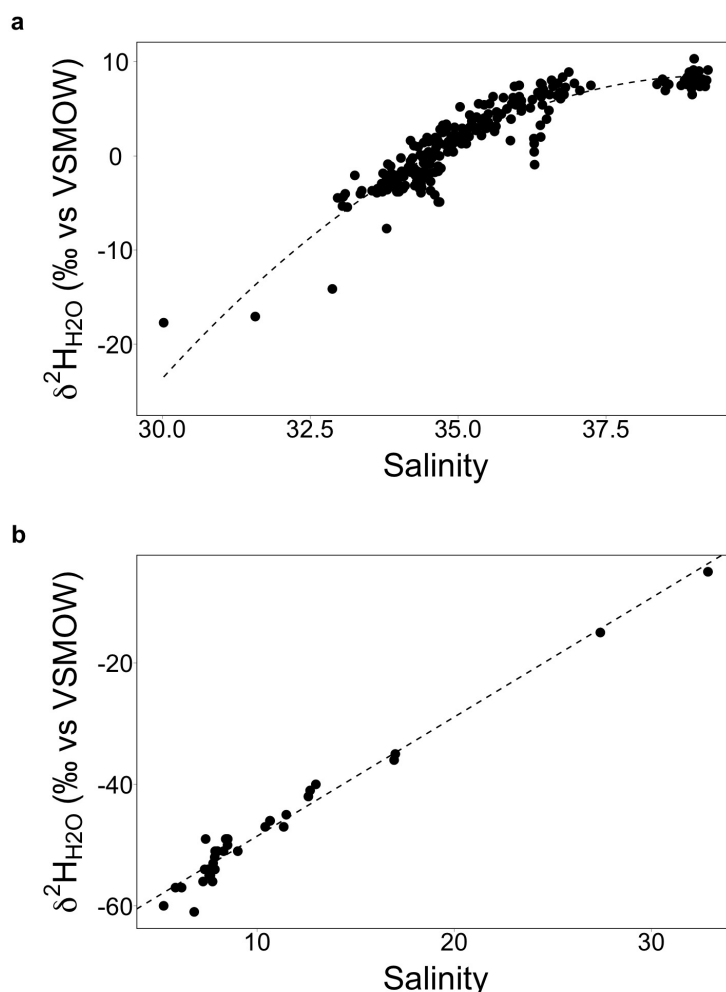


Figure 3: Hydrogen isotopes of water plotted against salinity from (a) various global locations (adapted from Rohling, 2007), and (b) the Baltic Sea (adapted from Fröhlich et al., 1988).

salinities, leading to enriched $\delta^2\text{H}_{\text{C}_{37}}$ ratios at higher salinities (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014; Fig. 4). This salinity effect has been demonstrated in four different haptophyte species: *Emiliania huxleyi*, *Gephyrocapsa oceanica*, *Isochrysis galbana* and *Ruttenella lamellosa*, albeit with different sensitivities ranging between approximately 2 to 5 ‰ change in $\delta^2\text{H}_{\text{C}_{37}}$ per salinity unit (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014; Fig. 4). This salinity effect works in the same direction as the correlation between $\delta^2\text{H}$ and salinity in environmental waters, where isotopic enrichment corresponds to higher salinities. Consequently, $\delta^2\text{H}$ ratios of long-chain alkenones are correlated to salinity as a result of the change in fractionation and the change in the $\delta^2\text{H}$ of sea water. It was on these grounds that $\delta^2\text{H}$ ratios of long-chain alkenones were explored as a promising new proxy for paleo sea surface salinity and initially provided convincing paleosalinity estimates (van der Meer et al., 2007, 2008). Interestingly, $\delta^2\text{H}$ ratios of other organic compounds from different phytoplankton species, such as sterols and fatty acids from diatoms and cyanobacteria, also appear to be affected in a similar fashion by salinity, albeit with different sensitivities (Sachse et al., 2012 and references cited therein).

As mentioned above, salinity is not the only parameter known to affect α , and a few other effects have been noted in culture (Schouten et al., 2006; Wolhowe et al., 2009; Chivall et

al., 2014; M'Boule et al., 2014; van der Meer et al., 2015). Culture experiments growing marine species *E. huxleyi* and *G. oceanica* showed that growth rate negatively correlates with hydrogen isotope fractionation, meaning faster growth led to more fractionation and more depleted $\delta^2\text{H}_{\text{C}_{37}}$ ratios (Schouten et al., 2006; Wolhowe et al., 2009). Growth phase was shown to cause variation in the amount of fractionation, leading to weakened sensitivity of $\delta^2\text{H}_{\text{C}_{37}}$ –salinity during stationary and death phases of growth relative to exponential growth phase in two marginal marine species, *I. galbana* and *R. lamellosa* (Chivall et al., 2014). External factors like light intensity, a vital resource for photosynthesizing organisms such as haptophytes, also caused changes in α values and $\delta^2\text{H}_{\text{C}_{37}}$ ratios, with increased variability in fractionation observed at lower light intensities (van der Meer et al., 2015; Wolhowe et al., 2015). Laboratory and field studies have shown that high light intensity above $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ might not be a significant source of variation in fractionation (van der Meer et al., 2015; Wolhowe et al., 2015), but the effect of high light conditions on the fractionation response to salinity has not been investigated.

Differences in α between haptophyte species is also substantial and is noted between $\delta^2\text{H}_{\text{C}_{37}}$ ratios from marginal (*I. galbana*) and open marine (*E. huxleyi*) species with an offset of 100 ‰ when grown at the same salinity (M'Boule et al., 2014). Results from another marginal marine species, *R. lamellosa*, have similar $\delta^2\text{H}_{\text{C}_{37}}$ ratios as *I. galbana* (Chivall et al., 2014), adding further evidence for an offset between coastal and open marine species (Fig. 4). Although good progress has been made toward constraining which factors affect $\delta^2\text{H}_{\text{C}_{37}}$ ratios, some environmental and biological factors influencing the $\delta^2\text{H}$ of alkenones have not yet been investigated. Furthermore, the precise mechanisms governing the relationship between isotopic fractionation and salinity remain unknown.

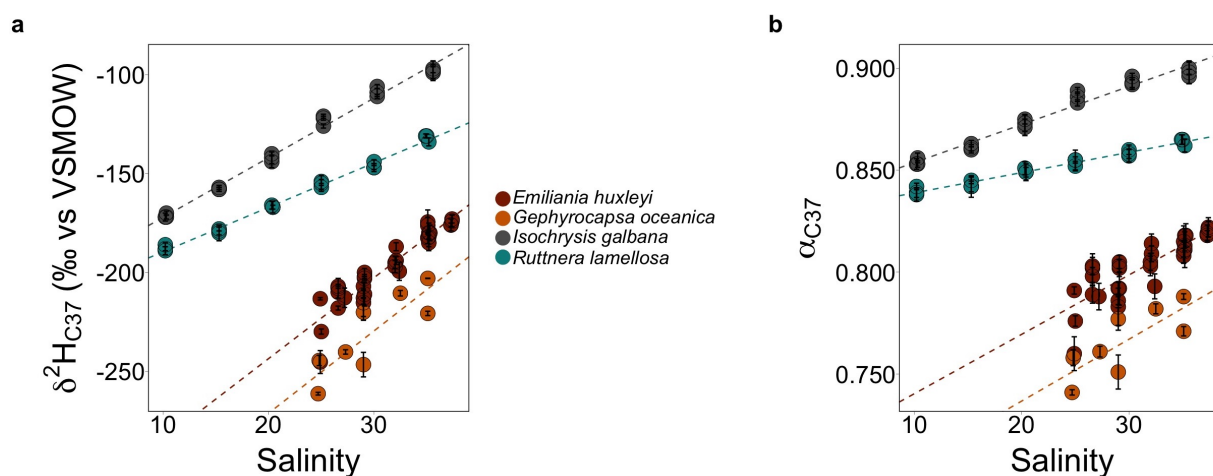


Figure 4: (a) Hydrogen isotope ratios and (b) hydrogen isotope fractionation factor α of long-chain alkenones plotted against salinity from laboratory culture experiments growing different species of haptophytes. There is a clear offset between *Isochrysis galbana* and *Ruttnera lamellosa* relative to *Emiliana huxleyi* and *Gephyrocapsa oceanica* for both parameters. Adapted from Schouten et al. (2006), Chivall et al. (2014), and M'boule et al. (2014).

4.2 Environmental studies and applications

In contrast to culture studies, surveys of the salinity effect on $\delta^2\text{H}$ ratios of alkenones in the natural environment are sparse, but the salinity effect has been noted for alkenones from the

Introduction

Chesapeake Bay estuary as well as saline to hypersaline ponds and lakes (Schwab and Sachs, 2011; Nelson and Sachs, 2014). No correlation between α and salinity was noted for long-chain alkenones from the Chesapeake Bay, potentially due to contributions from different species along the Chesapeake Bay salinity gradient (Schwab and Sachs, 2011). However, the $\delta^2\text{H}$ -salinity relationship has yet to be studied for open marine environments.

A few attempts have been made to use $\delta^2\text{H}_{C37}$ ratios in different marine sediment records to understand salinity changes over time scales spanning a couple hundred years (Pahnke et al., 2007) to longer records of 500 kyrs (Petrick et al., 2015), and even the Messinian Salinity Crisis between 5 - 6 Ma (Vasiliev et al., 2017). Measurements of $\delta^2\text{H}_{C37}$ ratios from down-core records centered around the African continent (Leduc et al., 2013; Kasper et al., 2014, 2015; Simon et al., 2015; Petrick et al., 2015), but also included records from the Mediterranean Sea and Black Sea (van der Meer et al., 2007, 2008), and one record from off-shore Colombia (Pahnke et al., 2007). Generally, these downcore records of $\delta^2\text{H}_{C37}$ ratios showed changes between glacial and interglacial time periods, with more enriched $\delta^2\text{H}_{C37}$ ratios reported for colder periods and more depleted $\delta^2\text{H}_{C37}$ ratios for warmer climates. These trends are in agreement with oxygen isotope trends. However, reconstructing absolute salinities from $\delta^2\text{H}_{C37}$ ratios was not possible due to the lack of an existing environmental calibration. Thus, salinity reconstructions relied on $\delta^2\text{H}_{C37}$ – salinity relationships established from culture studies. However, culture calibrations are species-specific, and it is rarely the case that only one species was contributing to the alkenone pool preserved in the geologic record. Consequently, a better understanding of the environmental $\delta^2\text{H}_{C37}$ – salinity relationship is necessary in order to more accurately reconstruct salinity in the geologic record.

5 Scope of the thesis

The discussion above highlights that a strong relationship has been observed between $\delta^2\text{H}$ ratios of alkenones and salinity in laboratory cultures, a couple modern settings and a few sediment records. While culture studies have focused on this relationship in detail, not all potential effects on the $\delta^2\text{H}_{C37}$ -salinity relationship have been investigated. Furthermore, particular attention has been placed on constraining the relationship between $\delta^2\text{H}$ ratios and salinity in culture studies, but this relationship has not been tested in present day marine settings. Therefore, the focus of this thesis was to better understand factors affecting the sensitivity of α_{C37} and $\delta^2\text{H}_{C37}$ ratios to salinity in laboratory cultures, and to characterize the relationship in present day marine environments, with the purpose of applying this knowledge to reconstruct salinity in down-core records.

In **Chapter two**, the impact of alkalinity and high light intensity on hydrogen isotopic fractionation was investigated. These two factors are important because alkalinity co-varies with salinity in natural waters, and haptophyte blooms occur under high irradiance, above 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Nanninga and Tyrrell, 1996). These high light blooms are thought to be responsible for a significant portion of alkenones exported to sediments. To constrain the effects of both alkalinity and high light intensity we performed two separate batch culture experiments with a non-calcifying strain of *E. huxleyi*. In these experiments, alkalinity was varied at constant salinity, salinity was varied at constant alkalinity, and a range of salinities were tested at an irradiance of 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Results confirmed that salinity is indeed responsible for the variation in $\delta^2\text{H}_{C37}$ ratios, and no correlation with alkalinity was noted for either $\delta^2\text{H}_{C37}$ ratios or α values. Furthermore, no decrease in the sensitivity of the $\delta^2\text{H}_{C37}$ – salinity relationship was observed under high light conditions. This suggests that

light intensity might not be a significant cause of variation in $\delta^2\text{H}_{\text{C}37}$ ratios from paleo archives if a majority of sedimentary alkenones are coming from large blooms occurring under high irradiance.

The $\delta^2\text{H}_{\text{C}37}$ ratios and fractionation effects have primarily been investigated in no longer calcifying or non-calcifying strains of haptophytes. Therefore, in **Chapter three**, a calcifying strain of *E. huxleyi* was grown at different alkalinities and salinities to investigate the effect of calcification on the hydrogen isotope fractionation response to salinity. Alkenones from this calcifying strain fractionated less and had more depleted $\delta^2\text{H}_{\text{C}37}$ ratios relative to non-calcifying strains. However, the $\delta^2\text{H}_{\text{C}37}$ response per change in salinity was statistically similar to the relationships reported for non-calcifying strains, leading to the conclusion that calcification should not significantly impact paleo applications of $\delta^2\text{H}_{\text{C}37}$ ratios.

While a number of environmental factors have been shown to affect the sensitivity of $\delta^2\text{H}$ ratios to salinity, the intracellular mechanisms controlling the correlation between $\delta^2\text{H}$ ratios and salinity for certain lipids are not well known. To gain further insight about these mechanisms, in **Chapter four**, the $\delta^2\text{H}$ ratios of C_{14} – C_{18} fatty acids, long-chain alkenones, brassicasterol and phytol measured for Group II and Group III alkenone producers grown at different salinities are reported. Analyses of the $\delta^2\text{H}$ of these different lipids suggested that compounds relying on cytosolic pools of nicotinamide adenine dinucleotide phosphate (NADPH; a substantial source of hydrogen for organic compounds) or synthesized from precursors relying on acetyl-coenzyme A retained the salinity signal. Compounds relying on pools of NADPH or pyruvate in the chloroplast have $\delta^2\text{H}$ ratios that did not change with salinity. In addition, the first characterization of $\delta^2\text{H}$ ratios and fractionation of alkenones from the Group II species *Tisochrysis lutea* showed that it responded similarly to salinity as other Group II species *I. galbana* and *R. lamellosa*, but was different from Group III species. These observations add further evidence to support a strong species offset between different haptophyte groups.

Little is known about the correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and salinity in the natural environment, especially in open marine settings. To gain insight into this relationship, in **Chapter five**, the $\delta^2\text{H}_{\text{C}37}$ ratios from surface sediments sampled from environments covering a sea surface salinity range from 7 – 39 were measured. $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios of surface waters were analyzed for each of the sampling locations in order to roughly estimate fractionation. A strong positive correlation was observed between $\delta^2\text{H}_{\text{C}37}$ ratios and salinity, but there was no correlation between fractionation factor α and salinity. The lack of a fractionation effect is most likely the result of mixing of low salinity, marginal marine and open marine alkenone-producing species along the combined transects. These findings indicate that understanding the composition of alkenone-producers is vital for interpreting $\delta^2\text{H}_{\text{C}37}$ ratios from sedimentary records.

The Baltic Sea is a dynamic marginal marine environment with an open connection to the North Sea at the present. The salinity gradient from the North Sea (34 psu) into the Baltic Sea proper (5 psu) made it an ideal setting to test the effects of mixing of different haptophyte species, which have been shown to vary along this particular salinity gradient. Furthermore, since the connection to the North Sea changed throughout the Holocene as a consequence of melting of the Scandinavian Ice Sheet and resultant continental uplift, the Baltic Sea experienced a number of different phase shifts which are defined by changes in salinity. Therefore, in **Chapter six**, alkenone distributions and $\delta^2\text{H}_{\text{C}37}$ ratios were measured from a piston core spanning the last 11 kyr. Alkenone distributions changed along with well-established Baltic Sea phases. Hydrogen isotope ratios show two time periods with major changes, a 50 ‰ enrichment in the middle of the freshwater Ancylus Lake phase (10.6 to 7.7

Introduction

kyr) and a 25 ‰ depletion at the boundary between the brackish Littorina Sea (7.2 to 3 kyr) and the fresher Modern Baltic phase (3 kyr to the present). The Ancyclus Lake enrichment was likely caused by a change in source water, whereas the Littorina into Modern Baltic depletion corresponded with a shift in alkenone distribution, meaning this was likely caused by a change in species. These findings led to the conclusion that $\delta^2\text{H}_{\text{C}_{37}}$ ratios can track shifts in source water as well as salinity and species changes.

To study the paleosensitivity of $\delta^2\text{H}_{\text{C}_{37}}$ ratios to salinity, in **Chapter seven**, $\delta^2\text{H}_{\text{C}_{37}}$ ratios were measured on a core from ODP site 1234 at the Chile Margin spanning the last 150 kyr. $\delta^2\text{H}_{\text{C}_{37}}$ ratios were more enriched during glacial stages and more depleted during interglacial stages, with the most depleted value during the Eemian interglacial and the most enriched value between marine isotope stages 2 and 3 at approximately 30 ka. Different calibrations for calculating salinity were explored, which all suggest salinity changes greater than 2 psu, larger than the previously suggested difference of global averages between the last glacial period (LGM, 21 ka) and the present. These comparisons suggest that either something crucial is missing from our understanding of the $\delta^2\text{H}_{\text{C}_{37}}$ – salinity relationship, or changes in surface ocean salinity in the past might have been larger than formerly hypothesized based on other proxy data and climate models.

The findings of this thesis emphasized the fact that the species effect and salinity are the most significant causes of variation in $\delta^2\text{H}_{\text{C}_{37}}$ ratios in laboratory and environmental settings. Mixing of different haptophyte species caused a dampening of the $\delta^2\text{H}_{\text{C}_{37}}$ – salinity response in modern marine environments and was also important for understanding the Baltic Sea climatic history. In contrast, alkalinity, calcification and high light intensity did not significantly impact the sensitivity of the $\delta^2\text{H}_{\text{C}_{37}}$ – salinity relationship in laboratory cultures. Mechanisms related to specific cellular compartments seem to be important for understanding which biomarkers retain a correlation with salinity, a finding which is useful for future studies employing $\delta^2\text{H}$ ratios to understand hydrological changes. Evidence from sediment cores suggested that reconstructed salinities based on existing $\delta^2\text{H}_{\text{C}_{37}}$ – salinity relationships show larger shifts than expected, which could mean that the paleosensitivity of $\delta^2\text{H}_{\text{C}_{37}}$ ratios to salinity is higher than observed in cultures and core tops or that salinity variations were larger in the past.. Thus, $\delta^2\text{H}_{\text{C}_{37}}$ ratios remain a good proxy for qualitative estimates of salinity change, but not quantitative at present. In future studies, emphasis should be placed on understanding which haptophyte species are contributing to the sedimentary alkenone pool in order to determine the most suitable calibration for estimating shifts in sea surface salinity. Better constraints on the paleosensitivity of this proxy are necessary, and generation of more paleo records and modern day surveys could help with this. Additionally, more efforts should be concentrated on conducting multiproxy research using both oxygen and hydrogen isotope ratios measured on samples from the same depths but on different organisms, in order to more confidently reconstruct water isotope shifts, and therefore, changes in salinity.



A multicore recovered from the Black Sea with well-preserved Coccolith ooze layers.
Photo by Rose Leahy.

Chapter 2

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

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Abstract

Over the last decade, hydrogen isotopes of long-chained 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 $\delta^2\text{H}_{C37}$ 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 under low light conditions. 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 use of hydrogen isotope fractionation as a paleosalinity proxy.

Keywords:

Alkenones, Hydrogen isotopes, Salinity, Alkalinity, High light intensity

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, $\delta^{18}\text{O}_{\text{foram}}$ (Emiliani, 1955), Mg/Ca (Elderfield and Ganssen, 2000), TEX_{86} (Schouten et al., 2002), U_{37}^K (Brassell et al., 1986), and LDI (Rampen et al., 2012). However, there are currently very few proxies for reconstructing sea surface salinity (SSS).

Evaporation, precipitation, continental runoff, and ice melt cause changes in seawater salinity, thereby influencing ocean circulation. The isotopic ratios of oxygen ($\delta^{18}\text{O}$) and hydrogen ($\delta^2\text{H}$) 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, a depleted isotopic signature is found for most precipitation-fed rivers and lakes (i.e. meteoric waters). As these waters drain into the ocean and mix with seawater, the sea surface salinity is lowered, as is the water isotope value. This leads to a strong linear correlation between $\delta^{18}\text{O}_{\text{water}}$ values and salinity in ocean water and therefore the $\delta^{18}\text{O}_{\text{water}}$ 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 $\delta^{18}\text{O}_{\text{water}}$ -S relationships and, thus, paleosalinity reconstructions. Therefore, constraining the correlation between $\delta^{18}\text{O}_{\text{water}}$ values and S currently poses a challenge in attempts to extract reliable palaeosalinity estimates from inferred $\delta^{18}\text{O}_{\text{water}}$ values.

Over the last decade, culture studies have shown that the hydrogen isotopic ratios of long chain alkenones ($\delta^2\text{H}_{\text{C}_{37}}$), 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 ($\delta^2\text{H}_{\text{H}_2\text{O}}$) (Englebrecht and Sachs, 2005; Paul, 2002), which in turn is correlated with salinity (Craig and Gordon, 1965). In addition to the observed relationship between $\delta^2\text{H}_{\text{C}_{37}}$ and $\delta^2\text{H}_{\text{H}_2\text{O}}$ values, biological hydrogen isotope fractionation has been shown to decrease with increasing salinity, thereby amplifying the salinity to seawater $\delta^2\text{H}$ 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 $\delta^2\text{H}_{\text{C}_{37}}$ has been proposed as an appropriate proxy for reconstructing sea surface salinity (SSS) (Englebrecht and Sachs, 2005; Schouten et al., 2006). For example, $\delta^2\text{H}_{\text{C}_{37}}$ values measured on alkenones extracted from Mediterranean sapropel S5 shows similar trends to $\delta^{18}\text{O}$ 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). $\delta^2\text{H}_{\text{C}_{37}}$ values 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 $\delta^2\text{H}_{\text{C}_{37}}$ values during glacial termination I and II and from the last glacial maximum into the Holocene, which align with $\delta^{18}\text{O}_{\text{foram}}$ values from the same region (Simon et al., 2015; Petrick et al., 2015; Kasper et al., 2014). Leduc et al. (2013) show a divergence in estimates of sea surface salinity between $\delta^2\text{H}_{\text{C}_{37}}$ derived and planktonic foraminifera derived proxies ($\delta^{18}\text{O}_{\text{sw}}$ and Ba/Ca ratios) across the Holocene in the Gulf of Guinea, which are attributed to differences in the isotopic ratios of rainfall over the time period.

Although a relationship between $\delta^2\text{H}_{\text{C}_{37}}$, $\delta^2\text{H}_{\text{H}_2\text{O}}$ 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 $\delta^2\text{H}_{\text{C}_{37}}$ values and salinity in suspended particulate matter from the Amazon Plume. In the Chesapeake Bay Estuary (USA), $\delta^2\text{H}_{\text{C}_{37}}$ values in sediments relate to $\delta^2\text{H}_{\text{C}_{37}}$ values from suspended particulate matter filters and $\delta^2\text{H}_{\text{H}_2\text{O}}$ values, but fractionation does not show a relationship with salinity (Schwab and Sachs, 2011). Nelson and Sachs (2014) also tested the use of $\delta^2\text{H}_{\text{C}_{37}}$ in North American lakes covering a salinity range of 10-133. In these North American lakes, there is a relationship between $\delta^2\text{H}_{\text{H}_2\text{O}}$ and $\delta^2\text{H}_{\text{C}_{37}}$, but no trend between fractionation and salinity (Nelson and Sachs, 2014). These environmental datasets suggest that there are other factors affecting hydrogen isotope fractionation, which complicate the use of $\delta^2\text{H}_{\text{C}_{37}}$ 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; Wolhowe et al., 2009; Sachs and Kawka, 2015), 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 $\text{C}_{37:3}$ and $\text{C}_{37:2}$ alkenones are integrated (van der Meer et al., 2013), the effect of temperature on hydrogen isotope fractionation has been shown to be negligible on the $\delta^2\text{H}_{\text{C}_{37}}$ SSS proxy, eliminating one impeding factor (Schouten et al., 2006). Growth rate and irradiance have also been proven to influence total carbon isotope fractionation of alkenones used as a $p\text{CO}_2$ proxy (Pagani, 2014 and references therein). Both of these factors are related and seem to play a significant role for

isotopic fractionation of alkenones, and the effects remain to be completely understood.

The effect of alkalinity on hydrogen isotope ratios and fractionation has not yet been tested. The effect of alkalinity on hydrogen isotope fractionation is unknown because some of the culture experiments (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014) investigating hydrogen isotopes from alkenones created media of different salinities by evaporation, which changed alkalinity together with salinity in the culture media. In the natural environment, precipitation and evaporation do not only influence 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). Alkalinity is essentially the ability of water to neutralize acid, which is linked to the amount of H^+ . H^+ is readily exchanged between extracellular and intracellular water, therefore, the amount of H^+ could potentially effect the hydrogen isotope composition of intracellular water, which is a source of hydrogen for 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 $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, but not above (van der Meer et al., 2015). However, some of the culture studies that reported a strong correlation between hydrogen isotope fractionation and salinity were performed at relatively low light intensities (Wolhowe et al., 2009; M'Boule et al., 2014; Chivall et al., 2014). Since algal blooms occur under high light conditions in surface waters across the globe (Nanninga and Tyrrell, 1996; Holligan et al., 1993), and hydrogen isotope fractionation is less variable at high light conditions (van der Meer et al., 2015), the effect of salinity on hydrogen isotope fractionation 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. Because the effects of alkalinity on hydrogen isotope fractionation were being assessed, a non-calcifying strain was chosen to avoid significant changes to the alkalinity of the media caused by the organisms, changes that have previously been shown to occur during large blooms of *E. huxleyi* (Holligan et al., 1993).

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. Salinity was measured using a VWR CO310 Portable Conductivity, Salinity and Temperature Instrument.

The alkalinity/salinity experiments consisted of batch cultures with a salinity range of 26 – 42 and constant A_T of 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, pH was kept constant (7.9 ± 0.07). 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. Alkalinity was determined by titration with 0.1 M HCl and calculated using Gran plots (Gran, 1952; Johansson et al., 1983; Hansson and Jagner, 1973). Temperature was a constant 15°C and light intensity was consistently kept at $75 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ 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 $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ using cool white fluorescent light, with a light:dark cycle of 16:8 h, and constant temperature of 18.5°C and constant alkalinity. 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. Growth rate was calculated as the slope of the linear fit of the natural logarithm of cell density ($\ln[\text{cell density}]$) in the exponential part of the growth curve. 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 (10-12 days). Cultures (600 mL for alkalinity/salinity experiment and 150 mL for high light experiment) were filtered over $0.7 \mu\text{m}$ GF/F filters to collect organic material and medium was subsequently collected following filtration to determine $\delta^2\text{H}$ of the growth water.

2.2 Water isotope analysis

Hydrogen isotopic ratios of the medium ($\delta^2\text{H}_{\text{H}_2\text{O}}$) were measured on water collected prior to the experiment and after the experiment concluded. $\delta^2\text{H}_{\text{H}_2\text{O}}$ was measured using elemental analysis-thermal conversion-isotope ratio monitoring mass spectrometry (EA/TC/irMS) (see Schouten et al., 2006). $1 \mu\text{L}$ of sample water was injected at least 10 times during a single analytical run. $\delta^2\text{H}_{\text{H}_2\text{O}}$ 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 ($\delta^2\text{H}_{\text{C}_{37}}$). Both the GC-FID and the GC/TC/irMS were equipped with an Agilent CP-Sil 5 column (25 m x 0.32 mm internal diameter; film thickness = $0.4 \mu\text{m}$). GC

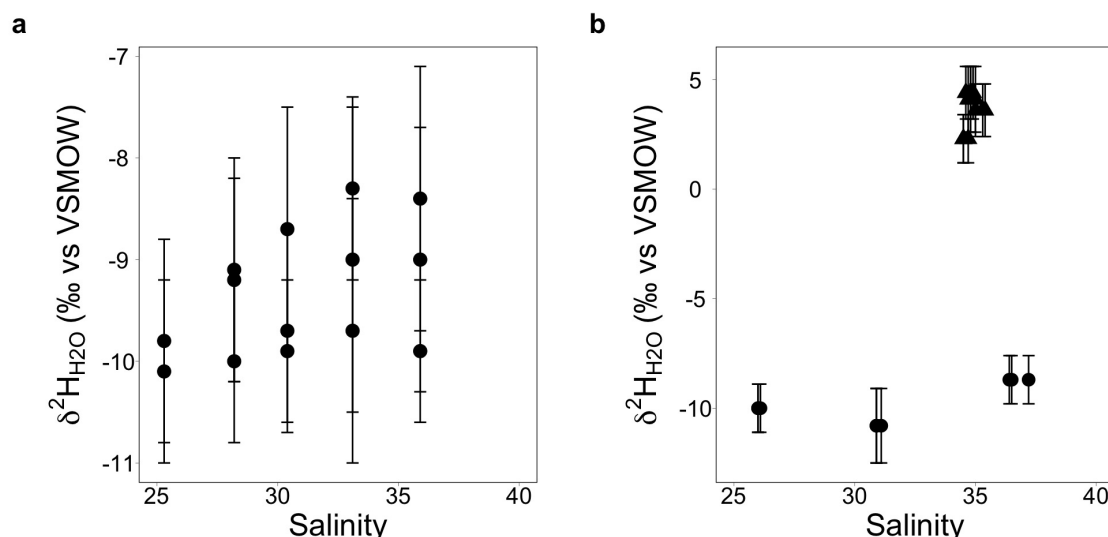


Figure 1: The figure shows the lack of correlation between $\delta^2\text{H}_{\text{H}_2\text{O}}$ and salinity of the culture media for both the (a) high light and the (b) alkalinity-salinity experiments. The triangles in the alkalinity-salinity plot are the $\delta^2\text{H}_{\text{H}_2\text{O}}$ values of the media covering a range of alkalinity, whereas the circles represent the $\delta^2\text{H}_{\text{H}_2\text{O}}$ values for the constant alkalinity media.

temperature programs were the same as discussed in M'Boule et al. (2014). 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 nA^{-1} for the alkalinity/salinity experiments and 5.4 and 5.5 ppm nA^{-1} for the high light experiments. A Mix B standard (supplied by A. Schimmelmann, Indiana University) was run to assess machine 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 $\text{C}_{37:2}$ and $\text{C}_{37:3}$ alkenones (van der Meer et al., 2013). The isotopic fractionation of alkenones compared to media is expressed as $\alpha_{\text{C}_{37}}$ and calculated using the equation:

$$\frac{\delta^2\text{H}_{\text{C}_{37}} + 1000}{\delta^2\text{H}_{\text{H}_2\text{O}} + 1000} \quad (1)$$

2.4 Statistics

Analysis of covariance (ANCOVA) was applied to test if a significant difference exists between equations of the linear regression models representative of the $\alpha_{\text{C}_{37}}$ -salinity relationship between this study and previous culture studies of *E. huxleyi*. All statistical analyses were run in R using the R stats package.

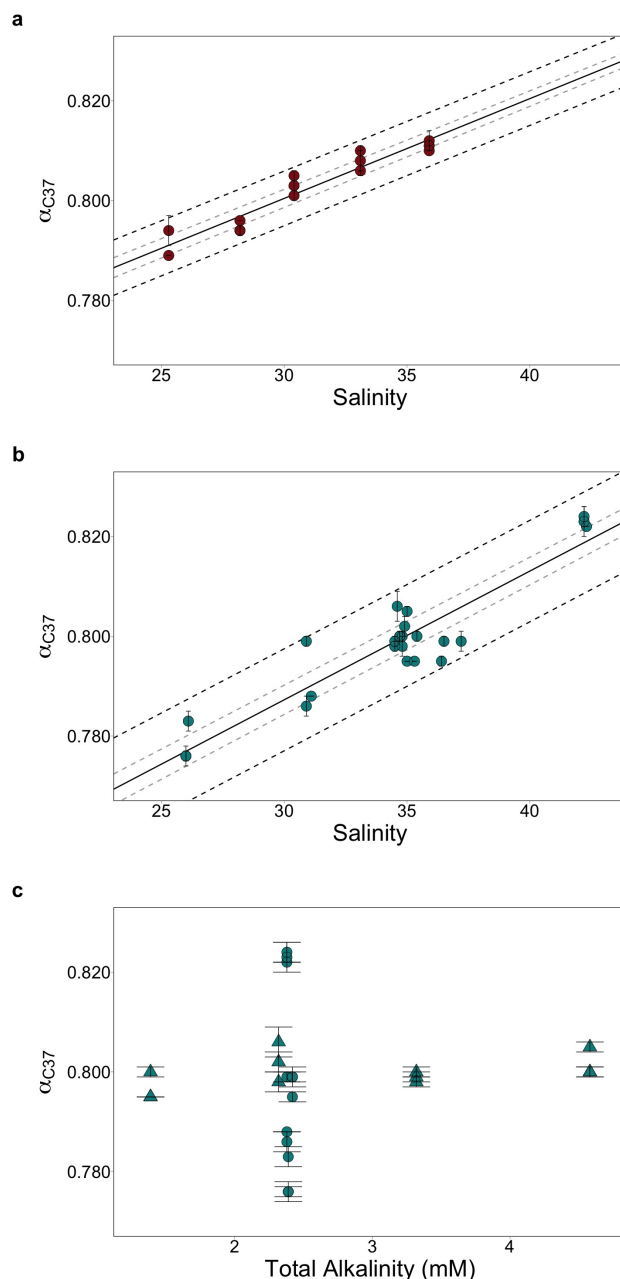


Figure 2: Hydrogen isotope fractionation factor α_{C37} plotted against salinity for the (a) high light experiment and (b) alkalinity-salinity experiment. The dotted lines represent the 1 and 2 σ confidence intervals for the linear regression model fit to the dataset. (c) shows hydrogen isotope fractionation factor α_{C37} plotted against total alkalinity (A_T) for the alkalinity-salinity experiment. The triangles represent the set of batch cultures grown at constant salinity (35) over a range of alkalinity (1.4 - 4.6 mM), and the circles represent the batch cultures grown at a constant alkalinity (2.4 mM) over a range of salinity (26 - 42).

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 relatively small over the course of the batch culture experiments (Table 1). Changes in $\delta^2\text{H}_{\text{H}_2\text{O}}$ values of the culture media sampled prior to beginning the experiments and at the end of the experiments were minimal, generally $<0.4\text{‰}$ (Table 1). Slightly larger changes in $\delta^2\text{H}_{\text{H}_2\text{O}}$ values occurred for media of the alkalinity/salinity experiments, but still $<1.3\text{‰}$ (Table 1) and were therefore ignored. Since the salinity of the media was not altered by evaporation, but by addition of NaCl in contrast to previous culture studies (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014), $\delta^2\text{H}_{\text{H}_2\text{O}}$ values were not correlated with salinity due to different procedures for creating of the media (Figure 1). Furthermore, the media was created separately for each experiment, causing differences between the original $\delta^2\text{H}_{\text{H}_2\text{O}}$ values (Figure 1).

$\delta^2\text{H}_{\text{C}_{37}}$ values ranged from -230.9‰ to -197‰ across all experiments, with more depleted values at lower salinities (Table 1). Growth rates (μ) ranged from $0.65 - 0.93\text{ d}^{-1}$ for the alkalinity/salinity experiment and $0.54 - 0.67\text{ d}^{-1}$ for the high light experiment, with lower growth rates at higher salinities (Table 1). Growth rate is weakly correlated with $\alpha_{\text{C}_{37}}$ for both experiments (Figure 3). Higher $\alpha_{\text{C}_{37}}$ values occur at higher salinities for both the alkalinity/salinity and high light experiments. A strong linear relationship between $\alpha_{\text{C}_{37}}$ values and salinity was observed in both experiments (Figure 2a,b): for the high light experiment, $\alpha_{\text{C}_{37}} = 0.002\text{S} + 0.7408$ ($R^2 = 0.92$, $n=14$; $p<0.001$), and for the alkalinity/salinity experiment, $\alpha_{\text{C}_{37}} = 0.0026\text{S} + 0.7098$ ($R^2 = 0.85$, $n=24$; $p<0.001$). For the alkalinity/salinity experiment, $\alpha_{\text{C}_{37}}$ remains relatively constant (0.799 ± 0.003) over the range of alkalinity, but covers a range of $0.776 - 0.824$ at constant alkalinity (Table 1, Figure 2c).

4 Discussion

4.1 Impact of alkalinity and light

In the alkalinity/salinity experiment, $\alpha_{\text{C}_{37}}$ values 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 1.4 to 4.6 (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 in fractionation 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 ($600\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) show a strong correlation between $\alpha_{\text{C}_{37}}$ values 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. Ocean surface light levels span a range from zero to over 800 PAR (over $1600\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) (Frouin and Murakami, 2007), and haptophytes are not believed to be photoinhibited, primarily blooming at light intensities above $500\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ (Nanninga and Tyrrell, 1996). Furthermore, *E. huxleyi* has been shown to adapt to different light conditions by expressing different genes under high and low light conditions (Rokitta et al., 2012), showing that growth is possible under different light regimes.

Table 1: Growth parameters and hydrogen isotope ratios of alkenones from alkalinity-salinity and high light batch cultures of *Emiliana huxleyi* CCMP1516.

Salinity	Temperature (°C)	Irradiance ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$)	Growth rate (d^{-1})	$\delta\text{D}_{\text{H}_2\text{O}}$ (‰ vs. VSMOW) initial	St. dev.	$\delta\text{D}_{\text{H}_2\text{O}}$ (‰ vs. VSMOW) end	St. dev.	$\delta\text{D}_{\text{C}_{37}}$ (‰ vs. VSMOW)	St. dev.	$\alpha_{\text{C}_{37}}$	Error	A _T mM initial	A _T mM end	pH initial	pH end
<i>Alkalinity and Salinity Experiment Strain CCMP1516</i>															
26.0	15	75	0.82	-10.0	1.1	-8.8	1.8	-230.9	1.8	0.776	0.002	2.39	2.49	8	8.8
26.0	15	75	0.87	-10.0	1.1	-9.6	1.6	-231.1	1.4	0.776	0.001	2.39	2.46	8	8.7
26.1	15	75	0.85	-10.0	1.1	-8.5	2.0	-224.3	2.1	0.783	0.002	2.39	2.46	8	8.7
30.9	15	75	0.93	-10.8	1.7	-10.1	2.6	-209.1	0.7	0.799	0.001	2.38	2.42	7.9	7.8
31.1	15	75	0.93	-10.8	1.7	-10.7	0.9	-220.3	0.2	0.788	0.000	2.38	2.47	7.9	8.7
30.9	15	75	0.93	-10.8	1.7	-9.6	1.1	-221.6	1.8	0.786	0.002	2.38	2.46	7.9	8.6
36.5	15	75	0.86	-8.7	1.1	-8.9	1.8	-207.8	0.5	0.799	0.001	2.42	2.48	7.8	8.7
37.2	15	75	0.83	-8.7	1.1	-11.1	1.5	-209.3	1.6	0.799	0.002	2.42	2.49	7.8	8.8
36.4	15	75	0.86	-8.7	1.1	-8.9	1.4	-212.5	1.1	0.795	0.001	2.42	2.45	7.8	8.6
42.2	15	75	0.69	-7.4	1.6	-7.4	1.6	-183.1	2.9	0.823	0.003	2.38	2.46	7.9	8.6
42.2	15	75	0.65	-7.4	1.6	-8.6	0.9	-182.8	2.3	0.824	0.002	2.38	2.45	7.9	8.6
42.3	15	75	0.68	-7.4	1.6	-7.3	1.0	-184.4	0.2	0.822	0.000	2.38	2.45	7.9	8.7
35.4	15	75	0.7	3.6	1.2	3.5	1.6	-197.0	1.4	0.800	0.001	1.39	1.5	7.8	8
35.3	15	75	0.7	3.6	1.2	4.0	1.7	-201.8	0.2	0.795	0.000	1.39	1.45	7.8	8.5
35.0	15	75	0.69	3.6	1.2	5.6	2.1	-201.5	0.3	0.795	0.000	1.39	1.48	7.8	8.5
34.8	15	75	0.86	4.4	1.2	5.0	1.1	-198.7	2.2	0.798	0.002	2.32	2.42	7.9	8.6
34.6	15	75	0.87	4.4	1.2	3.8	1.0	-190.2	3.0	0.806	0.003	2.32	2.39	7.9	8.5
34.9	15	75	0.9	4.4	1.2	4.8	1.7	-194.5	2.1	0.802	0.002	2.32	2.39	7.9	8.6
34.7	15	75	0.82	2.3	1.1	3.8	1.2	-197.1	1.0	0.800	0.001	3.32	3.45	8	8.6
34.5	15	75	0.8	2.3	1.1	3.3	1.0	-199.8	0.7	0.798	0.001	3.32	3.52	8	8.8
34.5	15	75	0.83	2.3	1.1	5.1	1.1	-198.1	1.4	0.799	0.001	3.32	3.69	8	8.7
35.0	15	75	0.93	4.1	1.5	4.2	1.3	-191.6	0.7	0.805	0.001	4.58	4.56	7.9	8.5
34.8	15	75	0.89	4.1	1.5	1.7	1.5	-197.5	0.6	0.800	0.001	4.58	4.63	7.9	8.6
34.7	15	75	0.87	4.1	1.5	4.0	1.4	-197.0	0.8	0.800	0.001	4.58	4.63	7.9	8.5
<i>High Light Experiment Strain CCMP1516</i>															
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				

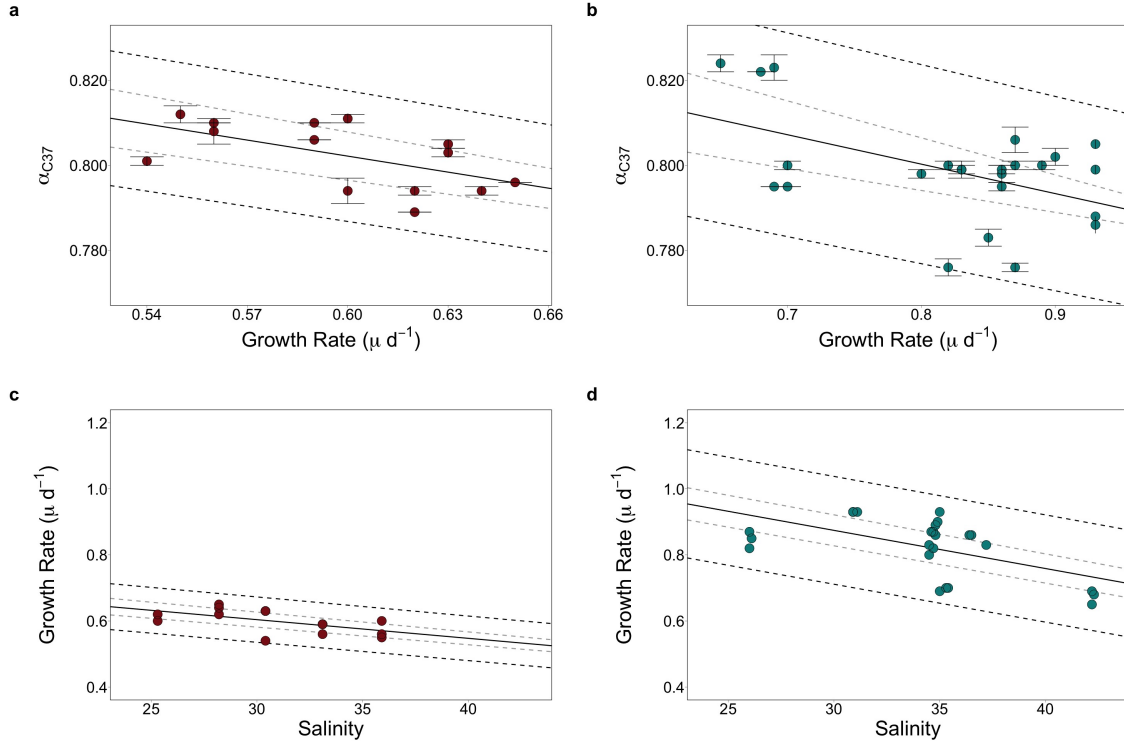


Figure 3: Relationship of growth rate (μd^{-1}) with hydrogen isotope fractionation factor α_{C37} and salinity. The dotted lines represent the 1 and 2 σ confidence intervals for the linear regression model applied to the data. (a) Growth rate and α_{C37} relationship for the high light experiment shows a weak negative correlation ($R^2 = 0.35$, $n = 14$, $p < 0.05$). (b) Growth rate and α_{C37} relationship for the alkalinity-salinity experiment also shows a weak negative correlation ($R^2 = 0.25$, $n = 24$, $p < 0.05$). (c) Salinity and growth rate for the high light experiment ($R^2 = 0.29$, $n = 14$, $p < 0.05$). (d) Salinity and growth rate for the alkalinity-salinity experiment ($R^2 = 0.29$, $n = 24$, $p < 0.05$).

