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Growth phase dependent hydrogen isotopic fractionation in alkenone-producing haptophytes

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Abstract

Several recent works have investigated use of the hydrogen isotopic composition of C_{37} alkenones (δD_{K37s}), lipid biomarkers of certain haptophyte microalgae, as an independent paleosalinity proxy. We discuss herein the factors impeding the success of such an application and identify the potential alternative use of δD_{K37s} measurements as a proxy for non-thermal, physiological stress impacts on the $U_{37}^{K'}$ paleotemperature index. Batch-culture experiments with the haptophyte *Emiliania huxleyi* (CCMP 1742) were conducted to determine the magnitude and variability of the isotopic contrasts between individual C_{37} alkenones, an analytical impediment to the use of δD_{K37s} in any paleoceanographic context. Further experiments were conducted with *Emiliania huxleyi* (CCMP 1742) and *Gephyrocapsa oceanica* (PZ3-1) to determine whether, and to what extent, δD_{K37s} varies between the physiological extremes of nutrient-replete exponential growth and nutrient-depleted senescence, the basis for our proposed use of the measurement as an indicator of stress. *Emiliania huxleyi* exhibited an isotopic contrast between di- and tri-unsaturated C_{37} alkenones ($\alpha_{K37:3-K37:2}\approx 0.97$) that is nearly identical to that reported recently by others for environmental samples. Furthermore, this contrast appears to be constant with growth stage. The consistency of the offset across different growth stages suggests that a single, well-defined value for $\alpha_{K37:3-K37:2}$ exists and that its use in an isotope mass-balance will allow accurate determination of δD values for individual alkenones without having to rely on time- and labor-intensive chemical separations. The isotopic fractionation between growth medium and C_{37} alkenones was observed to increase dramatically upon the onset of nutrient-depletion-induced senescence, suggesting that δD_{K37s} may serve as an objective tool for recognizing and potentially correcting, at least semi-quantitatively, for the effects of nutrient stress on $U_{37}^{K'}$ temperature records.

1 Introduction

1.1 The premise of alkenone δD as a water composition proxy

Englebrecht and Sachs (2005) employed measurements of C_{37} alkenone hydrogen isotopic composition (δD_{K37s}) in suspended particulate matter (SPM) and surface sediments around the Bermuda Rise to quantify the impact of lateral particle transport on the paleoSST record encoded by alkenone unsaturation patterns ($U_{37}^{K'}$) in an underlying drift deposit. Comparison of δD_{K37s} measurements in sediment samples with the δD of the water in the overlying surface ocean revealed that hydrogen isotopic fractionation factors ($\alpha_{K37s-\text{water}}$) were quite consistent site to site. Given this finding and results from a batch culture experiment with the alkenone producer *Emiliania huxleyi*, they argued that alkenones provide a reliable paleoproxy for the δD composition of surface water.

Non-exchangeable hydrogen in photosynthesized organic matter (e.g. from lipids) necessarily reflects the isotopic composition of the water from which it was produced (e.g. Yakir and DeNiro, 1990). Interpretation of bulk lipid δD measurements is complicated, however, by the differences in net isotopic fractionation exhibited by different organisms and by different individual compounds. These differences are due to a host of factors, including differences in water transport into and out of the cell, biosynthetic pathways and relative synthetic rates (Sessions et al., 1999). Alkenones, though, are a species-specific biomarker, unique to a small number of haptophyte algae and usually assumed, in marine sediments younger than ~280 ky, to be produced predominantly by *Emiliania huxleyi* (Herbert, 2003). If the assumption is made that a single measured hydrogen isotopic fractionation factor, α, defined as:

$$\alpha_{K37s-\text{water}} = \frac{\delta D_{K37s} + 1000}{\delta D_{\text{water}} + 1000} \quad (1)$$

is applicable to alkenone synthesis by *E. huxleyi*, then fossil alkenones (younger than 280 ky) could provide a useful proxy for the isotopic composition of surface water.

Alkenones are an attractive basis for such a proxy because of their recalcitrance (Volkman et al., 1980) and utility as a coeval paleothermometer (Brassell, 1993). Given certain constraints on the regional isotopic composition of precipitation and runoff (Craig and Gordon, 1965), the δD of surface water may serve, in turn, as a quantitative proxy
5 for salinity.