The slope of the α -salinity correlation, or fractionation response per unit salinity, is statistically similar ($p < 0.05$) for both the alkalinity/salinity and the high light experiments, based on analysis of covariance (ANCOVA) between the linear regression models fit to each dataset. There is a weak negative correlation of growth rate 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 3), which aligns with findings of Sachs and Kawka (2015) who report a negative correlation between growth rate and fractionation, albeit a stronger relationship. Growth rate is also negatively correlated with salinity in both experiments (Table 1; Figure 3), which is consistent with earlier work of Schouten et al. (2006). 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; Sachs et al., 2016; Table 2) and our experiments. Sachs et al. (2016) report δ^2H

values for individual alkenones, thus we used a weighted mean average of the $\delta^2\text{H}_{C37:3}$ and $\delta^2\text{H}_{C37:2}$ values to compare with other results reporting integrated $\delta^2\text{H}_{C37}$ values. The slopes of the α_{C37} -salinity relationships are not statistically different from each other ($p > 0.05$), with the exception of three comparisons: Sachs et al. (2016) was statistically different ($p \leq 0.05$) from Schouten et al. (2006), M'Boule et al. (2014) and the Alkalinity/Salinity experiment (Table 2). A possible explanation for the statistical difference between the α_{C37} -salinity relationship of Sachs et al. (2016) and the other three experiments could be due to the fact that the Sachs et al. (2016) experiment was conducted using chemostats with a controlled growth rate, whereas, the other experiments were batch culture experiments where growth rate varied. Growth rate has been shown to effect hydrogen isotope fractionation of alkenones (Schouten et al., 2006; Wolhowe et al., 2009; Sachs and Kawka et al., 2015), and therefore could account for the difference between reported fractionation responses to salinity. Although the slopes are statistically similar ($p > 0.05$), different strains used in the experiments, and the individual experiments themselves (i.e., conducted by different labs using different techniques) do likely play a large role in the observed differences between slopes. Furthermore, the intercepts of the regression models applied to the α_{C37} -salinity relationships for the *E. huxleyi* culture data are all significantly different ($p \leq 0.05$), i.e. the absolute fractionation differs between the different studies, except for the relationship reported by M'Boule et al. (2014) and our high light experiment. 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 experiments, as each strain would respond in a similar fashion to salinity changes, but fractionate to a different extent, similar to differences seen between species (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014). This could be due to differences in fractionation between intra- and extracellular sources of hydrogen or differences in lipid synthesis rates. Another explanation for part of the discrepancies in intercepts could be analytical differences between laboratories, i.e. small offsets in measured absolute values of C_{37} alkenones.

With the exception of the high light experiment, all other culture experiments with *E. huxleyi* being discussed here were grown at light intensities between 50-300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; alkalinity/salinity experiment). The fact that the strong α_{C37} -salinity response is also identified in *E. huxleyi* grown 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. Van der Meer et al. (2015) suggested that at light intensities above 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, α_{C37} responds differently to changes in light intensity than below, with a larger reported range in fractionation values at light intensities below 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Wolhowe et al. (2015) also show this trend in α_{C37} measured on alkenones in suspended particulate matter from the Gulf of California and Eastern Tropical North Pacific. Krumhardt et al. (2016) indicate that although haptophyte indicative pigments were high below surface water layers in the subtropical North Atlantic, they were also abundant in the upper 30 m of the water column, especially during spring. Based on these findings and the $U_{37}^{K'}$ core-top calibration, we can be confident that alkenones preserved in the sediments are largely reflecting surface water temperatures during the time of the year that haptophytes are known to bloom (Müller et al., 1998). Furthermore, haptophytes are thought primarily to bloom at light intensities above 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in the surface ocean (Nanninga and Tyrrell, 1996), leading to the conclusion that the light and depth effects discussed previously (van der Meer et al., 2015; Wolhowe et al., 2015) might not have such a large effect on the α_{C37} -salinity response as previously believed.

Table 2: Linear regression equations for hydrogen isotope fractionation-salinity (α_{C37} -salinity) relationship for a compilation of culture experiments growing different strains of *Emiliana huxleyi*

Reference	Strain	α_{C37} -Salinity relationship	R ²	Number of points
Schouten et al. (2006)	<i>E. huxleyi</i> PML B92/11	$\alpha_{C37} = 0.0033 * S + 0.6928$	0.74	11
M'Boule et al. (2014)	<i>E. huxleyi</i> CCMP 1516	$\alpha_{C37} = 0.0021 * S + 0.7401$	0.80	20
Sachs et al. (2016)	<i>E. huxleyi</i> CCMP 347	$\alpha_{C37} = 0.0015 * S + 0.7770$	0.88	9
Alkalinity and Salinity	<i>E. huxleyi</i> CCMP 1516	$\alpha_{C37} = 0.0026 * S + 0.7098$	0.86	24
High Light	<i>E. huxleyi</i> CCMP 1516	$\alpha_{C37} = 0.0020 * S + 0.7408$	0.92	14

4.3 Potential mechanisms for salinity and light responses

As mentioned above, our results show that the response in hydrogen isotopic fractionation of alkenones to salinity is statistically 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 and references therein). The effect of salinity on hydrogen isotope fractionation seems to be a general feature recorded in alkenones, fatty acids, sterols, phytene and diploptene produced by algal photoautotrophs (Heinzelmann et al., 2015; Schouten et al., 2006; Sachse and Sachs, 2008; Sachs and Schwab, 2011; Nelson and Sachs, 2014). Nicotinamide adenine dinucleotide phosphate (NADPH) is associated with large isotope fractionation values, larger than the fractionation between extracellular and intracellular water, but both are used as sources of H for synthesis of organic compounds (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). NADPH derived via ferredoxin-NADP⁺ reductase (FNR) in photosystem 1 (photosynthetically derived) tends to be depleted by 600 ‰ in ²H when compared to intracellular water (Luo et al., 1991), whereas NADPH produced via the OPP pathway is also depleted compared to intracellular water, but much less than photosynthetically derived 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 causes depletion in ²H which is further enhanced during continued biosynthesis, meaning organic compounds containing H largely derived from metabolically reduced NADPH are characterized by depletion in ²H. 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 (FNR derived, for instance) could, therefore, cause differences in the amount of ²H 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). Danevčič and Stopar (2011) also found that intracellular production of L-proline, an osmoregulating amino acid, increased. The advantage of up regulating the OPP derived NADPH would be tied to this increase in L-proline, which helps continue growth and biosynthesis at higher salinities in *Vibrio sp.* 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 if the OPP pathway exists in the same location as the site of alkenone synthesis. However, in photoautotrophic organisms, the reduction of NADP^+ to NADPH is also directly linked to photosystem activity (FNR) and, therefore, light intensity (Allen, 2002), and as previously mentioned, this initial reduction is characterized by a very large fractionation step (Schmidt et al., 2003; Maloney et al., 2016; Luo et al., 1991). 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, but the effect of changing light intensity on hydrogen isotope fractionation is much lower at light intensities $>200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Furthermore, Sachs et al. (2017) showed a light effect on hydrogen isotope fractionation of $\text{C}_{14:0}$ fatty acid, but no effect of light was observed on hydrogen fractionation of $\text{C}_{16:0}$ and $\text{C}_{16:1}$ fatty acids from the diatom *Thalassiosira pseudonana* grown over a low light range from 6 – 47 $\mu\text{mol m}^{-2} \text{s}^{-1}$. 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 $\text{C}_{14:0}$ (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; our alkalinity/salinity experiment), 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 the OPP pathway at higher salinities, cells could also produce more ^2H -depleted compounds, osmolytes for instance (Dickson et al., 1982; Sachs et al., 2016 and references therein; Sachse et al., 2008; Danevčič and Stopar, 2011), which would leave the intracellular NADPH pool more enriched, and would result in ^2H 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 $\alpha_{\text{C}37}$ -salinity are expected for cells grown at high light and low light conditions, which is in contrast to what our results show.

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. Due to balancing between ATP and NADPH production and consumption within the cell (Walker et al., 2014), NADPH formation dominates at high light levels, whereas ATP synthesis dominates at lower light levels (Beardall et al., 2003), leading to the idea of a larger pool of photosynthetically derived NADPH inside the cell under high light conditions, would, in turn, cause differences in hydrogen isotope fractionation during the synthesis of alkenones, but this is not what the data shows. Transhydrogenase enzyme activity can remove NADPH when in excess by reducing NAD^+ to NADH using NADPH (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 ^2H enriched pool of NADPH behind. At high light conditions, an excess of NADPH is expected, and therefore, increased transhydrogenase activity, something also seen with increasing salinity (Danevčič and Stopar, 2011). 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, similar to what was reported for different fatty acids by Sachs et al. (2017). 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 or is counteracted by enzymatic activity (Sachs et al., 2017). A better understanding of the hydrogen isotopic composition of different relevant hydrogen pools and how the alkenone synthesis process works is required for more accurate determinations of the mechanism responsible for the strong salinity response to hydrogen isotope fractionation of alkenones.

5 Conclusions

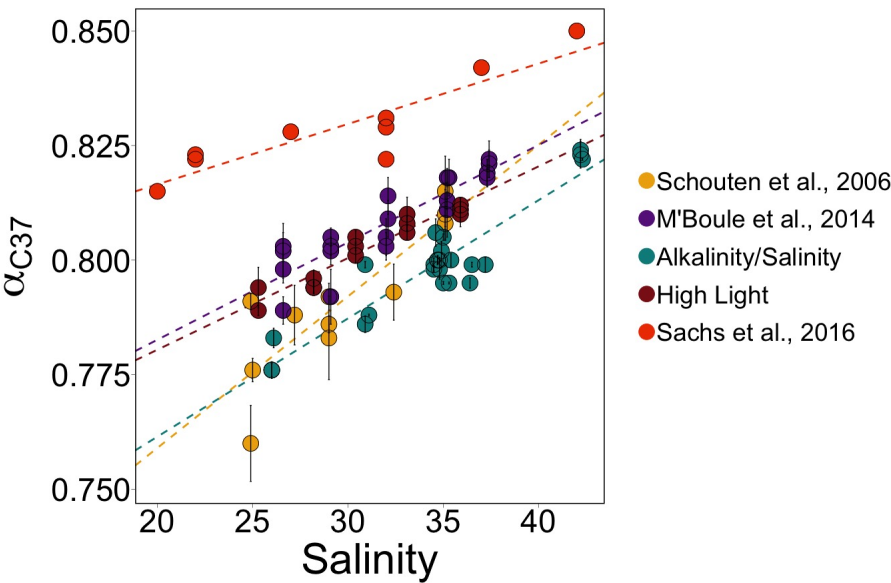
Our results show that salinity has a strong effect on hydrogen isotope fractionation of alkenones in cultivated *E. huxleyi* at high light intensities. In contrast, alkalinity, although co-varying with salinity in environmental waters, does not affect hydrogen isotope fractionation between alkenones 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, and further supports the use of α_{C37} for reconstructing paleo sea surface salinity.

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Supplementary material



Supplementary Figure 1: Hydrogen isotope fractionation factor α_{C37} -salinity relationship for cultures growing different strains of *Emiliana huxleyi* used in the statistical comparison in section 4.2. The dotted lines represent the linear regression model applied to the α_{C37} -salinity relationship for each individual experiment.

Supplementary Table 1: Analysis of covariance (ANCOVA) data for comparison of the α_{C37} -salinity relationship for different culture studies of *Emiliana huxleyi*.

Analysis of Covariance (ANCOVA)	
Experiment	Significance
Schouten vs M'Boule	0.05
Schouten vs Alkalinity/Salinity	0.19
Schouten vs High Light	0.05
Schouten vs Sachs	0.01
M'Boule vs Alkalinity/Salinity	0.19
M'Boule vs High Light	0.73
M'Boule vs Sachs	0.05
Alkalinity/Salinity vs High Light	0.12
Alkalinity/Salinity vs Sachs	0.00
High Light vs Sachs	0.07
ALL	0.00



Artwork of a calcifying *Emiliana huxleyi* cell created by Dr. Lisa Roberts. This artwork arose from her PhD entitled: "Antarctic Animation: How animation can be used to combine scientific data and subjective responses". 2010, UNSW, Australia.

Chapter 3

Hydrogen isotope fractionation response to salinity and alkalinity in a calcifying strain of *Emiliana huxleyi*

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Abstract

Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{C37}$) correlate with water isotope ratios and salinity, albeit with varying degrees of biological fractionation between alkenones and water. These differences in fractionation are the result of environmental and species related effects, which, in some cases, have consequences for the magnitude of the $\delta^2\text{H}_{C37}$ response per unit increase in salinity. Earlier culture experiments have focused on constraining isotope fractionation factor α in non-calcifying strains of *Emiliana huxleyi*. Here we studied isotopic fractionation in a calcifying strain of *E. huxleyi* and show that although absolute fractionation is different, the response to changes in salinity and alkalinity is similar to those of non-calcifying species. This suggests that calcification does not alter the $\delta^2\text{H}_{C37}$ response to salinity significantly.

Keywords:

Hydrogen isotopes, Alkenones, Calcification, *Emiliana huxleyi*

1 Introduction

Haptophyte algae are one of the most abundant phytoplankton groups in the modern ocean (Monteiro et al., 2016). Certain species of haptophytes create tiny plates of calcium carbonate called coccoliths. These calcifying haptophyte algae are extremely important for the global carbon cycle, and are believed to have contributed most of the precipitated marine calcium carbonate across the Cenozoic (Monteiro et al., 2016). Particular groups of haptophytes also synthesize long-chain alkenones (Volkman et al., 1980; De Leeuw et al., 1980), which are methyl and ethyl ketones typically with a chain length between 35 and 40 carbon atoms (Longo et al., 2013). Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{C37}$) correlate significantly with salinity in cultures, and this relationship appears to be largely related to a salinity response of biological hydrogen isotope fractionation (α) between alkenone and water $\delta^2\text{H}$ ratios (Schouten et al., 2006, M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). Haptophytes, including *Emiliana huxleyi*, generally produce coccoliths in the natural environment, but can also be found in non-calcifying or naked forms believed to be caused by mutations (Paasche, 2002). Additionally, calcifying haptophytes have diploid (calcifying) and haploid (non-calcifying) life stages that are not only morphologically distinct, but also have different responses to environmental conditions (Fiorini et al., 2010). However, so far, the majority of previous cultures have focused on the $\delta^2\text{H}_{C37}$ ratios and α_{C37} values of non-calcifying haptophyte strains. Here we present data for a calcifying strain of *E. huxleyi* to identify potential impacts of coccolithophorid calcification on α_{C37} in relation to salinity and alkalinity.

2 Materials and Methods

2.1 Media Conditions

Batch cultures of a calcifying strain of *Emiliana huxleyi*, RCC2050, isolated from the Mediterranean Sea, were grown in media created from filtered North Sea water with added

vitamins and trace metals following the K-medium recipe from Roscoff Culture Collection. From a stock of filtered North Sea water, salinities above and below 34 were produced by adding NaCl and ultra-pure water, respectively. KHCO_3 and K_2CO_3 were added to change the alkalinity of the media. In the final media, nitrogen and phosphate were at K/10, but vitamin and trace metal amounts were at K/2 concentrations (following Keller et al., 1987). Alkalinity was measured spectrophotometrically using an automated spectrophotometric alkalinity system (ASAS) as described in Liu et al. (2015). Temperature and salinity were measured using a VWR CO310 portable conductivity, salinity and temperature instrument, and pH was measured using a Metrohm pH meter.

2.2 Experiments

Before each experiment, cells were acclimated for 4 generations. Experiments were conducted in triplicate in 500 mL of media at six different conditions (Table 1). Light intensity was kept between $170 - 200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 16:8 light:dark cycle at a temperature of 15°C . Cells were counted using an Accuri C6 flow cytometer. Cell densities were kept at or below 100,000 cells / mL in both acclimation phase and final experiment to avoid major changes to the alkalinity of the media. All cultures were checked for continued calcification using phase contrast light microscopy. Growth rates were ascertained by determining the slope of the linear fit of the natural logarithm of cell density in the exponential part of the growth curve. Cells were harvested by filtration over pre-combusted GF75 $0.3 \mu\text{m}$ GF/F filters. Filters were freeze-dried and biomass was extracted ultrasonically using dichloromethane : methanol 2:1 (v : v). Extracts were further separated into three fractions following methods described in Weiss et al. (2017).

2.3 Isotope Measurements

Hydrogen isotope ratios of the culture media were measured on TC/EA/irMS following Weiss et al. (2017). Hydrogen isotope ratios of long-chain alkenones were measured on GC/TC/irMS using an RTX-200 60 m GC column with the following GC temperature program: 70°C to 250°C at 18°C / min , 250 to 320°C at 1.5°C / min , and kept at 320°C for 25 min with a flow rate of 1.5 mL / min . H_3^+ correction was made at the start of each day ($2.929 - 3.165 \text{ ppm nA}^{-1}$) and an *n*-alkane mix (Mix B) supplied by A. Schimmelmann (Indiana University) was measured prior to sample analysis. Samples were run only when average and standard deviation for the Mix B standard were within 5 ‰. ^2H monitoring gas of predetermined isotopic composition was introduced into the ion source at the start and finish of each analytical run. Squalane ($-164 \pm 3 \text{ ‰}$) was co-injected with each sample to ensure machine stability and fits with the predetermined value of $-170 \pm 4 \text{ ‰}$. Error bars for $\delta^2\text{H}_{\text{C}37}$ ratios are the result of duplicate measurements, and thus represent reproducibility. We report both the individual $\delta^2\text{H}_{\text{C}37:3}$ and $\delta^2\text{H}_{\text{C}37:2}$ as well as integrated $\delta^2\text{H}_{\text{C}37}$ ratios (Table 1), but use the integrated values for comparison with previously published results.

3 Results and Discussion

Hydrogen isotope ratios of alkenones of the calcifying *E. huxleyi* strain RCC2050 grown at salinities of 32 to 40 and alkalinities of 2043 to $3579 \mu\text{mol kg}^{-1}$ (Table 1) span from -248 to -216 ‰ . There is a strong positive linear correlation between $\alpha_{\text{C}37}$ – salinity ($r = 0.77$, $p <$

0.005; Fig. 1a), but there is no significant relationship between α_{C37} – alkalinity ($r = 0.18$, $p > 0.05$). The latter is in agreement with previous results (Weiss et al., 2017). Furthermore, no relationship between growth rate (varying between 0.45 and 0.69 divisions per d^{-1}) and alkalinity is observed. The linear correlation between α_{C37} – salinity is in line with previous results for non-calcifying strains of *E. huxleyi* (Fig. 1a). The magnitude of this response is statistically similar across all experiments: ranging between 0.001 – 0.003 change in α_{C37} per salinity change (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017; this study).

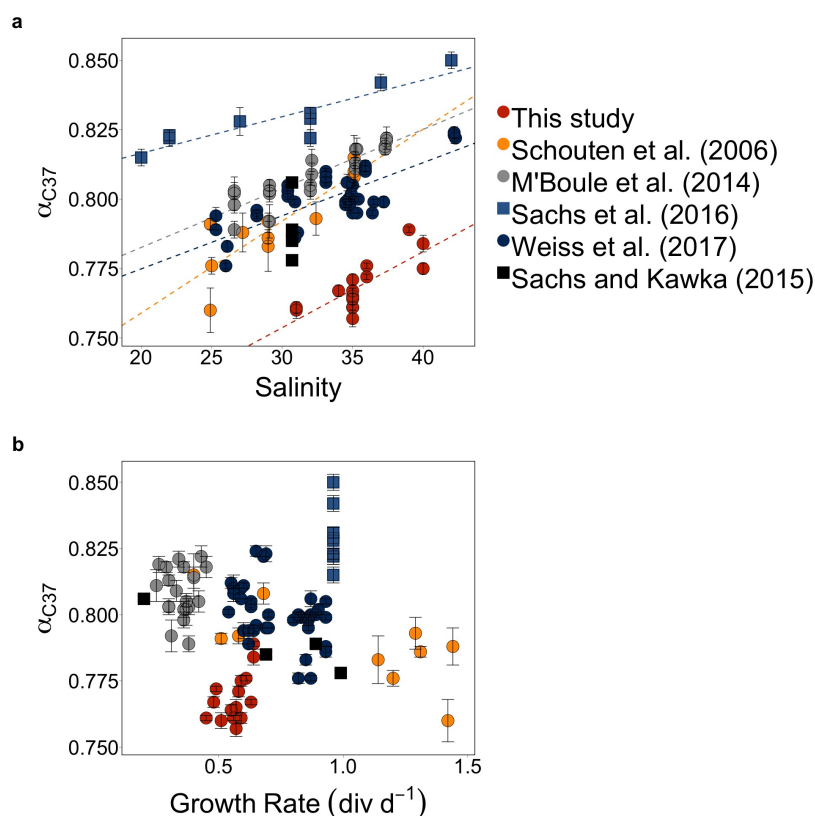


Figure 1: (a) Hydrogen isotope fractionation between alkenones and water (α_{C37}) versus salinity. (b) Hydrogen isotope fractionation factor α_{C37} versus growth rate. (c) Hydrogen isotope ratios of alkenones (δ^2H_{C37}) versus salinity. Results are from batch (circles) and continuous (squares) culture experiments of *Emiliania huxleyi*.

However, some differences are observed. First, δ^2H_{C37} ratios from RCC2050 are more depleted and α_{C37} values are lower, implying that calcification might result in more fractionation during alkenone synthesis. Calcification occurs in a closed vesicle (coccolith vesicle) where conditions are tightly regulated (Sviben et al., 2016). H^+ is generated during calcification, and is transported through the cytosol (Taylor et al., 2011; Monteiro et al., 2016). This H^+ might be more abundant in calcifying strains, leading to more fractionation and depleted alkenones. Additionally, if this calcification derived H^+ pool is isotopically depleted as a result of increased concentration in calcifying haptophytes with respect to non-calcifying cells, it might contribute to a more isotopically depleted intracellular pool of H^+ available for biosynthesis of organic compounds (alkenones) in calcifying haptophytes. Alkenones are thought to be synthesized by chain elongation from fatty acids in the cytosol (Rontani et al., 2006), and are thus heavily influenced by cytosolic pools of NADPH. It is possible that

these larger fluxes of H^+ into the cytoplasm in calcifying coccolithophores are responsible for the enhanced hydrogen isotope fractionation (lower α_{C37}) observed here. Second, RCC2050 shows a significant positive correlation between α_{C37} and growth rate ($r = 0.55$, $p < 0.05$; Fig. 1b). This relationship is in contrast with results from previous culture experiments which report a negative correlation between α_{C37} and growth rate (Schouten et al., 2006; M'Boule et al., 2014; Sachs and Kawka, 2015; Weiss et al., 2017). One possibility could be that in calcifying haptophytes, enhanced growth is associated with increased calcification, a phenomenon noted for blooms of coccolithophores during which cells are known to increase calcification and create liths in greater abundance than necessary, resulting in multiple layers of liths in some cases (Paasche, 2002; Monteiro et al., 2016). Increased calcification would generate more H^+ that is then pumped into the cytoplasm. This potentially enhanced H^+ generation at higher growth rates might result in reduced fractionation relative to fractionation at lower growth rates, leading to relatively more enriched cytosolic pool of H^+ available for synthesis of organic compounds, under faster growth, causing this positive correlation. Alternatively, the range of growth rates in this study is rather narrow, and perhaps with a larger range in growth rates or in chemostat cultures, the correlation might be different. Nevertheless, the slope of the linear regression of δ^2H_{C37} – salinity response for RCC2050 (2.7 ± 0.6) is not statistically different from the responses reported for other haptophyte species (Fig. 1c). Therefore, while fractionation is increased in this calcifying strain, our results suggest that calcification does not appear to significantly affect the δ^2H_{C37} – salinity response. Considering the fact that a majority of alkenone-producing haptophytes in the natural environment are calcifying, this finding is important for the use of δ^2H_{C37} ratios to reconstruct salinity, i.e., sedimentary alkenones produced by both calcifying and non-calcifying haptophytes should not result in significantly different salinity estimates.

4 Conclusions

New results from a calcifying strain of *E. huxleyi* show that alkalinity does not have an effect on hydrogen isotope ratios and fractionation, similar to findings from non-calcifying strains. Calcification appears to have an effect on hydrogen isotope fractionation of long-chain alkenones, but the α_{C37} -salinity response and δ^2H_{C37} response per unit salinity change is similar to that of non-calcifying strains. These findings suggest that application of δ^2H_{C37} ratios to reconstruct salinity should not be significantly impacted by a mixing of calcifying and non-calcifying *E. huxleyi* in the geologic record.

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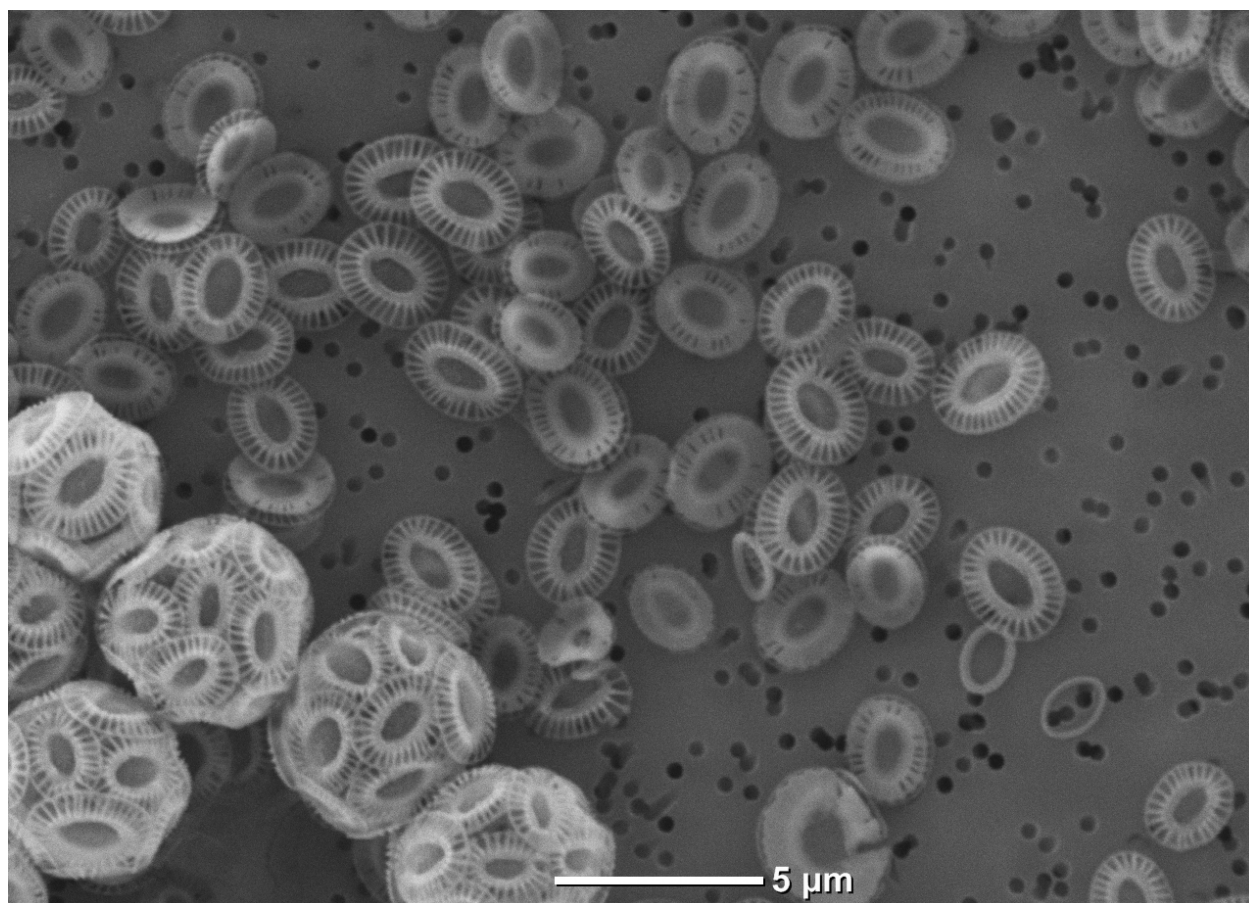
Table 1: Growth water parameters, hydrogen isotope ratios and fractionation values for batch cultures of *Emiliania huxleyi* strain RCC2050. For two samples, alkenone concentrations were not sufficient for hydrogen isotope analyses, thus were not measured, indicated by "n.d.".

Condition	Replicate	pH pre	pH post	A _T pre ($\mu\text{mol kg}^{-1}$)	A _T post ($\mu\text{mol kg}^{-1}$)	Salinity pre	Salinity post	Growth Rate (div d ⁻¹)	C ₃₇ (pg cell ⁻¹)	$\delta^2\text{H}_{\text{H}_2\text{O}}$ (‰) vs VSMOW	$\delta^2\text{H}_{\text{C}_{37:2}}$ (‰) vs VSMOW	$\delta^2\text{H}_{\text{C}_{37:3}}$ (‰) vs VSMOW	$\delta^2\text{H}_{\text{C}_{37}}$ (‰) vs VSMOW	$\alpha_{\text{C}_{37}}$
1	A	8.6	8.4	3579	3325	35	35	0.63	0.65	-8 ± 1	-231 ± 0	-237 ± 1	-235 ± 1	0.771 ± 0.001
	B	8.6	8.1	3579	3223	35	35	0.57	0.09	-8 ± 1	-232 ± 6	-240 ± 2	-238 ± 2	0.769 ± 0.003
2	A	8.5	8.3	2554	2315	40	40	0.64	0.23	-8 ± 1	-223 ± 3	-224 ± 2	-224 ± 2	0.783 ± 0.003
	B	8.5	8.3	2554	2293	40	39	0.64	0.22	-8 ± 1	-216 ± 1	-217 ± 0	-216 ± 1	0.790 ± 0.001
	C	8.5	8.3	2554	2206	40	40	0.59	0.10	-7 ± 1	-227 ± 2	-230 ± 1	-229 ± 2	0.777 ± 0.002
3	A	8.3	8.2	2043	1859	35	35	0.45	0.30	-12 ± 1	-245 ± 1	-249 ± 0	-248 ± 1	0.761 ± 0.001
	B	8.3	8.2	2043	1854	35	35	0.57	0.12	-9 ± 2	-240 ± 5	-250 ± 1	-248 ± 2	0.759 ± 0.003
	C	8.3	8.2	2043	1848	35	35	0.56	0.22	-9 ± 1	-239 ± 1	-249 ± 1	-245 ± 1	0.762 ± 0.001
4	A	8.4	8.2	2278	2109	36	35	0.58	0.06	-8 ± 1	-232 ± 1	-238 ± 2	-235 ± 2	0.771 ± 0.002
	B	8.4	8.2	2278	2076	36	35	0.55	0.10	-7 ± 1	-236 ± 2	-245 ± 2	-241 ± 1	0.764 ± 0.002
	C	8.4	7.8	2278	2045	36	34	0.48	0.48	-7 ± 1	-234 ± 2	-243 ± 1	-240 ± 1	0.766 ± 0.002
5	A	8.5	8.3	2712	2529	32	31	0.53	0.01	-6 ± 1	n.d.	n.d.	n.d.	n.d.
	B	8.5	8.3	2712	2469	32	31	0.51	0.08	-7 ± 1	-236 ± 2	-249 ± 2	-246 ± 2	0.760 ± 0.003
	C	8.5	8.3	2712	2441	32	31	0.59	0.11	-6 ± 1	-239 ± 0	-246 ± 1	-243 ± 1	0.761 ± 0.002
6	A	8.2	8.4	3013	2919	36	36	0.61	0.06	-9 ± 1	-227 ± 1	-232 ± 1	-230 ± 0	0.776 ± 0.001
	B	8.2	8.3	3013	2939	36	36	0.49	0.09	-8 ± 1	-231 ± 1	-236 ± 0	-234 ± 0	0.772 ± 0.001
	C	8.2	8.3	3013	2914	36	36	0.69	0.01	-9 ± 1	n.d.	n.d.	n.d.	n.d.

Table 2: List of culture conditions for studies investigating growth and salinity effects on hydrogen isotope fractionation of long-chain alkenones in *Emiliana huxleyi*.

Experiment	Strain	Light $\mu\text{mol photons m}^{-2}\text{ s}^{-1}$	Temperature $^{\circ}\text{C}$	Salinity	Growth rate (div d ⁻¹)	$\delta^2\text{H}$ - salinity relationship		
						Growth phase	Slope	Intercept
Schouten et al., 2006	PML B92/11	300	15	24.9 to 35.1	0.4 to 1.4	Exponential	0.003 ± 0.0007	0.693 ± 0.019
M'Boule et al., 2014	CCMP 1516	50	15	26.6 to 37.4	0.25 to 0.45	Exponential	0.002 ± 0.0003	0.740 ± 0.008
Sachs et al., 2016	CCMP 374	225	19	20 to 42	0.96	Continuous	0.001 ± 0.0002	0.790 ± 0.007
Weiss et al., 2017	CCMP 1516	70 & 600	15 to 18.5	25.3 to 42.3	0.65 to 0.93	Exponential	0.002 ± 0.0003	0.737 ± 0.008
This Study	RCC 2050	170 to 200	15	31 to 40	0.45 to 0.69	Exponential	0.003 ± 0.0006	0.672 ± 0.021
Sachs and Kawka, 2015	CCMP 374	200	20	30.2 to 31.4	0.2 to 0.99	Continuous	-	-

Supplementary Material



Supplementary Figure 1: Scanning electron microscope image showing calcification of the *Emiliana huxleyi* strain RCC2050 investigated in this study.

Chapter 4

Impact of metabolic pathways and salinity on the hydrogen isotope ratios of haptophyte lipids

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Abstract

Hydrogen isotope ratios of biomarkers have been shown to reflect water isotope ratios, and in some cases correlate significantly with salinity. The $\delta^2\text{H}$ -salinity relationship is best studied for long-chain alkenones, biomarkers for haptophyte algae, and is known to be influenced by a number of different environmental parameters. It is not fully known why $\delta^2\text{H}$ ratios of lipids retain a correlation to salinity, and whether this is a general feature for other lipids produced by haptophyte algae. Here, we analyzed $\delta^2\text{H}$ ratios of three fatty acids, brassicasterol, long-chain C_{37} alkenones and phytol from three different haptophyte species grown over a range of salinities. Lipids synthesized in the cytosol, or relying on precursors of cytosolic origin, show a correlation between their $\delta^2\text{H}$ ratios and salinity. In contrast, biosynthesis in the chloroplast, or utilizing precursors created in the chloroplast, yields lipids that do not show a correlation between $\delta^2\text{H}$ ratios and salinity. This leads to the conclusion that location of metabolism is the first-order control on the salinity signal retained in $\delta^2\text{H}$ ratios of certain lipids. Additionally, we found that $\delta^2\text{H}$ ratios of alkenones from batch cultures of the Group II haptophyte species *Tisochrysis lutea* correlate positively with temperature, contrary to findings from cultures of Group III haptophytes, but retain a similar response to nutrient availability in line with other Group III haptophytes.

Keywords:

Hydrogen isotopes, Lipid biosynthesis, Haptophytes, salinity

1 Introduction

One of the best studied lipids for hydrogen isotope fractionation are long-chain alkenones, which are ethyl and methyl ketones with chain lengths of 35 - 42 carbon atoms and two to four degrees of unsaturation, found globally in lakes and oceans (De Leeuw et al., 1980; Volkman et al., 1980; Cranwell, 1985). Long-chain alkenones are synthesized by specific haptophyte algae from the order Isochrysidales, and are believed to be created from fatty acids via chain elongation and subsequent decarboxylation (Rontani et al., 2006). Haptophytes are genetically divided into three distinct groups: Groups I and II encompass haptophytes preferring lacustrine and coastal environments (although some Group II haptophytes can also be found in hypersaline lakes), and Group III comprises marine alkenone producers (Theroux et al., 2010). Hydrogen isotope ratios of C_{37} alkenones ($\delta^2\text{H}_{\text{C}_{37}}$) and fractionation factor ($\alpha_{\text{C}_{37}}$) values both have a significant positive correlation to salinity in cultures of Group II and Group III haptophytes (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014; Sachs et al., 2016; Weiss et al., 2017); Group I haptophytes are presently uncultured and thus nothing is known about how they fractionate hydrogen. $\delta^2\text{H}_{\text{C}_{37}}$ ratios have been applied to reconstruct salinity across different timescales and marine environments (Pahnke et al., 2007; Leduc et al., 2013; Kasper et al., 2014, 2015; Petrick et al., 2015; Simon et al., 2015; Weiss et al., in review). In addition to alkenones, a strong correlation between $\delta^2\text{H}$ ratios and salinity has also been shown for fatty acids and sterols from haptophytes and other phytoplankton in laboratory cultures (e.g. Sachs and Kawka, 2015; Sachs et al., 2016; Maloney et al., 2016), and is also found for other biomarkers such as C_{17} *n*-alkane, diploptene, and phytene (see compilation in Sachse et al., 2012).

Despite the strong relationship noted between $\delta^2\text{H}$ ratios and salinity, the precise intracellular mechanisms controlling the salinity relationship are not well constrained. Furthermore, a number of extracellular factors have been shown to affect $\alpha_{\text{biomarker}}$ and $\delta^2\text{H}_{\text{biomarker}}$ ratios in culture (Schouten et al., 2006; Wolhowe et al., 2009; Chivall et al., 2014; M'Boule et al., 2014; Sachs and Kawka, 2015; van der Meer et al., 2015; Sachs et al., 2016; Maloney et al., 2016; Weiss et al., 2017). For example, growth rate is shown to negatively correlate with $\delta^2\text{H}_{\text{C}_{37}}$ ratios and $\alpha_{\text{C}_{37}}$ values in Group III haptophyte species and is thought to be a limiting factor for reconstructions of paleosalinity (Schouten et al., 2006; Wolhowe et al., 2009; Sachs and Kawka, 2015). Additionally, the effect of temperature on $\delta^2\text{H}_{\text{C}_{37}}$ ratios and $\alpha_{\text{C}_{37}}$ values in culture is not well constrained, with some studies showing an effect (Wolhowe et al., 2009), and others not (Schouten et al., 2006). These extracellular parameters have primarily been investigated for Group III marine haptophytes, with little known about Group II species.

Here, we investigate the relationship between salinity and $\delta^2\text{H}$ ratios for a variety of haptophyte lipids to explore the impact of biosynthetic pathways. For this purpose, Group II and Group III haptophytes were grown at different salinities and $\delta^2\text{H}$ ratios of long-chain alkenones, $\text{C}_{14:0}$ – $\text{C}_{18:1}$ fatty acids, brassicasterol, and phytol were measured. A secondary aim is to provide the first characterization of $\delta^2\text{H}_{\text{C}_{37}}$ ratios from the Group II haptophyte *Tisochrysis lutea*, which was grown in batch cultures to assess effects of temperature and nutrient concentrations.

2 Methods

2.1 Batch cultures of *Emiliania huxleyi*, *Isochrysis galbana* and *Ruttnera lamellosa*

Batch cultures of *E. huxleyi* (CCMP 1516), *I. galbana* (CCMP 1323), and *R. lamellosa* (formerly *Chrysotila lamellosa*, CCMP 1307) were grown over a range of salinities from 26 – 37 for *E. huxleyi*, and 10 – 35 for both *I. galbana* and *R. lamellosa*. Cultures were grown at a temperature of 15°C, and light intensity of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ supplied by cool white fluorescent light with a light:dark cycle of 16:8 h. Specific media conditions are outlined in Chivall et al. (2014 – *R. lamellosa*) and M'Boule et al. (2014 – *E. huxleyi* and *I. galbana*). Aliquots from the starter culture were transferred to fresh media of each salinity five times prior to the experiment in order to acclimate algae to the new conditions and remove any potential memory effects. Biomass was filtered onto pre-combusted GF/F filters during exponential growth phase when cell concentrations were above 1×10^6 cells / mL. Filters were freeze-dried, and organics were extracted ultrasonically using dichloromethane / methanol 2:1 (v/v) to obtain total lipid extracts (TLE). TLEs were split in two for *E. huxleyi* and *I. galbana*. One part of the TLE was separated using an aluminium oxide column into three fractions: apolar (eluted with three column volumes of 9:1 (v/v) hexane / DCM), alkenone (eluted with four column volumes of 1:1 (v/v) hexane / DCM) and polar (eluted with four column volumes of 1:1 (v/v) DCM / MeOH; containing phytol and brassicasterol).

To obtain clean fractions for phytol and brassicasterol analyses, an aliquot of polar fractions from the TLE was saponified using 1N KOH / MeOH (10 mL; 1 h reflux). After cooling, the solvent was neutralised with HCl / MeOH and transferred into a separating funnel with 3 mL of bidistilled water. Lipids were extracted from the aqueous solution into 3×3 mL DCM. The solvent was removed under vacuum and the extracts dried over Na_2SO_4 . An aliquot of

this saponified polar fraction was cleaned over an aluminium oxide column, eluting with 1:1 (v/v) DCM/MeOH. Phytol and brassicasterol, contained in the saponified polar fraction, were acetylated with 100 μL acetic anhydride ($\delta^2\text{H} = -126\text{‰}$) at 60°C for 30 min in the presence of a small amount of $\text{Ag}(\text{CF}_3\text{SO}_3)$ (Das and Chakraborty, 2011). After cooling, 2 mL of a saturated aqueous solution of NaHCO_3 was added and the acetylated lipids extracted into $3 \times 1\text{ mL}$ ethyl acetate. The acetylated lipids were then cleaned over an aluminium oxide column, using 1:1 (v/v) hexane / DCM. Fatty acid extraction was conducted as described by Heinzelmann et al. (2015) for both *E. huxleyi* and *I. galbana*. Fatty acids were not analyzed for *R. lamellosa*.

2.2 Batch cultures of *Tisochrysis lutea*

Two strains of *T. lutea*, CCMP463 and NIES-2590, were grown in artificial seawater with added nutrients at a salinity of 30, light intensity of $100\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ and a range of temperatures from 10 to 35°C . Cells were harvested during late linear and early stationary phases. Alkenones were extracted as described in Nakamura et al. (2016). *T. lutea* strain CCAP 927/14 was grown in triplicate in filtered seawater under N-replete and N-limited conditions with salinity of 34 – 35, temperature from $20\text{--}23^\circ\text{C}$, and light intensity of $180\text{--}220\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ supplied by cool white fluorescent light. Cells were harvested after 4 and 10 days of growth. Neutral lipid fractions were extracted as described in da Costa et al. (2017), and ketone fractions were isolated over an aluminum oxide column using 1:1 (v/v) hexane:DCM.

2.3 Hydrogen isotope ratios

Hydrogen isotope ratios of growth water were measured prior to starting experiments and after filtration for *E. huxleyi*, *I. galbana*, and *R. lamellosa*. Culture water for the *T. lutea* nutrient experiment was measured prior to starting the experiments only, and no culture water was available for isotope analysis from the *T. lutea* temperature cultures. Water isotope ratios were measured following methods outlined in Weiss et al. (2017). Biomarkers were quantified using a gas-chromatograph coupled to a flame ionization detector (GC-FID) before measuring hydrogen isotope ratios on a Thermo Scientific Delta V GC/TC/irMS. For biomarkers from *E. huxleyi*, *I. galbana* and *R. lamellosa*, the GC was equipped with a 25 m CPSil 5 (Agilent, $25\text{ m} \times 0.32\text{ mm} \times 0.4\text{ }\mu\text{m}$) GC column following M'Boule et al. (2014). Due to lack of baseline separation when using a 25 m CPSil 5 GC column, alkenone $\delta^2\text{H}$ ratios from these three species are the integrated $\text{C}_{37:3}$ and $\text{C}_{37:2}$ values ($\delta^2\text{H}_{\text{C}_{37}}$). Alkenones from *T. lutea* were measured by GC/TC/irMS equipped with a RTX-200 60 m column (Restek, $60\text{ m} \times 0.32\text{ mm} \times 0.5\text{ }\mu\text{m}$), allowing for determination of $\delta^2\text{H}$ ratios of individual alkenones. The GC temperature program is as follows: 70°C to 250°C at $18^\circ\text{C}/\text{min}$, 250°C to 320°C at $1.5^\circ\text{C}/\text{min}$, then kept at 320°C for 25 min. Helium was used as a carrier gas and the flow rate was $1.5\text{ mL}/\text{min}$. The integrated $\delta^2\text{H}_{\text{C}_{37}}$ ratios, determined by a second peak integration encompassing both the $\text{C}_{37:3}$ and $\text{C}_{37:2}$ alkenone peaks, are also reported for comparison with alkenones from *E. huxleyi*, *I. galbana* and *R. lamellosa* measured on the CPSil 5 GC column. Prior to running samples each day, the H_3^+ factor was measured and corrected for. Values for the H_3^+ factor were $2.765 \pm 0.468\text{ ppm nA}^{-1}$ for analytical runs of *R. lamellosa* lipids, $2.752 \pm 0.428\text{ ppm nA}^{-1}$ for *I. galbana* lipids, and $4.644 \pm 0.648\text{ ppm nA}^{-1}$ for *E. huxleyi* lipids and $3.229 \pm 0.261\text{ ppm nA}^{-1}$ for *T. lutea* lipids. An *n*-alkane standard, Mix B, supplied by A. Schimmelmann (Indiana University) was measured, and samples were only run once the

Biosynthesis pathways & growth parameters

average deviation from the offline determined value and standard deviation for the Mix B were both less than 5 ‰. H² gas of known isotopic composition was measured at the beginning and end of each analytical run to further monitor machine stability. Squalane and C₃₀ *n*-alkane were co-injected with each run as another control on machine accuracy. Values for squalane were -167 ± 4 ‰ and for C₃₀ were -71 ± 4 ‰ for the entire sample set (n = 327). Standard deviations for $\delta^2\text{H}$ ratios represent the reproducibility between duplicate or triplicate analytical runs, and generally fall within the 3 ‰ precision window for the Thermo Scientific Delta V.

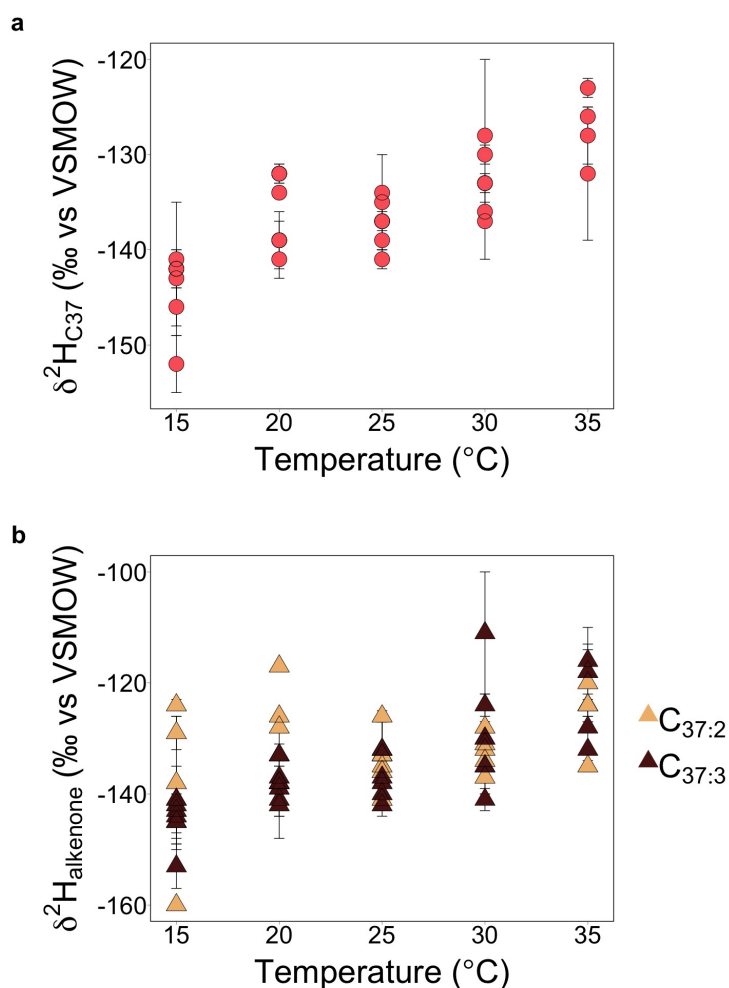


Figure 1: Hydrogen isotope ratios of (a) combined and (b) individual C₃₇ alkenones ($\delta^2\text{H}_{\text{C}_{37}}$) from Group II species *Tisochrysis lutea* plotted against temperature. Both integrated and individual $\delta^2\text{H}$ ratios show a positive linear correlation to temperature. Integrated: $r = 0.80$, $p < 0.001$; C_{37:3}: $r = 0.75$, $p < 0.001$; C_{37:2}: $r = 0.39$, $p < 0.05$; $n = 28$.

3 Results

Hydrogen isotope ratios and fractionation of six different lipids were measured and calculated, respectively, from extracts of batch cultures of four different haptophyte species (Supplementary Table 1). The $\delta^2\text{H}_{\text{C}_{37}}$ ratios from the Group III haptophyte *E. huxleyi* and the Group II haptophytes *I. galbana* and *R. lamellosa* were previously published by M'Boule

et al. (2014) and Chivall et al. (2014) and the $\delta^2\text{H}$ ratios of $\text{C}_{14:0}$ – $\text{C}_{18:1}$ fatty acids for *I. galbana* were previously published by Heinzelmann et al. (2015). Here we report the $\delta^2\text{H}_{\text{C}37}$ ratios from the Group II haptophyte *T. lutea* cultivated at different salinities, temperature and nutrient conditions. Furthermore, we analyzed the $\delta^2\text{H}$ ratios of $\text{C}_{14:0}$ – $\text{C}_{18:1}$ fatty acids from *E. huxleyi*, and brassicasterol and phytol from *E. huxleyi*, *I. galbana*, and *R. lamellosa* from the same culture material as that of M'Boule et al. (2014) and Chivall et al. (2014) (Supplementary Table 1). Fractionation factor α values were calculated for all compounds except for alkenones from the *T. lutea* temperature experiment because culture water was no longer available at the time of analyses.

3.1 Hydrogen isotope ratios of alkenones from *T. lutea*

Temperature experiment

Group II haptophyte *T. lutea* was grown over a temperature range of 15 – 35°C. Combined $\delta^2\text{H}_{\text{C}37}$ ratios ranged from -123 ‰ to -152 ‰, while isotope ratios of individual alkenones ranged from -111 ‰ to -153 ‰ for $\text{C}_{37:3}$ and -117 ‰ to -160 ‰ for $\text{C}_{37:2}$ (Table 1, Fig. 1). Growth was monitored by chlorophyll fluorescence, thus growth rates cannot be easily compared with rates calculated from daily cell counts as done for the other species. However, cells appeared to grow fastest at 35°C and slowest at 15°C (Nakamura et al., 2016). Similarly, alkenone concentrations were stable between 20°C and 30°C, and slightly lower at 35°C and 15°C (Nakamura et al., 2016). The hydrogen isotopic composition of the integrated C_{37} alkenones has a significant positive correlation with temperature ($r = 0.80$, $n = 28$, $p < 0.001$), as does the $\text{C}_{37:3}$ ($r = 0.75$, $p < 0.001$), while the $\text{C}_{37:2}$ only has a moderate positive correlation with temperature ($r = 0.39$, $p < 0.05$).

Nutrient experiment

To understand the impact of nutrients on hydrogen isotope ratios of alkenones from Group II haptophytes, *T. lutea* was grown under N-replete (nitrate concentration of 1.2 mM) and N-reduced (nitrate concentration of 0.6 mM) conditions. Cells were harvested in exponential and stationary growth phases (after 4 and 10 days of growth respectively). Growth rates for N-replete conditions were 0.21 div. d⁻¹, and N-reduced conditions were 0.14 div. d⁻¹. Under N-replete conditions, $\delta^2\text{H}_{\text{C}37}$ ratios ranged from -123 ‰ to -134 ‰, and were more depleted than under N-reduced conditions by around 10 ‰, where $\delta^2\text{H}_{\text{C}37}$ ratios ranged from -113 ‰ to -121 ‰ (Table 2; Fig. 2a). There is also a clear distinction between growth phases, with more depleted $\delta^2\text{H}_{\text{C}37}$ ratios corresponding to exponential growth phase (Fig. 2a). Fractionation factor $\alpha_{\text{C}37}$ ranged from 0.860 to 0.880, with higher fractionation (lower $\alpha_{\text{C}37}$) for higher nutrient conditions (Fig 2b). Higher fractionation was also observed during exponential growth phase compared to stationary growth phase (Fig. 2b). Higher nutrient concentrations led to faster growth, thus a negative correlation between fractionation and growth rate is noted (Fig. 2c). $\delta^2\text{H}_{\text{C}37}$ ratios from both *T. lutea* experiments align well with other Group II species *I. galbana* and *R. lamellosa*, even when temperature and nutrient concentrations caused a 50 ‰ range in $\delta^2\text{H}_{\text{C}37}$ ratios (Fig. 3). Hydrogen isotope ratios of individual C_{37} alkenones follow the same trends as the integrated C_{37} alkenones (Table 2). Integrated ratios are used for comparison with $\delta^2\text{H}$ ratios of *I. galbana* and *R. lamellosa* measured on a shorter GC column which prohibited baseline separation of the individual alkenones.

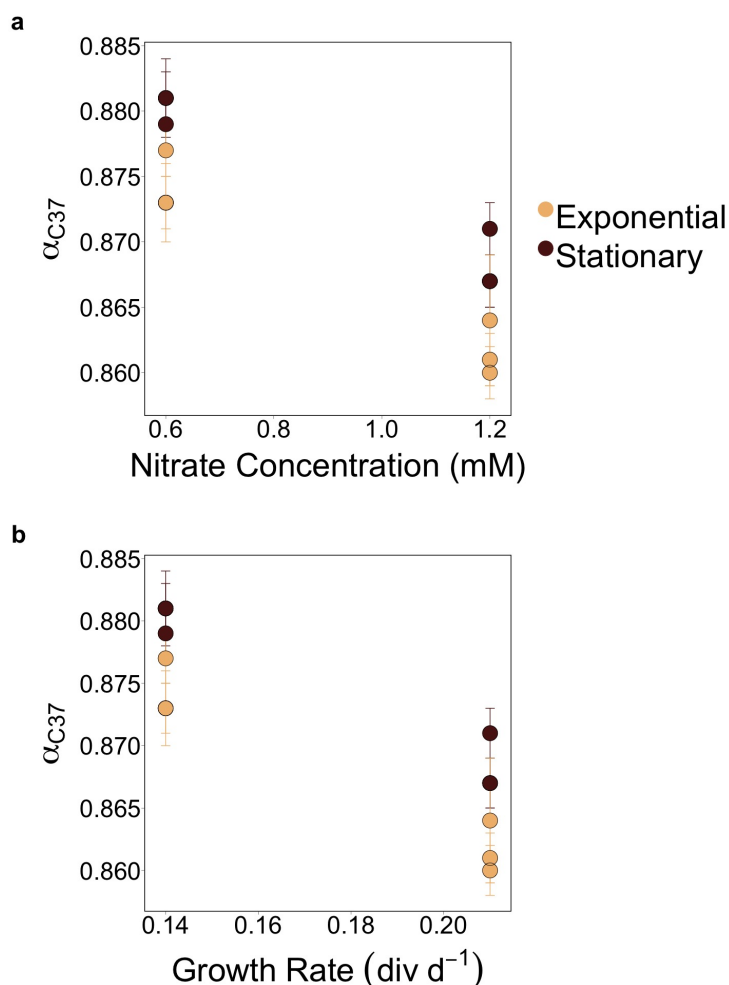


Figure 2: Results from batch cultures of *Tisochrysis lutea* investigating effect of nutrient concentrations, growth phase and growth rate on hydrogen isotope fractionation of long-chain alkenones (α_{C37}). Hydrogen isotope fractionation factor α_{C37} shows (a) a negative trend with nutrient concentration, and (b) a negative trend with growth rate. Relative isotopic enrichment is noted for stationary growth phase compared to exponential growth phase.

3.2 Hydrogen isotope ratios of lipids from *E. huxleyi*, *I. galbana* and *R. lamellosa*

For *I. galbana* and *R. lamellosa*, alkenones were the most enriched lipids by an average of 60 ‰ and 110 ‰, respectively, compared to the other lipids (Fig. 4a,b). In *E. huxleyi*, δ^2H ratios of $C_{14:0}$ and $C_{16:0}$ fatty acids were more depleted by 10 to 30 ‰ relative to alkenones, while the $C_{18:1}$ fatty acid was the most 2H -enriched compound by around 50 ‰ relative to the other fatty acids and alkenones (Fig. 4c). Fractionation for *E. huxleyi* fatty acids varied between 0.762 – 0.872, and correlates significantly with salinity for $C_{14:0}$ and $C_{18:1}$ fatty acids (Fig. 4c), but the correlation between fractionation and salinity is not statistically significant for $C_{16:0}$ ($r = 0.84$, $n = 4$, $p = 0.16$). However, we treat these significant correlations with caution as they are based on only four data points. The δ^2H ratios of both brassicasterol and phytol are more depleted than δ^2H ratios of alkenones by an average of 120 ‰ and 230 ‰, respectively, for all three species (Fig. 4; Supplementary Table 1). The δ^2H ratios and α of brassicasterol correlate

significantly with salinity in all three species (Fig. 4). There is no significant relationship with salinity for phytol from *E. huxleyi* and *I. galbana*, but both $\delta^2\text{H}_{\text{phytol}}$ ratios and α_{phytol} from *R. lamellosa* do correlate significantly with salinity ($\delta^2\text{H}_{\text{phytol}}$: $r = 0.90$, $n = 28$, $p < 0.001$; α_{phytol} : $r = 0.54$, $n = 28$, $p < 0.05$).

Table 1: Hydrogen isotope ratios of long-chain alkenones from the *Tisochrysis lutea* temperature experiment. Alkenone concentrations are reported in Nakamura et al. (2016).

<i>Tisochrysis lutea</i> -- Temperature experiment									
Strain: CCMP 463					Strain: NIES 2590				
Replicate	Temperature	$\delta^2\text{H}_{\text{C}_{37}}$ (‰ vs VSMOW)	$\delta^2\text{H}_{\text{C}_{37:2}}$ (‰ vs VSMOW)	$\delta^2\text{H}_{\text{C}_{37:3}}$ (‰ vs VSMOW)	Replicate	Temperature	$\delta^2\text{H}_{\text{C}_{37}}$ (‰ vs VSMOW)	$\delta^2\text{H}_{\text{C}_{37:2}}$ (‰ vs VSMOW)	$\delta^2\text{H}_{\text{C}_{37:3}}$ (‰ vs VSMOW)
A	15	-142 ± 7	-138 ± 12	-141 ± 6	A	15	-141 ± 0	-124 ± 1	-145 ± 0
B	15	-143 ± 3	-141 ± 3	-144 ± 4	B	15	-142 ± 2	-129 ± 3	-143 ± 1
C	15	-152 ± 3	-145 ± 4	-153 ± 4	C	15	-146 ± 2	-160 ± 0	-142 ± 2
A	20	-139 ± 3	-138 ± 10	-138 ± 1	A	20	-132 ± 1	-128 ± 0	-137 ± 3
B	20	-132 ± 1	-117 ± 0	-133 ± 0	B	20	-134 ± 0	-126 ± 0	-139 ± 2
C	20	-139 ± 2	-133 ± 2	-141 ± 3	C	20	-141 ± 2	-138 ± 3	-142 ± 2
A	25	-135 ± 1	-135 ± 1	-132 ± 5	A	25	-134 ± 4	-126 ± 1	-132 ± 5
B	25	-137 ± 3	-136 ± 2	-137 ± 3	B	25	-141 ± 1	-141 ± 2	-140 ± 0
C	25	-139 ± 2	-133 ± 1	-142 ± 2	C	25	-137 ± 3	-133 ± 6	-138 ± 2
A	30	-133 ± 0	-132 ± 1	-141 ± 2	A	30	-137 ± 4	-137 ± 3	-135 ± 5
B	30	-133 ± 1	-134 ± 2	-130 ± 2	B	30	-130 ± 1	-128 ± 1	-124 ± 2
C	30	-128 ± 8	-130 ± 6	-111 ± 11	C	30	-136 ± 1	-131 ± 3	-135 ± 3
A	35	-132 ± 7	-135 ± 1	-116 ± 2	-	-	-	-	-
B	35	-128 ± 3	-120 ± 7	-132 ± 3	-	-	-	-	-
C	35	-123 ± 1	-124 ± 1	-118 ± 8	-	-	-	-	-
D	35	-126 ± 0	-124 ± 2	-128 ± 2	-	-	-	-	-

4 Discussion

4.1 Effects of temperature for *T. lutea* alkenones

The influence of temperature on $\delta^2\text{H}_{\text{C}_{37}}$ ratios in haptophyte algae is not well constrained. Previous results from cultures investigating the effect of temperature on $\delta^2\text{H}_{\text{C}_{37}}$ ratios in Group III haptophyte species are contradictory (Schouten et al., 2006; Wolhowe et al., 2009), and there has been no characterization of the temperature effect on $\delta^2\text{H}_{\text{C}_{37}}$ ratios for Group II haptophytes to our knowledge. Our results show that individual alkenones, as well as integrated C_{37} alkenones, show a significant positive correlation with temperature in Group II species *T. lutea* (Fig. 1). The positive linear correlation for the integrated alkenones seems to be driven by the $\text{C}_{37:3}$ alkenone, begging the question whether the observed correlation is really a temperature effect, or primarily a function of relative abundance and therefore an indirect temperature effect (c.f. van der Meer et al., 2013).

Table 2: Hydrogen isotope ratios and fractionation factor α_{C37} values from the *Tisochrysis lutea* nutrient and growth phase experiment. Alkenone concentrations are reported in da Costa et al. (2017).

Tisochrysis lutea CCAP 927/14 -- Nutrient Experiment										
Replicate	Nitrate (nM)	$\delta^2H_{H_2O}$ (‰ vs VSMOW)	Growth Rate. (div d ⁻¹)	Growth Phase	$\delta^2H_{C37:3}$ (‰ vs VSMOW)	$\delta^2H_{C37:2}$ (‰ vs VSMOW)	δ^2H_{C37} (‰ vs VSMOW)	$\alpha_{C37:3}$	$\alpha_{C37:2}$	α_{C37}
A	0.6	7 ± 2	0.14	Exponential	-121 ± 3	-119 ± 2	-121 ± 2	0.873 ± 0.003	0.875 ± 0.002	0.873 ± 0.003
B					-119 ± 0	-122 ± 1	-121 ± 0	0.881 ± 0.000	0.878 ± 0.001	0.879 ± 0.000
C					-117 ± 1	-117 ± 0	-117 ± 1	0.883 ± 0.001	0.883 ± 0.000	0.883 ± 0.001
A	1.2	7 ± 2	0.21		-135 ± 1	-129 ± 4	-134 ± 1	0.865 ± 0.001	0.871 ± 0.005	0.866 ± 0.001
B					-132 ± 1	-130 ± 1	-133 ± 1	0.868 ± 0.002	0.870 ± 0.001	0.867 ± 0.002
C					-130 ± 6	-128 ± 3	-130 ± 4	0.870 ± 0.007	0.872 ± 0.004	0.870 ± 0.005
A	0.6	7 ± 2		Stationary	-116 ± 1	-110 ± 0	-113 ± 0	0.884 ± 0.001	0.890 ± 0.000	0.887 ± 0.000
B					-118 ± 0	-110 ± 2	-115 ± 1	0.882 ± 0.001	0.890 ± 0.002	0.885 ± 0.001
C					-117 ± 2	-110 ± 2	-113 ± 2	0.883 ± 0.002	0.890 ± 0.003	0.887 ± 0.002
A	1.2	7 ± 2			-125 ± 2	-118 ± 1	-123 ± 1	0.875 ± 0.002	0.882 ± 0.001	0.877 ± 0.001
B					-128 ± 1	-124 ± 5	-127 ± 0	0.872 ± 0.001	0.876 ± 0.005	0.873 ± 0.000
C					-129 ± 0	-120 ± 3	-127 ± 1	0.871 ± 0.000	0.880 ± 0.003	0.873 ± 0.001

The relative abundance of the $C_{37:3}$ and $C_{37:2}$ alkenones are strongly coupled to temperature, with higher amounts of the $C_{37:2}$ at higher temperatures (Prahl and Wakeham, 1987). The $C_{37:3}$ is synthesized from the $C_{37:2}$ by a desaturation step (Rontani et al., 2006; Endo et al., 2018; Kitamura et al., 2018), which occurs less often at higher temperatures. Desaturation is associated with 2H depletion (Chikaraishi et al., 2004), thus it follows that the $C_{37:3}$ alkenone should be more depleted than its $C_{37:2}$ precursor, as observed here (Fig. 1). At lower temperatures, when there is a higher abundance of $C_{37:3}$, and much lower relative abundance of $C_{37:2}$, the $C_{37:2}$ should be comparatively enriched. For the *T. lutea* temperature experiment, the $C_{37:3}$ was depleted relative to the $C_{37:2}$ from 15 – 25°C, but from 30 – 35°C, where the $C_{37:2}$ was much more abundant than the $C_{37:3}$, a relative depletion of the $C_{37:2}$ compared to the $C_{37:3}$ was observed (Fig. 1). It appears that 2H depletion shifts in favor of the dominant alkenone, and when the difference in abundance of both alkenones is close to zero, the offset between the δ^2H ratios of the two alkenones is also close to zero. These findings suggest that the temperature correlation observed for alkenones from *T. lutea* is likely related to relative abundance shifts, which in turn has an effect on δ^2H_{C37} ratios, implying an indirect temperature effect on δ^2H_{C37} ratios. Growth rate might also influence these correlations, but due to the fact that growth was monitored by chlorophyll fluorescence in these experiments, we cannot compare absolute values with growth rates obtained by daily cell counts. However, alkenone concentrations did not vary substantially over the temperature range (5-10 $\mu g / mL$ at 15° C and 35°C vs 10-15 $\mu g / mL$ from 20 -30°C; Fig. 2b from Nakamura et al., 2016), leading us to conclude that growth rates did not significantly impact our δ^2H_{C37} ratios.

4.2 Effects of nutrients and growth phase for *T. lutea* alkenones

T. lutea was also grown under two different nutrient concentrations and harvested during exponential and stationary growth phases to assess growth-related effects on α_{C37} values. N-reduced and N-replete media led to different growth rates: 0.14 μd^{-1} for N-reduced and 0.21 μd^{-1} for N-replete. While these two growth rates are not extremely different, they led to a distinct offset in isotope ratios, with more fractionation under higher nutrient concentrations (i.e. higher growth rate) and a relative isotope depletion (Fig. 2). The negative correlation between α_{C37} values and growth rate was previously recognized in Group III species *E. huxleyi* and *G. oceanica* (Schouten et al., 2006; Wolhowe et al., 2009). The same negative relationship between α_{C37} values and growth rate was noted for our *T. lutea* experiment (Fig. 2), confirming that similar growth effects on δ^2H_{C37} ratios also occur in Group II species and are tightly coupled to nutrient availability. Sachs and Kawka (2015) hypothesized that lower growth rate leads to an up-regulation of OPP derived NADPH causing lipids to be relatively enriched; at higher growth rates, photosynthetically derived NADPH becomes the dominant H source, leading to a relative depletion of lipid δ^2H ratios. This mechanism works in reverse to the salinity mechanism, where increased salinity causes an up-regulation of OPP derived NADPH, and therefore might account for the reduced sensitivity of δ^2H_{C37} -salinity in the natural environment (e.g. Schwab and Sachs, 2011; Weiss et al., 2019). The δ^2H_{C37} ratios from *T. lutea* (temperature and nutrient experiments) fit well with values noted for other Group II species *I. galbana* and *R. lamellosa* (Fig.3). Three Group II species now show similar δ^2H_{C37} ratios which are more enriched than values reported for the Group III species *E. huxleyi* and *G. oceanica*, corroborating the hypothesis of a distinct Group II and Group III δ^2H_{C37} signal (M'Boule et al., 2014).

4.3 Impact of salinity on haptophyte lipids

Fatty acids and long-chain alkenones

Fatty acids from *E. huxleyi* in our experiment showed ^2H -enrichment with chain elongation (Supplementary Table 1, Fig. 4). The same has previously been observed for fatty acids from *E. huxleyi* (Sachs and Kawka, 2015), and *I. galbana* (Heinzelmann et al., 2015), for the diatom *Thalassiosira pseudonana* (Maloney et al., 2016), as well as in some marine macroalgae belonging to Heterokontophyta and Rhodophyta (Chikaraishi et al., 2004). Subsequent depletion is reported after desaturation from $\text{C}_{18:0}$ to $\text{C}_{18:1}$ of around 40 ‰ for Heterokontophyta and approximately 70 ‰ for Rhodophyta, but the overall combined effect of elongation and desaturation from $\text{C}_{16:0}$ to $\text{C}_{18:1}$ results in isotopic enrichment of $\text{C}_{18:1}$ relative to $\text{C}_{16:0}$ (Chikaraishi et al., 2004). In general, these observations also hold true for fatty acids from *E. huxleyi* and *I. galbana* discussed here (Supplementary Table 1, Fig. 4). Both species show a significant positive correlation between $\delta^2\text{H}$ ratios of fatty acids and salinity (Fig. 4),

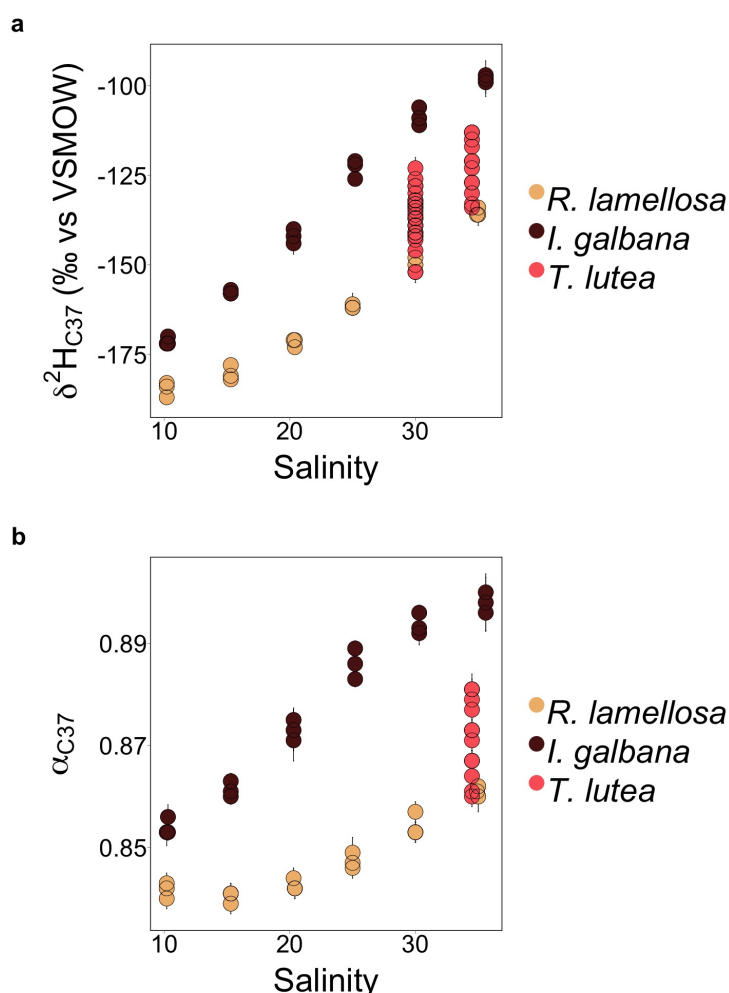


Figure 3: (a) Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{\text{C}37}$) and (b) hydrogen isotope fractionation factor ($\alpha_{\text{C}37}$) plotted against salinity for three Group II species: *Ruttenra lamellosa* (Chivall et al., 2014), *Isochrysis galbana* (M'Boule et al., 2014), and *Tisochrysis lutea* (This study). New results presented here of $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values from *T. lutea* align with the other two species, confirming a distinct Group II signal.

except for the α - salinity correlation for $C_{16:0}$ from *E. huxleyi* and $C_{18:1}$ from *I. galbana* ($C_{16:0}$: $r = 0.84$, $p > 0.05$; $C_{18:1}$: $r = -0.34$, $p > 0.05$). However, the correlations for fatty acids from *E. huxleyi* are based on a low number of data points ($n = 4$ for each fatty acid), thus we treat these significant correlations with caution. The lack of correlation for $C_{18:1}$ in *I. galbana* might partially be explained by the desaturation step since desaturation is associated with ^2H depletion, which has the potential to counteract the ^2H enrichment associated with higher salinities.

The $\delta^2\text{H}$ ratios and α values of alkenones from *I. galbana* (M'Boule et al., 2014), *R. lamellosa* (Chivall et al., 2014), *E. huxleyi* (M'Boule et al., 2014) all show strong positive correlations with salinity (Supplementary Table 1, Fig. 4). Interestingly, for *E. huxleyi*, the $C_{18:1}$ is the most ^2H -enriched compound out of the six, in contrast to *I. galbana*, where alkenones are the most ^2H -enriched (Supplementary Table 1, Fig. 4). This highlights another potential offset between haptophyte Groups II and III. Species related differences aside, there is a correlation between $\delta^2\text{H}$ ratios and salinity for both fatty acids and alkenones. In algae, the first two steps of fatty acid biosynthesis (formation of malonyl-CoA and initial elongation by fatty acid synthases) occur in the chloroplast, and additional elongation beyond C_{16} or C_{18} relies directly on cytosolic sources (Cook and McMaster, 2002; Huerlimann and Heimann, 2013). Fatty acid elongation occurs via the stepwise addition of two carbon atoms and four hydrogen atoms, and desaturation removes two hydrogen atoms, allowing for substantial isotopic change (Chikaraishi et al., 2004), which can explain the isotopic differences between fatty acids and alkenones. Furthermore, alkenones are thought to be synthesized from these shorter chain fatty acids by elongation and subsequent decarboxylation in the chloroplast (Rontani et al., 2006). Fatty acids, although mainly synthesized in the chloroplast, also rely on acetyl-CoA as a precursor. Acetyl-CoA might be responsible for the salinity signal noted for fatty acids and alkenones, since de novo synthesis of acetyl-CoA comes from pyruvate produced in the cytosol (DeNiro and Epstein, 1977).

Brassicasterol and phytol

The $\delta^2\text{H}$ ratios and α values of brassicasterol correlate significantly with salinity for all three species, whereas $\delta^2\text{H}$ ratios of phytol do not correlate with salinity for either *E. huxleyi* or *I. galbana* (Fig. 4). Brassicasterol and phytol are isoprenoid lipids formed with either isopentenyl phosphate (IPP) or dimethylallyl pyrophosphate (DMAPP) as precursors. Two pathways exist for the synthesis of IPP and DMAPP. The Mevalonate (MVA) Pathway generates isoprenoid precursors in the cytosol using acetyl-CoA (Schmidt et al., 2003; Eisenreich et al., 2004). In contrast, the Methylerythritol pathway (MEP) creates IPP and DMAPP in the plastids using pyruvate and glyceraldehyde-3-phosphate (G3P) (Schmidt et al., 2003; Guggisberg et al., 2014; Lohr et al., 2012; Sachs et al., 2016). Since phytol is synthesized by precursors generated via MEP and brassicasterol is synthesized by precursors from MVA (Lichtenthaler et al., 1997; Bohlmann et al., 1999; Lichtenthaler, 1999; Chikaraishi et al., 2009), the offset of ~ 100 ‰ between the two compounds likely stems from differences associated with these two pathways. Synthesis of isoprenoid precursors via the MVA pathway involves two reduction steps, but the MEP involves three reduction steps. One of the three reduction steps in the MEP is catalyzed by the IspH enzyme that supplies IPP and/or DMAPP with depleted H (Schmidt et al., 2003), potentially explaining why phytol is more ^2H depleted than brassicasterol.

Further evidence to confirm different precursor pathways and cellular compartments for phytol versus sterol comes from a light intensity experiment conducted by Sachs et al.

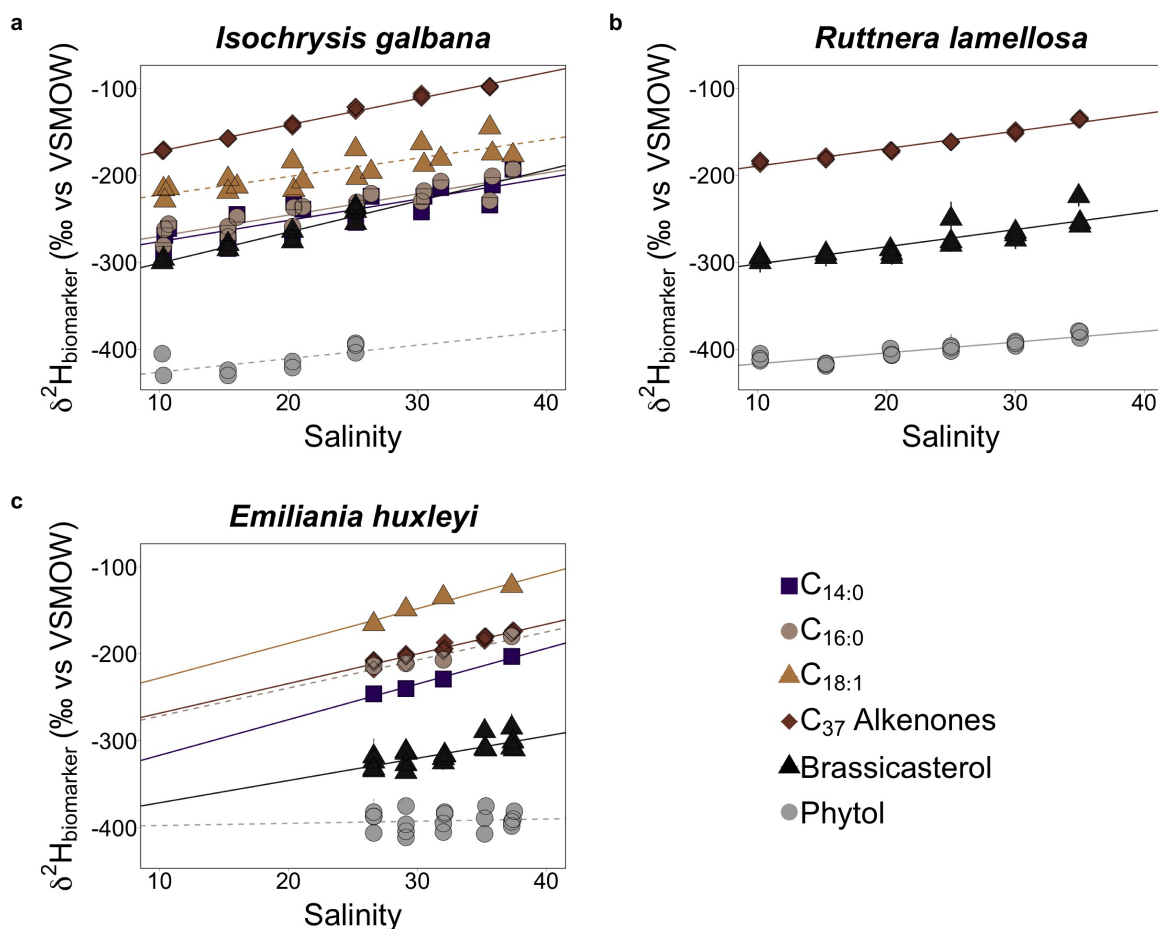


Figure 4: Hydrogen isotope ratios ($\delta^2\text{H}$) of six biomarker lipids plotted against salinity for Group II species *Isochrysis galbana* (a) and *Ruttnera lamellosa* (b) and Group III species *Emiliana huxleyi* (c). Long-chain alkenones and $\text{C}_{14:0}$ – $\text{C}_{18:1}$ fatty acids from *I. galbana* published by M’Boule et al. (2014) and Heinzelmann et al. (2015) respectively. Long-chain alkenones from *R. lamellosa* and *E. huxleyi* published in Chivall et al. (2014) and M’Boule et al. (2014) respectively. Positive correlations between $\delta^2\text{H}$ ratios and salinity are noted for all lipids except for phytol synthesized by *I. galbana* and *E. huxleyi*.

Significant, positive correlations between $\delta^2\text{H}$ ratios and salinity are noted by solid regression lines and insignificant correlations are indicated with dashed lines.

(2017) assessing effects of irradiance on organic compounds from the diatom *Thalassiosira pseudonana*. The $\delta^2\text{H}$ ratios of phytol showed a strong correlation with light intensity ($R^2 = 0.90$, $p < 0.0001$), while the $\delta^2\text{H}$ ratio of 24-methyl-cholesta-5,24(28)-dien-3 β -ol was not affected by light intensity. These results support the idea that phytol is synthesized by precursors in the chloroplast, especially since phytol is a component of chlorophyll (de Souza and Nes, 1969). Sterols, on the other hand, can be synthesized by cytosolic precursors and rely on a H source that is not connected to light availability, but instead potentially correlated to salinity, either directly or indirectly.

Acetyl-CoA used in the MVA pathway is synthesized from pyruvate or β -oxidation of excess fatty acids in the cytosol (Nelson and Cox, 2017). Pyruvate can be synthesized in the chloroplast, and used directly in MEP, but it can also be synthesized in the cytosol, where it can be converted to acetyl-CoA and used in MVA (Williams and Randall, 1979; Disch et al., 1998;

Wallace, 2013). Differences in cellular compartment and biosynthetic precursors (acetyl-CoA and pyruvate) associated with metabolic reactions provide an explanation for why $\delta^2\text{H}$ ratios of brassicasterol correlate to salinity and $\delta^2\text{H}$ ratios of phytol do not. However, in *R. lamellosa*, there is a positive linear correlation between phytol $\delta^2\text{H}$ and salinity ($\delta^2\text{H}$: $r = 0.90$, $n = 18$, $p < 0.001$), and a much weaker correlation for α ($r = 0.54$, $n = 18$, $p < 0.05$). The correlation with salinity observed for phytol from *R. lamellosa* is puzzling. It might be related to an addition of pyruvate synthesized in the cytosol mixing with pyruvate synthesized in the chloroplast, since pyruvate is reported to flow between the two cellular compartments (Williams and Randall, 1979).

4.4 Mechanisms for salinity impact on hydrogen isotope fractionation

The general trend observed between $\delta^2\text{H}$ ratios of organic compounds (excluding phytol) and salinity is $\delta^2\text{H}$ enrichment with increasing salinity. As explained above, the absence of a correlation between $\delta^2\text{H}$ ratios of phytol and salinity is presumably the result of the location of phytol biosynthesis and biosynthesis of the key intermediate, pyruvate. Fatty acids, alkenones and brassicasterol are all connected more directly to H pools in the cytosol because of their reliance on acetyl-CoA as a precursor, and all show relative ^2H -enrichment at increased salinities (Fig. 4). While the precise mechanism controlling the correlation between $\delta^2\text{H}$ ratios and salinity is unknown, certain concepts related to hydrogen isotope fractionation and intracellular pools of hydrogen are known.

NADPH is an essential cofactor that acts as a significant source of H in biosynthesis reactions (Sessions et al., 1999; Zhang et al., 2009 and references cited therein). The hydrogen isotopic composition of NADPH can vary substantially depending on the source or location of reduction within the cell, which can then be evidenced down-stream in the hydrogen isotope ratios of resultant organic compounds (Zhang et al., 2009). NADPH can be reduced via two main sources: light reactions in the chloroplast (photosynthetically-derived) and the oxidative pentose phosphate (OPP) pathway in the cytosol (metabolically-derived). Photosynthetically-derived NADPH is the product of the reduction of NADP^+ by the redox protein ferredoxin (Shin, 2004; Zhang et al., 2009; Cormier et al., 2018). The resultant NADPH is ^2H -depleted compared to growth water. The $\delta^2\text{H}$ ratios of NADPH reduced by light reactions of photosynthesis are dependent on light intensity below $\sim 115 \mu \text{mol photons m}^{-2} \text{s}^{-1}$ and large fractionation is associated with lower light intensities (Cormier et al., 2018). In the cytosol, the OPP pathway supplies the cell with NADPH by reducing NADP^+ during conversion of glucose-6-phosphate (Wamelink et al., 2008), a reaction that is not linked to photosynthetic activity, but potentially linked to salinity (Sachs et al., 2016). It has been speculated that at lower light intensities, the OPP pathway supplies a larger portion of NADPH for biosynthesis since NADPH from light reactions of photosynthesis is limited (Cormier et al., 2018). Additionally, up-regulation of the OPP pathway is hypothesized at increased salinities, which is associated with relative ^2H -enrichment (Maloney et al., 2016; Sachs et al., 2016). A dominance of metabolically-derived H for biosynthesis would yield more enriched organic compounds relative to a when a photosynthetically-derived H source is dominant, and this enrichment is enhanced at increased salinities. Isotopic enrichment is observed at increased salinities in a majority of the lipids discussed here (Fig. 4), leading to the hypothesis that OPP is likely the dominant H source of NADPH used for synthesis of these lipids. For haptophytes not growing under light-limited conditions, but growing over a range of salinities, a balance between photosynthetically reduced and OPP derived NADPH is expected at lower salinities, and a larger proportion of OPP derived NADPH is predicted

at higher salinities. Phytol synthesis occurs in the chloroplast, thus a significant portion of H should come from photosynthetically-derived NADPH, explaining why phytol $\delta^2\text{H}$ ratios are significantly depleted relative to other organic compounds, and $\delta^2\text{H}$ ratios of phytol might be more sensitive to light rather than salinity.

The amount of H derived from water for biosynthesis is an equally important control on why some lipids retain a correlation to salinity. For example, when pyruvate is formed by the tricarboxylic acid (TCA) cycle, there is more exchange of H with relatively enriched surrounding water, which causes a relative isotopic enrichment (Sachs et al., 2016; Cormier et al., 2018). In contrast, when pyruvate is formed by glycolysis, only one third of the H comes from water, resulting in a relative depletion (Sachs et al., 2016). However, as explained above, NADPH sources are thought to exert a strong control on $\delta^2\text{H}$ ratios of lipids, thus these differences between pyruvate from TCA compared to glycolysis might be of secondary importance in resultant lipid $\delta^2\text{H}$ ratios. When larger amounts of H derived directly from water is used in lipid synthesis, there is a more direct connection between lipid $\delta^2\text{H}$ ratios and growth water $\delta^2\text{H}$ ratios, and therefore less fractionation. Less fractionation is observed at higher salinities for the majority of biomarkers presented here, therefore it is possible that up-regulation of certain pathways incorporates proportionally more H directly from intracellular water, or potentially employing more acetyl-CoA from TCA-derived pyruvate, at higher salinities causes a closer connection to extracellular $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios.

At higher salinities cells must maintain osmotic pressure to avoid lysis, and create organic compounds known as osmolytes for this purpose (Dickson et al., 1982; Dickson and Kirst, 1987). Indeed, higher amounts of osmolytes (most notably DMSP) are observed at higher salinities in some haptophyte species (Dickson and Kirst, 1987). It has been previously proposed that enhanced production of osmolytes might cause the intracellular pool of H to become more ^2H enriched as a result of increased recycling of intracellular water (Sachse and Sachs, 2008; Maloney et al., 2016), leading to enriched $\delta^2\text{H}$ ratios of lipids at higher salinities. *I. galbana* is adapted to grow at a large range of salinities, while *E. huxleyi* is adapted to grow at a higher, but more restricted range of salinities. Because *I. galbana* is more cosmopolitan, it might be more metabolically active as a consequence of salinity stress over the much larger range of salinities at which it grows, and could likely be shifting more precursors and reducing power into osmolyte production than *E. huxleyi* at the same salinity. This would cause relative enrichment of the intracellular H pool in *I. galbana* compared to *E. huxleyi*, explaining offset in $\delta^2\text{H}$ ratios observed between these two species. The enrichment associated with increased osmolyte production might not be as strongly evidenced in fatty acids because fatty acids are initially synthesized in the chloroplast, up to C_{14} or C_{16} , thus are not exposed directly to cytosolic pools of H. Chain elongation leading to long-chain alkenones does take place in the cytosol, thus $\delta^2\text{H}$ ratios of alkenones reflect changes in salinity.

The above mechanisms suggest that the site of NADPH reduction is the primary driver of the hydrogen isotope composition of lipids, but the source of the salinity signal recorded in haptophyte lipids potentially comes from biosynthetic precursors tied to specific cellular compartments. The hydrogen isotopic composition of compounds formed from cytosol-derived precursors, and those synthesized directly in the cytosol, change with salinity. Lipids that are synthesized in the chloroplast and depend more on chloroplastic precursors in conjunction with photosynthetically-derived NADPH, do not show variation in $\delta^2\text{H}$ ratios with salinity (e.g. phytol in *E. huxleyi* and *I. galbana*), or greatly diminished (e.g. $\text{C}_{16:0}$ in *E. huxleyi*).

5 Conclusions

New results presented here showed a positive effect of temperature on $\delta^2\text{H}$ ratios of long-chain alkenones for Group II species *T. lutea* which is likely coupled with changes in relative abundance of individual alkenones. Nutrient concentration showed a similar effect on Group II species *T. lutea* as previously reported for Group III species *E. huxleyi*, with depletion of $\delta^2\text{H}$ ratios associated with stationary growth phase and higher nutrient concentrations. The effect of nutrients and growth on $\delta^2\text{H}$ ratios noted here is opposite to the effect of salinity, and might cause changes in the sensitivity of the $\delta^2\text{H}$ -salinity relationship in the natural environment.

Investigation of $\delta^2\text{H}$ ratios of multiple lipids produced by the same haptophyte algae showed that biosynthesis occurring in the cytosol, and relying on precursors of cytosolic origin, yields lipids with $\delta^2\text{H}$ ratios that are sensitive to salinity. In contrast, the $\delta^2\text{H}$ of lipids produced in the chloroplast, and dependent upon precursors partially or whole produced in the chloroplast, did not change with salinity. Therefore, alkenones, brassicasterol and fatty acids with a chain length of at least 16 carbon atoms, or diagenetic products derived from these lipids, should be selected instead of phytol or shorter chain fatty acids for paleosalinity reconstructions. The offset between $\delta^2\text{H}$ ratios of lipids synthesized by Group II and III haptophytes is likely a result of differing mechanisms for regulating salt stress. Group III haptophytes are plausibly more adapted to growth at higher salinities than Group II haptophytes, which are adapted for growth at a more variable range of salinities. The offset between species, which can be on the order of 100 ‰, is an essential factor to consider when employing $\delta^2\text{H}$ ratios to reconstruct salinity in areas where mixing of species is anticipated.

Author contributions

D. C and S. K. designed and carried out the *E. huxleyi*, *I. galbana* and *R. lamellosa* experiments and the respective hydrogen isotope analyses. H. N. provided the extracts for the *T. lutea* temperature experiment. F. D. C. and P. S. provided the extracts for the *T. lutea* nutrient and growth phase experiment. G. M. W. conducted hydrogen isotope analyses for both *T. lutea* experiments and prepared the manuscript with contributions from all co-authors.

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Supplementary Table 1: Hydrogen isotope ratios ($\delta^2\text{H}_{\text{C}_{37}}$) and fractionation factor $\alpha_{\text{C}_{37}}$ values for lipids from *Emiliania huxleyi*, *Isochrysis galbana* and *Ruttnera lamellosa*. Alkenones from *E. huxleyi* and *I. galbana* previously published in M'Boule et al. (2014). Fatty acids from *I. galbana* are from Heinzlmann et al. (2015). Alkenones from *R. lamellosa* were previously published in Chivall et al. (2014).

Emiliania huxleyi CCMP 1516																						
Salinity		26.6	26.6	26.6	26.6	29.1	29.1	29.1	29.1	32	32.1	32	32.1	35.2	35.2	35.2	35.3	37.3	37.4	37.3	37.5	
Replicate		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
Water		-11	-10	-9	-12	-6	-6	-5	-5	0	-3	0	-1	2	3	3	2	7	7	6	6	
SD		1	1	2	2	2	1	1	1	1	2	2	2	0	1	1	2	2	2	1	2	
Reference	Lipid																					
M'Boule et al. (2014)	C ₃₇	δ ² H ‰	-208	-210	-218	-207	-203	-202	-211	-200	-195	-194	-197	-187	-185	-180	-182	-180	-176	-173	-176	-174
		SD	5	3	1	1	1	1	6	2	3	2	3	2	2	1	7	4	1	2	3	3
		alpha	0.802	0.798	0.789	0.802	0.802	0.803	0.792	0.805	0.805	0.809	0.803	0.814	0.813	0.818	0.811	0.818	0.818	0.821	0.819	0.822
		SD	0.006	0.003	0.003	0.003	0.003	0.002	0.006	0.002	0.004	0.004	0.003	0.004	0.002	0.002	0.006	0.004	0.002	0.003	0.003	0.004
This study	Sterol	δ ² H ‰	-318	-324	-334	-332	-312	-336	-327	-314	-325	-316	-320	-317	-	-309	-289	-310	-308	-301	-285	-310
		SD	20	1	4	4	1	1	1	4	8	1	1	1	-	7	0	3	2	1	16	0
		alpha	0.690	0.683	0.672	0.675	0.693	0.669	0.676	0.689	0.675	0.687	0.681	0.684	-	0.689	0.709	0.689	0.687	0.693	0.710	0.686
		SD	0.014	0.004	0.006	0.006	0.004	0.003	0.003	0.006	0.009	0.003	0.003	0.003	-	0.008	0.002	0.006	0.005	0.004	0.014	0.002
This study	Phytol	δ ² H ‰	-387	-387	-406	-382	-396	-411	-404	-375	-405	-382	-395	-384	-	-389	-407	-375	-393	-390	-398	-381
		SD	20	13	1	7	2	0	1	6	3	2	2	2	-	8	1	10	0	3	4	1
		alpha	0.620	0.619	0.600	0.625	0.608	0.593	0.599	0.629	0.595	0.620	0.605	0.616	-	0.609	0.591	0.624	0.603	0.605	0.598	0.615
		SD	0.007	0.006	0.002	0.005	0.003	0.002	0.002	0.004	0.003	0.003	0.003	0.003	-	0.005	0.002	0.005	0.002	0.003	0.004	0.002
This study	C _{14:0}	δ ² H ‰	-246	-	-	-	-240	-	-	-	-229	-	-	-	-	-	-	-	-204	-	-	-
		SD	1	-	-	-	1	-	-	-	0	-	-	-	-	-	-	-	1	-	-	-
		alpha	0.762	-	-	-	0.764	-	-	-	0.771	-	-	-	-	-	-	-	0.791	-	-	-
		SD	0.002	-	-	-	0.003	-	-	-	0.001	-	-	-	-	-	-	-	0.002	-	-	-
This study	C _{16:0}	δ ² H ‰	-214	-	-	-	-211	-	-	-	-207	-	-	-	-	-	-	-	-180	-	-	-
		SD	2	-	-	-	1	-	-	-	0	-	-	-	-	-	-	-	1	-	-	-
		alpha	0.795	-	-	-	0.793	-	-	-	0.793	-	-	-	-	-	-	-	0.814	-	-	-
		SD	0.007	-	-	-	0.004	-	-	-	0.002	-	-	-	-	-	-	-	0.005	-	-	-
This study	C _{18:1}	δ ² H ‰	-166	-	-	-	-149	-	-	-	-135	-	-	-	-	-	-	-	-122	-	-	-
		SD	2	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	2	-	-	-
		alpha	0.844	-	-	-	0.856	-	-	-	0.865	-	-	-	-	-	-	-	0.872	-	-	-
		SD	0.02	-	-	-	0.002	-	-	-	0.001	-	-	-	-	-	-	-	0.002	-	-	-

Supplementary Table 1: (continued) Hydrogen isotope ratios ($\delta^2\text{H}_{\text{C}_{37}}$) and fractionation factor $a_{\text{C}_{37}}$ values for lipids from *Emiliania huxleyi*, *Isochrysis galbana* and *Ruttnera lamellosa*. Alkenones from *E. huxleyi* and *I. galbana* previously published in M'Boule et al. (2014). Fatty acids from *I. galbana* are from Heinzlmann et al. (2015). Alkenones from *R. lamellosa* were previously published in Chivall et al. (2014).

Isochrysis galbana CCMP 1323																				
Salinity		10.3	10.2	10.3	15.3	15.3	15.3	20.3	20.3	20.3	25.2	25.2	25.2	30.3	30.3	30.3	35.6	35.6	35.6	
Replicate		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	
Water		-29	-30	-30	-22	-22	-22	-17	-17	-16	-9	-11	-10	-3	-3	-2	4	5	5	
SD		1	1	2	1	1	1	2	2	2	2	1	1	2	1	1	2	1	1	
Reference	Lipid																			
M'Boule et al. (2014)	C ₃₇	δ ² H ‰	-172	-172	-170	-158	-157	-158	-142	-140	-144	-122	-126	-121	-111	-106	-109	-97	-99	-98
		SD	1	2	1	1	1	1	3	1	3	1	1	1	1	1	2	2	3	5
		alpha	0.853	0.853	0.856	0.861	0.863	0.86	0.873	0.875	0.871	0.886	0.883	0.889	0.892	0.896	0.893	0.9	0.896	0.898
		SD	0.002	0.003	0.002	0.002	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.006
This study	Sterol	δ ² H ‰	-296	-300	-	-279	-	-285	-264	-276	-	-255	-241	-236	-	-	-	-	-	-
		SD	2	2	-	3	-	6	8	3	-	1	5	5	-	-	-	-	-	-
		alpha	0.724	0.720	-	0.736	-	0.731	0.748	0.736	-	0.752	0.766	0.771	-	-	-	-	-	-
		SD	0.004	0.003	-	0.004	-	0.009	0.011	0.005	-	0.002	0.007	0.006	-	-	-	-	-	-
This study	Phytol	δ ² H ‰	-430	-405	-	-424	-	-430	-414	-421	-	-404	-395	-393	-	-	-	-	-	-
		SD	5	4	-	5	-	4	5	4	-	2	4	1	-	-	-	-	-	-
		alpha	0.586	0.612	-	0.588	-	0.583	0.596	0.589	-	0.601	0.611	0.613	-	-	-	-	-	-
		SD	0.008	0.007	-	0.009	-	0.008	0.008	0.008	-	0.004	0.007	0.002	-	-	-	-	-	-
Heinzelmann et al. (2015)	C _{14:0}	δ ² H ‰	-290	-269	-261	-284	-266	-245	-272	-231	-239	-254	-237	-224	-242	-224	-214	-234	-211	-193
		SD	0	1	1	1	1	2	1	6	0	5	2	3	0	0	2	1	1	1
		alpha	0.731	0.732	0.740	0.753	0.761	0.763	0.752	0.749	0.780	0.771	0.777	0.785	0.758	0.770	0.772	0.780	0.784	0.799
		SD	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.001	0.002	0.002	0.002	0.002
Heinzelmann et al. (2015)	C _{16:0}	δ ² H ‰	-281	-262	-256	-271	-259	-248	-259	-237	-236	-241	-231	-221	-230	-218	-207	-229	-201	-193
		SD	2	2	1	1	2	1	3	1	2	6	2	2	2	1	1	0	1	1
		alpha	0.740	0.746	0.754	0.766	0.772	0.767	0.758	0.756	0.775	0.776	0.784	0.795	0.762	0.767	0.775	0.783	0.791	0.800
		SD	0.003	0.003	0.001	0.001	0.003	0.001	0.004	0.001	0.003	0.008	0.003	0.003	0.003	0.001	0.001	0.000	0.001	0.001
Heinzelmann et al. (2015)	C _{18:1}	δ ² H ‰	-216	-229	-215	-204	-219	-213	-184	-217	-207	-170	-203	-196	-163	-188	-181	-145	-175	-177
		SD	1	2	1	1	0	0	2	1	1	6	2	1	1	1	3	4	1	1
		alpha	0.808	0.814	0.830	0.838	0.839	0.851	0.793	0.797	0.795	0.805	0.814	0.821	0.805	0.803	0.804	0.808	0.818	0.816
		SD	0.001	0.003	0.001	0.001	0.000	0.000	0.002	0.001	0.001	0.007	0.003	0.001	0.001	0.001	0.001	0.004	0.005	0.001

Supplementary Table 1: (continued) Hydrogen isotope ratios ($\delta^2\text{H}_{\text{C}_{37}}$) and fractionation factor $\alpha_{\text{C}_{37}}$ values for lipids from *Emiliania huxleyi*, *Isochrysis galbana* and *Ruttenella lamellosa*. Alkenones from *E. huxleyi* and *I. galbana* previously published in M'Boule et al. (2014). Fatty acids from *I. galbana* are from Heinzlmann et al. (2015). Alkenones from *R. lamellosa* were previously published in Chivall et al. (2014).

<i>Ruttenera lamellosa</i> CCMP 1307																				
Salinity			10.2	10.2	10.2	15.3	15.3	15.3	20.4	20.4	20.3	25	25	25	30	30	30	35	34.9	35
Replicate			A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Water			-29	-31	-33	-23	-26	-25	-18	-16	-19	-11	-9	-11	-6	-6	-3	4	3	5
SD			1	2	2	1	2	1	2	2	2	1	2	1	2	2	2	2	2	3
Reference	Lipid																			
Chivall et al. (2014)	C ₃₇	δ ² H ‰	-183	-184	-187	-178	-181	-182	-173	-171	-171	-161	-162	-162	-148	-152	-150	-136	-136	-134
		SD	2	1	1	2	2	2	1	1	1	3	2	1	1	1	1	3	1	1
		alpha	0.842	0.843	0.840	0.841	0.841	0.839	0.842	0.842	0.844	0.849	0.846	0.847	0.857	0.853	0.853	0.860	0.861	0.862
		SD	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.001	0.002	0.002	0.002	0.003	0.002	0.002
This study	Sterol	δ ² H ‰	-293	-300	-294	-294	-294	-290	-290	-294	-285	-280	-250	-276	-274	-268	-265	-254	-224	-258
		SD	2	2	17	5	6	14	12	6	8	7	19	7	10	2	1	4	11	0
		alpha	0.728	0.723	0.730	0.723	0.725	0.728	0.723	0.717	0.728	0.728	0.757	0.732	0.730	0.737	0.737	0.743	0.774	0.739
		SD	0.003	0.003	0.017	0.005	0.006	0.014	0.013	0.006	0.008	0.007	0.018	0.007	0.010	0.003	0.002	0.005	0.010	0.003
This study	Phytol	δ ² H ‰	-413	-405	-411	-417	-416	-419	-407	-406	-399	-398	-396	-402	-391	-396	-393	-380	-379	-387
		SD	0	4	12	2	3	2	5	3	5	7	13	1	8	4	7	1	2	3
		alpha	0.604	0.614	0.609	0.597	0.600	0.596	0.604	0.604	0.612	0.608	0.609	0.605	0.612	0.608	0.608	0.617	0.619	0.610
		SD	0.002	0.005	0.015	0.003	0.004	0.003	0.007	0.005	0.006	0.008	0.015	0.002	0.009	0.005	0.008	0.002	0.003	0.005



Total lipid extracts from the Baltic Sea.

Chapter 5

Constraining the application of hydrogen isotopic composition of long-chain alkenones as a salinity proxy using marine surface sediments

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Abstract

Sea surface salinity is an essential environmental parameter necessary to understand past changes in global climate. However, reconstructing absolute salinity of the surface ocean with high enough accuracy and precision remains a complicated task. Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{C37}$) have been shown to reflect salinity in culture studies and have been proposed as a tool to reconstruct sea surface salinity in the geologic record. The correlation between $\delta^2\text{H}_{C37}$ – salinity in culture is prominently caused by the relationship between $\delta^2\text{H}_{\text{H}_2\text{O}}$ and salinity, as well as the increase in fractionation factor α with increasing salinity. The $\delta^2\text{H}_{C37}$ – salinity relationship in the natural environment is poorly understood. Here, surface sediments from a variety of environments covering a wide range of salinities were analyzed to constrain the environmental relationship between salinity and hydrogen isotopes of alkenones. $\delta^2\text{H}_{C37}$ correlates significantly ($r = 0.75$, $p < 0.0001$) with annual mean salinity. Interestingly, the biological hydrogen isotope fractionation (α_{C37}) seems independent of salinity. These findings are different from what has previously been observed in culture experiments, but align with other environmental datasets and suggest that the salinity effect on biological hydrogen isotope fractionation observed in culture is not apparent in sediments. The absence of a correlation between α_{C37} and salinity for marine surface sediments might be best explained by a mixing of multiple alkenone-producing species contributing to the sedimentary alkenone signal that fractionate in distinct ways. Nevertheless, sedimentary $\delta^2\text{H}_{C37}$ ratios still correlate with salinity and $\delta^2\text{H}_{\text{H}_2\text{O}}$, suggesting that $\delta^2\text{H}_{C37}$ ratios are useful for paleosalinity reconstructions. Our surface sediment calibration presented here can be used when different species contribute to the sedimentary alkenone pool and substantial changes in salinity are expected.

Keywords

Hydrogen isotope, alkenones, surface sediments, salinity

1 Introduction

Understanding changes in surface ocean salinity in the past would greatly increase knowledge about ocean circulation and heat transport around the globe with respect to global climate changes. A reliable tool to reconstruct sea surface salinity has long been sought after by the paleoceanographic community. A number of possibilities have been explored, but the reconstruction of sea surface salinity changes can be quite complicated (Rohling, 2007 and references therein). Thus, no direct proxy to reconstruct absolute paleosalinities exists to date. Attempts to understand past salinity changes have employed dinoflagellate (dinocysts) (Wall and Dale, 1968) and diatom assemblages in combination with transfer functions (Fritz et al., 1991; Wilson et al., 1994), but these are qualitative rather than quantitative. Both oxygen and hydrogen isotopes of water have been shown to have a strong quantitative relationship with salinity (Craig and Gordon, 1965). After a correction for calcification temperature using Mg/Ca ratios, oxygen isotope ratios of foraminiferal calcite can be used to reconstruct water oxygen isotope ratios and in turn salinity (e.g. Duplessy et al., 1991; Rohling, 2000). This method is not without issue since temporal changes in the salinity-water isotope ratio relationship occur through time, causing large uncertainties to be associated with these paleosalinity estimates (Rohling et al., 2007, and references cited therein). Hydrogen

isotope ratios can be used to estimate salinity in complement to oxygen isotope ratios since the two are strongly correlated to one another, most commonly evidenced by the global meteoric water line (Craig and Gordon, 1965). Hydrogen isotope ratios of biomarker lipids have been shown to relate to the hydrogen isotope ratios of water, both in culture and natural waters (i.e., lakes), albeit with a large biological fractionation effect (Sachs et al., 2012 and references cited therein). Thus, organic molecules could potentially be valuable proxy carriers for reconstructing the hydrogen isotopic composition of paleo water once the effect of biological fractionation is constrained.

For the marine environment, hydrogen isotope ratios measured on long-chain alkenones have been proposed as a tool to reconstruct changes in salinity (e.g. Schouten et al., 2006; van der Meer et al., 2007; Wolhowe et al., 2009; Leduc et al., 2013; Kasper et al., 2014; Sachs et al., 2016). Long-chain alkenones are produced exclusively by haptophyte algae of the order Isochrysidales (Volkman et al., 1980; de Leeuw et al., 1980). Alkenones are an attractive compound of interest due to their species specificity and the fact that the hydrogen they contain is non-exchangeable. Culture experiments, most prominently with the common modern-day marine alkenone-producing haptophyte, *Emiliania huxleyi*, show a strong linear trend between hydrogen isotope ratios of C_{37} alkenones (δ^2H_{C37}) and salinity (Schouten et al., 2006; Wolhowe et al., 2009; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). The relationship between δ^2H_{C37} and salinity has a steeper slope than the $\delta^2H_{H_2O}$ -salinity relationship showing that biological hydrogen isotope fractionation between alkenones and growth water is decreasing and the fractionation factor α_{C37} is increasing with salinity (Schouten et al., 2006; Wolhowe et al., 2009; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). As a result, the correlation between δ^2H_{C37} ratios and salinity in culture studies is the effect of both the hydrogen isotopic composition of the water and biological hydrogen isotope fractionation. Previous culture experiments have focused on the effects of different environmental parameters on α_{C37} values (Schouten et al., 2006; Wolhowe et al., 2009; M'Boule et al., 2014; Chivall et al., 2014; Sachs and Kawka, 2015; Sachs et al., 2016; Weiss et al., 2017). Several variables have been highlighted that appear to have an influence on α_{C37} values, e.g., salinity, growth rate, growth phase, light intensity, and species (Schouten et al., 2006; Wolhowe et al., 2009; Chivall et al., 2014; van der Meer et al., 2015; M'Boule et al., 2014). Alkalinity and temperature have been shown to have a negligible effect on α_{C37} values (Weiss et al., 2017; Schouten et al., 2006). However, the influence of these parameters on α_{C37} values, and therefore δ^2H_{C37} ratios, in the natural environment is poorly understood.

Despite this, there have been several paleo reconstructions of salinity using δ^2H_{C37} ratios. These reconstructions all show reasonable relative changes in salinity suggesting that δ^2H_{C37} ratios are a suitable paleoceanographic tool (van der Meer et al., 2007; Pahnke et al., 2007; Leduc et al., 2013; Kasper et al., 2014; Simon et al., 2015; Petrick et al., 2015). Nevertheless, an environmental calibration for the δ^2H_{C37} -salinity relationship is necessary to bridge the gap between culture studies and paleo reconstructions. δ^2H_{C37} ratios from surface sediments in the Chesapeake Bay Estuary, USA, were analyzed for hydrogen isotope ratios of alkenones (Schwab and Sachs, 2011). A positive linear correlation between $\delta^2H_{alkenone}$ and salinity is observed, but there is no relationship between $\alpha_{alkenone}$ and salinity, in contrast to culture results (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). One explanation proposed by Schwab and Sachs (2011) for the lack of correlation between $\alpha_{alkenone}$ and salinity is the presence of different alkenone-producing species in the Chesapeake Bay Estuary. Indeed, coastal alkenone-producing haptophyte species are characterized by more enriched δ^2H_{C37} ratios and higher $\alpha_{alkenone}$ values (less fractionation) compared to marine alkenone-producing haptophytes (M'Boule et al., 2014; Chivall et al., 2014). Therefore,

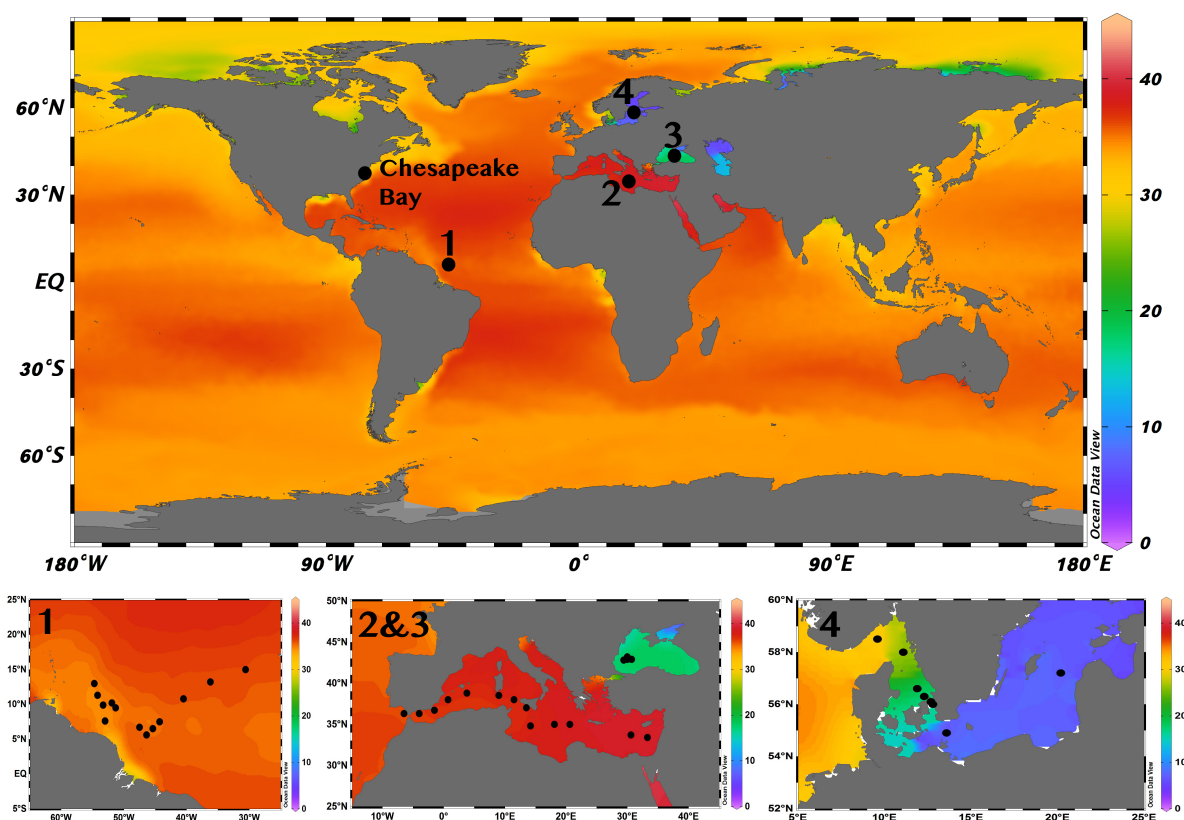


Figure 1: Global map of annual mean salinity values from the World Ocean Atlas 2013 with 0.25° grid resolution with locations of transects. (1) North Atlantic transect (North Atlantic) into Amazon River outflow with annual mean salinity from 33 to 36.5. (2) Mediterranean Sea (Mediterranean) transect covering annual mean salinity of 36.5 to 39. (3) Black Sea with an annual mean salinity of 18. (4) North Sea into the Baltic Sea transect (Baltic) covering annual mean salinity from 30 (N. Sea) to 7 (Baltic). Previously published data for North Sea into the Baltic Sea (Kaiser et al., 2017) and Chesapeake Bay Estuary (Schwab and Sachs, 2011) also included. Specific sampling locations shown in lower panel.

contributions from both groups of alkenone-producers can mute the correlation between $\alpha_{alkenone}$ and salinity. Suspended particulate matter (SPM) was also analyzed for $\delta^2\text{H}_{C37}$ ratios along a transect in the North Atlantic and shows that both $\delta^2\text{H}_{C37}$ ratios and α_{C37} values correlate with salinity, but only for SPM with an alkenone concentration $> 10 \text{ ng L}^{-1}$ (Häggi et al., 2015). The correlation with higher alkenone concentrations suggests that alkenones have been produced predominantly in situ when concentrations are higher. No correlation is observed for concentrations $< 10 \text{ ng L}^{-1}$ because the alkenones potentially represent a recalcitrant alkenone pool transported from elsewhere. Alkenones are known to be relatively recalcitrant and can therefore be transported laterally over rather long distances (Ohkouchi et al., 2002).

Here we test the relationship between hydrogen isotope ratios of alkenones and salinity by measuring hydrogen isotope ratios of alkenones preserved in surface sediments from four marine environments covering an annual mean sea surface salinity range from 7 - 39. Because these sediments are from areas covering a large salinity gradient, they also cover a range of

temperature and light conditions, as well as differences in nutrient composition, evaporation and precipitation and species composition. This allows us to test if these variables affect the relationship between hydrogen isotopic composition of alkenones and salinity in the natural marine environment.

2 Methods

Surface sediments (0-1 cm) were collected in triplicate using a multicorer aboard the RV Pelagia during six separate cruises: (1) to the Southeast North Atlantic (North Atlantic), (2) the Mediterranean Sea (in two legs) from Cyprus to Cadiz, Spain (Mediterranean), (3) the North Sea through the Skaggeak, Kattegat and into the Baltic Sea (Baltic), and the (4) the two campaigns in the Black Sea (Fig. 1). The surface sediments were freeze-dried and lipids were extracted via Accelerated Solvent Extraction (Dionex ASE 200) using dichloromethane:methanol (9:1, v/v) at 100 °C with a pressure of 7.6×10^6 Pa. Total lipid extracts were separated into apolar, ketone and polar fractions using a small aluminum oxide column following Weiss et al. (2017).

2.1 North Atlantic

Alkenones from North Atlantic surface sediments were quantified using an Agilent 6890 gas chromatograph coupled to a flame ionization detector (GC-FID) with helium as a carrier gas. Hydrogen isotope measurements were conducted using Thermo Finnigan DELTA V GC/TC/irMS. Both GCs were equipped with a CPSil5 column (Agilent, 25 m x 0.32 mm x 0.4 μ m), using the same temperature program as M'Boule et al. (2014). Prior to running samples for isotope ratios each day, the H_3^+ factor was measured and ranged between 4.4 – 5.4 ppm nA^{-1} , with a variation of less than 0.2 ppm nA^{-1} from day to day. An external standard, Mix B (supplied by A. Schimmelmann, Indiana University), was run each day prior to running samples. Samples were only analyzed when the average difference from the offline values and standard deviation of the Mix B were less than 5 ‰. Squalane and a C_{30} *n*-alkane were co-injected with each hydrogen isotope run, and values had an average δ^2H ratio of -158.7 ± 5.8 ‰ (squalane) and -77.9 ± 8.0 ‰ (C_{30} *n*-alkane); standard deviations are relatively high for this sample set due to the comparatively high and variable background with co-eluting compounds in these samples at or close to the time squalane and C_{30} elute from the column. Ketone fractions from the same sampling station were combined prior to hydrogen isotope measurement when alkenone abundance was too low (based on GC-FID quantification). The reported isotope ratios and standard deviations are the average of duplicate measurements. Due to the lack of baseline separation with the CPSil5 GC column, we report only the isotope ratios of combined C_{37} alkenones for these samples (following van der Meer et al., 2013).

2.2 Mediterranean Sea, Baltic Sea and Black Sea

For alkenone quantification of the Mediterranean Sea, Baltic Sea and Black Sea sediments, ketone fractions were run on an Agilent 6890 GC-FID equipped with an RTX-200 column (Restek, 60 m x 0.32 mm x 0.5 μ m), which allowed improved separation of alkenones with different chain lengths and degrees of unsaturation, similar to Longo et al. (2013), but with slightly different column dimensions (see Supplementary Fig. 1 for comparison of separated

and integrated $\delta^2\text{H}$ ratios). The GC temperature program was as follows: 70 °C to 250 at 18 °C/min, 250-320 °C at 1.5°C/min, then 320 °C for 25 min at a flow rate of 1.5mL/min.

For additional cleanup of the alkenones prior to $\delta^2\text{H}$ measurements, we separated the ketone fractions over a silver nitrate impregnated silica column using the following solvents: 100 % dichloromethane, dichloromethane / ethyl acetate (1:1, v/v). and 100 % ethyl acetate, with the alkenones ending up in the dichloromethane / ethyl acetate fraction. This clean-up step was especially necessary for the Mediterranean samples which contained high and variable backgrounds.

Alkenone quantification (Supplementary Table 1) was sufficient for determination of the $U_{37}^{K'}$ index (Fig. 2, Supplementary Table 2), but was often too low for duplicate $\delta^2\text{H}$ measurements. For this reason, ketone fractions from the same sampling station were combined when necessary prior to measurements of isotope ratios. Ketone fractions from Mediterranean Sea sediment extracts were run on GC-FID using both CPSil5 and RTX-200 columns to determine whether significant differences occur for calculation of the $U_{37}^{K'}$ index between columns (Supplementary Table 3).

Hydrogen isotope ratios of alkenones were measured on a Thermo Finnigan DELTA V GC/TC/irMS with an RTX-200 column using the same GC temperature program as described above for GC-FID. The H_3^+ factor varied from 4.2 – 5.2 ppm nA^{-1} for the Mediterranean Sea, 2.9 – 3.0 ppm nA^{-1} for the Black Sea, and 2.4 – 4.5 for the Baltic Sea. The larger ranges are due to cleaning and tuning of the ion source in between analyzing sample batches while day to day variation was < 0.2 ppm nA^{-1} . As with the North Atlantic samples, an external Mix B standard was run each day before measuring samples, and we only proceeded with running samples once the average difference from the offline values and standard deviation of the Mix B were less than 5 ‰. An in house alkenone mix was run periodically to further assess machine stability. Squalane and a C_{30} *n*-alkane were co-injected with each run; $\delta^2\text{H}$ values for the Mediterranean sample series had an average of -171.9 ± 3.9 ‰ (squalane) and -78.0 ± 4.6 ‰ (C_{30} *n*-alkane), the Baltic Sea sample series had an average of -166.2 ± 2.9 ‰ (squalane) and -71.3 ± 2.5 ‰ (C_{30} *n*-alkane), and the Black Sea sample series had an average of -165.8 ± 1.9 ‰ (squalane) and -69.2 ± 1.4 ‰ (C_{30} *n*-alkane). Isotope ratios for the individual alkenones are reported (Table 1, Supplementary Fig. 1), but we use the values of the integrated C_{37} alkenones for statistical comparisons with the North Atlantic samples and previously published Baltic Sea data (Kaiser et al., 2017). Reported isotope ratios and standard deviations are the average of duplicate analytical runs.

2.3 Sea surface water isotopes, salinity and temperature

Water samples were collected from surface waters at the same location that the sediment samples were taken, and salinity, temperature and $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios were measured. $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios were measured using TC/EA/irMS following methods of M'Boule et al. (2014). Water was not collected for isotopic analyses along the North Atlantic transect, therefore, we estimated $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios using the Schmidt et al. (1999) Global Seawater Oxygen isotope database and the global meteoric waterline equation following Craig and Gordon (1965):

$$\delta^2\text{H} = 8 \times \delta^{18}\text{O} + 10 \quad (1)$$

Hydrogen isotope fractionation factor $\alpha_{\text{C}_{37}}$ was calculated for all samples using the equation:

$$\frac{\delta^2 H_{C37} + 1000}{\delta^2 H_{H2O} + 1000} \quad (2)$$

Seasonal and annual mean sea surface temperatures and salinity were taken from the World Ocean Atlas (Locarnini et al., 2010; Antonov et al., 2010).

3 Results

3.1 Sea water

$\delta^2 H_{H2O}$ ratios, temperature, and salinity of surface waters were measured at each sampling station where sediment was collected, except for the North Atlantic transect where only temperature and salinity were measured. Temperature ranged from 27.1 to 29.4 °C for the North Atlantic, 14.0 to 19.0 °C for the Mediterranean, 8.3 °C for the Black Sea, and 10.6 to 11.9 °C for the Baltic; salinity ranged from 31.1 to 36.4 for the North Atlantic, 36 to 39 for the Mediterranean, 18.5 for the Black Sea and 7 to 30 for the Baltic; $\delta^2 H_{H2O}$ ratios ranged from 6 to 10 ‰ for the North Atlantic, 7 to 14 ‰ for the Mediterranean, -18 ‰ for the Black Sea, and -51 to -9 ‰ for the Baltic Sea (Table 1). As mentioned in the introduction, a strong correlation between $\delta^2 H_{H2O}$ and salinity is expected for surface ocean waters, as both are impacted in a similar fashion by the hydrological cycle (Craig and Gordon, 1965; Frölich et al., 1988). Indeed, for the combined Mediterranean, Black Sea, and Baltic Sea transects, where we measured both $\delta^2 H_{H2O}$ and salinity at each sampling location, there is a strong significant relationship between measured salinity and $\delta^2 H_{H2O}$ ($r = 0.99$, $p < 0.001$; Fig. 3a). When using the Schmidt et al. (1999) database to estimate $\delta^2 H_{H2O}$ for the North Atlantic transect (where only salinity was measured at each sampling location), the correlation still remains highly significant ($r = 0.98$, $p < 0.001$).

3.2 $U_{37}^{K'}$ values

$U_{37}^{K'}$ index values were determined on a standard CPSil5 25 m as well as on a 60 m RTX-200 GC column, which we also used for compound specific hydrogen isotope analysis. To determine if the $U_{37}^{K'}$ index values measured on the two GC columns were different, we ran all Mediterranean samples on both columns (Supplementary Table 3). Using the student t-test, we determined that the differences between the $U_{37}^{K'}$ index calculated based on analyses from both columns are not statistically different. Therefore, we can compare the $U_{37}^{K'}$ index from the North Atlantic (measured on the CPSil5 column) with those from the other transects (measured on the RTX-200 column). The $U_{37}^{K'}$ index ranges from 0.74 – 0.99 for the North Atlantic, from 0.50 – 0.76 for the Mediterranean from 0.45 – 0.53 for the Black Sea, and 0.07 – 0.52 for the Baltic Sea (Supplementary Table 2). $U_{37}^{K'}$ index was converted to temperatures using the BAYSPLINE calibration (Tierney and Tingley, 2018). Temperatures range from 20.3 °C to 30.0 °C for the North Atlantic, 11.1 °C to 20.7 °C for the Mediterranean, 11.8 °C to 14.1 °C for the Black Sea, and 0.6 °C to 13.8 °C for the Baltic Sea (Fig. 2).

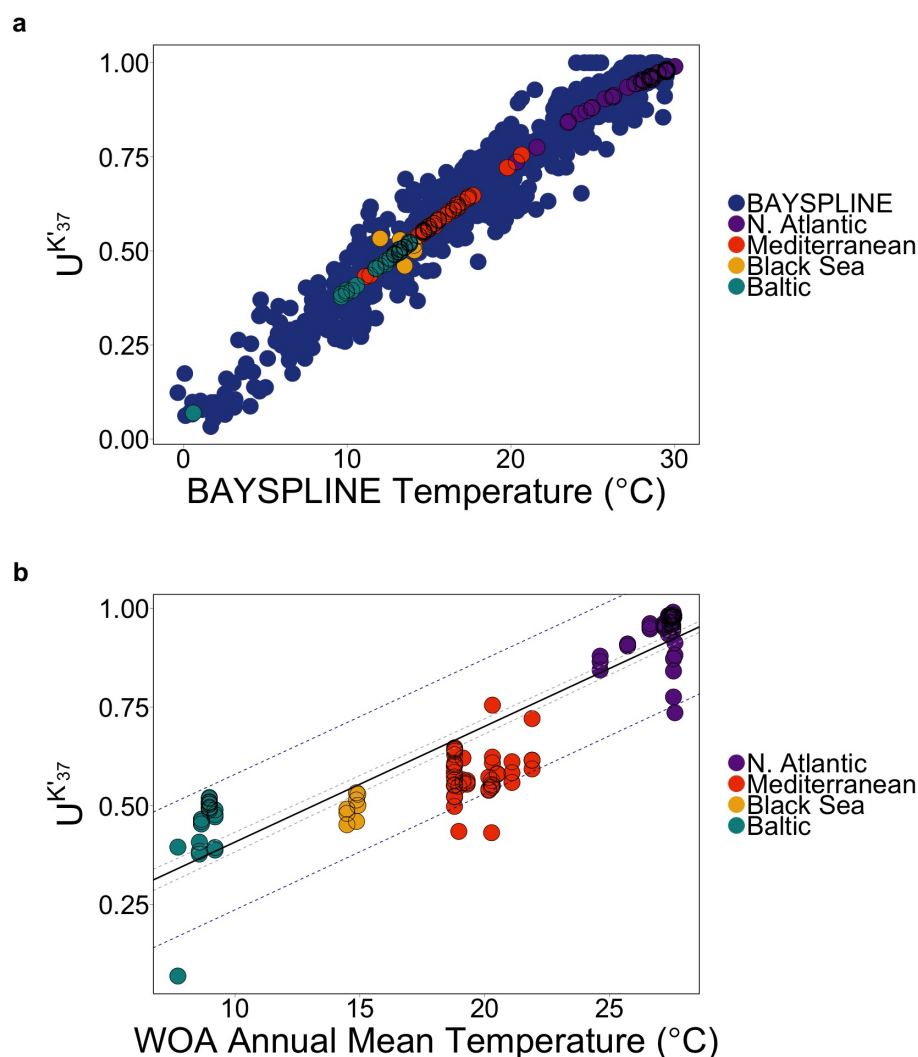


Figure 2: (a) $U_{37}^{K'}$ index calculated for our dataset calibrated to temperature using BAYSPLINE (Tierney and Tingley, 2018), and colored by transect. (b) $U_{37}^{K'}$ values for our dataset are plotted with core-top values from the BAYSPLINE calibration. The fact that our values align well with the BAYSPLINE compilation confirms that our dataset strongly reflects surface water conditions.

3.3 Correlations of hydrogen isotope ratios and fractionation with salinity

Correlations between $\delta^2\text{H}_{C37}$ ratios and salinity for individual transects are all poor with p values that are not significant (Table 2). The relationships between α_{C37} and salinity are likewise not statistically significant for the individual transects. The Baltic Sea is an exception, probably as a result of the larger salinity gradient covered by this transect. There is a significant positive correlation between $\delta^2\text{H}_{C37}$ ratios with annual mean salinity ($r = 0.83$, $p < 0.001$). When previously published marine surface sediment data from the Chesapeake Bay Estuary, USA (transformed to the weighted mean C_{37} value by using reported $U_{37}^{K'}$ and individual $\delta^2\text{H}_{C37}$ ratios from Schwab and Sachs, 2011) and the Baltic Sea (Kaiser et al., 2017) are included ($n = 18$), there remains a significant correlation with annual mean salinity

($r = 0.75$, $p < 0.001$). The correlation between $\delta^2\text{H}_{C37}$ – annual mean salinity is characterized by the linear regression equation:

$$\delta^2\text{H}_{C37} = 1.3 \times S + 227 \quad (R^2 = 0.55, p < 0.001, n = 95, \text{Fig. 4a}) \quad (3)$$

For the combined dataset, there is a weak negative correlation between α_{C37} and annual mean salinity ($r = -0.27$, $p < 0.05$; Fig. 4b).

4 Discussion

4.1 $U_{37}^{K'}$ temperatures

The $U_{37}^{K'}$ index is a well-known and well-studied proxy for sea surface temperature (SST) (Brassell et al., 1986; Müller et al., 1998; Tierney and Tingley, 2018). Although $U_{37}^{K'}$ is shown to vary with depth of alkenone production, alkenones preserved in sediments are strongly reflecting surface mixed layer conditions (Prah et al., 2005). To calibrate $U_{37}^{K'}$ values to SSTs, we used the BAYSPLINE regression model, which is more accurate than previous calibrations for temperatures above 24 °C (Tierney and Tingley, 2018). The $U_{37}^{K'}$ -SST relationship for this dataset aligns nicely with the compilation of global core-top $U_{37}^{K'}$ SST values (Tierney and Tingley, 2018; Fig. 2), which leads to the conclusion that alkenones along these transects are also recording surface water values. In general, nannoplankton blooms, which include haptophytes, occur during the spring, following a bloom of diatoms (Lochte et al., 1993). In the Eastern Mediterranean, coccolithophore production reaches a maximum between late winter and early spring (Ziveri et al., 2000) and the largest haptophyte blooms in the Western Mediterranean occur in fall (Knappertsbusch, 1993; Cacho et al., 1999). To determine if the sedimentary alkenones from the compiled dataset are affected by differences in seasonality due to the seasonal production of alkenones, we tested how well the $U_{37}^{K'}$ index correlates with annual mean and seasonal mean surface water temperatures from the World Ocean Atlas. $U_{37}^{K'}$ values correlate positively to both spring and annual mean temperatures ($r = 0.89$ for annual and $r = 0.92$ for spring), allowing us to conclude that alkenones preserved along these transects are reflecting surface water conditions and $\delta^2\text{H}_{C37}$ ratios do not contain a strong seasonality signal.

4.2 Hydrogen isotope ratios of alkenones

To test how accurately $\delta^2\text{H}_{C37}$ ratios are tracking surface water isotope ratios, we first compared the $\delta^2\text{H}_{C37}$ ratios of the sediments with the $\delta^2\text{H}_{H2O}$ ratios measured in surface water at the sampling locations. Sedimentary alkenones represent possible lateral transport (Ohkouchi et al., 2002) and accumulation over several decades to centuries, while the water signal covers several weeks to months. Nonetheless, there is a positive linear correlation ($r = 0.80$, $p < 0.001$) between surface water isotope ratios and $\delta^2\text{H}_{C37}$, represented by the linear regression equation:

$$\delta^2\text{H}_{C37} = 0.84 \times \delta^2\text{H}_{H2O} - 186 \quad (R^2 = 0.64, p < 0.001, n = 75, \text{Fig. 3b}) \quad (4)$$

This confirms that our $\delta^2\text{H}_{C37}$ ratios are plausibly recording surface water conditions. Since the $\delta^2\text{H}_{C37}$ ratios of alkenones seem to be recording the $\delta^2\text{H}_{H2O}$ ratios of surface water, which

Surface sediment calibration

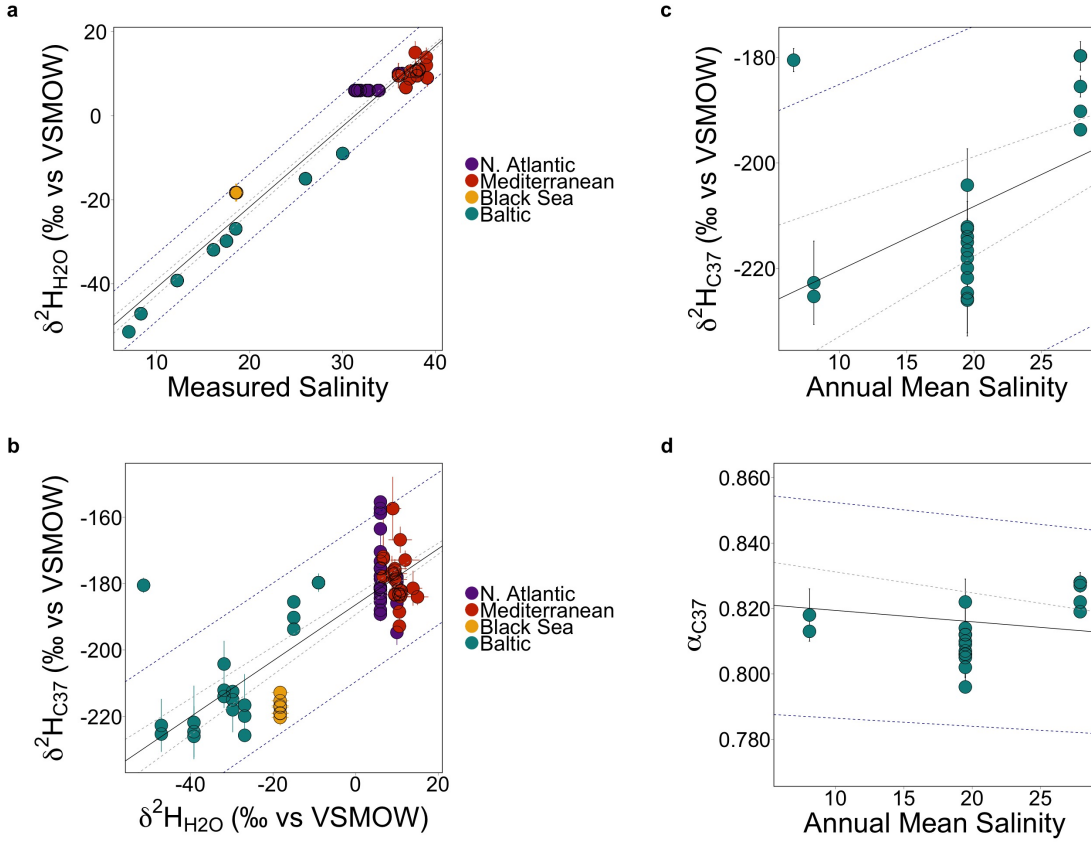


Figure 3: Surface water salinity measured during sampling plotted against hydrogen isotope ratios of surface water also collected during sampling for all transects are shown in (a), with estimated water isotope values for the North Atlantic Transect from Schmidt et al., 1999. (b) shows water isotope ratios of surface water plotted against hydrogen isotope ratios of alkenones extracted from surface sediments for all transects. Results for the correlation between annual mean salinity and $\delta^2\text{H}_{\text{C}37}$ for the Baltic Sea transect are shown in (c) and $\alpha_{\text{C}37}$ is plotted against annual mean salinity for the Baltic Sea transect in (d). Annual mean salinity values were taken from the World Ocean Atlas (Antonov et al., 2010; Locarnini et al., 2010). Y-axis errors are the standard deviations of duplicate analyses and X-axis errors are the standard errors of the statistical mean for the 0.25° gridded WOA annual mean salinity dataset.

itself is strongly correlated to salinity, a strong correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and salinity is expected. Rather than using measured salinity from each sampling station, we used the seasonal mean and annual mean salinities from the World Ocean Atlas to better align with an integrated sedimentary signal (Locarnini et al., 2010; Antonov et al., 2010). Because each transect covers a relatively small range of salinities, we focus on the combined transect. Our $U_{37}^{K'}$ values suggest that there is not a strong seasonal signal recorded by the alkenones for the combined dataset, and we observed the same for $\delta^2\text{H}_{\text{C}37}$ ratios (Table 2). The Baltic Sea transect covers a relatively large salinity range from approximately 7 – 30, and the $\delta^2\text{H}_{\text{C}37}$ ratios from the Baltic Sea transect correlate best with spring salinity ($r = 0.52$, $p < 0.05$; Fig. 3c). However, at lower salinities this overall positive correlation deviates from what is observed in culture studies (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016). $\delta^2\text{H}_{\text{C}37}$ ratios are more enriched at lowest salinities, hinting that $\delta^2\text{H}_{\text{C}37}$ ratios might be

influenced by some other variable. As mentioned above, we combined new data presented here with previously published data to extend the salinity gradient from 7 - 39. For the combined dataset, there is a significant, positive linear correlation between $\delta^2\text{H}_{\text{C}37}$ ratios with annual mean salinity (see equation 3, Table 2, Fig. 4).

In cultures, a strong correlation between hydrogen isotope fractionation factor $\alpha_{\text{C}37}$ values and salinity is reported (Schouten et al., 2006; M'Boule et al., 2014; Chivall et al., 2014; Sachs et al., 2016; Weiss et al., 2017). Calculating $\alpha_{\text{C}37}$ values from sediment data is difficult as we do not have the $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios over multiple years to decades. As a substitute, we used the $\delta^2\text{H}_{\text{H}_2\text{O}}$ ratios of surface waters taken at the surface sediment sampling locations to roughly estimate $\alpha_{\text{C}37}$. Surprisingly, we do not find a significant correlation between $\alpha_{\text{C}37}$ and annual mean or seasonal salinity (Table 2, Fig 4b); seasonal correlations can be found in Table 2). A lack of correlation between $\alpha_{\text{C}37}$ and salinity has also been observed for alkenones in surface sediments of hypersaline lakes (Nelson and Sachs, 2014), and in the Chesapeake Bay Estuary (Schwab and Sachs, 2011) as well as in suspended particulate organic matter (SPOM) from a combined Atlantic and Pacific Ocean survey (Gould et al., 2019). The latter dataset includes alkenones produced by the two main open ocean haptophytes, *G. oceanica* and *E. huxleyi*, which are both shown to fractionate differently (Schouten et al., 2006). For the surface sediment datasets, the lack of correlation between $\alpha_{\text{C}37}$ and salinity is attributed to mixing of alkenones produced by haptophyte species from both marine and marginal marine environments. We explore the potential species effect on our dataset in section 4.4.

4.3 Environmental factors impacting $\delta^2\text{H}$ ratios of alkenones

Based on the $\text{U}_{37}^{K'}$ index, we infer that our dataset is reflecting surface water conditions. Indeed, we see a significant correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and sea surface salinity, but no such correlation is observed between $\alpha_{\text{C}37}$ and salinity, a thought-provoking observation warranting further investigation. The North Atlantic surface sediments appear to have a much larger range in both $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values than expected based on the small range of annual mean salinity (34.8 – 36.5). In fact, the measured surface salinity is much larger (31 – 36.5) than annual mean salinity and captures the effect of the Amazon river outflow at the time of sampling. Freshwater from the Amazon river not only lowers surface salinities, but also likely transports haptophytes and the alkenones they produce from elsewhere. This may explain the $> 20 \text{ ‰}$ variation in $\delta^2\text{H}_{\text{C}37}$ ratios observed for the North Atlantic transect. A similar range in $\delta^2\text{H}_{\text{C}37}$ ratios was identified in suspended particulate matter along a nearby transect (Häggi et al., 2015). The large range in $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values might be a regional artefact and the result of lateral transport. Furthermore, as already noted, Baltic Sea sediments from lower salinities have more enriched $\delta^2\text{H}_{\text{C}37}$ ratios and deviate from the $\delta^2\text{H}_{\text{C}37}$ – salinity trend. As a consequence, factors other than salinity are conceivably affecting $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values and obscuring correlations. Therefore, we compared $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values with other environmental factors, like mean annual and seasonal temperatures and nutrients derived from the World Ocean Atlas (Locarnini et al., 2010; Antonov et al., 2010; Garcia et al., 2010) to determine the impacts of other environmental parameters on $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values.

In the natural environment, higher nutrient concentrations provide the potential for higher growth rates. To assess whether growth rate has an influence on our dataset, we tested the correlation between our $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values with annual mean nitrate (N) and phosphate (P) from the World Ocean Atlas (Antonov et al., 2010; Garcia et al., 2010). For our dataset, there is no correlation between $\alpha_{\text{C}37}$ values and either annual mean N or P, but

there is a negative correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and both annual mean N and P (Table 2). Higher nutrient values correspond to more depleted isotope ratios (Fig. 5), prompting further investigation about how growth rate effects might influence our dataset. Similar effects have been noted in chemostats of *E. huxleyi* via a correlation between higher nutrient composition and more depleted isotope ratios (Sachs and Kawka, 2015). To better understand the correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and annual mean P, we examined the relationship between annual mean P and annual mean salinity. There is a strong negative correlation between annual mean salinity and annual mean P ($r = -0.84$, $p < 0.001$; Fig. 5), implying that the relationship between $\delta^2\text{H}_{\text{C}37}$ ratios and annual mean P is probably controlled by salinity. The correlation between annual mean salinity and annual mean P is anti-correlated to the $\delta^2\text{H}_{\text{C}37}$ -annual mean salinity correlation. Because of this, we propose that growth rate probably does not strongly influence the $\delta^2\text{H}_{\text{C}37}$ ratios for our dataset, and we suggest that this cannot explain the apparent lack of correlation between $\alpha_{\text{C}37}$ values and salinity.

Interestingly, there is a positive linear correlation with $\delta^2\text{H}_{\text{C}37}$ ratios and temperature, with more enriched values corresponding to higher temperatures (Table 2). The best correlations are with fall and winter temperature (fall: $r = 0.75$, $p < 0.0001$, winter: $r = 0.74$, $p < 0.0001$; Table 2). A moderate correlation is observed between $\alpha_{\text{C}37}$ values and temperature (Table 2). In culture, the effect of temperature is not well constrained. Some results suggest no effect of temperature on either $\delta^2\text{H}_{\text{C}37}$ ratios or $\alpha_{\text{C}37}$ values (Schouten et al., 2006). For other batch cultures, a negative correlation between temperature and both $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values is reported, meaning more enriched isotope ratios and less fractionation correspond to lower temperatures (Wolhowe et al., 2009). Because the culture results on the temperature effect are contradictory to one another and to what is observed in the field, it is difficult to confidently conclude that the temperature effect observed for our sample set is a true temperature effect. Furthermore, temperature and salinity are correlated with each other ($r = 0.75$, $p < 0.001$). The warmer regions have higher salinities and vice versa. Differences between evaporation and precipitation for the separate environments, for example between the evaporative Mediterranean Sea and the other transects, could be the cause of the strong correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and temperature. Thus, the observed temperature relationship could possibly be an indirect correlation and not actually a temperature effect.

Light intensity has been shown to influence both $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values in culture and in the water column (van der Meer et al., 2015; Wolhowe et al., 2015). Wolhowe et al. (2015) report $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values from suspended particulate matter from the Gulf of California and Eastern Tropical North Pacific, and results show a depth effect on $\alpha_{\text{C}37}$ values, with lower depths corresponding to higher fractionation (lower $\alpha_{\text{C}37}$). This depth effect is actually in all likelihood the effect of light intensity, since light intensities are lower at deeper depths. The trend between $\alpha_{\text{C}37}$ and depth in suspended particulate matter reported by Wolhowe et al. (2015) agrees with culture data assessing the effect of light intensity on $\alpha_{\text{C}37}$ values (van der Meer et al., 2015). $\alpha_{\text{C}37}$ values from our samples fall in the higher light range, above $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, where there is a negligible effect of light intensity (Fig. 5). We cannot completely rule out the effects of light and water depth on our sample set, but because $\alpha_{\text{C}37}$ values for our dataset align with $\alpha_{\text{C}37}$ values from higher light intensities ($> 200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), we assume that the light and depth effects are minimal. Recent results from SPM in the North Pacific also suggest that light availability and nutrient limitation have a smaller effect on both $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values than has been shown in culture (Wolfshorndl et al., 2019), aligning with our conclusions that light and growth rate effects are negligible for the data presented here.

Table 1: Hydrogen isotope ratios of surface water and long-chain alkenones, along with surface water parameters for the North Atlantic, Mediterranean, Black Sea and Baltic Sea transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$\delta^2\text{H}_{\text{C}_{37:3}}$ (‰ vs. VSMOW)			$\delta^2\text{H}_{\text{C}_{37:2}}$ (‰ vs. VSMOW)			Combine $\text{d } \delta^2\text{H}_{\text{C}_{37:2}}$ (‰ vs. VSMOW)			$\delta^2\text{H}_{\text{H}_{2}\text{O}}$ (‰ vs. VSMOW)			$\alpha_{\text{C}_{37}}$	S.E.	WOA Annual mean salinity	S.E.
					S.D.			S.D.			S.D.			S.D.						
North Atlantic	1	1a	15.0	-30.6	-	-	-	-	-	-	-178	0	36.4	27.1	10	-	0.806	-	36.3	0.0
		1b			-	-	-	-	-	-	-186	1				-	0.814	-	36.3	0.0
	3	3a	13.2	-36.2	-	-	-	-	-	-	-180	0	36.4	27.3	10	-	0.812	-	36.3	0.1
		5a	10.8	-40.5	-	-	-	-	-	-	-179	0	36.0	28.0	10	-	0.813	-	35.9	0.1
	7	5b			-	-	-	-	-	-	-195	4				-	0.797	-	35.9	0.1
		7a	7.5	-44.3	-	-	-	-	-	-	-173	1	32.6	29.2	6	-	0.822	-	35.6	0.1
	8	7b			-	-	-	-	-	-	-159	2				-	0.836	-	35.6	0.1
		8a	6.5	-45.4	-	-	-	-	-	-	-164	0	31.9	29.2	6	-	0.832	-	35.0	0.4
	9	8b			-	-	-	-	-	-	-176	5				-	0.82	-	35.0	0.4
		9a	5.6	-46.4	-	-	-	-	-	-	-170	7	31.4	28.9	6	-	0.825	-	35.7	0.2
	10	9b			-	-	-	-	-	-	-178	2				-	0.817	-	35.7	0.2
		10a	6.7	-47.5	-	-	-	-	-	-	-182	7	31.3	29.4	6	-	0.814	-	35.4	0.1
	13	10b			-	-	-	-	-	-	-179	0				-	0.817	-	35.4	0.1
		13a	7.6	-53.0	-	-	-	-	-	-	-188	0	32.8	28.0	6	-	0.807	-	34.3	0.3
		13b			-	-	-	-	-	-	-182	1				-	0.813	-	34.3	0.3
		13c			-	-	-	-	-	-	-189	2				-	0.807	-	34.3	0.3
	14	14a	9.5	-51.3	-	-	-	-	-	-	-185	0	31.6	28.5	6	-	0.814	-	35.0	0.2
		14b			-	-	-	-	-	-	-181	2				-	0.81	-	35.0	0.2
	16	16a	10.2	-51.9	-	-	-	-	-	-	-183	2	33.9	29.2	6	-	0.812	-	35.1	0.1
		17a	9.9	-53.3	-	-	-	-	-	-	-181	1	31.6	29.0	6	-	0.814	-	34.9	0.3
	20	17b			-	-	-	-	-	-	-175	2				-	0.82	-	34.9	0.3
		20a	11.3	-54.2	-	-	-	-	-	-	-155	2	33.9	29.2	6	-	0.811	-	35.0	0.4
	21	20b			-	-	-	-	-	-	-184	1				-	0.84	-	35.0	0.4
		21a	11.3	-54.2	-	-	-	-	-	-	-157	2	33.8	28.9	6	-	0.838	-	35.0	0.4

Surface sediment calibration

Table 1: (continued) Hydrogen isotope ratios of surface water and long-chain alkenones, along with surface water parameters for the North Atlantic, Mediterranean, Black Sea and Baltic Sea transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$\delta^2\text{H}_{\text{C}_{37:3}}$ (‰ vs. VSMOW)		$\delta^2\text{H}_{\text{C}_{37:2}}$ (‰ vs. VSMOW)		Combine $d\delta^2\text{H}_{\text{C}_{37}}$ (‰ vs. VSMOW)		Measured		$\delta^2\text{H}_{120}$ (‰ vs. VSMOW)		$\alpha_{\text{C}_{37}}$	S.E.	WOA Annual mean salinity	S.E.
					S.D.		S.D.		S.D.		Salinity	Temperature	S.D.					
Mediterranean	406 - 1	E_1a	33.3	33.4	-178	2	-180	2	-181	5	39.0	19.0	14	2	0.807	0.003	39.1	0.0
	406 - 2	E_2a	33.7	30.6	-156	9	-170	1	-173	3	39.0	18.0	12	4	0.817	0.003	39.0	0.0
	406 - 5	E_5a	35.0	20.6	-175	1	-176	3	-177	2	39.1	17.0	9	2	0.815	0.002	38.6	0.0
		E_5b			-146	14	-155	7	-157	10				2	0.835	0.004	38.6	0.0
	407 - 1	W_1a	34.8	14.2	-176	6	-186	3	-184	2	37.8	17.0	15	3	0.804	0.002	37.9	0.1
	407 - 2	W_2a	37.0	13.5	-184	8	-177	1	-183	6	38.2	15.2	11	2	0.808	0.003	37.3	0.0
		W_2b			-183	1	-177	6	-182	5				2	0.809	0.003	37.3	0.0
		W_2c			-180	3	-183	4	-182	4				2	0.809	0.003	37.3	0.0
	407 - 3	W_3a	38.0	11.5	-180	4	-179	1	-176	1	38.0	15.0	10	2	0.816	0.002	37.6	0.0
		W_3b			-186	3	-180	2	-183	3				2	0.809	0.002	37.6	0.0
	407 - 4	W_4a	38.5	9.0	-186	5	-170	7	-184	5	38.2	14.6	11	3	0.807	0.003	37.7	0.0
		W_4b			-166	5	-170	5	-167	4				3	0.824	0.003	37.7	0.0
	407 - 6	W_6a	38.8	3.8	-177	5	-177	3	-177	5	37.2	15.5	9	2	0.816	0.003	37.3	0.0
	407 - 7	W_7a	38.0	0.7	-194	1	-194	2	-193	1	37.3	15.5	11	2	0.799	0.002	37.5	0.0
		W_7b			-187	4	-179	21	-189	3				2	0.803	0.002	37.5	0.0
	407 - 8	W_8a	36.7	-1.5	-185	2	-172	3	-182	0	37.9	14.0	11	2	0.810	0.002	37.0	0.0
		W_8b			-184	6	-184	4	-183	5				2	0.808	0.003	37.0	0.0
		W_8c			-183	0	-186	1	-183	1				2	0.808	0.002	37.0	0.0
	407 - 9	W_9a	36.3	-4.0	-178	1	-179	2	-178	1	36.8	15.2	7	1	0.817	0.002	37.0	0.0
		W_9b			-170	6	-172	12	-172	6				1	0.823	0.003	37.0	0.0
		W_9c			-173	7	-167	20	-173	12				1	0.822	0.004	37.0	0.0
	407 - 10	W_10a	36.3	-6.5	-179	2	-179	9	-179	3	36.0	15.0	10	3	0.813	0.003	36.1	0.0
		W_10b			-179	4	-185	3	-183	0				3	0.809	0.002	36.1	0.0
		W_10c			-177	3	-171	12	-176	3				3	0.817	0.003	36.1	0.0
		W_10d			-173	0	-180	1	-177	1				3	0.815	0.002	36.1	0.0

Table 1: (continued) Hydrogen isotope ratios of surface water and long-chain alkenones, along with surface water parameters for the North Atlantic, Mediterranean, Black Sea and Baltic Sea transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$\delta^2\text{H}_{\text{C37:3}}$ (‰ vs. VSMOW)	S.D.	$\delta^2\text{H}_{\text{C37:2}}$ (‰ vs. VSMOW)	S.D.	Combined $\delta^2\text{H}_{\text{C37}}$ (‰ vs. VSMOW)	S.D.	Measured Salinity	Measured Temperature e	$\delta^2\text{H}_{\text{HDO}}$ (‰ vs. VSMOW)	S.D.	α_{C37}	S.E.	WOA Annual mean salinity	S.E.
Black	12	12a	42.9	30.0	-218	0	-221	1	-219	0	18.5	8.3	-18	2	0.796	0.002	18.2	0.2
		12b			-211	1	-215	2	-213	2				2	0.802	0.002	18.2	0.2
	2	2a	42.9	30.7	-215	1	-216	1	-215	1	18.5	8.3	-18	2	0.799	0.002	18.2	0.2
		2b			-217	0	-218	0	-217	0				2	0.797	0.002	18.2	0.2
	4	2c			-220	2	-221	2	-220	2				2	0.794	0.002	18.2	0.2
		4a	42.8	29.4	-218	3	-220	3	-219	3	18.5	9.4	-18	2	0.795	0.003	18.2	0.4
		4b			-214	0	-219	1	-217	1				2	0.798	0.002	18.2	0.4
Baltic	1	1a	58.5	9.6	-182	2	-177	1	-180	3	30.0	11.5	-9	1	0.828	0.002	27.9	0.1
		1b			-181	2	-181	4	-180	1				1	0.828	0.002	27.9	0.1
	2	2a	58.0	11.1	-190	0	-192	2	-190	1	26.0	11.5	-15	1	0.822	0.002	27.9	0.1
		2b			-189	1	-199	0	-194	1				1	0.819	0.001	27.9	0.1
	3	2c			-188	1	-184	4	-186	2				1	0.827	0.002	27.9	0.1
		3a	56.6	11.9	-215	1	-208	4	-212	1	16.1	11.5	-32	1	0.814	0.001	19.5	0.1
	3	3b			-206	7	-203	7	-204	7				1	0.822	0.003	19.5	0.1
		3c			-211	5	-218	2	-214	3				1	0.812	0.003	19.5	0.1
	4	4a	56.3	12.3	-213	5	-217	4	-215	4	17.5	10.7	-30	1	0.809	0.003	19.5	0.1
		4b			-209	0	-215	1	-213	1				1	0.812	0.002	19.5	0.1
	4	4c			-216	7	-220	7	-218	7				1	0.806	0.003	19.5	0.1
		5a	56.1	12.7	-214	8	-210	6	-217	9	18.5	11.2	-27	1	0.805	0.004	19.5	0.1
	5	5b			-225	0	-227	1	-226	1				1	0.796	0.001	19.5	0.1
		5c			-221	0	-221	1	-220	1				1	0.802	0.001	19.5	0.1
	6	6a	56.0	12.8	-222	11	-221	12	-222	11	12.2	12.0	-39	1	0.810	0.004	19.5	0.1
		6b			-224	8	-232	1	-225	8				1	0.807	0.004	19.5	0.1
	7	6c			-223	2	-227	1	-226	0				1	0.806	0.001	19.5	0.1
		7a	54.9	13.6	-222	8	-221	11	-223	8	8.3	11.9	-47	0	0.818	0.004	8.1	0.0

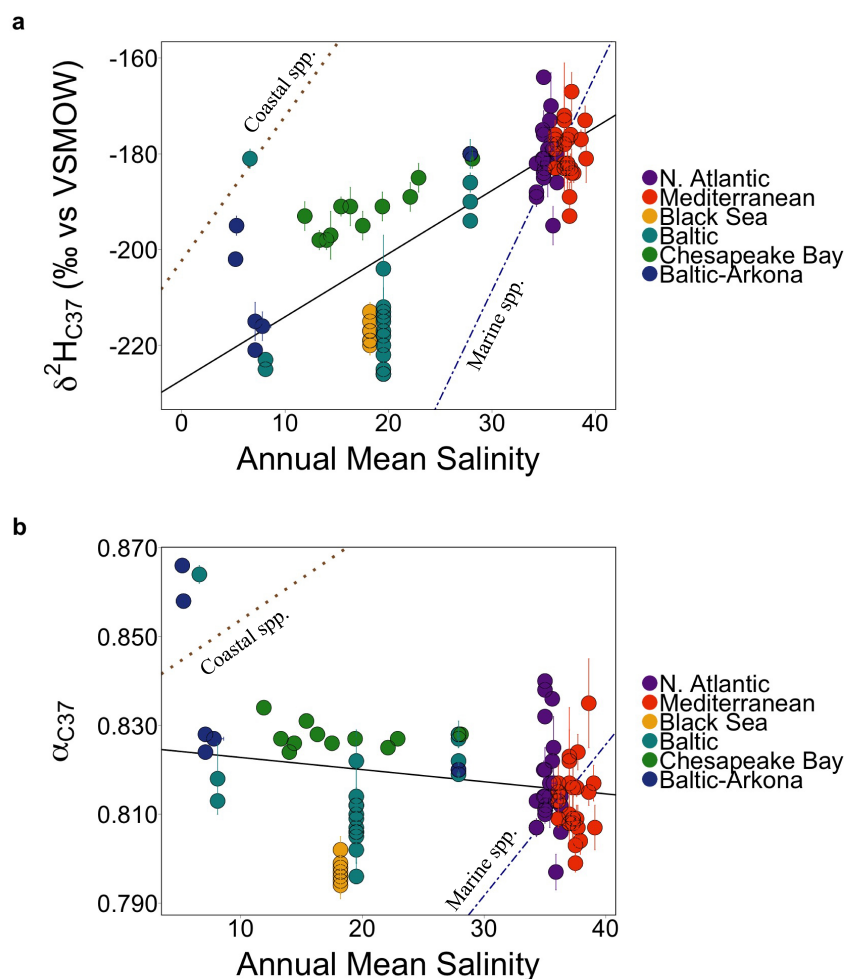


Figure 4: Annual mean salinity plotted against (A) $\delta^2\text{H}_{\text{C}37}$ and (B) $\alpha_{\text{C}37}$ for all surface sediments (this study combined with previously published data from Schwab and Sachs, 2011 and Kaiser et al., 2017) with linear regression equations from culture experiments with marine haptophyte alkenone-producers denoted by the dashed line (compiled in Weiss et al., 2017; includes data from Schouten et al., 2006, M'Boule et al., 2014, Sachs et al., 2016) and coastal haptophyte alkenone-producers designated by the dotted line (Chivall et al., 2014; M'Boule et al., 2014). Y-axis errors are the standard deviations of duplicate analyses and X-axis errors are the standard errors of the statistical mean for the 0.25° gridded WOA annual mean salinity dataset. North Atlantic and Mediterranean $\delta^2\text{H}_{\text{C}37}$ ratios fit with marine haptophyte culture data, and the lower salinity Baltic Sea and Chesapeake Bay Estuary $\delta^2\text{H}_{\text{C}37}$ ratios fit with coastal haptophyte culture values. $\delta^2\text{H}_{\text{C}37}$ and annual mean salinity for the World Ocean Atlas are strongly positively correlated ($r = 0.75$, $p < 0.001$). The linear regression equation that best fits the combined surface sediment data is: $\delta^2\text{H}_{\text{C}37} = 1.3 \text{ S} - 227$ ($R^2 = 0.55$, $p < 0.001$, $n = 95$). There is no significant correlation between salinity and $\alpha_{\text{C}37}$.

4.4 Species effect on $\delta^2\text{H}$ ratios of alkenones

As mentioned above, it has been suggested that contributions from different alkenone-producing species caused the lack of correlation between $\alpha_{\text{C}37}$ and salinity in the Chesapeake Bay Estuary (Schwab and Sachs, 2011). Data from hypersaline lakes also show diminished sensitivity due to differences between lacustrine and marine alkenone producers (Nelson and

Sachs, 214). Our findings are quite similar to these two datasets, i.e. a strong correlation of $\delta^2\text{H}_{\text{C}37}$ with salinity, but not of $\alpha_{\text{C}37}$ with salinity. The combination of data presented in this study and previous published results (Fig. 4) show that at higher salinities both $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values are similar to those found in *E. huxleyi* and *G. oceanica* culture experiments (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). At the lowest salinities, $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ values fit with culture experiments of *I. galbana* and *R. lamellosa* (M'Boule et al., 2014; Chivall et al., 2014), implying that differences between alkenone-producing haptophyte species could be important for our surface sediment dataset.

The Mediterranean and North Atlantic are characterized by high salinity (34–39) and the presence of *E. huxleyi* and *G. oceanica* (Knappertsbusch, 1993; McIntyre and Bé, 1967; Lomas and Bates, 2004), explaining why Mediterranean and North Atlantic $\delta^2\text{H}_{\text{C}37}$ ratios are in good agreement with *E. huxleyi* and *G. oceanica* culture data. For the modern day Black Sea, *E. huxleyi* is the only reported alkenone-producer (Moncheva et al., 2001), but both *E. huxleyi* and *G. oceanica* are absent from the present-day Baltic Sea (Tyrrell et al., 2008). Haptophytes have been reported to live in the Baltic Sea (Vepsäläinen et al., 2005). Previously reported alkenone patterns resemble those produced by *Ruttenra* and *Isochrysis* species (Conte et al., 1994; Schulz et al., 2000), and 18S rRNA analyses confirm the presence of both marine and coastal haptophyte groups in the Baltic Sea (Kaiser et al., 2019). Additionally, Baltic Sea alkenone distributions have a reported absence of C_{38} methyl alkenones, which are generally present in *E. huxleyi* and *G. oceanica* (Schulz et al., 2000). We also observe the absence of C_{38} methyl alkenones in some of our Gotland Basin sediments, suggesting a source other than *E. huxleyi* or *G. oceanica*. Indeed, the observed environmental salinity range for *E. huxleyi* is 11–41 (Schulz et al., 2000; Brand, 1994), and *G. oceanica* does not reproduce in salinities below 15 (Brand, 1984), so it is expected that *E. huxleyi* and *G. oceanica* will not live in the Baltic Sea. Moreover, the $\text{C}_{36:2}$ alkenone was detected in surface sediments from the Arkona basin in the Baltic Sea, as well as in Black Sea sediments. While the origin of the $\text{C}_{36:2}$ alkenone is not known, its presence always coincides with lower salinities, and is hypothesized to be from either *I. galbana* or a low salinity strain of *E. huxleyi* (Xu et al., 2001; Coolen et al., 2009; Warden et al., 2016). Based on the presence of the $\text{C}_{36:2}$ alkenone and the absence of C_{38} methyl alkenones, we can conclude that different haptophyte species are contributing to the sedimentary alkenones in the Baltic Sea.

4.5 Implications

Our results show that $\alpha_{\text{C}37}$ values in marine surface sediments are not sensitive to salinity, opposite to observations from culture studies (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017) and some paleo settings (e.g. van der Meer et al., 2007). It appears that mixing of alkenones produced by different species is responsible for the lack of correlation between $\alpha_{\text{C}37}$ and salinity across our dataset. Alternatively, $\delta^2\text{H}_{\text{C}37}$ ratios from marine surface sediments compliment what has been observed in culture studies, and correlate significantly with sea surface salinity. Indeed, $\delta^2\text{H}_{\text{C}37}$ ratios have been used to track salinity changes for a number of paleo records (Pahnke et al., 2007; Leduc et al., 2013; Kasper et al., 2014; Kasper et al., 2015; Simon et al., 2015; Petrick et al., 2015). For these records, salinity is most commonly reconstructed using the slope of the $\delta^2\text{H}_{\text{C}37}$ -salinity relationship from Schouten et al. (2006) of 4.8 for *E. huxleyi* and 4.2 for *G. oceanica*, which are both much larger than the slope of 1.3 obtained for this dataset. To test the applicability of our surface sediment calibration for paleo settings, we have applied our calibration of $\delta^2\text{H}_{\text{C}37}$ -salinity to the $\delta^2\text{H}_{\text{C}37}$ record generated from a sediment core in the Agulhas leakage area (Kasper et al.,

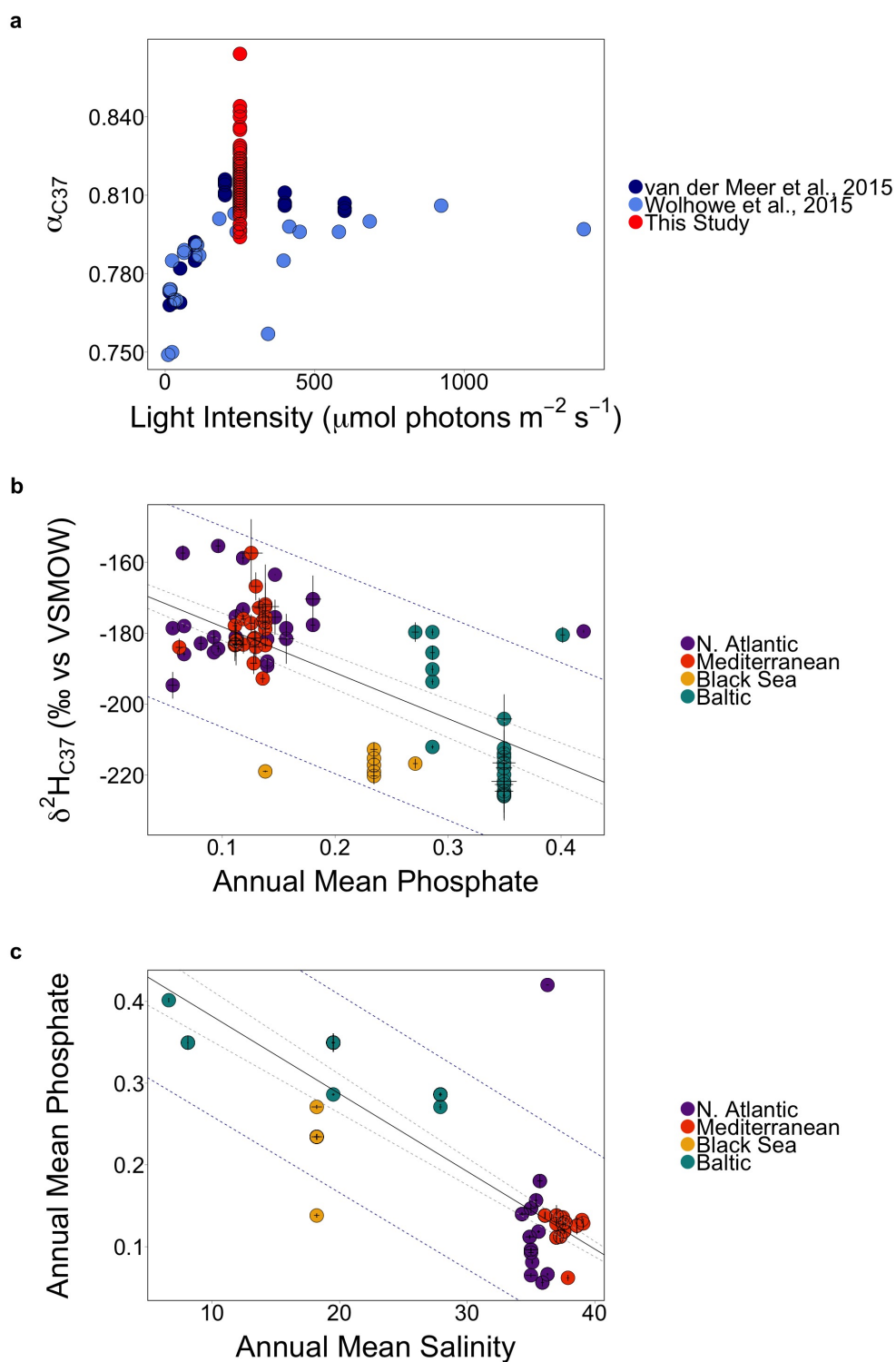


Figure 5: Light intensity plotted against α_{C37} from cultures (van der Meer et al., 2015), suspended particulate matter (Wolhowe et al., 2015) and surface sediments (A). Annual mean phosphate is plotted against $\delta^2\text{H}_{C37}$ (B) and annual mean salinity (C). Annual mean phosphate and salinity values were taken from the World Ocean Atlas (Antonov et al., 2010, Locarnini et al., 2010; Garcia et al., 2010).

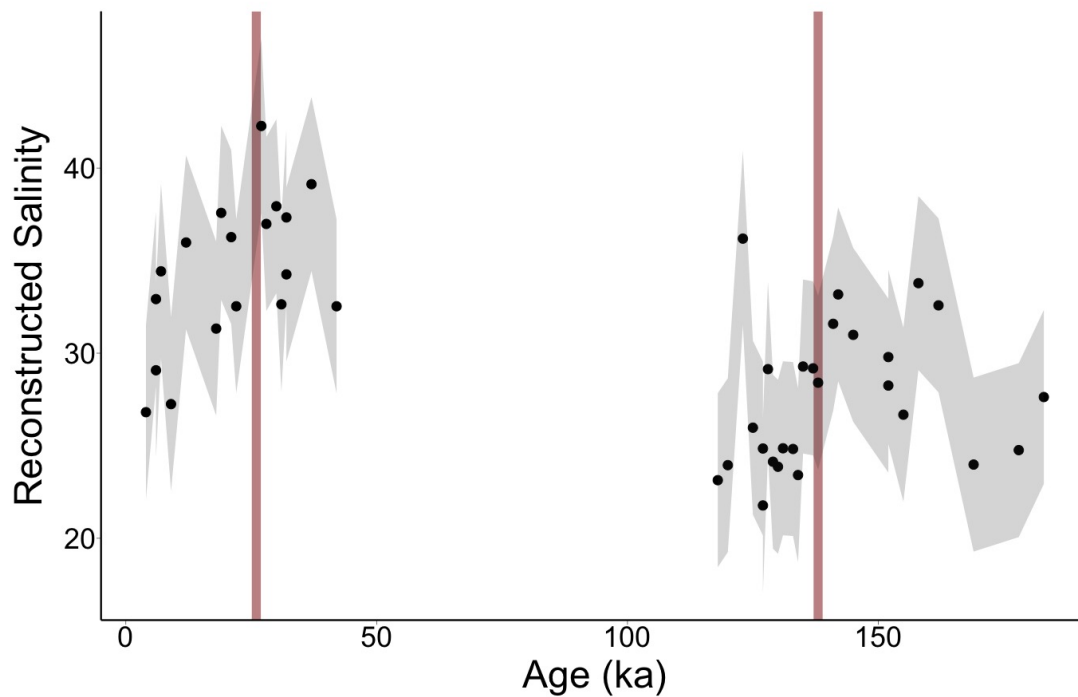


Figure 6: Reconstructed salinities for the Agulhas Leakage ($\delta^2\text{H}_{C37}$ ratios published in Kasper et al., 2014) using the linear regression equation from the combined surface sediment dataset presented here. The surface sediment calibration results in a salinity shift of 9 psu across Termination I and 5 psu across Termination II.

2014). As the amount of ice in ice sheets and glaciers around the globe varies (characterized by isotopically depleted values), the isotopic composition of the ocean (typically isotopically enriched) changes as a result (Chappell and Shackleton, 1986). Thus, in order to use $\delta^2\text{H}_{C37}$ ratios to calculate salinity values, the $\delta^2\text{H}_{C37}$ ratios must be corrected for global ice volume at the particular time period of interest. Kasper et al. (2014) corrected their $\delta^2\text{H}_{C37}$ ratios for global ice volume and calculated salinity changes using the slope of 4.8 from Schouten et al. (2006) to get a salinity change of 1.9 for Termination I and 1.7 for Termination II, which fits with salinity reconstructed from $\delta^{18}\text{O}_{\text{foram}}$ and Mg/Ca ratios measured on the same core. If we apply the slope of 1.3 from our surface sediment calibration, we obtain a salinity change of 9 across Termination I and 5 across Termination II (Fig. 6), much higher than reported values and $\delta^{18}\text{O}_{\text{foram}}$ and Mg/Ca reconstructions (Kasper et al., 2014). Not only does the surface sediment calibration result in large changes in salinity, but the propagated error for the calibration is 4.7, making it difficult to reconstruct the small changes in salinity expected for open ocean settings. This shows that our surface sediment calibration is substantially over-estimating changes in salinity. The surface sediment calibration presented here likely captures the effect of different haptophyte species, and therefore over-estimates paleo salinity shifts in open ocean settings where only marine haptophytes are presumed to contribute to the sedimentary alkenone record.

5 Conclusions

Based on the compilation of surface sediments from a range of environmental settings, we have shown that $\delta^2\text{H}_{\text{C}37}$ ratios have a strong positive linear correlation with annual mean sea surface salinity. However, $\alpha_{\text{C}37}$ values do not correlate with sea surface salinity, in contrast to previous findings from culture experiments. Other environmental factors, like temperature and nutrients, correlate with $\delta^2\text{H}_{\text{C}37}$ ratios, but also with salinity, implying an indirect relationship between these parameters and $\delta^2\text{H}_{\text{C}37}$ ratios, and possibly no effect on $\delta^2\text{H}_{\text{C}37}$ ratios. The most plausible explanation for the lack of correlation between $\alpha_{\text{C}37}$ and sea surface salinity is the difference in alkenone-producing haptophytes present in different locations. The $\delta^2\text{H}_{\text{C}37}$ ratios corresponding to lower salinities align nicely with culture data from coastal haptophyte species which are also reported to live in the low salinity environments along the different transects. The $\delta^2\text{H}_{\text{C}37}$ ratios corresponding to higher salinities fit nicely with the reported values for marine alkenone-producing species. Our results suggest that $\delta^2\text{H}_{\text{C}37}$ ratios might be a suitable proxy for sea surface salinity, especially in marginal marine environments where large ranges of salinity and mixing of alkenones from different alkenone-producing species is common. However, when applying $\delta^2\text{H}_{\text{C}37}$ ratios to estimate salinity for down-core records from open marine sites where smaller shifts in salinity and a stable alkenone-producing population are anticipated, the slope from our calibration provides salinity changes that are much larger than expected. While $\delta^2\text{H}_{\text{C}37}$ ratios can provide valuable information about past shifts in salinity, we emphasize that it can presently only be used to obtain qualitative estimates of salinity, rather than quantitative.

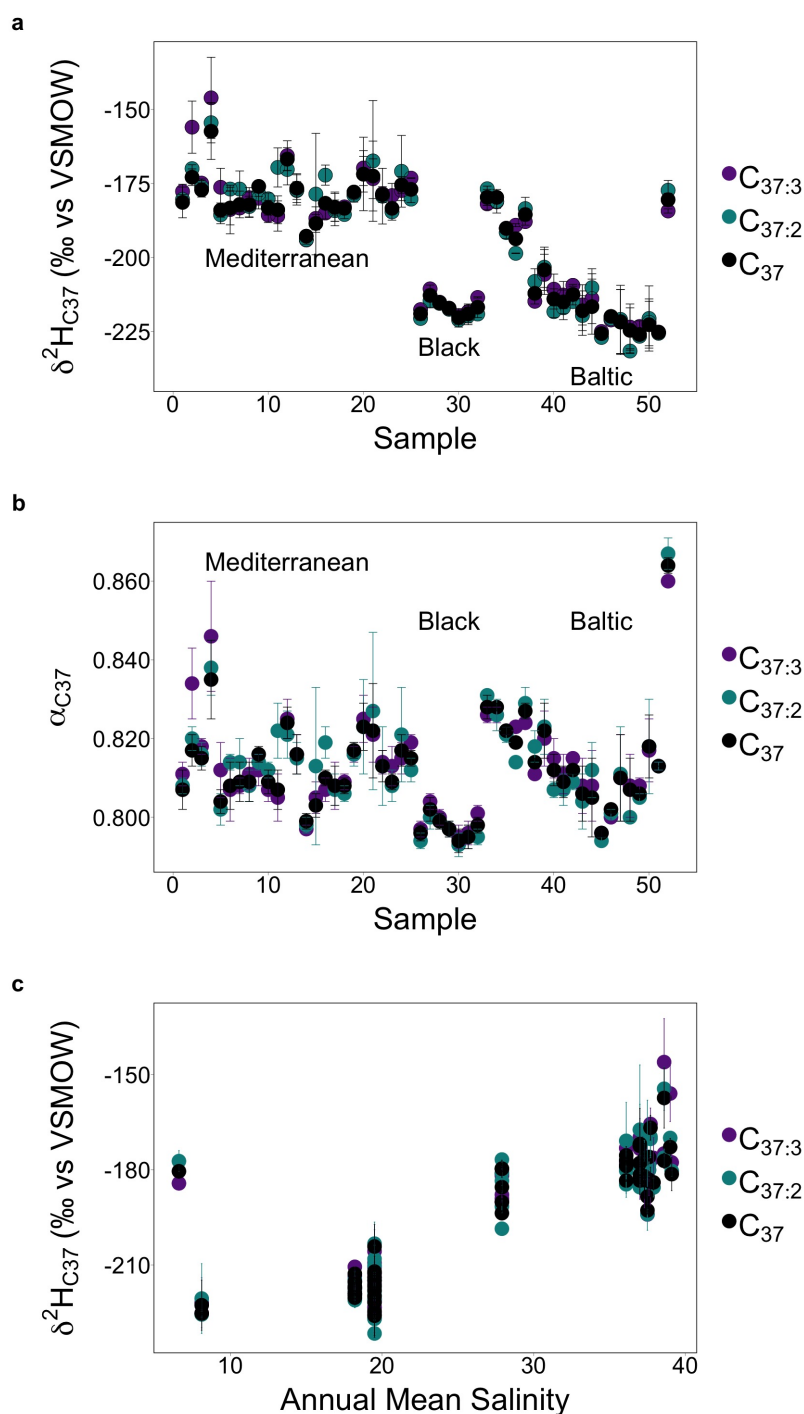
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Table 2: Pearson's correlation coefficient (r) and p-values for strongest correlations between $\delta^2\text{H}_{\text{C}37}$ and $\alpha_{\text{C}37}$ with annual mean and seasonal salinity for individual transects. Correlations between $\delta^2\text{H}_{\text{C}37}$ and $\alpha_{\text{C}37}$ with annual mean and seasonal salinity, temperature and nutrients for the combined dataset, including previously published data from Schwab and Sachs (2011) and Kaiser et al. (2017). Table 2 also shows r and p-values for U^{K}_{37} values correlated to annual mean and seasonal temperatures.

	Parameters	R	p value
North Atlantic	$\delta^2\text{H}_{\text{C}37}$ vs. spring salinity	-0.17	> 0.05
	$\alpha_{\text{C}37}$ vs. spring salinity	-0.20	> 0.05
Mediterranean	$\delta^2\text{H}_{\text{C}37}$ vs. summer salinity	0.27	> 0.05
	$\alpha_{\text{C}37}$ vs. spring salinity	0.15	> 0.05
	$\alpha_{\text{C}37}$ vs. summer salinity	0.15	> 0.05
Black Sea	$\delta^2\text{H}_{\text{C}37}$ vs. fall salinity	0.35	> 0.05
	$\delta^2\text{H}_{\text{C}37}$ vs. winter salinity	0.35	> 0.05
	$\alpha_{\text{C}37}$ vs. fall salinity	0.44	> 0.05
	$\alpha_{\text{C}37}$ vs. winter salinity	0.44	> 0.05
Baltic Sea	$\delta^2\text{H}_{\text{C}37}$ vs. spring salinity	0.52	< 0.05
	$\alpha_{\text{C}37}$ vs. fall salinity	-0.63	< 0.01
This study and previously published results from Schwab and Sachs (2011) & Kaiser et al. (2017)	$\delta^2\text{H}_{\text{C}37}$ vs. annual mean salinity	0.75	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. spring salinity	0.75	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. summer salinity	0.75	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. fall salinity	0.71	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. winter salinity	0.70	< 0.001
	$\alpha_{\text{C}37}$ vs. annual mean salinity	-0.27	< 0.05
	$\alpha_{\text{C}37}$ vs. spring salinity	-0.28	< 0.05
	$\alpha_{\text{C}37}$ vs. summer salinity	-0.27	< 0.05
	$\alpha_{\text{C}37}$ vs. fall salinity	-0.31	< 0.05
	$\alpha_{\text{C}37}$ vs. winter salinity	-0.31	< 0.05
Temperature	$\delta^2\text{H}_{\text{C}37}$ vs. fall temperature	0.75	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. winter temperature	0.74	< 0.001
	$\alpha_{\text{C}37}$ vs. fall temperature	0.32	< 0.01
	$\alpha_{\text{C}37}$ vs. winter temperature	0.34	< 0.01
Nutrients	$\delta^2\text{H}_{\text{C}37}$ vs. annual mean N	-0.52	< 0.001
	$\delta^2\text{H}_{\text{C}37}$ vs. annual mean P	-0.75	< 0.001
	$\alpha_{\text{C}37}$ vs. annual mean N	-0.12	> 0.05
	$\alpha_{\text{C}37}$ vs. annual mean P	-0.14	> 0.05
	Annual mean S vs. annual mean P	-0.84	< 0.001
U^{K}_{37}	U^{K}_{37} vs. annual mean temperature	0.89	p < 0.001
	U^{K}_{37} vs. spring temperature	0.92	p < 0.001

Supplementary material



Supplementary Figure 1: Separated $C_{37:3}$ and $C_{37:2}$ alkenones compared to integrated C_{37} alkenones. (a) δ^2H_{C37} ratios for $C_{37:3}$, $C_{37:2}$ and integrated C_{37} alkenones for each sample. (b) α_{C37} values for $C_{37:3}$, $C_{37:2}$ and integrated C_{37} alkenones for each sample. (c) shows δ^2H_{C37} ratios plotted against annual mean salinity (from the World Ocean Atlas – Antonov et al., 2010; Locarnini et al., 2010) for both individual and integrated C_{37} alkenones. Values for the separated alkenones in all three panels generally overlap and no large differences between values for the two separated alkenones exist.

Surface sediment calibration

Supplementary Table 1: Concentrations of long-chain alkenones from marine surface sediments from the North Atlantic, Mediterranean Sea, Black Sea, and Baltic Sea.

Location	Station		CONCENTRATIONS (ng / g sediment)							
			C _{36:2}	C _{37:4}	C _{37:3}	C _{37:2}	C _{38:3} Et	C _{38:2} Et	C _{38:3} Me	C _{38:2} Me
North Atlantic	1	1a	-	-	63	406	236	116	-	-
		1b	-	-	46	331	226	0	-	-
		1c	-	-	62	336	211	95	-	-
	3	3a	-	-	32	303	-	-	-	-
		3b	-	-	19	189	120	60	-	-
		3c	-	-	38	385	240	126	-	-
	5	5a	-	-	8	164	100	52	-	-
		5b	-	-	13	314	-	-	-	-
		5c	-	-	6	104	61	33	-	-
	7	7a	-	-	12	489	288	148	-	-
		7b	-	-	7	315	157	95	-	-
		7c	-	-	6	208	137	62	-	-
	8	8a	-	-	8	375	206	118	-	-
		8b	-	-	6	260	131	75	-	-
		8c	-	-	8	375	192	113	-	-
	9	9a	-	-	7	333	188	100	-	-
		9b	-	-	12	588	292	171	-	-
		9c	-	-	4	185	98	52	-	-
	10	10a	-	-	6	158	79	43	-	-
		10b	-	-	13	364	182	104	-	-
		10c	-	-	12	383	187	106	-	-
	11	11a	-	-	1	13	-	-	-	-
		11b	-	-	2	16	-	-	-	-
		11c	-	-	2	5	-	-	-	-
	12	12a	-	-	2	9	-	-	-	-
		12b	-	-	2	5	-	-	-	-
		12c	-	-	1	8	-	-	-	-
	13	13a	-	-	57	1192	778	353	-	-
		13b	-	-	63	1378	804	392	-	-
		13c	-	-	69	1173	702	338	-	-
	14	14a	-	-	5	497	343	154	-	-
		14b	-	-	13	576	336	179	-	-
		14c	-	-	2	117	-	-	-	-
	16	16a	-	-	5	75	-	-	-	-
		16b	-	-	16	824	480	239	-	-
		16c	-	-	6	229	213	61	-	-
	17	17a	-	-	10	556	370	162	-	-
		17b	-	-	11	494	269	144	-	-
	20	20a	-	-	9	377	235	106	-	-
		20b	-	-	12	542	301	165	-	-
		20c	-	-	10	561	261	129	-	-
	21	21a	-	-	5	119	107	34	-	-
		21b	-	-	8	158	88	46	-	-
		21c	-	-	3	73	107	19	-	-
	22	22a	-	-	1	15	0	0	-	-
		22b	-	-	5	114	108	37	-	-
		22c	-	-	1	19	0	0	-	-
	23	23a	-	-	3	58	126	20	-	-
		23b	-	-	5	96	88	30	-	-
		23c	-	-	3	54	63	14	-	-

Surface sediment calibration

Supplementary Table 1: (continued) Concentrations of long-chain alkenones from marine surface sediments from the North Atlantic, Mediterranean Sea, Black Sea, and Baltic Sea.

Location	Station		CONCENTRATIONS (ng / g sediment)							
			C _{36:2}	C _{37:4}	C _{37:3}	C _{37:2}	C _{38:3} Et	C _{38:2} Et	C _{38:3} Me	C _{38:2} Me
Mediterranean	1	E_1a	-	-	41	66	26	56	20	23
		E_1b	-	-	37	55	22	46	17	19
		E_1c	-	-	20	51	17	57	10	19
		E_1d	-	-	106	170	49	154	54	58
	2	E_2a	-	-	72	101	38	89	37	37
		E_2b	-	-	39	50	26	44	19	17
		E_2c	-	-	71	111	33	97	42	31
	3	E_3a	-	-	16	27	9	24	9	9
		E_3b	-	-	24	37	14	31	12	14
		E_3c	-	-	17	53	18	47	17	19
		E_3d	-	-	189	231	83	187	87	78
	5	E_5a	-	-	34	45	19	41	16	17
		E_5b	-	-	32	38	19	35	16	13
		E_5c	-	-	181	214	84	206	88	82
	6	E_6a	-	-	70	84	44	72	34	30
		E_6b	-	-	53	66	36	64	26	25
		E_6c	-	-	117	89	40	80	38	30
		E_6d	-	-	108	140	53	116	55	54
	1	W_1a	-	-	120	167	47	134	57	57
		W_1b	-	-	69	95	33	31	78	33
	2	W_2a	-	-	347	520	223	176	520	187
		W_2b	-	-	313	440	165	145	389	142
		W_2c	-	-	372	456	211	171	404	148
	3	W_3a	-	-	351	270	183	171	214	86
		W_3b	-	-	691	842	312	303	672	265
	4	W_4a	-	-	161	159	101	83	133	53
		W_4b	-	-	287	385	164	135	276	111
		W_4c	-	-	196	215	101	85	166	75
	6	W_6a	-	-	179	222	64	166	77	63
		W_6b	-	-	46	59	27	22	46	18
		W_6c	-	-	226	286	119	105	226	89
	7	W_7a	-	-	51	83	41	25	91	27
		W_7b	-	-	300	379	141	137	284	124
		W_7c	-	-	164	214	81	78	165	68
	8	W_8a	-	-	170	221	83	78	163	68
		W_8b	-	-	66	82	32	29	61	28
		W_8c	-	-	218	239	81	96	173	74
	9	W_9a	-	-	974	1488	432	409	1088	417
		W_9b	-	-	796	1350	236	306	693	292
		W_9c	-	-	896	1388	283	383	945	397
	10	W_10a	-	-	512	926	234	210	800	270
		W_10b	-	-	278	490	97	121	356	145
		W_10c	-	-	402	736	138	173	542	206
		W_10d	-	-	99	177	36	125	44	56

Surface sediment calibration

Supplementary Table 1: (continued) Concentrations of long-chain alkenones from marine surface sediments from the North Atlantic, Mediterranean Sea, Black Sea, and Baltic Sea.

Location	Station		CONCENTRATIONS (ng / g sediment)							
			C _{36:2}	C _{37:4}	C _{37:3}	C _{37:2}	C _{38:3} Et	C _{38:2} Et	C _{38:3} Me	C _{38:2} Me
Black	12	12_1	-	-	146	120	68	107	86	47
		12a	252245	339	7110	6583	2931	5628	3681	2581
		12b	344550	269	5876	5681	2598	4977	3224	2280
	2	2a	1281529	643	13023	11106	5055	8965	6836	4180
		2b	449816	777	21070	24045	8763	19607	11505	9777
		2c	554297	317	6692	6714	2813	5839	3522	2586
	4	4a	518686	435	10087	11340	4054	9214	5397	4359
		4b	960947	2244	53186	56378	21059	46459	28828	22584
Baltic	2	2a	-	183	3081	2821	1182	2145	1196	855
	3	3c	-	48	1406	1429	626	1301	695	530
	4	4a	-	77	1860	1827	713	1404	892	671
		4b	-	47	1305	1358	507	1061	602	491
	5	5b	-	79	1592	1654	547	1163	660	557
	5	5c	-	28	870	883	293	618	376	306
	6	6a	-	175	5566	5750	2162	4315	2439	2005
		6b	-	43	1523	1655	536	1181	671	589

Surface sediment calibration

Supplementary Table 2: $U_{37}^{K'}$ values, reconstructed sea surface temperatures (SST) and C_{37}/C_{38} ratios for samples from all four transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$U_{37}^{K'}$	SST °C (reconstructed)	C_{37}/C_{38} ratio
North Atlantic	1	1a	15.0	-30.6	0.86	24.2	1.3
		1b			0.88	24.9	1.7
		1c			0.84	23.5	1.3
	3	3a	13.2	-36.2	0.90	25.7	-
		3b			0.91	26.3	1.2
		3c			0.91	26.2	1.2
	5	5a	10.8	-40.5	0.95	28.1	1.1
		5b			0.96	28.7	-
		5c			0.95	28.0	1.2
	7	7a	7.5	-44.3	0.98	29.5	1.1
		7b			0.98	29.6	1.3
		7c			0.97	29.0	1.1
	8	8a	6.5	-45.4	0.98	29.5	1.2
		8b			0.98	29.5	1.3
		8c			0.98	29.5	1.3
	9	9a	5.6	-46.4	0.98	29.5	1.2
		9b			0.98	29.6	1.3
		9c			0.98	29.5	1.3
	10	10a	6.7	-47.5	0.96	28.5	1.4
		10b			0.96	28.5	1.3
		10c			0.97	29.0	1.3
	11	11a	5.5	-51.5	0.91	26.3	-
		11b			0.88	25.0	-
		11c			0.74	20.3	-
	12	12a	6.1	-52.5	0.84	23.5	-
		12b			0.78	21.6	-
		12c			0.87	24.6	-
	13	13a	7.6	-53.0	0.95	28.0	1.1
		13b			0.96	28.7	1.2
		13c			0.94	27.7	1.2
	14	14a	9.5	-51.3	0.99	30.0	1.0
		14b			0.98	29.4	1.1
		14c			0.98	29.5	-
	16	16a	10.2	-51.9	0.93	27.1	-
		16b			0.98	29.6	1.2
		16c			0.97	29.0	0.9
	17	17a	9.9	-53.3	0.98	29.5	1.1
		17b			0.98	29.5	1.2
	20	20a	9.9	-53.3	0.98	29.4	1.1
		20b			0.98	29.5	1.2
		20c			0.98	29.5	1.5
	21	21a	11.3	-54.2	0.96	28.6	0.9
		21b			0.95	28.1	1.2
		21c			0.96	28.5	0.6
	22	22a	14.8	-55.2	0.94	27.6	-
		22b			0.96	28.5	0.8
		22c			0.96	28.5	-
	23	23a	15.8	-57.1	0.96	28.6	0.4
		23b			0.95	28.1	0.9
		23c			0.95	28.2	0.7

Surface sediment calibration

Supplementary Table 2: (continued) $U_{37}^{K'}$ values, reconstructed sea surface temperatures (SST) and C_{37}/C_{38} ratios for samples from all four transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$U_{37}^{K'}$	SST °C (reconstructed)	C_{37}/C_{38} ratio
Mediterranean Sea	1	E_1a	33.3	33.4	0.62	16.8	0.9
		E_1b			0.59	16.0	0.9
		E_1c			0.72	19.8	0.7
		E_1d			0.62	16.7	0.9
	2	E_2a	33.7	30.6	0.58	15.6	0.9
		E_2b			0.56	14.9	0.8
		E_2c			0.61	16.5	0.9
	3	E_3a	34.1	27.6	0.62	16.7	0.9
		E_3b			0.61	16.5	0.8
		E_3c			0.75	20.7	0.7
		E_3d			0.55	14.6	1.0
	5	E_5a	35.0	20.6	0.57	15.3	0.8
		E_5b			0.54	14.4	0.8
		E_5c			0.54	14.4	0.9
	6	E_6a	35.0	18.1	0.55	14.7	0.9
		E_6b			0.55	14.7	0.8
		E_6c			0.43	11.1	1.1
		E_6d			0.56	15.0	0.9
	1	W_1a	34.8	14.2	0.58	15.6	1.0
		W_1b			0.58	15.6	0.9
	2	W_2a	37.0	13.5	0.60	16.2	0.8
		W_2b			0.58	15.6	0.9
		W_2c			0.55	14.7	0.9
	3	W_3a	38.0	11.5	0.44	11.4	0.9
		W_3b			0.55	14.6	1.0
	4	W_4a	38.5	9.0	0.50	13.2	0.9
		W_4b			0.57	15.3	1.0
		W_4c			0.52	13.8	1.0
	6	W_6a	38.8	3.8	0.55	14.7	1.1
		W_6b			0.56	15.0	0.9
		W_6c			0.56	15.0	1.0
	7	W_7a	38.0	0.7	0.62	16.7	0.7
		W_7b			0.56	15.0	1.0
		W_7c			0.57	15.3	1.0
	8	W_8a	36.7	-1.5	0.56	15.0	1.0
		W_8b			0.55	14.7	1.0
		W_8c			0.52	13.8	1.1
	9	W_9a	36.3	-4.0	0.60	16.2	1.0
		W_9b			0.63	17.0	1.4
		W_9c			0.61	16.5	1.1
	10	W_10a	36.3	-6.5	0.64	17.4	0.9
		W_10b			0.64	17.4	1.1
		W_10c			0.65	17.7	1.1
		W_10d			0.64	17.4	1.1

Surface sediment calibration

Supplementary Table 2: (continued) $U_{37}^{K'}$ values, reconstructed sea surface temperatures (SST) and C_{37}/C_{38} ratios for samples from all four transects.

Location	Station	Sample	Latitude (°N)	Longitude (°E)	$U_{37}^{K'}$	SST °C (reconstructed)	C_{37}/C_{38} ratio
Black Sea	2	12_1	42.9	30.7	0.45	11.8	0.9
		12a			0.48	12.6	0.9
		12b			0.49	12.9	0.9
		2a			0.51	13.5	0.9
	12	2b	42.9	30.0	0.46	12.0	1.0
		2c			0.53	14.1	0.9
	4	4a	42.8	29.4	0.50	13.2	0.9
		4b			0.53	14.1	0.9
Baltic Sea	1	1a	58.5	9.6	0.39	9.9	0.9
		1b			0.39	9.9	0.9
	2	2a	58.0	11.1	0.49	12.9	1.1
		2b			0.48	12.6	1.1
		2c			0.47	12.4	1.1
	3	3a	56.6	11.9	0.50	13.2	1.0
		3b			0.49	12.9	1.0
		3c			0.49	12.9	1.0
	4	4a	56.3	12.3	0.50	13.2	0.9
		4b			0.50	13.2	1.0
		4c			0.51	13.5	1.0
	5	5a	56.1	12.7	0.50	13.3	1.1
		5b			0.52	13.8	1.1
		5c			0.51	13.5	1.1
	6	6a	56.0	12.8	0.50	13.2	1.1
		6b			0.51	13.5	1.0
		6c			0.52	13.8	1.1
	7	7a	54.9	13.6	0.45	11.7	1.1
		7b			0.46	12.0	1.1
		7c			0.47	12.4	1.0
	9	9a	55.5	15.6	0.38	9.7	1.0
		9b			0.41	10.6	1.1
		9c			0.38	9.6	1.2
	10	10a	57.2	20.2	0.07	0.6	2.1
		10b			0.40	10.3	0.9

Surface sediment calibration

Supplementary Table 3: $U_{37}^{K'}$ values for both Cpsil5 25 m column and RTX-200 60 m column for validation of use of RTX-200 60 m column for calculations of $U_{37}^{K'}$ index.

Station	Sample	$U_{37}^{K'}$ Cpsil5	$U_{37}^{K'}$ RTX-200
406 - 1	E_1a	0.76	0.59
	E_1b	0.67	0.72
	E_1c	0.62	0.62
406 - 2	E_2a	0.84	0.58
	E_2b	0.56	0.56
	E_2c	0.61	0.61
406 - 3	E_3a	0.78	0.62
	E_3b	0.58	0.61
	E_3c	0.74	0.75
	E_3d	0.55	0.55
406 - 5	E_5a	0.59	0.57
	E_5b	0.69	0.54
	E_5c	0.55	0.54
406 - 6	E_6a	0.78	0.55
	E_6b	0.52	0.55
	E_6c	0.75	0.43
	E_6d	0.57	0.56
407 - 1	W_1a	0.55	0.58
	W_1b	0.75	0.58
407 - 2	W_2a	0.65	0.60
	W_2b	0.56	0.58
	W_2c	0.53	0.55
407 - 3	W_3a	0.40	0.44
	W_3b	0.58	0.55
407 - 4	W_4a	0.49	0.50
	W_4b	0.83	0.52
407 - 6	W_6a	0.55	0.55
	W_6b	0.55	0.56
407 - 7	W_7a	0.94	0.62
	W_7b	0.56	0.56
	W_7c	0.57	0.57
407 - 8	W_8a	0.55	0.56
	W_8b	0.58	0.55
	W_8c	0.49	0.52
407 - 9	W_9a	0.60	0.60
407 - 10	W_10a	0.64	0.64



Freeze-dried sediment from the Black Sea from 'Microbiocene: Ancient ooze to future myths' by Baum & Leahy in collaboration with Julie Lattaud, Laura Schreuder & Gabriella Weiss exhibited during London Design Week.

Chapter 6

Alkenone distributions and hydrogen isotope ratios show changes in haptophyte species and source water in the Holocene Baltic Sea

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Abstract

The Baltic Sea is a dynamic, marginal marine basin which has experienced a number of large changes in salinity during the Holocene as a result of fluctuations in global and local sea level related to melting of glacial ice sheets and isostatic rebound because of the melting of the Scandinavian Ice Sheet. These changes likely had pronounced effects on the species composition of haptophytes, a common phytoplankton group found in the Baltic Sea. This dynamic environment provides the ideal setting to study the impact of species composition on the distribution and hydrogen isotope ratios of long-chain alkenones, haptophyte-specific biomarkers. Here we analyzed the distribution and hydrogen isotopic composition of alkenones in Holocene sediments that cover the contrasting hydrological phases of the Baltic Sea. Alkenone distributions changed along the different salinity phases of the Baltic Sea, suggesting that species shifts coincide with changes in salinity. Hydrogen isotope ratios show two major shifts: the first shift takes place in the middle of the freshwater Ancylus Lake phase (10.6 to 7.7 ka) and the second shift which coincides with the transition from the brackish Littorina Sea phase (7.2 to 3 ka) into the fresher Modern Baltic phase (3 ka to the present). The first shift represents a significant enrichment of 50 ‰, which cannot be explained by salinity or species changes only, but is more likely caused by a change in source water. With the more isotopically depleted ice sheets melted, the hydrogen isotopic signature of the fresh water end-member shifts to much higher values. The second shift is likely caused by a change in species composition alone, because it coincides with a change in alkenone distribution. These findings show that hydrogen isotope ratios of long-chain alkenones, when combined with their relative distribution, can be used to reconstruct changes in source water of glacier-fed lakes.

Keywords:

Alkenones, Hydrogen isotopes, Baltic Sea, Haptophytes

1 Introduction

Haptophyte algae are important primary producers globally, inhabiting both lacustrine and marine environments (Theroux et al., 2010). Haptophytes are extremely diverse, with over 300 documented species (Jordan and Chamberlain, 1997). Certain species of the order Isochrysidales are the only known producers of long-chain alkenones, polyunsaturated methyl and ethyl ketones with chain lengths between 35 and 40 carbon atoms (De Leeuw et al., 1980; Volkman et al., 1980; Theroux et al., 2010). These long-chain alkenones are often well preserved in sediments and are highly useful proxies to understand past changes in global climate, in particular sea surface temperature using the $U_{37}^{K'}$ index (Brassell et al., 1986; Prahl and Wakeham, 1987; Müller et al., 1998; Tierney and Tingley, 2018). Furthermore, the hydrogen isotope composition of long-chain alkenones (δ^2H_{C37}) has been shown to strongly relate to salinity and the δ^2H of water (e.g. Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017), which has led to the use of δ^2H_{C37} ratios to reconstruct paleosalinity in a number of different marine environments (Pahnke et al., 2007; van der Meer et al., 2007, 2008; Leduc et al., 2013; Kasper et al., 2014, 2015; Petrick et al., 2015; Simon et al., 2015; Weiss et al., 2019a). However, other factors such as growth rate, (Sachs and Kawka, 2015), growth phase (Wolhowe et al., 2009; Chivall et al., 2014), and light

intensity (van der Meer et al., 2015; Weiss et al., 2017), are also known to affect the $\delta^2\text{H}$ ratios of alkenones.

One particular factor that is not well constrained yet is the impact of species composition on the $\delta^2\text{H}$ ratios of alkenones. Alkenone-producing haptophytes are separated into three distinct groups based on 18S ribosomal RNA (Theroux et al., 2010), and seem to prefer niches with a distinct salinity range. Group I haptophytes are presently uncultured, but have been found in freshwater, lacustrine environments in the Northern Hemisphere (Theroux et al., 2010; Longo et al., 2016, 2018). Group II haptophytes are the most cosmopolitan, found in both oligohaline and hypersaline lakes (Theroux et al., 2010; Longo et al., 2016), and cultured representatives include *Isochrysis galbana* and *Ruttenella lamellosa*. Marine species fall under Group III haptophytes, which include *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Theroux et al., 2010).

Only recently, analytical advances have allowed for better constraint on the contributions of each of these three groups to the pool of sedimentary alkenones. Groups can now be distinguished because of the improved separation and detection of tri-unsaturated alkenone positional isomers with different double bond positions at $\Delta^{14,21,28}$ instead of the common $\Delta^{7,14,21}$ configuration (Longo et al., 2013; Dillon et al., 2016; Longo et al., 2016). These so-called “tri-unsaturated isomers” have been detected alongside the traditional $\text{C}_{37:3}$ methyl, $\text{C}_{38:3}$ ethyl and methyl, and $\text{C}_{39:3}$ ethyl alkenones, and allow for better distinction between haptophyte Groups. Each haptophyte group appears to have different alkenone distributions: Group I is characterized by the presence of the novel tri-unsaturated isomers of C_{37} - C_{39} , in addition to the regular di-, tri- and tetra-unsaturated C_{37} - C_{39} alkenones; Group II does not synthesize the novel tri-unsaturated isomers, but instead synthesizes the standard suite of C_{37} - C_{39} , and are also thought to produce the shorter chain C_{35} and C_{36} alkenones. The latter alkenones have been detected in marginal marine environments generally characterized by having low salinities compared to the open ocean (Xu et al., 2001; Rontani et al., 2001; Fujine et al., 2006; van Soelen et al., 2014; Warden et al., 2016; Weiss et al., 2019b). These shorter chain alkenones have also appeared in an *E. huxleyi* culture after being kept in cultivation for a few years (Prahl et al., 2006). In contrast to the C_{37} - C_{40} alkenones, the C_{35} and C_{36} are thought to be synthesized from the $\text{C}_{37:2}$ and $\text{C}_{38:2}$ respectively via β -oxidation and produced by mutants of Group II species in the natural environment (Zheng et al., 2016). Finally, Group III produce the standard suite of C_{37} methyl and C_{38} ethyl and methyl alkenones, with low abundance of the C_{39} ethyl alkenones in some cases (Longo et al., 2013).

Besides different alkenone distributions, the hydrogen isotope ratios of long-chain alkenones also differ by group; more depleted $\delta^2\text{H}_{\text{C}_{37}}$ ratios are noted for Group III haptophytes compared to Group II when both are grown at the same salinity (Chivall et al., 2014; M'Boule et al., 2014). Based on distributions and hydrogen isotope ratios of alkenones, mixed contributions of Groups I and II as well as Groups II and III has been noted in some environments (Schwab and Sachs, 2011; Nelson and Sachs, 2014; Weiss et al., 2019b), but a mixing of all three Groups based on distributions has only been observed for surface sediments of the Baltic Sea (Kaiser et al., 2019). In that case, a transect from the Kattegat into the Baltic Sea basin showed the presence of all three haptophyte groups as reflected in changing alkenone distributions. This was confirmed by 18S rDNA preserved in surface sediments (Kaiser et al., 2019).

The study of Kaiser et al. (2019) has potentially large consequences for interpreting the sedimentary record of the Baltic Sea with respect to $\delta^2\text{H}_{\text{C}_{37}}$ ratios, as the Baltic Sea has experienced strongly varying salinities throughout the Holocene. The Baltic Sea began as the freshwater Baltic Ice Lake at around 13 ka, and as sea levels rose as a result of the

deglaciation, transitioned into a brackish basin known as the Yoldia Sea (from 11.6 to 10.6 ka, Moros et al., 2002). Continental uplift as a result of the isostatic adjustment to the melting of the Scandinavian Ice Sheet, closed off the Baltic Sea from the North Sea, causing the freshwater Ancylus Lake phase (from 10.6 until approximately 7.7 ka, Moros et al., 2002). This freshwater phase ended by a large marine transgression, shifting the Baltic Sea into the brackish Littorina Sea phase (Moros et al., 2002). The Littorina Sea lasted for a few thousand years, from 7.2 to around 3 ka, with a gradual freshening into the Modern Baltic phase as a consequence of continued continental uplift, which was mainly noted in the Baltic proper (Björck, 1995; Andrén et al., 2000). Initial work from a Holocene sediment core has shown that hydrogen isotope ratios of long-chain alkenones from the Baltic Sea might reflect changes in species composition (Warden et al., 2016), but techniques used in that study did not allow for detection of tri-unsaturated isomers and alkenones of lower abundance, thus no detailed reconstructions of species changes could be made.

Here we generated a comprehensive down-core record of $\delta^2\text{H}_{\text{C}_{37}}$ ratios and alkenone distributions from the Arkona Basin in the Baltic Sea analyzed on an RTX-200 60 m GC column, allowing for the separation and detection of individual alkenones and isomers. Detection of these isomers allowed for changes in species composition to be determined based on alkenone distributions related to the different Baltic Sea phase shifts. This also provided an ideal setting for testing how well hydrogen isotope ratios of long-chain alkenones are recording salinity changes in marginal marine environments, and how they relate to species shifts.

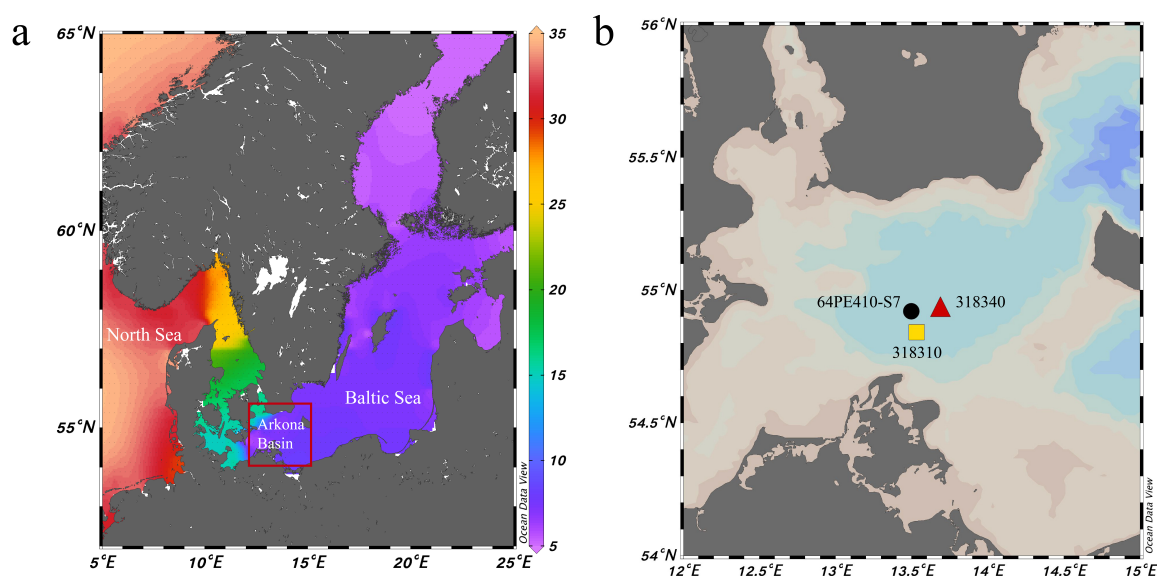


Figure 1: (a) Map showing annual mean salinity from the World Ocean Atlas 13 (Zweng et al., 2013) for the North Sea into the Baltic Sea. The Arkona Basin is noted by the red box. (b) The location ($54^{\circ}55.208' \text{ N}$, $13^{\circ}29.992' \text{ E}$) of core 64PE410-S7 (black circle) in the Arkona Basin of the Baltic Sea. The age model for 64PE410-S7 was determined by radiocarbon dating and comparison with two neighboring cores, 318310 ($54^{\circ}50.34' \text{ N}$, $13^{\circ}32.03' \text{ E}$) and 318340 ($54^{\circ}55.77' \text{ N}$, $13^{\circ}41.44' \text{ E}$), noted by the yellow square and red triangle respectively, from Warden et al. (2016).

2 Methods

2.1 Location and climate history

A 12 m piston core (64PE410-S7) was recovered from the Arkona Basin of the Baltic Sea (54°55.208 N, 13°29.992 E) with RV Pelagia in May 2016. The Arkona Basin is located in the western edge of the Baltic Sea next to the open connection with the North Sea via the Kattegat and Skagerrak (Fig. 1). Situated at the western edge of the Baltic Sea, the Arkona Basin is perfectly suited for finding evidence of major marine transgressions and regressions occurring throughout the Baltic Sea climate history.

The phases in the development of the Baltic Sea during the Holocene are relatively well-established (Björck, 1995), but the timing of these events in the Arkona Basin differs from timing reported for the Baltic proper (Warden et al., 2016). At the end of the last glacial, the Baltic Ice Lake formed, a cold freshwater lake that resulted from melting of the Scandinavian Ice Sheet (Mörner, 1995). The Scandinavian Ice Sheet caused a dam between the Baltic Sea and the connection with the North Sea, which disappeared as the Scandinavian Ice Sheet melted; the connection to the North Sea was re-established at around 11.6 ka (Moros et al., 2002). Re-opening of the connection with the North Sea caused the higher salinity water of the North Sea to mix with the fresh Baltic Sea, causing the brackish phase known as the Yoldia Sea. The Scandinavian Ice Sheet continued to melt, and the release of the enormous weight of the ice caused continental uplift, leading to a perceived lowering of sea level and an event known as the Yoldia Regression at ~10.6 ka (Moros et al., 2002). During the Yoldia Regression, the basin was again cut off from the sea. Without significant marine inflow, only freshwater entered the basin, resulting in the freshwater Ancylus Lake phase (Moros et al., 2002). Around 7.7 ka, a large marine transgression occurred, bringing saline water into the basin, and permanently restoring the connection to the sea (Moros et al., 2002; Warden et al., 2016). This phase is known as the brackish Littorina Sea phase. Continued continental uplift subsequently caused the Baltic Sea to become increasingly fresh, transitioning into the Modern Baltic phase at around 3 ka, where conditions are akin to those observed in the Baltic Sea today (Björck, 1995; Andrén et al., 2000). The Arkona Basin has slightly higher salinities at present (~8 psu) compared to the Baltic proper as a result of an open connection with the Kattegat.

2.2 Long-chain alkenones

Samples for long-chain alkenone analyses were taken every ~100 cm in the top section of the core, then every 10 cm between 930 cm and the bottom (1216 cm). In total 41 sediment samples were freeze-dried and organics were extracted using a Dionex Accelerated Solvent Extractor (ASE). Following ASE, total lipid extracts were collected and separated into three fractions using small aluminum oxide columns: an apolar fraction was separated using hexane : dichloromethane 9 : 1 (v : v), a ketone fraction using hexane : dichloromethane 1 : 1 (v : v), and a polar fraction using dichloromethane : methanol 1 : 1 (v : v). Following separation over an aluminum oxide column, alkenones ended up in the ketone fraction, and were cleaned over a silver nitrate impregnated silica gel column using 100 % dichloromethane to remove co-eluting compounds, and alkenones were eluted using dichloromethane : ethyl acetate 1 : 1 (v : v). Alkenone concentrations were determined by using a gas chromatograph with a flame ionization detector equipped with an RTX-200 column (Restek, 60 m x 0.32 mm x 0.5 μ m), and calculated based on a known concentration of a C₁₉ ketone internal standard (Table

1). Alkenones were identified using a gas chromatograph coupled to a mass spectrometer (GC-MS) equipped with the same 60 m RTX-200 column. The GC temperature program was as follows: 70 °C to 250 °C at 18 °C/min, 250 – 320 °C at 1.5 °C/min, then kept at 320 °C for 25 min at a flow rate of 1.5 mL/min. Two indices have been proposed to trace the presence of Group I haptophytes (Longo et al., 2016):

$$RIK_{37} = \frac{C_{37:3a}}{C_{37:3a} + C_{37:3b}} \quad (1)$$

$$RIK_{38E} = \frac{C_{38:3a}Et}{C_{38:3a}Et + C_{38:3b}Et} \quad (2)$$

In these equations, a represents the traditional $C_{37:3}$ and $C_{38:3}$ alkenones and b represents to novel tri-unsaturated isomers. These indices, along with the binary mixing model from Longo et al. (2018),

$$\%GroupII = \frac{RIK_{37} - 0.56}{0.44} \quad (3)$$

were used to determine the percentage of Group I and Group II haptophytes in samples containing tri-unsaturated isomers. When Group I species were present, temperature was calculated using R3b index:

$$R3b = \frac{C_{37:3b}}{C_{38:3b} + C_{37:3b}} \quad (4)$$

and subsequent temperature calibration from Longo et al. (2018):

$$T = \frac{R3b - 0.42}{0.032} \quad (5)$$

Salinity was calculated using the $\%C_{37:4}$ calibration from Kaiser et al. (2017):

$$\%C_{37:4} = -5.8353 \cdot S + 51.8028 \quad (6)$$

with $\%C_{37:4}$ being the concentration of $C_{37:4}$ as a percentage of total C_{37} alkenones. Furthermore, salinity was estimated from the RIK_{37} salinity calibration from Kaiser et al. (2019):

$$S = \frac{RIK_{37} + 0.5319}{0.0677} \quad (7)$$

2.3 Hydrogen isotope analyses

Hydrogen isotope ratios of alkenones were measured using gas chromatography coupled to an isotope ratio mass spectrometer via a high temperature conversion reactor. The GC was also equipped with an RTX-200 60 m column using the temperature program described above. At the start of each day, the H_3^+ factor was calculated and corrected for, which ranged from 2.5 to 2.6 ppm nA^{-1} , 3.1 to 3.3 ppm nA^{-1} , and 6.8 to 6.9 ppm nA^{-1} , with a day to day variation of < 0.1 ppm nA^{-1} . Tuning of the ion source is responsible for the observed differences in H_3^+ factor over the entire sample measuring period. Prior to measuring samples, an *n*-alkane mixture (Mix B – supplied by A. Schimmelmann, Indiana University), which covers a δ^2H range from approximately -50 ‰ to -250 ‰, was measured. Sample analyses were only conducted when the average difference and standard deviation between online and offline

values was less than 5 ‰. Squalane and C₃₀ *n*-alkane were co-injected with each sample run for further control on the stability of the system, and ranged from -169 ± 3 ‰ and -78 ± 8 ‰ respectively, fitting well with their offline established values of -170 ± 4 ‰ (squalane), and -79 ± 5 ‰ (C₃₀). High standard deviation for the C₃₀ *n*-alkane is attributed to co-eluting compounds. Samples were run in duplicate, and error bars represent the standard deviations of duplicate isotope ratio analyses.

2.4 XRF scanning

The geochemical composition of core 64PE410-S7 was obtained with an Avaatech X-ray fluorescence (XRF) core scanner at the Royal Netherlands Institute for Sea Research (NIOZ). This XRF core scanner contains a 100W rhodium X-ray tube and a Rayspec cubed SiriusSD silicon drift detector. Prior to the XRF-core-scan measurements, the sediment surfaces of the core sections were flattened and covered with a 4- μ m thin Ultralene foil. For calcium (Ca) and titanium (Ti) the used settings were 10 kV, 0.4 mA, 10 s, without a primary beam filter. For bromine (Br) the used settings were 30 kV, 0.15 mA, 10 s, with a thin palladium primary beam filter. The core sections were measured continuously with a 1-cm resolution, excluding some gaps that were caused by gas expansion during core retrieval. The irradiated surface area of the X-Ray beam was set at 1 cm (“down core”) by 1.2 cm (“cross core”) using the slits of the scanner. All spectral data were processed using the bAxil spectrum analysis software to calculate the element intensities (in counts). The standard tablet SARM-4 was measured before and after each core section, showing that precision was high (relative standard deviation <10%) for all elements targeted here.

2.5 Chronological framework

The age model for core 64PE410-S7 is based on a combination of ¹⁴C-dating and correlation of Ca/Ti and Br records to previously dated, similar records close by (318310: 54°50.34' N; 13°32.03' E and 318340: 54°55.77' N; 13°41.44' E) (Warden et al., 2016, Supp. Fig. 1). Bromine is considered to be a good indicator of marine organic carbon content (Ziegler et al., 2008), which can thus be linked to the total organic carbon content obtained through loss on ignition (LOI) in close-by core 318310 (Warden et al., 2016). Five important transitions are present within sediments obtained from the Arkona basin (see Warden et al., 2016), which are clearly distinguishable in the carbonate (Ca/Ti) and organic matter (Br and LOI) contents of the sediment cores (numbered 1 to 5 in Supp. Fig. 1). First, the occurrence of a peak in organic matter during the Medieval Climate Anomaly (MCA) at ~0.9 ka. Second, the transgression maximum indicated by the carbonate maximum at ~7.2 ka. Third, the start of the Ancylus Lake/Littorina Sea transitional phase defined by the increase in organic matter contents at ~7.7 ka. Fourth, the Ancylus Lake regression pinpointed by a peak in organic matter at ~10.2 ka. Fifth, the transition between the Yoldia Sea and the Ancylus Lake phase at ~10.6 ka. The latter is not present in the here studied core, however, the dip in Ca/Ti content at ~10.5 ka in core 318340 (Warden et al., 2016) can be linked to that in core 64PE410-S7 (marked orange in Supp. Fig. 1). Additionally, we obtained three ¹⁴C dates on mollusk shells to improve age control for the interval younger than 7 ka (Supp. Fig. 1). Shells were radiocarbon dated by Beta Analytic, corrected for the local reservoir effects and calibrated using CALIB (Stuiver et al., 2018). The final age model for all depths was created using the Bacon software package in R (Blaauw and Christen, 2011). In Bacon, the error estimates for ¹⁴C ages were also applied to the XRF tie-points (as these are also based on correlation to the nearby ¹⁴C dated cores),

and student's t-test values were set to 33 and 34 to allow for more narrow error estimates as suggested in the Bacon manual. The resulting age model suggested that sedimentation rates varied between 55 and 250 cm / kyr.

3 Results and Discussion

3.1 Alkenone distributions

Sediments from the Arkona Basin covering the last 10.6 kyr were analyzed for long-chain alkenones. Nineteen different alkenones were detected, with chain lengths ranging from 35 to 40 carbon atoms (Supp. Table 1). Tri-unsaturated isomers of C_{37:3} methyl and C_{38:3} ethyl alkenones were detected in addition to the conventional C_{37:3} methyl and C_{38:3} ethyl alkenones in some samples. These tri-unsaturated isomers have previously only been reported for lacustrine sediments (Longo et al., 2013, 2016; Dillon et al., 2016) and surface sediments in the Baltic Sea basin (Kaiser et al., 2019). Changes in alkenone distribution patterns align with Baltic Sea phase boundaries (Fig. 2). Alkenone distributions for the Yoldia Sea phase are typical for marine Group III, i.e., lacking tri-unsaturated isomers as well as C₃₉ and C₄₀ alkenones (cf Longo et al., 2013). The first emergence of tri-unsaturated isomers and C₃₉ ethyl and methyl alkenones occurs at the Yoldia Sea to Ancylus Lake boundary (Fig. 2), signifying the first occurrence of Group I species in this record. There is a brief return to Group III distribution (absence of tri-unsaturated isomers and lack of C₃₉ and C₄₀ alkenones), prior to the start of the Ancylus Lake distribution. The Ancylus Lake distribution is characterized by the presence of tri-unsaturated isomers, C₃₉ ethyl and methyl, as well as C₄₀ ethyl alkenones (Fig. 2). This pattern points to a mixing of Group I and II haptophytes throughout the Ancylus Lake phase, and distributions are stable across the entire phase.

To constrain the species mixing of Group I and II haptophytes, the percentage of Group I and Group II producers was determined using equation 3, which is based on alkenone distributions in Northern Hemisphere lacustrine sediments and suspended particulate matter (Longo et al., 2018). Group I is indeed dominant throughout the Ancylus Lake phase (57 to 75 %), with Group II only contributing substantially at the beginning and the end of the phase (60 to 75 %). This is supported by the RIK38E index (equation 2, Table 2), another indicator of Group II and Group I haptophytes (Longo et al., 2016), as it remains below 0.75 characteristic of Group I haptophytes.

The transition between the Ancylus Lake and the brackish Littorina Sea phase lasts for ~0.5 kyr. Distributions are distinctly Group III at the start of the Littorina Sea phase, with only conventional C₃₇ methyl and C₃₈ ethyl alkenones, but in the latter half of the transition, the C_{35:2} and C_{36:2} alkenones appear for the first time (Fig. 2). The Littorina Sea phase is distinguished from other phases by the presence of these shorter chain alkenones as well as the absence of tri-unsaturated isomers, C₃₉ and C₄₀ alkenones. The presence of the shorter chain alkenones indicates the presence of Group II haptophytes (Zheng et al., 2016). The final shift in alkenone distribution corresponds to the change from the Littorina Sea into the Modern Baltic phase, and represents a mixed Group II and Group III signal indicated by the presence of the C_{35:2}, C_{36:2} and C₃₉ ethyl alkenones (Fig. 2).

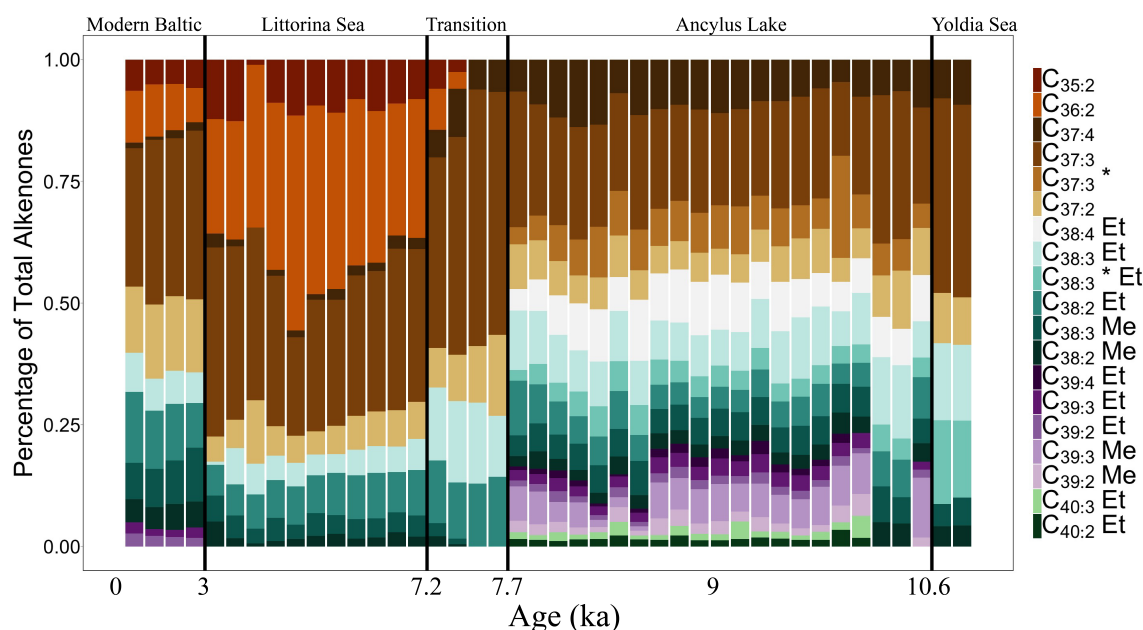


Figure 2: Percent distributions of individual long-chain alkenones present in each sample. The star indicated the novel tri-unsaturated isomers. Alkenones show distinct distributions for each of the Baltic Sea phases.

3.2 Temperature and salinity estimates

Temperature

The U_{37}^K and $U_{37}^{K'}$ indices are well-established sea surface temperature proxies (Brassell et al., 1986; Prahl and Wakeham, 1987; Rosell-Melé et al., 1995; Müller et al., 1998). The latter excludes the tetra-unsaturated C₃₇ alkenone in the equation, whereas the former does not. Application of the temperature calibration for the U_{37}^K using Atlantic surface sediments (Rosell-Melé et al., 1995) gives negative temperature estimates here, while the culture calibration from Prahl and Wakeham (1987) gives a temperature range between 0.3 and 2.3 °C, much lower than present day spring mean SST of 8 °C (Locarnini et al., 2013). When excluding the tetra-unsaturated C₃₇ alkenones from the equation ($U_{37}^{K'}$ index), and using the global temperature calibration for the $U_{37}^{K'}$ index (Müller et al., 1998), temperature estimates range from 2.2 – 8.5 °C (Fig. 3, Table 2). Haptophytes in the present day Baltic Sea are known to bloom during the months of April to June (Hällfors, 2004; Blanz et al., 2005), so it is reasonable to expect that alkenones should be reflecting spring temperature values. Modern spring mean temperature is 8 °C (Locarnini et al., 2013), and the most modern sample has a temperature of 8.5 °C using the global $U_{37}^{K'}$ calibration, which is hence in good agreement with spring mean. Therefore, the $U_{37}^{K'}$ index seems to be the more reliable of the two indices for reconstructing temperatures.

Nevertheless, the presence of different Group I and Group II alkenone producers is known to complicate the use of these well-established U_{37}^K and $U_{37}^{K'}$ indices in lacustrine and brackish environments (Zheng et al., 2016b). New indices have been proposed based on Group I and II distributions that include novel tri-unsaturated isomers in the temperature ratios (Longo et al., 2016, 2018). Since Group I haptophytes are the most abundant in the Ancylus Lake phase (10.6 to 7.7 kyr), the R3b index seems more applicable for temperature reconstructions during this time period. Using the R3b temperature calibration from Longo et al. (2018), temperature estimates range from 4.0 – 9.2 °C throughout the Ancylus Lake phase, similar to estimates

using the Müller et al. (1998) calibration for the $U_{37}^{K'}$ index, which range from 4.7 – 7.7 °C (Fig. 3, Table 2).

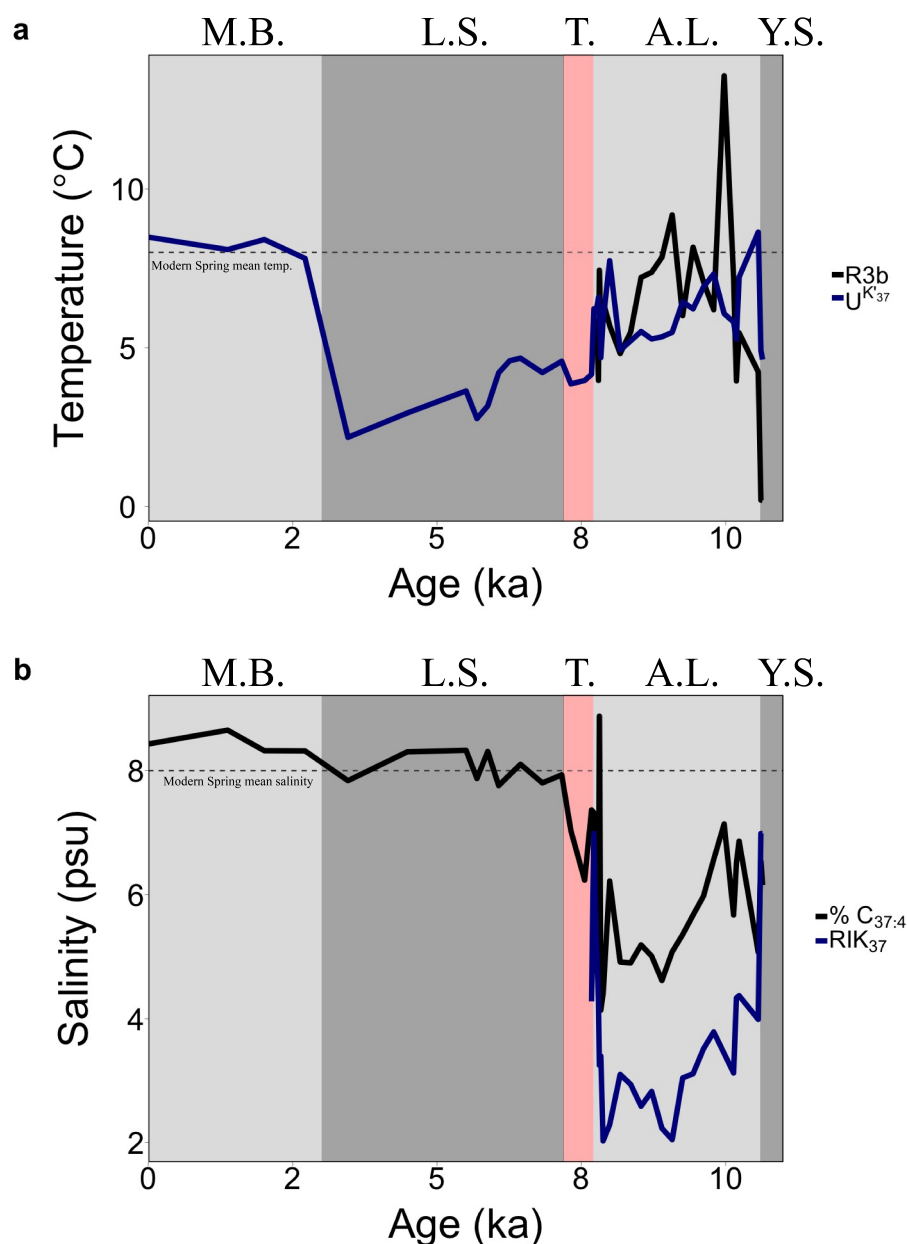


Figure 3: (a) Comparison of reconstructed temperatures based on the well-established $U_{37}^{K'}$ index and the newly proposed R3b index (Longo et al., 2018). The R3b index includes the tri-unsaturated C_{37} and C_{38} isomers and was therefore only applied to samples containing said isomers. (b) Reconstructed salinities using the Baltic Sea % $C_{37:4}$ calibration (Kaiser et al., 2017) and the RIK_{37} calibration (Kaiser et al., 2019), both from Baltic Sea surface sediments. Similar to the R3b temperature index, the RIK_{37} index includes the tri-unsaturated C_{37} isomer, thus was only applied to samples containing the tri-unsaturated C_{37} isomer. Present day spring mean temperature (8 °C) and salinity (8.1 psu) from the World Ocean Atlas 2013 (Locarnini et al., 2013; Zweng et al., 2013) are denoted by the dotted line.

Table 1: Hydrogen isotope ratios of C_{36:2}, and integrated C₃₇ and C₃₈ alkenones.

Depth (cm)	Age (kyr)	$\delta^2\text{H}_{\text{C}_{36}}$ (‰)		$\delta^2\text{H}_{\text{C}_{37}}$ (‰)		$\delta^2\text{H}_{\text{C}_{38}}$ (‰)	
		vs VSMOW)	S.D.	vs VSMOW)	S.D.	vs VSMOW)	S.D.
120	1.37	-173	2	-212	4	-208	1
320	2.71	-182	3	-209	1	-191	7
420	3.46	-188	2	-184	2	-173	1
600	5.31	-176	0	-181	3	-159	10
620	5.50	-179	1	-190	1	-182	4
640	5.69	-176	1	-180	5	-160	19
660	5.90	-178	0	-187	2	-167	12
700	6.26	-184	2	-193	3	-183	7
760	6.83	-191	3	-190	6	-172	3
800	7.16	-186	3	-191	3	-180	4
840	7.32	-172	1	-189	8	-189	1
900	7.56	-	-	-192	1	-200	3
930	7.68	-	-	-196	2	-193	2
940	7.72	-	-	-197	3	-189	2
950	7.76	-	-	-199	0	-193	4
960	7.80	-	-	-188	2	-195	1
964	7.81	-	-	-191	4	-191	2
970	7.84	-	-	-190	1	-196	3
980	7.88	-	-	-191	1	-193	14
1000	8.17	-	-	-186	1	-178	4
1010	8.35	-	-	-184	0	-177	0
1020	8.54	-	-	-183	1	-177	3
1030	8.72	-	-	-194	3	-182	0
1040	8.90	-	-	-187	1	-179	5
1050	9.07	-	-	-197	2	-202	2
1060	9.26	-	-	-193	1	-205	1
1070	9.44	-	-	-206	0	-201	0
1080	9.62	-	-	-233	3	-238	2
1090	9.79	-	-	-242	2	-252	3
1100	9.97	-	-	-231	4	-251	4
1110	10.14	-	-	-241	0	-233	7
1120	10.19	-	-	-239	2	-233	1
1200	10.56	-	-	-230	1	-224	2
1210	10.61	-	-	-228	3	-214	4
1216	10.64	-	-	-233	5	-211	1

Salinity

Salinities were reconstructed using the $\%C_{37:4}$ for the entire record, and the RIK_{37} based equation for the Ancyclus Lake phase since it was the only phase to contain C_{37} tri-unsaturated isomers. (equations 6 and 7, respectively; Kaiser et al., 2017, 2019). Values range from 4.1 to 8.7 ($\%C_{37:4}$), and 2.0 to 5.2 (RIK_{37}), with the $\%C_{37:4}$ yielding slightly higher values (Table 2). The reconstructed salinities based on $\%C_{37:4}$ start to rise at the end of the Ancyclus Lake phase leading into the Littorina Sea phase, and both $\%C_{37:4}$ and RIK_{37} calibrations show an increase in salinities around 7.7 ka when the marine transgression took place (Fig. 3). In general, $\%C_{37:4}$ gives a salinity value closest to the modern day spring mean of 8.1 psu (Zweng et al., 2013), suggesting it may be the most appropriate for our record. However, due to the presence of tri-unsaturated isomers during the Ancyclus Lake phase, the RIK_{37} salinity calibration might provide better estimates, since it includes these novel isomers. Indeed, using the RIK_{37} calibration yields an average salinity of 3 ± 0.4 , which seems more logical than an average of 6 ± 0.5 (obtained from the $\%C_{37:4}$ calibration) for this relatively fresh phase in the Baltic Sea history (Jensen et al., 1999). The Ancyclus Lake phase in the Arkona Basin is dominated by lacustrine macrofossils, diatoms and sedimentary deposits (Jensen et al., 1999), reinforcing a lower salinity to freshwater environment during this phase. Furthermore, the $\%C_{37:4}$ shows more fluctuations in salinity, whereas the RIK_{37} has a more steady decrease in salinity, in line with a shift from a brackish into a lacustrine, freshwater environment. In general, when tri-unsaturated isomers are present, indices accounting for these isomers seem more appropriate for reconstructing temperature and salinity. The above observations confirm the necessity of obtaining optimal separation of individual alkenones to enable detection of these informative isomers.

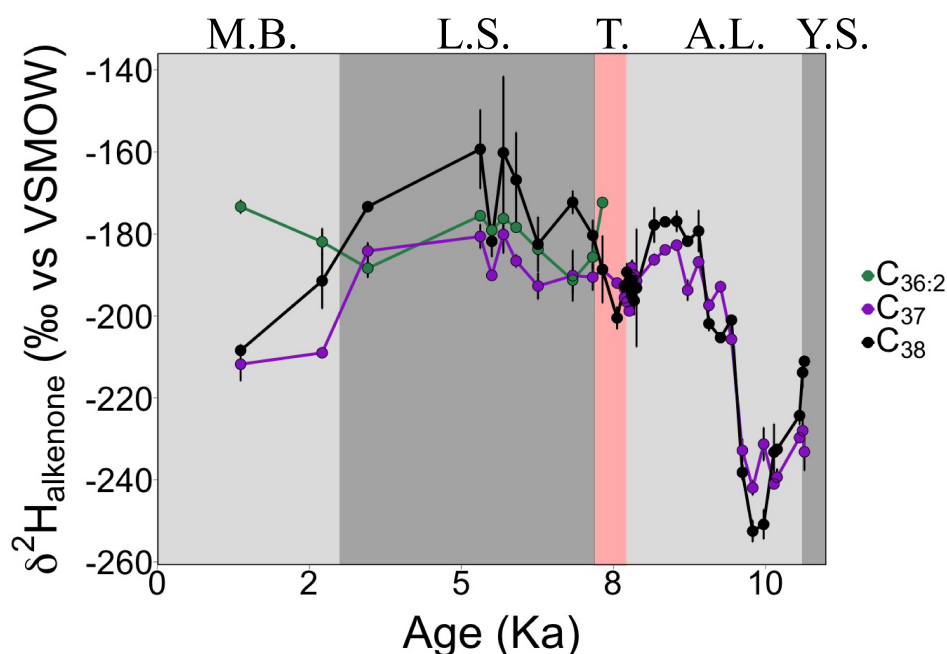


Figure 4: Hydrogen isotope ratios of $C_{36:2}$, and integrated C_{37} and C_{38} alkenones over the last 10.6 kyr in the Arkona Basin. Two major isotope shifts occur: the C_{37} and C_{38} alkenones become significantly enriched in the middle of the Ancyclus Lake phase, and the $C_{36:2}$ deviates from the C_{37} and C_{38} at the Littorina Sea to Modern Baltic phase shift. Error bars represent reproducibility of duplicate hydrogen isotope analyses.

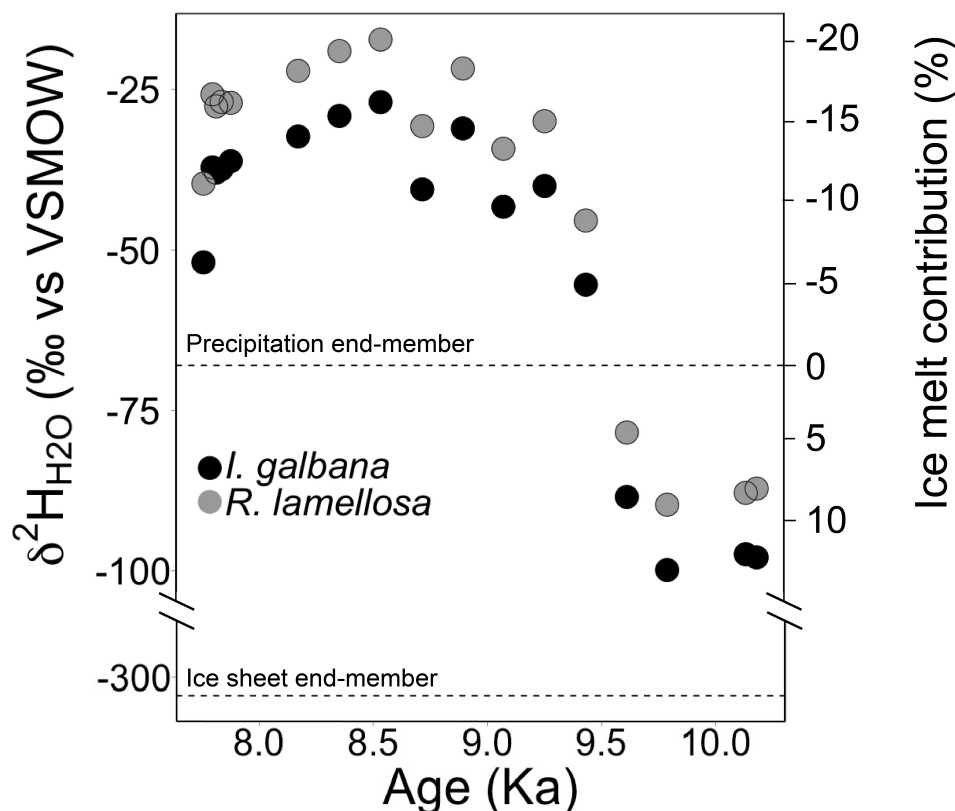


Figure 5: Reconstructed hydrogen isotope ratios of water plotted alongside the corresponding ice melt contribution for the Ancylus Lake phase. Ice melt contribution was calculated using a simple two end-member mixing model, with modeled Scandinavian Ice Sheet values and modern precipitation ratios as the two end-members. We accounted for fractionation values using results from cultures of two different Group II species *Isochrysis galbana* and *Ruttnera lamellosa*. Percent contribution of ice becomes negative, suggesting that the Scandinavian Ice Sheet no longer contributed water to the Baltic Sea after 9.5 ka and was replaced by a much lighter source.

3.3 Hydrogen isotope ratios

Hydrogen isotope ratios of integrated C_{37} and C_{38} alkenones were measured, and for the $C_{36:2}$ alkenones when present (Fig. 4). Isotope ratios for both C_{37} and C_{38} were integrated because separation was not sufficient to adequately separate the novel tri-unsaturated isomers from the standard tri-unsaturated alkenones for reliable isotope measurements. δ^2H ratios of C_{37} and C_{38} show two major shifts: a substantial enrichment of ~ 50 ‰ starting at approximately 10 ka (mid-Ancylus Lake phase), and a depletion of ~ 25 ‰ at around 3 ka (Littorina Sea to Modern Baltic phase boundary). The $C_{36:2}$ first occurs at the start of the Littorina Sea phase, and δ^2H ratios are similar to those of the C_{37} alkenones. The δ^2H ratios of the $C_{36:2}$ become enriched across the transition from the Littorina Sea into the Modern Baltic phase by around 15 ‰, deviating from the C_{37} and C_{38} alkenones, which become increasingly depleted by as much as 40 ‰ in the most modern sample at 1.37 ka (Fig. 4). It has been hypothesized that the $C_{36:2}$ ethyl is synthesized from the $C_{38:2}$ ethyl alkenones by β -oxidation (Zheng et al., 2016a). During the Littorina Sea phase, there is a relative enrichment of the C_{38} compared to the C_{36} , but this reverses in the Modern Baltic phase. It might be the case that when $C_{36:2}$ is synthesized

from $C_{38:2}$, the loss of four hydrogens via β -oxidation causes the C_{36} to become relatively 2H depleted. In the more modern phase, there might be a greater amount of C_{38} produced by a different species (Group III), causing relative depletion of the C_{38} pool which overwrites this degradation effect.

Table 2: Alkenone indices for the Ancylus Lake phase. Grey shading indicates when Group II species are dominant and no shading indicates that Group I species are dominant based on the binary mixing model from Longo et al. (2018).

Depth (cm)	Age (Kyr)	RIK ₃₇	RIK _{38E}	% Group I	% Group II	% $C_{37:4}$ Salinity (Kaiser et al., 2017)	R3b Salinity (Kaiser et al., 2019)	U ^K ₃₇ Temperature (Müller et al., 1998)	R3b Temperature (Longo et al., 2018)
950	7.76	0.89	0.84	0.25	0.75	6.5	5.3	6.1	5.9
960	7.80	0.82	0.72	0.41	0.59	5.4	4.3	6.6	4.0
964	7.81	0.75	0.72	0.57	0.43	8.9	3.2	6.0	7.4
970	7.84	0.76	0.63	0.55	0.45	4.1	3.4	4.7	5.4
980	7.88	0.67	0.60	0.75	0.25	4.4	2.0	5.8	6.5
990	7.99	0.69	0.63	0.71	0.29	6.2	2.3	7.7	5.7
1000	8.17	0.74	0.60	0.59	0.41	4.9	3.1	4.9	4.8
1010	8.35	0.73	0.61	0.62	0.38	4.9	2.9	5.2	5.5
1020	8.54	0.71	0.64	0.67	0.33	5.2	2.6	5.5	7.2
1030	8.72	0.72	0.66	0.63	0.37	5.0	2.8	5.3	7.4
1040	8.90	0.68	0.63	0.72	0.28	4.6	2.2	5.3	7.8
1050	9.07	0.67	0.67	0.75	0.25	5.1	2.1	5.5	9.2
1060	9.26	0.74	0.69	0.60	0.40	5.4	3.0	6.4	6.0
1070	9.44	0.74	0.75	0.59	0.41	5.7	3.1	6.2	8.2
1080	9.62	0.77	0.77	0.53	0.47	6.0	3.5	6.9	7.1
1090	9.79	0.79	0.76	0.49	0.51	6.6	3.8	7.3	6.2
1110	10.14	0.74	0.74	0.59	0.41	5.7	3.1	5.8	7.2
1120	10.19	0.82	0.72	0.40	0.60	6.5	4.3	5.3	4.0
1130	10.23	0.82	0.77	0.40	0.60	6.9	4.4	7.2	5.5

The two shifts observed in the C_{37} and C_{38} alkenones (at approximately 3 ka and 10 ka) reveal two separate effects that do not coincide with large shifts in reconstructed salinity values. Reconstructed salinities are stable from the Littorina Sea into the Modern Baltic phase at the Arkona Basin. The shift towards more negative values at 3 ka aligns with the phase boundary between the Littorina Sea and the Modern Baltic phase and coincides with a shift in the alkenone producing population from Group II dominance to a mixed Group II and Group III signal noted by changes in distributions (Fig. 2). As explained above, the C_{37} and C_{38} alkenones differ from the $C_{36:2}$ by up to 40 ‰ in the most modern sample. Our isotope data from the Modern Baltic phase cannot confirm the synthesis of $C_{36:2}$ from $C_{38:2}$ via β -oxidation, and instead suggests mixing of different haptophyte groups contributing alkenones to the sedimentary alkenone pool. Furthermore, as salinity becomes lower, more depleted isotope ratios for C_{37} and C_{38} alkenones are expected due to a lowering of the δ^2H of the water as well as increased isotopic fractionation (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). Compiled Group III culture data (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017) suggests that salinities of 7 to 8 psu result in isotope ratios of around -218 ‰, close to measured values for this interval. Group II species, on the other hand, tend to have more enriched isotope ratios at these salinities (around -186 ‰; based on results from Chivall et al., 2014 and M'Boule et al., 2014). The presence of the $C_{36:2}$ implies Group II contribution to the sedimentary alkenone

pool, and while the $C_{36:2}$ isotope ratios have not been reported from culture experiments, the similarity between $C_{36:2}$ with both the C_{37} and C_{38} during the Yoldia Sea phase, suggests that when produced by Group II species, the three do not differ by more than $\sim 15\text{‰}$. Collectively, these observations suggests that the isotope shift at 3 ka is caused by a change in species composition.

The large isotope enrichment of $\sim 50\text{‰}$ starting at 10 ka occurs in the middle of the Ancylus lake phase and does not coincide with a change in alkenone distributions, and hence the alkenone distribution-based salinity estimates also show no large fluctuations (Fig. 2 and 3). Thus, an alternative mechanism to explain the observed shift in δ^2H ratios has to be invoked. Source water isotope ratios also exhibit a strong control on δ^2H ratios (Paul, 2002; Englebrecht and Sachs, 2005). During this time period, the Scandinavian Ice Sheet, acting as an additional water source for the Baltic Sea, was decreasing in size as the result of an increase in high latitude summer insolation and changes in ablation rates (Cuzzone et al., 2016). Ice is generally known to have significantly depleted hydrogen isotope ratios, and model data suggests that the Scandinavian Ice Sheet, which formed during glacial conditions, had a δ^2H value of around -310‰ (de Boer et al., 2014). Modern day Baltic Sea precipitation has a value of $-68.1 \pm 0.7\text{‰}$ (Fröhlich et al., 1988), significantly more enriched compared to the melting Scandinavian Ice Sheet at this time. The difference between these two sources is large enough that a substantial contribution of ice melt derived source water would be reflected in δ^2H_{C37} ratios, resulting in significantly depleted values (Fig. 4).

To constrain this, the δ^2H ratios of source water can be reconstructed using calibration data from haptophyte cultures and a simple end-member mixing model with melt water and precipitation together contributing to the Baltic Sea water. The offset between δ^2H ratios of water and the δ^2H ratios of alkenones is not constant, and is affected by biological fractionation (fractionation factor α) occurring during alkenone synthesis (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). The fractionation factor α from two Group II species *I. galbana* and *R. lamellosa* at a salinity of around 3 (value obtained from the alkenone estimates; Fig. 3) is 0.841 and 0.832 respectively (M'Boule et al., 2014; Chivall et al., 2014). Assuming this fractionation value and using the measured δ^2H ratios of alkenones across the $\sim 50\text{‰}$ shift, we calculate that the δ^2H ratios of the water should change from -99‰ to -40‰ using *I. galbana* α values and -89‰ to -30‰ using *R. lamellosa* α values, respectively. Following this, we can estimate the percent contribution of both ice melt derived source water and precipitation using -310‰ and -68‰ as end members. As expected, it shows that there is a decrease in the percent contribution from ice melt derived source water starting at approximately 10 ka (Fig. 5). However, reconstructed contributions exceed 100 % (precipitation) or go into negative values (ice) at some points, suggesting that a freshwater source with a heavier δ^2H ratio than that of modern precipitation must have been present. Indeed, a rise in water level during this time period coincides with the creation of the Dana River, suggesting another freshwater source arose between 9.5 and 9.0 ka (Björck, 1995). Thus, a more 2H -enriched freshwater end-member, might not be unrealistic. Still, these values cannot fully explain the here observed range in δ^2H_{H2O} values. This would either require a heavier precipitation source, which is not likely in view of the reconstructed temperatures, or admixing of sea water. The sea water either somehow entered the basin (although it was closed off from the North Sea during this time), or some sea water remained from a more saline earlier phase in the basin (i.e. Yoldia Sea). Regardless of the δ^2H ratios of both end members (or three when including sea water), it can be estimated that the freshwater input directly from the melting Scandinavian Ice Sheet ceased to contribute to the freshwater budget around 9.5 ka. The R3b index indicates a spike in temperature coincident with the shift in source water,

suggesting that temperatures might have locally increased in response to the ice sheet being fully melted.

4 Conclusions

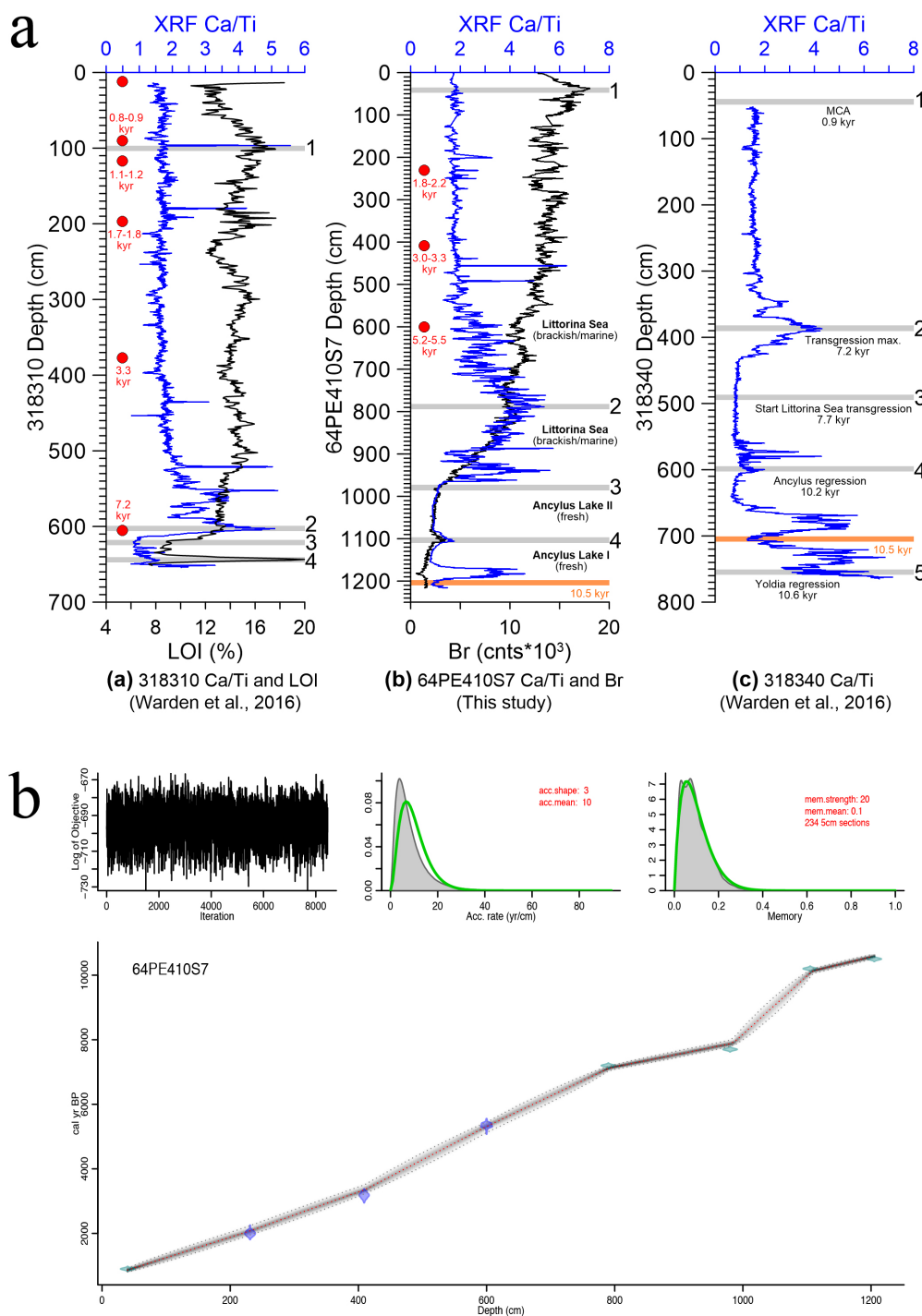
A large number of alkenone isomers were detected in Holocene age sediments from the Arkona Basin using improved gas chromatography separation, allowing for better determination of the haptophyte population in the Holocene Baltic. Tri-unsaturated isomers, indicative of Group I alkenone producers, are prominent during the Ancylus Lake freshwater phase from 10.6 to 7.7 kyr. For this reason, temperature and salinity calibrations based on indices including tri-unsaturated isomers provide more reliable estimates during this time period, circumventing issues associated with traditional alkenone temperature indices (i.e. anomalously high or low temperatures). Changes in distribution show mixing between Group III and II in the Yoldia Sea, Littorina Sea and Modern Baltic phases.

Hydrogen isotope ratios of long-chain alkenones inform about changes in salinity and species composition, but also provide insight into shifts in the dominant freshwater sources at the Arkona Basin. Results presented here show that analysis of well-separated alkenone distributions in conjunction with hydrogen isotope ratios provides a more in-depth understanding of species, salinity and hydrological changes in paleoenvironmental settings, especially in marginal marine environments.

Acknowledgements

The captain and crew of the RV Pelagia are thanked for their help with obtaining the piston core in the present investigation. This study received funding from the Netherlands Earth System Science Center (NESSC) through a Gravitation grant (024.002.001) from the Dutch Ministry for Education, Culture and Science. All acquired data will be stored in the Pangaea database.

Supplementary material



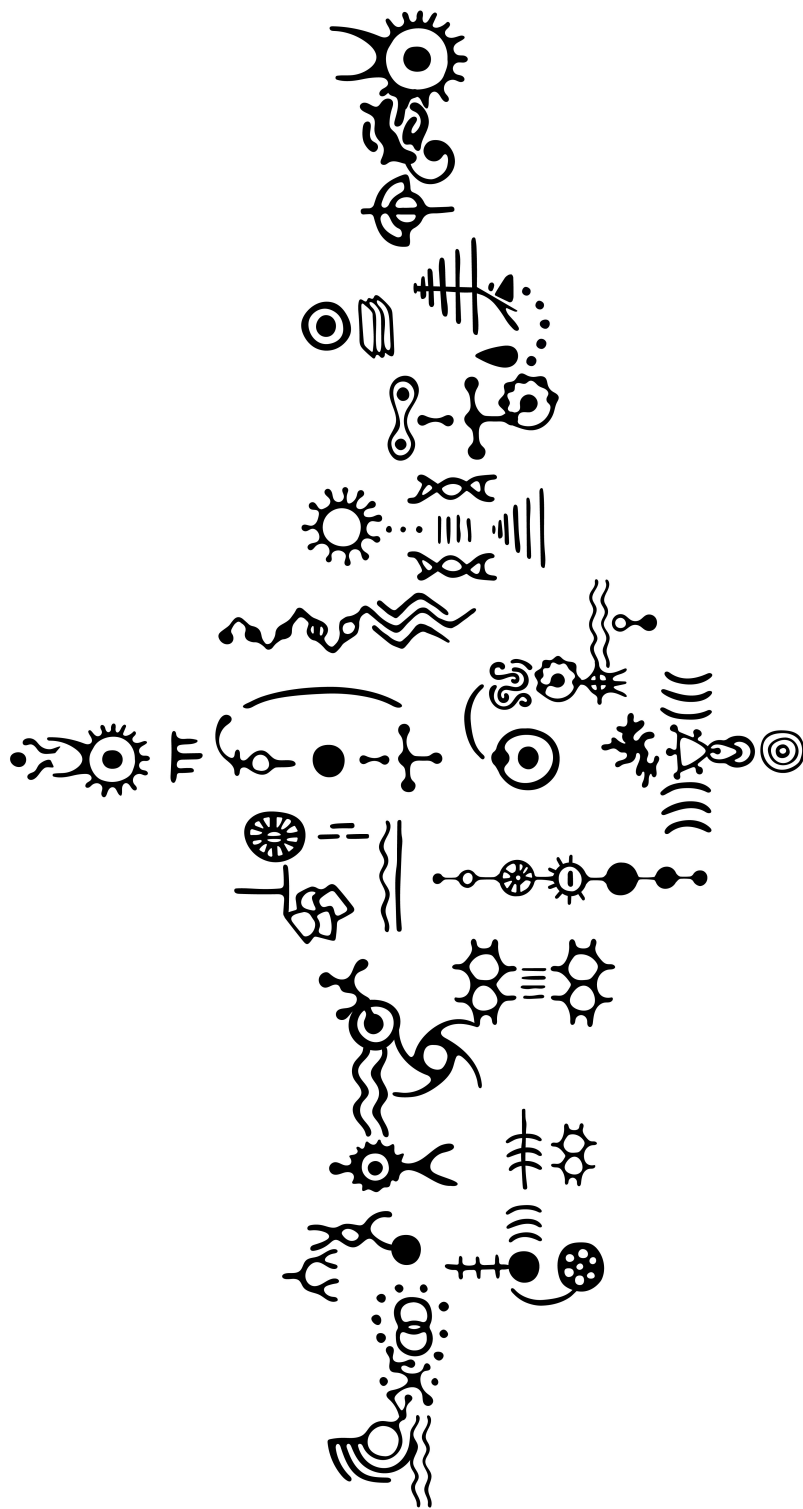
Supplementary Figure 1: (a) Correlation based on XRF Ca/Ti and Br between core 64PE410-S7 (this study) and two neighboring cores from Warden et al. (2016). Five tie-points were established: (1) the Medieval Climate Anomaly at 0.9 ka, (2) the transgression maximum at 7.2 ka, (3) the start of the Ancyclus Lake / Littorina Sea transition, (4) the Ancyclus Lake regression at 10.2 ka, and (5) the boundary between the Yoldia Sea and Ancyclus Lake at 10.6 ka. Red dots represent ¹⁴C dates. (b) The age-depth model created using the Bacon package in R.

Supplementary Table 1: Alkenone concentrations for sediment samples for core 64PE410-S7 in the Arkona Basin.

Depth (cm)	Age (kyr)	C _{35:2} (ng / g)	C _{36:2} (ng / g)	C _{37:4} (ng / g)	C _{37:3} (ng / g)	C _{37:3} * (ng / g)	C _{37:2} (ng / g)	C _{38:4} Ethyl (ng / g)	C _{38:3} Ethyl (ng / g)	C _{38:3} * Ethyl (ng / g)
120	1.38	157	325	20	1029	-	464	-	201	-
220	2.01	159	304	51	1033	-	489	-	216	-
320	2.71	202	242	58	1195	-	516	-	215	-
520	4.49	150	290	17	424	-	70	-	89	-
620	5.50	90	348	12	312	-	61	-	51	-
640	5.69	101	388	13	178	-	49	-	43	-
660	5.88	103	425	12	297	-	52	-	46	-
680	6.07	112	370	23	265	-	59	-	39	-
700	6.25	76	320	20	269	-	65	-	50	-
720	6.45	108	317	17	294	-	73	-	56	-
760	6.83	71	211	21	258	-	58	-	41	-
800	7.16	76	269	21	295	-	71	-	60	-
840	7.32	68	95	64	442	-	91	-	170	-
900	7.56	90	122	350	1583	-	336	-	593	-
930	7.68	-	-	529	4478	-	992	-	1417	-
940	7.73	-	-	660	4958	-	1647	-	1246	-
950	7.76	-	-	552	2349	295	769	376	1021	189
960	7.80	-	-	458	1136	253	402	320	540	209
970	7.84	-	-	656	1096	347	272	458	402	240
1000	8.17	-	-	201	414	146	108	220	160	108
1010	8.35	-	-	218	437	162	120	206	172	110
1020	8.53	-	-	139	297	124	87	164	118	67
1030	8.71	-	-	159	329	127	92	171	129	67
1040	8.90	-	-	63	108	51	31	65	42	25
1050	9.08	-	-	164	322	159	94	164	128	64
1060	9.25	-	-	182	413	148	142	162	214	94
1070	9.43	-	-	117	303	106	101	139	148	50
1080	9.61	-	-	119	349	106	131	124	197	58
1090	9.80	-	-	98	376	103	150	115	199	63
1100	9.98	-	-	113	370	511	120	149	174	87
1110	10.14	-	-	105	276	97	85	97	147	52
1120	10.19	-	-	47	198	43	55	53	90	35
1130	10.23	-	-	17	78	17	31	19	39	11
1200	10.56	-	-	28	56	14	27	27	21	11
1210	10.61	-	-	22	108	-	28	-	43	47
1216	10.64	-	-	31	134	-	33	-	53	54

Supplementary Table 1: (continued) Alkenone concentrations for sediment samples for core 64PE410-S7 in the Arkona Basin.

Depth (cm)	Age (kyr)	C _{38:2}	C _{38:3}	C _{38:2}	C _{39:4}	C _{39:3}	C _{39:2}	C _{39:3}	C _{39:2}	C _{40:3}	C _{40:2}
		Ethyl (ng / g)	Methyl (ng / g)	Methyl (ng / g)	Ethyl (ng / g)	Ethyl (ng / g)	Ethyl (ng / g)	Methyl (ng / g)	Methyl (ng / g)	Ethyl (ng / g)	Ethyl (ng / g)
120	1.38	364	239	138	-	42	66	-	-	-	-
220	2.01	371	282	169	-	47	62	-	-	-	-
320	2.71	317	382	182	-	72	62	-	-	-	-
520	4.49	76	56	20	-	-	-	-	-	-	-
620	5.50	79	48	11	-	-	-	-	-	-	-
640	5.69	69	27	13	-	-	-	-	-	-	-
660	5.88	86	51	24	-	-	-	-	-	-	-
680	6.07	89	39	26	-	-	-	-	-	-	-
700	6.25	84	38	15	-	-	-	-	-	-	-
720	6.45	95	40	19	-	-	-	-	-	-	-
760	6.83	64	32	23	-	-	-	-	-	-	-
800	7.16	88	41	19	-	-	-	-	-	-	-
840	7.32	145	30	24	-	-	-	-	-	-	-
900	7.56	409	40	16	-	-	-	-	-	-	-
930	7.68	1098	-	-	-	-	-	-	-	-	-
940	7.73	1418	-	-	-	-	-	-	-	-	-
950	7.76	948	358	179	60	176	108	588	205	113	130
960	7.80	401	284	180	53	104	77	334	110	49	67
970	7.84	269	254	105	56	99	55	245	72	46	69
1000	8.17	93	123	49	16	32	17	15	18	14	25
1010	8.35	106	108	68	37	68	41	157	73	21	29
1020	8.53	69	78	42	28	44	34	97	50	30	34
1030	8.71	66	85	41	31	43	28	109	33	21	19
1040	8.90	26	32	15	12	20	10	44	15	7	7
1050	9.08	74	93	49	20	49	25	93	32	58	25
1060	9.25	121	122	69	58	80	49	146	63	25	40
1070	9.43	74	70	34	23	41	22	82	24	19	22
1080	9.61	93	94	44	22	49	27	95	23	13	21
1090	9.80	110	97	51	23	47	24	130	23	26	23
1100	9.98	101	144	105	46	66	49	200	84	38	82
1110	10.14	67	84	47	-	43	15	115	62	63	24
1120	10.19	47	48	32	-	-	-	-	-	-	-
1130	10.23	20	14	12	-	-	-	-	-	-	-
1200	10.56	24	14	11	-	5	5	35	5	-	-
1210	10.61	-	13	11	-	-	-	-	-	-	-
1216	10.64	-	19	15	-	-	-	-	-	-	-



Microglyphs from 'Microbiocene: Ancient ooze to future myths' by Baum & Leahy in collaboration with Julie Lattaud, Laura Schreuder & Gabriella Weiss

Chapter 7

Paleosensitivity of hydrogen isotope ratios of long-chain alkenones to salinity changes at the Chile Margin

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Abstract

Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{\text{C}_{37}}$ ratios) have been shown to correlate with salinity in several culture studies. However, it is uncertain how applicable the $\delta^2\text{H}_{\text{C}_{37}}$ -salinity relationship is to reconstruct past salinity. $\delta^2\text{H}_{\text{C}_{37}}$ ratios were measured on sediments from a drill core (ODP site 1234) at the Chilean Margin covering the last 150 kyr. High $\delta^2\text{H}_{\text{C}_{37}}$ values corresponded to glacial time periods and interglacial periods are characterized by lower $\delta^2\text{H}_{\text{C}_{37}}$ values, aligning with $\delta^{18}\text{O}$ ratios measured on planktonic foraminifera from the same core. Effects of parameters such as species composition, the $\delta^2\text{H}$ - $\delta^{18}\text{O}$ relationship used for ice volume corrections, and nutrient or light controlled growth rate, did not appear to significantly alter $\delta^2\text{H}_{\text{C}_{37}}$ ratios at ODP 1234. We used linear regression equations from batch culture experiments, marine surface sediments, and suspended particulate organic matter to quantitatively characterize salinity changes over the last 150 kyr at ODP 1234. However, most of these equations yielded larger salinity shifts than previously suggested, a phenomenon also observed for other $\delta^2\text{H}_{\text{C}_{37}}$ records. This suggests that the paleosensitivity of $\delta^2\text{H}_{\text{C}_{37}}$ ratios to salinity was larger in the geologic record than has been observed in any modern environment or laboratory settings, or that glacial to interglacial salinity shifts might have been larger than currently believed.

Keywords

Hydrogen isotopes, alkenones, paleosalinity, Chile Margin

1 Introduction

Continental climate is regulated considerably by our global oceans. Ocean circulation transfers heat around the planet as a result of density driven changes caused in part by fluctuations in temperature and salinity (Rahmstorf, 2002). A robust understanding of global and regional climate shifts requires estimates of both parameters. Various organic and inorganic proxies are applied to reconstruct temperature throughout the geologic record (Emiliani, 1955; Brassell et al., 1986; Elderfield and Ganssen, 2000; Schouten et al., 2002). Reconstructions of salinity changes, on the other hand, are more complicated (Rohling, 2007). Inorganic proxies based on foraminiferal calcite include Ba/Ca ratios (Wilde et al., 2007), $\delta^{18}\text{O}$ ratios corrected for calcification temperature using Mg/Ca ratios (Duplessy et al., 1991), and Na/Ca (Wit et al., 2013; Mezger et al., 2018). Paleosalinity is also inferred from the hydrogen isotope composition of long-chain alkenones ($\delta^2\text{H}_{\text{C}_{37}}$), biomarker lipids synthesized by a select group of haptophyte algae of the order Isochrysidales (Marlowe et al., 1984). Marine haptophytes synthesize alkenones with 37 – 39 carbon atoms, and two to four degrees of unsaturation (de Leeuw et al., 1980; Volkman et al., 1980). The ratio of the tri-unsaturated C_{37} alkenone to the di-unsaturated C_{37} alkenone corresponds to changes in temperature, making it a useful proxy for understanding surface ocean temperature anomalies in the past (Brassell et al., 1986; Prahl and Wakeham, 1987). Hydrogen isotope ratios of long-chain alkenones correlated significantly with salinity in laboratory culture experiments, with more enriched isotope ratios corresponding to higher salinities (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). These studies have also provided insight about other environmental factors affecting hydrogen isotope fractionation in long-chain alkenones ($\alpha_{\text{C}_{37}}$), and how these factors might complicate the salinity signal recorded by alkenone $\delta^2\text{H}$ ratios ($\delta^2\text{H}_{\text{C}_{37}}$). For

example, growth rate, an important variable that affects lipid production, negatively correlated with the hydrogen isotope fractionation factor α (Schouten et al., 2006; Wolhowe et al., 2009; Sachs and Kawka, 2015). When growth was kept constant, the correlation between $\delta^2\text{H}_{\text{C}37}$ ratios and salinity remained (Sachs et al., 2016), suggesting that growth rate might exhibit an insignificant control on $\delta^2\text{H}_{\text{C}37}$ ratios relative to salinity. Irradiance is a vital resource for photosynthesizing organisms like haptophytes, and a parameter that can control algal growth rate. Culture and open ocean datasets showed that irradiance affects $\delta^2\text{H}_{\text{C}37}$ ratios and $\alpha_{\text{C}37}$ at light intensities below $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, but above 200 the effect is negligible (van der Meer et al., 2015; Wolhowe et al., 2015). In addition to parameters like growth rate and irradiance, different alkenone-producing haptophyte species are known to produce alkenones with different $\delta^2\text{H}_{\text{C}37}$ ratios resulting in distinct $\alpha_{\text{C}37}$ values under identical growth conditions in both cultures and the natural environment (Schwab and Sachs, 2011; Chivall et al., 2014; M'Boule et al., 2014; Nelson and Sachs, 2014; Weiss et al., 2019). Alkenone producing haptophyte species are genetically divided into three distinct groups: Group I are lacustrine alkenone producers with no cultured representatives at the present inhabiting fresh water and oligohaline lakes (Theroux et al., 2010). Group II haptophytes live in lacustrine to hypersaline lakes and coastal environments, and Group III are open marine species (Theroux et al., 2010). In batch culture, there is approximately 100 ‰ difference between hydrogen isotope ratios of alkenones Group II and III species grown at the same salinity (Chivall et al., 2014; M'Boule et al., 2014). Group III species *Emiliania huxleyi* and *Gephyrocapsa oceanica* also diverge (ca 25 ‰), but to a lesser degree than observed between Group II and III (Schouten et al., 2006).

Despite the fact that a number of environmental factors affected $\delta^2\text{H}_{\text{C}37}$ ratios in culture, salinity always showed a significant positive correlation with $\delta^2\text{H}_{\text{C}37}$ ratios in cultures (Schouten et al., 2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). For this reason, $\delta^2\text{H}_{\text{C}37}$ ratios have been used to infer qualitative information about salinity changes in the geologic record (Pahnke et al., 2007; van der Meer et al., 2007, 2008; Leduc et al., 2013; Vasiliev et al., 2013; Kasper et al., 2014, 2015; Petrick et al., 2015; Simon et al., 2015). However, the slopes of the linear regression equations for the relationship between $\delta^2\text{H}_{\text{C}37}$ ratios and salinity vary considerably between culture experiments, ranging from 4.8 (Schouten et al., 2006) to 1.3 (Sachs et al., 2016), equating to a 4.8 ‰ or 1.3 ‰ change in $\delta^2\text{H}_{\text{C}37}$ ratio per practical salinity unit (psu), respectively. Alkenones in marine surface sediments had a 1.3 ‰ response to salinity (Weiss et al., 2019), and alkenones in suspended particulate organic matter (SPOM) from the Atlantic and Pacific oceans had a $\delta^2\text{H}_{\text{C}37}$ – salinity response of 4.2 (Gould et al., 2019). These large differences make it difficult to quantitatively constrain past salinity changes.

To understand the sensitivity of the $\delta^2\text{H}_{\text{C}37}$ – salinity relationship in past settings, we measured $\delta^2\text{H}_{\text{C}37}$ ratios on a down-core record from ODP site 1234, off the coast of Chile. This system is important because of the influence of surface and deep water currents (which cause upwelling and contribute to productivity), freshwater runoff, and southern westerly winds, making this setting a dynamic test site for enhancing our understanding of the controlling factors on $\delta^2\text{H}_{\text{C}37}$ ratios in the geologic record. For example, the convergence of different water masses and the input of continental runoff could potentially be recorded in $\delta^2\text{H}_{\text{C}37}$ values. The site was previously studied for past upwelling conditions and sea surface temperature evolution using long-chain diols and other lipid biomarkers (de Bar et al., 2018), and covers the last 150 kyr and six marine isotope stages (Lisiecki and Raymo, 2005). We compared the ODP 1234 $\delta^2\text{H}_{\text{C}37}$ record to other sediment records of $\delta^2\text{H}_{\text{C}37}$ ratios to infer how sensitive $\delta^2\text{H}_{\text{C}37}$ ratios are to past salinity changes overall.

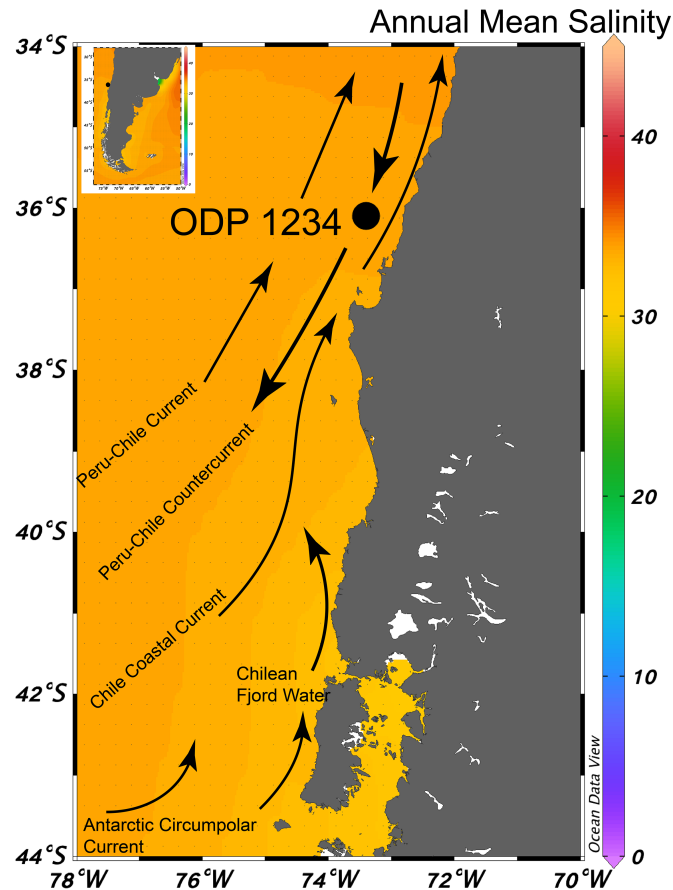


Figure 1. Map showing the environmental setting of ODP 1234. The core location is influenced by the Northward Peru-Chile Current and Chilean Coastal Current, and the southward Peru-Chile Countercurrent. In the south, Antarctic Circumpolar waters potentially feed into the PCC, CCC, and PCCC, and Chilean Fjord Waters can also enter the system from the southeast.

2 Materials and Methods

2.1 Geographic Setting

The Peru-Chile upwelling system has been the subject of a number of studies (Dugdale et al., 1977; Busch and Keller, 1981; Vargas et al., 2007), and has been referred to as a “one of the world’s high productivity regions of great significance” (Marchant et al., 1999). Site ODP 1234 (36°13.153’ S, 73°40.902’ W) was drilled on the continental slope ~ 70 km away from the coast at 1015 m water depth, just off the continental shelf which extends to about 60 km (Strub et al., 1998). The major currents at this site are the Peru-Chile Countercurrent (PCCC) which flows southward, surrounded on the east by the northward flowing Peru-Chile Current (PCC) and on the west by the northward flowing Chile Coastal Current (CC). The Antarctic Circumpolar Current (ACC) feeds into the PCCC and Chilean Fjord Water (CFW) feeds into the CC (Strub et al., 1998; Lamy et al., 2002), potentially adding isotopically depleted water into the system (Fig. 1). Isotopically depleted water is also introduced via freshwater runoff coming from the nearby Río Bio-Bio and Río Itata (Muratli et al., 2010). Upwelling brings the nutrient rich, cold water to the surface, resulting in high productivity (Bakun, 1990). The modern day surface salinity at the site is 33.8 psu, with higher salinity of 34.2 psu to the northwest of the site and lower salinity of 33 psu at the south near the Los Lagos region (Mix

et al., 2003), covering a range slightly larger than 1 psu for the region.

2.2 Long-chain alkenones

Samples were taken in ~ 2 kyr resolution ($n = 72$). Age model specifics are described in de Bar et al. (2018), who used a modified version of the age model from Heusser et al. (2006). The age-depth model was constructed based on benthic $\delta^{18}\text{O}$ correlated to the Vostok ice core chronology and core MD95204 from the Atlantic in conjunction with radiocarbon dating. Two age models were constructed for the upper and lower halves of the sections, and these were connected by linear interpolation between ca. 83 to 90 ka. Organics were extracted from the sediments and ketone fractions (de Bar et al., 2018) were used for hydrogen isotope measurements of alkenones (Weiss et al., 2019). Hydrogen isotope ratios were measured on alkenones in duplicate using on-column injection on a gas chromatograph coupled to a Thermo Delta V isotope ratio mass spectrometer via a high temperature conversion reactor (Isolink I) and Conflo IV. The GC was equipped with an RTX-200 column (Restek, 60 m x 0.32 mm x 0.5 μm). The GC temperature program is as follows: 70 °C to 250 °C at 18 °C/min, 250 – 320 °C at 1.5 °C/min, then 320 °C for 25 min at a flow rate of 1.5 mL / min. The RTX-200 60 m column allows for better separation and determination of isotope ratios of individual alkenones, and thus we report the individual $\text{C}_{37:3}$ and $\text{C}_{37:2}$ alkenones, as well as the integrated C_{37} alkenones. The integrated C_{37} $\delta^2\text{H}$ ratios were determined by a manual peak integration combining the two $\text{C}_{37:3}$ and $\text{C}_{37:2}$ peaks and used for better comparison with previously published records of $\delta^2\text{H}$ ratios which used combined $\text{C}_{37:3}$ and $\text{C}_{37:2}$ isotope ratios. The H_3^+ factor was measured and corrected for at the start of each day before measuring standards and samples, and varied from 2.633 – 3.195 ppm nA^{-1} , with differences of less than 0.1 ppm nA^{-1} from day to day. To monitor machine performance and stability, an n -alkane standard, Mix B (supplied by A. Schimmelmann, Indiana University), was run at the start of each day and samples were only run when the average difference and standard deviation between online and offline values was less than 5 ‰. Squalane and C_{30} n -alkane were co-injected with each $\delta^2\text{H}$ sample run to further monitor machine performance. Values for squalane were -165 ± 3 ‰, and C_{30} were -75 ± 4 ‰ over the entire sample set, which fit well with predetermined values of -170 ± 4 ‰ and -79 ± 5 ‰ respectively. Isotope ratios presented here are based on single-point referencing to a calibrated gas cylinder, and error bars represent the reproducibility between duplicate or triplicate analytical runs.

2.3 Paleosalinity calculation

A global ice volume correction for $\delta^2\text{H}_{\text{C}_{37}}$ ratios was taken from the ANICE fully coupled 3D ice-sheet-sea-level model (de Boer et al., 2014). Since the ice volume correction from ANICE is for oxygen isotope ratios, we converted these to hydrogen isotope ratios using the global meteoric water line from Craig and Gordon (1965):

$$\delta^2\text{H} = 8 \times \delta^{18}\text{O} + 10 \quad (1)$$

Salinity was calculated from ice volume corrected $\delta^2\text{H}_{\text{C}_{37}}$ ratios using a surface sediment calibration (Weiss et al., 2019):

$$\delta^2\text{H}_{\text{C}_{37}} = 1.3 \times S - 227 \quad (2)$$

and, to contrast with the smaller sensitivity, the culture calibration from Schouten et al. (2006) for *Emiliania huxleyi*:

$$\delta^2 H_{C37} = 4.8 \times S - 347 \quad (3)$$

and the SPOM calibration from Gould et al. (2019):

$$\delta^2 H_{C37} = 4.2 \times S - 340 \quad (4)$$

We selected the Schouten et al. (2006) equation because *E. huxleyi* is the dominant modern alkenone producing haptophyte in the south eastern Pacific (Hagino and Okada, 2004, 2006). The SPOM calibration represents the environmental $\delta^2 H_{C37}$ response to salinity in the open ocean at the time of sampling, and therefore captures a snapshot of the marine signal, which can be juxtaposed with the surface sediment calibration.

3 Results and Discussion

3.1 Salinity reconstructions

$\delta^2 H_{C37}$ ratios ranged from -195 ‰ to -167 ‰, covering a range of 28 ‰ over the last ~150 kyr at ODP 1234 (Table 1; Fig. 2a). The $\delta^2 H_{C37}$ ratios correlated well with different marine isotope stages MIS 1 – 6, and even the substages of MIS 5 (Fig. 2a). The most enriched $\delta^2 H_{C37}$ ratios fall on the boundary between MIS 2 and 3 and in MIS 6; the most depleted $\delta^2 H_{C37}$ ratios correspond to MIS 5, and specifically MIS 5e, the Eemian interglacial. Oxygen isotope ratios measured on the planktonic foraminifera *G. bulloides* for this site (de Bar et al., 2018) likewise recorded more enriched values during colder time periods and more depleted values during warmer climates (Fig. 2b). Isotope ratios for individual tri and di-unsaturated C_{37} alkenones were also determined (Table 1; Supp. Fig. 1). In general, both alkenones followed the same trend as the integrated C_{37} alkenones. Interestingly, the two diverge at the LGM, and the tri-unsaturated alkenones were more enriched in 2H , while the di-unsaturated alkenones became more depleted. This divergence is likely the result of a change in the relative abundance of the two alkenones. The difference in $\delta^2 H$ ratios between the two alkenones is known to be quite variable (D'Andrea et al., 2007; Schwab and Sachs, 2009; Wolhowe et al., 2009; van der Meer et al., 2013). The $C_{37:3}$ is synthesized from the $C_{37:2}$, and at colder temperatures there is a lower relative abundance of the $C_{37:2}$ (Endo et al., 2018; Kitamura et al., 2018). Normally, the synthesis of $C_{37:3}$ from a $C_{37:2}$ causes a depletion of the $C_{37:3}$, leaving the $C_{37:2}$ relatively enriched, since desaturation is associated with isotopic depletion (Chikaraishi et al., 2004). However, at colder temperatures, where low abundances of the $C_{37:2}$ and higher amounts of $C_{37:3}$ are expected, the synthesis of $C_{37:3}$ would become dominant and actually result in enrichment of the $C_{37:3}$ and depletion of the $C_{37:2}$ (van der Meer et al., 2013). Since most studies and salinity calibrations are based on the integrated C_{37} alkenones, we focused on these for the following discussions.

$\delta^2 H_{C37}$ ratios were corrected for global ice volume using the ANICE model (de Boer et al., 2014) and the global meteoric water line (Craig and Gordon, 1965; equation 1). From these ice volume corrected $\delta^2 H_{C37}$ ratios, salinity shifts were calculated using three different equations: the surface sediment calibration of Weiss et al. (2019; equation 2), the *E. huxleyi* batch culture calibration (Schouten et al., 2006, equation 3), and the SPOM calibration from Gould et al. (2019; equation 4). Equation 2 yielded a substantial salinity range from 23 to 42 psu, with a value of 24 for the most modern sample (Fig. 3). Since ODP 1234 is

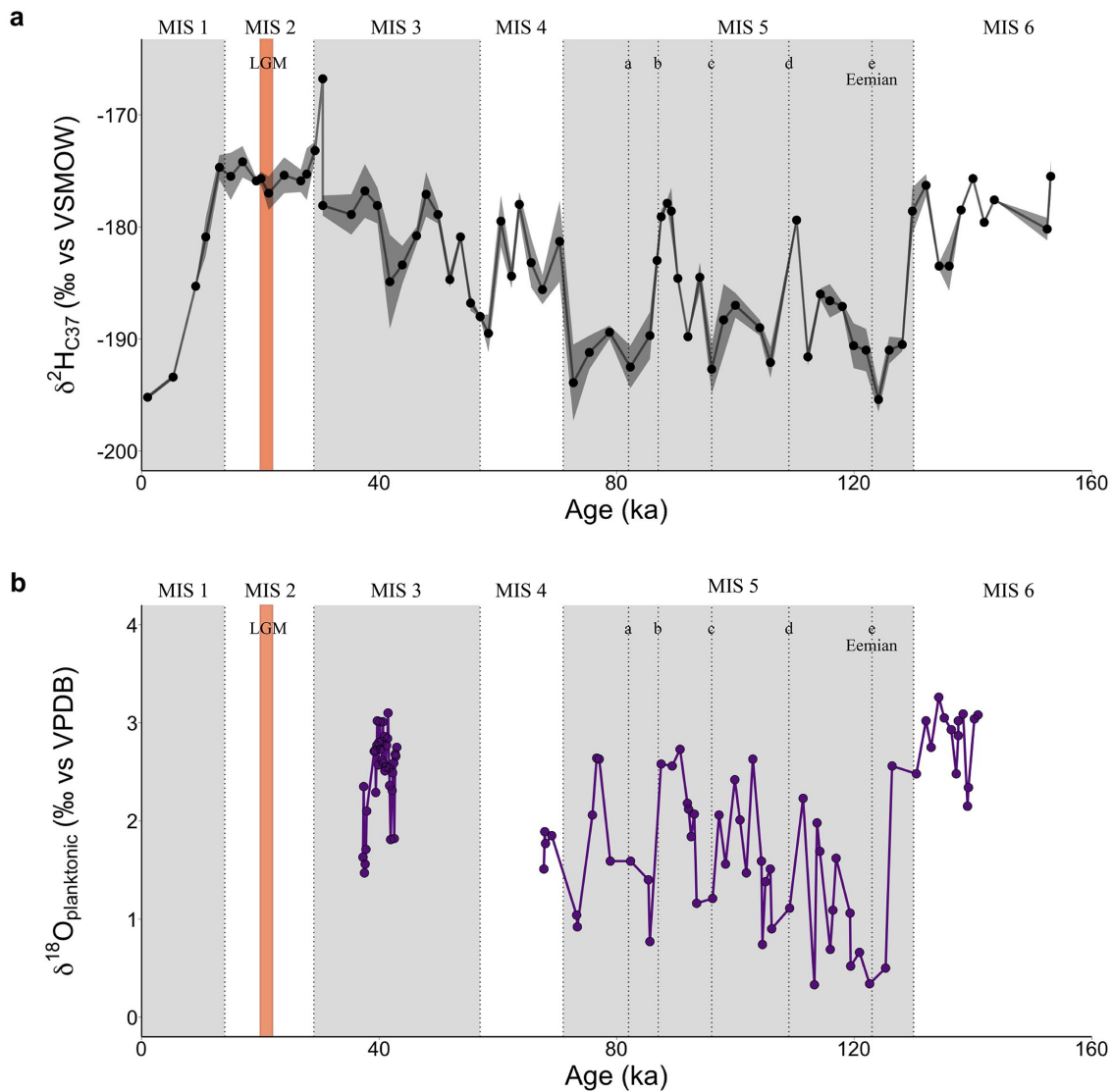


Figure 2. (a) Record of $\delta^2\text{H}_{\text{C}37}$ ratios and (b) $\delta^{18}\text{O}$ ratios of planktonic foraminifera over the last ~150 kyr (adapted from de Bar et al., 2018) plotted with marine isotope stages 1-6. Grey shading in (a) represents the standard deviation of duplicate isotope measurements. $\delta^2\text{H}_{\text{C}37}$ and $\delta^{18}\text{O}$ ratios follow the same trends, with more enriched isotope ratios corresponding to colder time periods and more depleted isotope ratios corresponding to warmer climates.

located ~70 km away from the coast with a water depth of 1015 m, and salinity variations are thought to be on the order of 1 – 2 psu between glacial and interglacial periods (Adkins et al., 2002; Broecker, 2002), the large range of salinity resulting from equation 2 seems unrealistic. Equation 2 most likely records mixing of alkenones from different haptophyte groups because it includes sediments from marginal marine environments with lower salinities (Weiss et al., 2019). Although these different haptophyte groups have a similar response to salinity, they each have characteristic isotope ratios, offset by ~100 ‰. At the present, the Group III haptophyte *E. huxleyi* is the dominant alkenone-producing haptophyte off the western coast of South America (Hagino and Okada, 2004; 2006). Furthermore, analysis of calcareous nannofossils in ODP 1234 showed a dominance of *E. huxleyi* and a very low abundance of *G. oceanica* throughout the studied interval (Mix et al., 2003). Indeed, alkenone distributions

at ODP 1234 were distinctly Group III since no isomers of tri-unsaturated alkenones were detected, nor were any significant changes in abundance of C_{38} methyl alkenones observed, both of which are characteristic of Group I or II haptophytes (Longo et al., 2013; Dillon et al., 2016; Longo et al., 2016; Zheng et al., 2016). Thus, the sediment calibration of Weiss et al. (2019) is likely not applicable and it may be more logical to use a species-specific calibration to calculate paleosalinity at ODP 1234. Consequently, equation 3 for *E. huxleyi* from Schouten et al. (2006) was applied and reconstructed salinities varied on the order of 5 psu instead of 19 psu (Fig. 3). Using equation 3 gave a value of 32 for the most recent sample, which is in better agreement with modern measured values from the site than equation 2, although still underestimating the modern value of 33.8. Applying the Atlantic and Pacific SPOM calibration from Gould et al. (2019) (equation 4) provided an equivalent range of 6 psu (Fig. 3). The most modern sample had a salinity of 34, which aligns nicely with the modern day salinity of 33.8 (Mix et al., 2003).

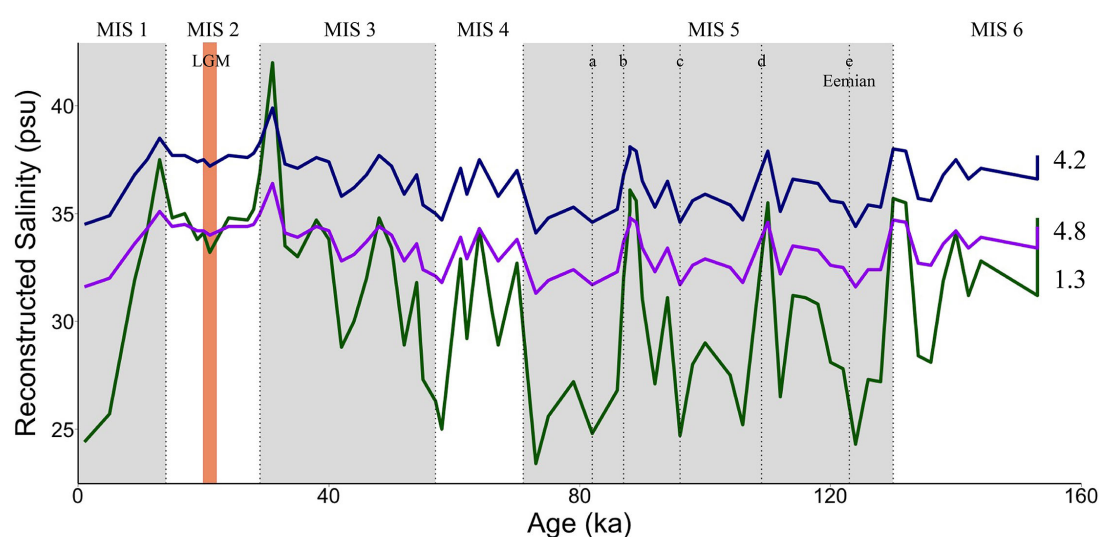


Figure 3. Reconstructed salinities (psu) for ODP 1234 using the marine surface sediment calibration from Weiss et al. (2019) in green with a slope of 1.3, the linear regression equation from Schouten et al. (2006) batch culture experiment growing *Emiliania huxleyi* in light purple with a slope of 4.8, and the SPOM calibration from Gould et al. (2019) in dark blue with a slope of 4.2. The surface sediment calibration yielded a salinity shift of ~ 19 psu over the entire record, whereas the *E. huxleyi* and SPOM equations covered a more reasonable salinity shift of ~ 5 psu across the record. The SPOM equation (Gould et al., 2019) provided the best estimate for the most modern sample of 34 psu, aligning with the measured salinity of 33.8 psu at ODP 1234.

Foraminiferal based proxies and climate models suggest that global ocean salinity changed on the order of 1 – 2 psu between now and the Last Glacial Maximum (LGM; Adkins et al., 2002; Broecker, 2002). The δ^2H_{C37} ratios of the Chilean Margin suggested a much larger change, regardless of the calibration used to reconstruct salinities. As outlined above, a number of environmental factors have been shown to affect δ^2H_{C37} ratios by causing more or less fractionation between alkenones and growth water (Schouten et al., 2006; Wolhowe et al., 2009; Chivall et al., 2014; M'Boule et al., 2014; Sachs and Kawka, 2015; van der Meer et al., 2015; Sachs et al., 2016; Weiss et al., 2017), which may have contributed to the large changes in δ^2H_{C37} ratios. These potential effects will be explored below.

3.2 Species and temperature effects on salinity reconstructions

Based on alkenone distribution patterns down-core, we found only alkenones synthesized by Group III haptophytes (i.e., $C_{37:3}$, $C_{37:2}$ methyl alkenones and $C_{38:3}$, $C_{38:2}$ ethyl and methyl alkenones). *E. huxleyi* is the most prevalent alkenone producing haptophyte in the modern Pacific Ocean around South America (Hagino and Okada, 2004, 2006). Nonetheless, as explained above, it is possible that fluctuations in dominant Group III alkenone producers *E. huxleyi* and *G. oceanica* occurred over the last 150 kyr, although nannofossil data for ODP 1234 showed a dominance of *E. huxleyi* and very sparse presence of *G. oceanica* throughout the last ~ 150 kyr (Mix et al., 2003). Although the differences in the slopes for the δ^2H_{C37} – salinity equation between *E. huxleyi* and *G. oceanica* are not substantial (4.8 versus 4.2 respectively, Schouten et al., 2006), *G. oceanica* fractionates hydrogen to a larger extent than *E. huxleyi*. Thus, if changes in the main alkenone producer took place in the past, additional shifts in δ^2H_{C37} , on top of those caused by salinity changes, could occur. *G. oceanica* is thought to be the principal alkenone producer in warm, high salinity marginal seas, while *E. huxleyi* is more dominant in open ocean settings (Okada and Honjo, 1975). It might have been the case that *G. oceanica* became the major contributor of alkenones into the sedimentary record during warmer time periods, and *E. huxleyi* supplied the majority of alkenones in colder climates. Furthermore, it has been suggested that temperature itself can affect the δ^2H_{C37} (Wolhowe et al., 2009). However, compiled results of temperature experiments with *E. huxleyi* and *G. oceanica* showed an insignificant negative correlation between δ^2H_{C37} and temperature ($r = -0.43$, $p > 0.05$; data from Schouten et al., 2006 and Wolhowe et al., 2009). In addition, analyses of SPM in the Pacific Ocean over a temperature change of 1 – 4 °C, showed no systematic changes in δ^2H_{C37} ratios or in alkenone producers (Wolfshorndl et al., 2019), suggesting that the potential effect of temperature on either relative abundance of alkenone producers or δ^2H_{C37} ratios hypothesized from culture studies might not actually exist in the natural environment. $U_{37}^{K'}$ ratios from ODP 1234 suggested a temperature change of 3 °C between the LGM and the most modern sample (de Bar et al., 2018), similar to the range of temperature observed in the Pacific Ocean study (Wolfshorndl et al., 2019). Thus, by extrapolating these results to our dataset, we concluded that the temperature shifts between the LGM and the modern are not large enough to cause substantial shifts in relative abundance of alkenone producers, nor should these temperature changes have had a significant effect on δ^2H_{C37} ratios.

3.3 Nutrients and light

Algal growth rate is regulated by nutrient availability and light intensity. Both parameters caused changes in fractionation in batch and continuous culture experiments of different haptophyte species (Sachs and Kawka, 2015; van der Meer et al., 2015). Growth rate negatively correlated with fractionation factor α_{C37} , meaning faster growth led to increased fractionation (Schouten et al., 2006; Sachs and Kawka, 2015). Light intensity caused significant variation in α_{C37} at irradiance levels below $\sim 200 \mu \text{mol photons m}^{-2} \text{s}^{-1}$, but above $200 \mu \text{mol photons m}^{-2} \text{s}^{-1}$ there was a negligible effect of light on fractionation (van der Meer et al., 2015). A field comparison between light-limited and nutrient-limited sites in the Pacific Ocean suggested that environmental variation in δ^2H ratios under different irradiance and nutrient regimes was smaller than observed in laboratory culture, and therefore may not be of significant concern for paleo studies (Wolfshorndl et al., 2019). Since the sediment samples used here encompass numerous years and growth seasons, we could not actually correct for

these parameters nor constrain them on a per event basis. Furthermore, the effects of irradiance and nutrient concentration were hypothesized to be negligible in the field (Wolfshorndl et al., 2019), leading to the assumption that they did not significantly contribute to the variation in the $\delta^2\text{H}_{C37}$ ratios at ODP 1234.

3.4 Influences of water isotope ratios

Mixing of different water masses has the potential to cause variation in $\delta^2\text{H}_{\text{H}_2\text{O}}$ and therefore, $\delta^2\text{H}_{C37}$ ratios. Antarctic waters are colder and isotopically lighter than water in the tropics (Lee et al., 2007). A strengthening of the ACC during glacial periods has been hypothesized (Pudsey and Howe, 1998; Barker and Thomas, 2004) and thus shifts in $\delta^2\text{H}_{C37}$ ratios could correspond to more input of these colder and relatively depleted waters via the ACC during glacials. However, the observed shifts in $\delta^2\text{H}_{C37}$ ratios were not large enough to be attributed to ACC as the dominant water mass during glacial periods because influence of isotopically lighter and lower salinity waters would cause a substantially depleted $\delta^2\text{H}_{C37}$ signal, similar to values noted for off-shore Colombia below -205 ‰ (Pahnke et al., 2007). For the same reason, we can also exclude the influence of large volumes of river runoff, which are similarly characterized by a significantly depleted $\delta^2\text{H}_{C37}$ signal. Such depleted values were not observed at ODP 1234, leading to the conclusion that neither the ACC nor substantial continental runoff could be evidenced in the $\delta^2\text{H}_{C37}$ ratios at ODP 1234 during the last ~150 kyr.

Past $\delta^2\text{H}$ water isotope ratios were calculated from oxygen isotope ratios, either from models or other proxy data, using the meteoric water line (equation 1), and used to correct for the impact of global ice volume changes. In a highly evaporative basin, such as the Mediterranean, the local meteoric water line has higher d excess, but the slope is the same as the global meteoric water line (Gat, 1980, 1982; Gat and Carmi, 1987). For the majority of the surface ocean, it is hypothesized that d excess is near zero and only reaches as high as six in highly evaporative regions (Rohling, 2007). Thus, potential differences in the $\delta^{18}\text{O}$ and $\delta^2\text{H}$ correlation in the surface ocean are not large enough to cause significant changes to the ice volume correction, and therefore would not substantially alter our paleosalinity reconstructions.

Thus, at this point the seemingly higher sensitivity of $\delta^2\text{H}_{C37}$ to paleosalinity changes compared to present day sensitivity in cultures and the natural environment cannot be explained by any of the parameters that have been shown to affect this sensitivity in culture or environmental datasets. For this reason, we conclude that the $\delta^2\text{H}_{C37}$ ratios at ODP 1234 were primarily reflecting changes in sea surface salinity, and these changes may either be larger than previously hypothesized (Adkins et al., 2002; Broecker, 2002), or reflect a greater sensitivity of $\delta^2\text{H}_{C37}$ to salinity.

3.5 Comparison with previous records

Paleosalinity estimates based on $\delta^{18}\text{O}$ ratios of the foraminifera *Neoglobobulimina pachyderma* (corrected for temperature using UK'37 temperature estimates) over the last 8 kyr at a nearby site showed a change from 33 – 36 psu over this short time period (Lamy et al., 2002) suggesting that this region might have larger fluctuations in salinity than the global average. While $\delta^2\text{H}_{C37}$ ratios suggest larger salinity shifts over longer time periods, both the Schouten et al. (2006) and Gould et al. (2019) calibrations suggest a salinity change of ~2 psu over the last 9 kyr. Estimates based on foraminiferal $\delta^{18}\text{O}$ ratios combined with $U_{37}^{K'}$ temperatures are subject to large uncertainties (Rohling, 2007), and might be biased by

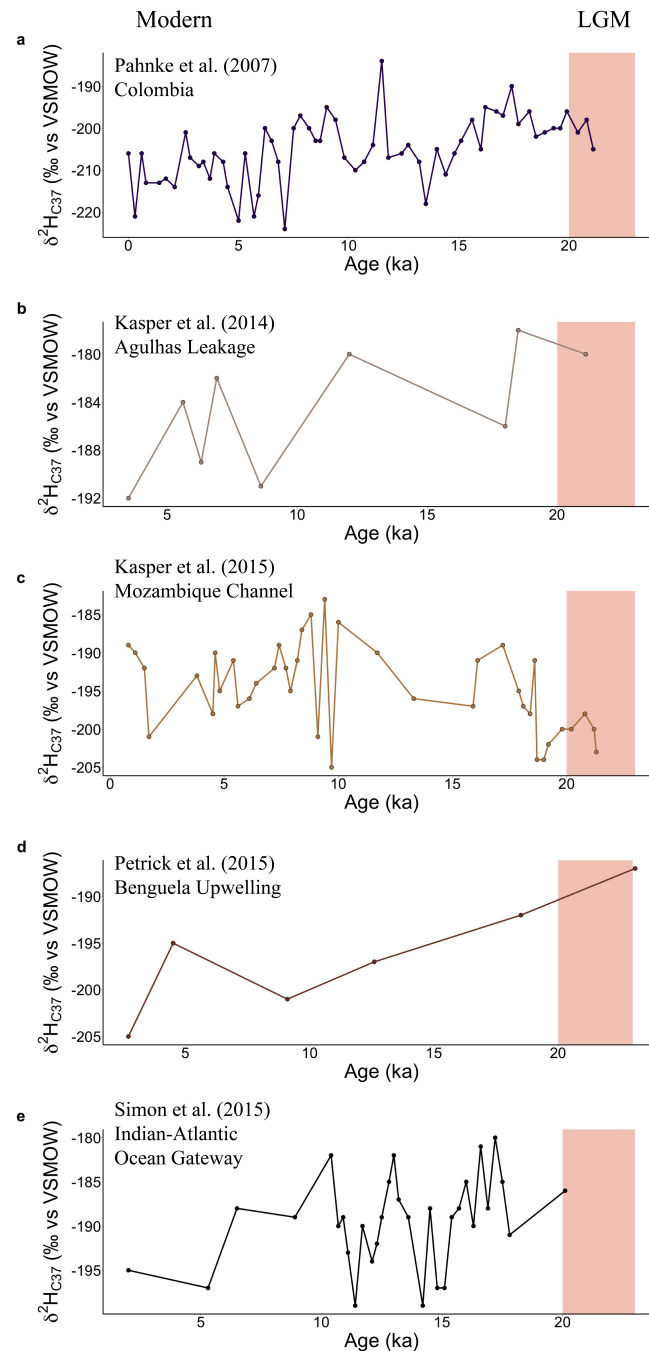


Figure 4. Records of $\delta^2\text{H}_{\text{C}37}$ ratios from previously published records (Pahnke et al., 2007; Kasper et al., 2014, 2015; Petrick et al., 2015; Simon et al., 2015) covering the Last Glacial Maximum (LGM) until the present. Note the different scales for the y-axis. All records show more enriched values for the LGM and more depleted values for the modern, except for (c) the Mozambique Channel record from Kasper et al. (2015).

differences between these two proxies. Thus, to investigate whether the higher paleosensitivity of $\delta^2\text{H}_{\text{C}37}$ to salinity at ODP site 1234 was also observed elsewhere, or is unique to the Chilean Margin, we examined other previously published $\delta^2\text{H}_{\text{C}37}$ records. For this exercise, we considered the difference in $\delta^2\text{H}_{\text{C}37}$ between the most modern value and that of the LGM at ~21 ka. We included records from off-shore Colombia (Pahnke et al., 2007), the Agulhas

leakage (Kasper et al., 2014), Benguela Upwelling (Petrick et al., 2015), and the Indian-Atlantic Ocean Gateway (Simon et al., 2015). The Mozambique Channel (Kasper et al., 2015) and Gulf of Guinea (Leduc et al., 2013) records were excluded from this exercise. The Mozambique Channel record does not record a depletion from the LGM to the modern, as a result of lower sea levels and higher freshwater influx during the LGM at the core location (values are included in Table 2). At the Mozambique Channel site, Kasper et al. (2015) proposed that mixing of alkenones from coastal and marine producers caused a dampening of the $\delta^2\text{H}_{C37}$ signal at this location. The Gulf of Guinea record only covers the Holocene, and therefore was also excluded from this exercise. The general trends in all previously published records matched well with each other (e.g. more enriched $\delta^2\text{H}_{C37}$ ratios for the LGM than for the most modern sediment), but the amplitude of the shifts and the absolute values were different for each location due to regional climate characteristics (Fig. 4). To estimate sensitivity, the difference between LGM and the modern was divided by two psu (Table 2), assuming that this is the general salinity difference between modern and LGM (Adkins et al., 2002; Broecker, 2002). The sensitivity from these records ranged from 4.5 to 9 ‰ per psu, with a value of 5.5 for ODP 1234. A sensitivity of 9 ‰ per psu is unprecedented in any environmental or laboratory culture investigation of $\delta^2\text{H}_{C37}$ ratios, and the closest, the *E. huxleyi* calibration of Schouten et al. (2006), is half of that. It holds, then, that either something is missing from our understanding of paleo $\delta^2\text{H}_{C37}$ ratios, or salinity shifts were actually larger than other proxies suggested by about a factor of 2. Further research employing multiproxy approaches using inorganic proxies like Na/Ca or paired $\delta^{18}\text{O}$ and Mg/Ca in conjunction with $\delta^2\text{H}_{C37}$ ratios may shed light on this enigma.

4 Conclusions

$\delta^2\text{H}_{C37}$ ratios for the last 150 kyr recorded global climate trends on glacial to interglacial timescales at ODP site 1234. Application of different calibrations for the $\delta^2\text{H}_{C37}$ – salinity relationship resulted in extremely large shifts in salinity that are unprecedented in open ocean sites based on present understanding of sea surface salinity. A larger slope for the $\delta^2\text{H}_{C37}$ – salinity relationship has to be invoked in order to reconstruct paleosalinities with changes of 1 – 2 psu between glacial and interglacial time periods. Based on other sediment records of $\delta^2\text{H}_{C37}$ ratios, a paleosensitivity between 4.5 and 9 provided estimates of relative changes in salinity for open ocean sites that align with current knowledge of glacial and interglacial global salinity shifts inferred from oxygen isotope based proxies and climate models. However, such a large slope has not previously been recorded in laboratory culture or natural environmental settings. This suggests that either our understanding of the salinity response to $\delta^2\text{H}_{C37}$ ratios is incomplete, or salinity shifts were actually larger than previously suggested.

Acknowledgements, samples and data

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Table 1: Hydrogen isotope ratios of long-chain alkenones (both individual C_{37:3} and C_{37:2} as well as integrated C₃₇ alkenones) measured on sediment extracts from ODP 1234 over the last ~150 kyr.

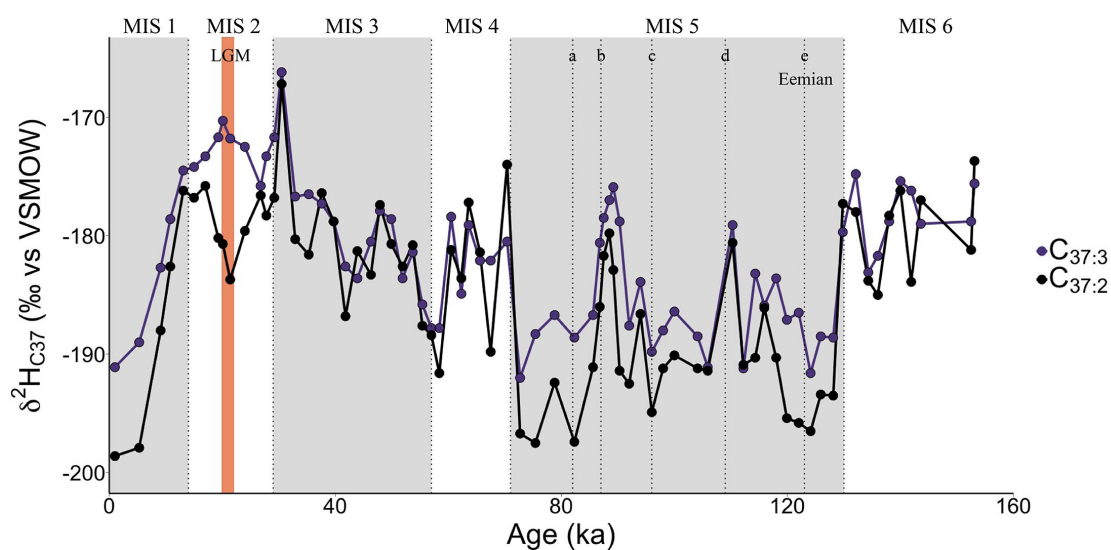
Age (kyr)	Depth (mcd)	C ₃₇ Alkenone Concentration (µg / g sediment)	δ ² H _{C_{37:3}} (‰ vs VSMOW)	S.D.	δ ² H _{C_{37:2}} (‰ vs VSMOW)	S.D.	δ ² H _{C₃₇} (‰ vs VSMOW)	S.D.	Ice volume corrected δ ² H _{C₃₇}
1.0	0.1	4.63	-191	0	-199	0	-195	0	-195
5.3	1.1	2.07	-189	0	-198	1	-193	0	-194
9.1	2.2	1.89	-183	0	-188	0	-185	0	-186
10.8	2.8	1.44	-179	2	-183	2	-181	2	-183
13.1	3.6	1.87	-175	1	-176	2	-175	1	-178
15.0	4.4	1.73	-174	2	-177	2	-176	2	-182
17.0	5.3	1.00	-173	2	-176	4	-174	1	-182
19.3	6.5	1.07	-172	1	-180	1	-176	0	-183
20.1	7.0	1.67	-170	1	-181	1	-176	1	-183
21.4	7.8	1.69	-172	2	-184	2	-177	2	-184
24.0	9.6	1.51	-173	1	-180	2	-175	2	-182
26.8	11.8	1.43	-176	0	-177	1	-176	1	-182
27.8	12.7	0.45	-173	2	-178	3	-175	2	-181
29.2	13.9	1.64	-172	1	-177	0	-173	1	-179
30.5	15.1	2.07	-166	1	-167	2	-167	2	-172
32.9	17.2	1.14	-177	1	-180	0	-178	1	-183
35.3	19.2	1.48	-177	1	-182	3	-179	2	-184
37.6	21.1	1.82	-177	2	-176	3	-177	2	-182
39.7	22.7	2.29	-179	1	-179	1	-178	2	-183
41.8	24.3	1.63	-183	4	-187	3	-185	4	-190
43.9	25.9	1.45	-184	2	-181	2	-183	2	-188
46.3	27.8	1.99	-181	1	-183	1	-181	1	-185
47.9	29.1	1.60	-178	2	-177	2	-177	2	-182
49.9	30.7	1.79	-179	1	-181	0	-179	1	-184
51.9	32.4	1.69	-184	1	-183	1	-185	1	-189
53.7	34.0	2.71	-181	0	-181	0	-181	0	-186
55.4	35.6	1.84	-186	1	-188	1	-187	1	-192
57.0	37.2	1.73	-188	0	-188	0	-188	0	-193
58.4	38.8	2.21	-188	2	-192	3	-190	2	-194
60.5	41.1	2.12	-178	2	-181	3	-180	2	-184
62.3	43.2	1.77	-185	1	-184	2	-184	1	-189
63.6	45.7	1.61	-179	1	-177	0	-178	1	-182
65.6	48.3	2.16	-182	2	-181	2	-183	2	-187
67.5	50.6	2.62	-182	1	-190	2	-185	2	-189
70.4	53.2	2.77	-181	3	-174	17	-181	4	-185
72.7	54.8	4.00	-192	3	-197	5	-194	3	-197
75.4	56.4	5.92	-188	1	-198	5	-191	2	-194
78.8	58.0	5.36	-187	2	-192	0	-189	1	-192
82.3	59.6	6.15	-189	1	-197	0	-193	2	-195
85.6	61.4	3.00	-187	2	-191	3	-190	2	-192
86.8	62.0	6.01	-181	1	-186	1	-183	1	-185
87.5	62.4	5.40	-179	0	-182	0	-179	1	-181
88.5	62.9	3.23	-177	1	-180	1	-178	0	-180
89.2	63.3	3.06	-176	2	-183	2	-179	2	-181
90.3	63.9	4.43	-179	0	-191	0	-185	0	-187
92.0	65.5	3.82	-188	0	-193	0	-190	0	-192
94.0	67.3	3.52	-184	1	-187	1	-185	1	-187
96.0	69.2	5.25	-190	2	-195	2	-193	2	-195
98.0	71.0	4.99	-188	3	-191	3	-188	3	-191
100.0	72.9	4.90	-186	2	-190	1	-187	1	-189
104.1	76.6	6.57	-189	0	-191	2	-189	1	-191
105.9	78.3	3.15	-191	1	-191	2	-192	1	-194
110.3	80.6	4.28	-179	0	-181	0	-179	0	-181
112.2	81.6	6.40	-191	1	-191	1	-192	1	-193
114.3	82.4	6.17	-183	1	-190	1	-186	1	-186
115.9	83.0	4.78	-186	3	-186	1	-187	2	-187
118.0	83.8	7.02	-184	2	-190	0	-187	0	-187
119.9	84.5	7.25	-187	1	-195	3	-191	2	-190
122.0	85.3	8.63	-187	1	-196	3	-191	2	-191
124.1	86.1	6.56	-192	1	-197	1	-195	1	-195
125.9	86.7	9.57	-189	0	-193	1	-191	1	-191
128.1	87.2	5.73	-189	0	-194	2	-191	1	-192
129.8	87.6	2.12	-180	2	-177	3	-179	2	-181
132.1	88.3	2.44	-175	1	-178	0	-176	1	-181
134.3	89.3	1.97	-183	0	-184	0	-184	0	-190
136.0	90.1	2.10	-182	2	-185	2	-184	2	-191
138.0	91.1	1.69	-179	0	-178	0	-179	0	-186
140.0	92.1	1.20	-175	1	-176	2	-176	0	-183
141.9	93.5	1.18	-176	1	-184	0	-180	0	-186
143.6	94.7	1.56	-179	1	-177	0	-178	0	-184
152.5	100.6	2.05	-179	1	-181	1	-180	1	-186
153.1	101.0	1.12	-176	2	-174	2	-176	2	-182

Table 2. Hydrogen isotope ratios of long-chain alkenones ($\delta^2\text{H}_{\text{C}_{37}}$) for the most recent sample and for Last Glacial Maximum (LGM; ~21 ka) from this study and previously published records of $\delta^2\text{H}_{\text{C}_{37}}$ ratios (Pahnke et al., 2007; Kasper et al., 2014; Kasper et al., 2015; Petrick et al., 2015; Simon et al., 2015). The differences in $\delta^2\text{H}_{\text{C}_{37}}$ ratios between LGM and the modern in all records suggest a larger $\delta^2\text{H}_{\text{C}_{37}}$ –salinity sensitivity that previously reported in culture or environmental analyses, except for the Mozambique Channel record (Kasper et al., 2015) which does not record glacial to interglacial shifts.

Modern			LGM			
Paper	Age (ka)	Ice volume corrected $\delta^2\text{H}_{\text{C}_{37}}$ (‰)	Age (ka)	Ice volume corrected $\delta^2\text{H}_{\text{C}_{37}}$ (‰)	Difference	Sensitivity for shift of 2 psu ⁻¹
		vs VSMOW)		vs VSMOW)		
Pahnke et al., 2007	0.3	-221	21.1	-205	16	8
Kasper et al., 2014	3.5	-192	21.1	-180	12	6
Kasper et al., 2015	0.8	-189	21.3	-203	-14	-7
Petrick et al., 2015	2.7	-205	23.1	-187	18	9
Simon et al., 2015	2.0	-195	20.1	-186	9	4.5
ODP 1234	1.0	-195	21.4	-184	11	5.5

Linear Regression Equations for $\delta^2\text{H}_{\text{C}_{37}}$ -salinity Relationship						
Paper	Type	Slope	SE	Intercept	SE	number
Schouten et al. (2006)	Culture	4.8	0.7	-347	19.4	11
Gould et al. (2019)	SPM	4.2	1.0	-340	37.0	63
Weiss et al. (2019)	Sediment	1.3	0.1	-227	3.6	95

Supplementary material



Supplementary Figure 1. Hydrogen isotope ratios of individual $C_{37:3}$ and $C_{37:2}$ alkenones over the last ~150 kyr from ODP 1234. In general, individual alkenones both follow the same trends as integrated C_{37} alkenones (more enriched values during glacials and more depleted values during interglacials). They diverge at the Last Glacial Maximum (LGM), which is likely the result of changes in relative abundance of the individual alkenones.



Seagulls flying on the beach in Cyprus.

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Texel sunset.

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Curriculum Vitae

Gabriella Montaña Weiss was born on the 8th of April, 1988 in Redwood City, California. In 2006, she graduated from Centennial High School in Corona, California and began her college education at Riverside Community College in Riverside, California. In 2008, she transferred to the University of California, Riverside (UCR) and obtained her Bachelors degree in Anthropology with a minor in Religious Studies in June 2010. During the subsequent three years, she worked as an English and Mathematics tutor for a non-profit organization, giving lessons to students, in addition to working as an administrative assistant for the company. In 2013, Gabriella enrolled in a Masters program at Queen's University Belfast, where she completed her MSc Dissertation in 2014 entitled, "A chronology of Holocene palaeosalinity in the eastern Limfjord, Denmark based on isotopic and micropalaeontological evidence" under the supervision of Dr. Paula J. Reimer. She was awarded the Kerr MSc Prize for best overall performance in the Masters and the Kerr MSc Dissertation Prize for dissertation with the highest mark. In March of 2015, she moved to the beautiful, windy island of Texel to begin her PhD research at NIOZ on hydrogen isotope ratios of long-chain alkenones as a tool for paleo sea surface salinity reconstructions under the supervision of Dr. Marcel T. J. van der Meer, Dr. Stefan Schouten and Dr. Jaap S. Sinninghe Damsté. During her four years on Texel, she had many wonderful opportunities to participate in scientific sampling expeditions, attend international conferences and courses, and take advantage of the wealth of knowledge present in the BGC / MMB department at NIOZ. She will continue her scientific career as a post doc investigating intramolecular isotopic variation in the group of Dr. Alex Sessions at Caltech and the group of Dr. Kate Freeman at Penn State.



Publications

Weiss, G.M., Pfannerstill, E.Y., Schouten, S., Sinninghe Damsté, J.S., van der Meer, M.T.J. (2017). Effects of alkalinity and salinity at low and high light intensity on hydrogen isotope fractionation of long-chain alkenones produced by *Emiliana huxleyi*. *Biogeosciences*, 14, 5693-5704. <http://dx.doi.org/10.5194/bg-14-5693-2017>

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