However, the assumption that a single, well-defined value of $\alpha_{K37s-water}$ actually exists is by no means a trivial one. The time- and space-variable environmental and physiological factors that unicellular phytoplankton encounter are numerous, and many of them may be expected to affect the net fractionation between synthesized compounds
10 and external water. Shortly after the work of Englebrecht and Sachs (2005) was published, Schouten et al. (2006) investigated the effects of temperature (T), salinity (S), and growth rate (μ) on $\alpha_{K37s-water}$ using a batch culturing approach. Both S and μ, but not T, were reported to effect hydrogen isotopic fractionation associated with alkenone biosynthesis by the haptophytes *E. huxleyi* and *G. oceanica*, the two most prominent
15 sources of these biomarkers in the modern ocean.

The reported salinity and growth-rate effects are problematic if δD_{K37s} measurements are to serve as the basis for a paleosalinity proxy. Although the apparent salinity effect could be beneficial, acting as an amplifier of the hydrologically-driven δD_{K37s} signal (Pahnke et al., 2007), its inclusion in the formulation of $\alpha_{K37s-water}$ as a func-
20 tion of salinity adds another source of uncertainty to the proxy calibration. Possible growth-rate effects bode even less well for the paleosalinity application of sedimentary δD_{K37s}. Variation in growth rate is nigh-impossible to constrain given the information currently available from sediment records. Consequently, concerns over its effect on carbon-isotopic fractionation in alkenones have played a large part in undermining this
25 measure's utility as a paleo-pCO_2 proxy (Benthien et al., 2002). Were stratigraphic variations in a "growth-rate δD_{K37s} signal" to appear down a core, they could not be deconvolved from changes in δD_{water}. For example, using the "ocean main sequence" $\delta^{18}O_{water}$ vs. salinity line ($\delta^{18}O_{water} \approx 0.5S-17$, Craig and Gordon, 1965) and assuming a "meteoric" relationship between δD and $\delta^{18}O$ ($\delta D_{water} \approx 8\delta^{18}O_{water}+10$, Craig, 1961),

a "typical whole ocean" salinity range of 32 to 37 would correspond to a δD_{water} range of ∼1 to 21‰ (vs. SMOW). Translated into a δD_{K37s} signal using the average value of $\alpha_{K37s-water}$ (0.790) reported by Schouten et al. (2006), δD_{K37s} measurements would span from −209‰ to −193‰, a range of ∼16‰. The range in $\alpha_{K37s-water}$ that Schouten et al. (2006) suggests is associated with an oceanographically reasonable variation in growth rate (∼0.2 and 0.8 day^{-1}, Bidigare et al., 1997) is 0.814 to 0.796. Within this range, a constant δD_{water} value of 11‰, the midpoint of the δD_{water} range above, would yield δD_{K37s} values falling anywhere between −177 and −195‰. This 18‰ range is greater than the 16‰ range expected for changes in open-ocean salinity! With fractionation factors this unconstrained, it is unlikely that δD_{K37s} measurements could provide unequivocal quantitative information about changes in δD_{water} for any oceanographic settings other than those characterized by the highest salinity contrasts, such as areas of periodic melt water discharge or variable river outflow (e.g. Friedman et al., 1964).

1.2 The premise of alkenone δD measurements as a physiological stress proxy

If δD_{K37s} values are to provide an effective proxy for any paleoreconstruction purpose (salinity or otherwise), it is essential that the effects of variable growth conditions on isotopic fractionation be evaluated and constrained. Schouten et al. (2006) have laid the groundwork in this regard, demonstrating that variation in cell physiology plays a significant role in setting δD_{K37s} values. Although these effects clearly complicate use of δD_{K37s} measurements for paleosalinity reconstruction, is it possible that this physiological "complication" could be exploited for paleoceanographic advantage?

Analysis of the molecular composition of alkenones preserved in surface sediments off the west coast of South America suggests the fossil signatures are characteristic of stressed, rather than exponentially dividing cells (Prahl et al., 2006). It was hypothesized that stress, in this setting, is imposed by the exposure of cells to a combination of nutrient depletion (prior to sedimentation) and light deprivation (during the export of viable cells). These results echo those of Conte et al. (1995), who observed the same biochemical cues in suspended particulate matter from North Atlantic surface waters.

Prahl et al. (2006) suggested that the effects that these stresses have on the alkenone unsaturation index ($U_{37}^{K'}$) contribute to the several-degree-Celsius "scatter" in the linear, global core-top calibration for $U_{37}^{K'}$ vs. mean annual SST (maSST, Müller et al., 1998).

5 If the fossil alkenone record does not strictly reflect the molecular composition of exponentially dividing cells, then to what extent might the δD composition of these biomarkers also deviate from that reported exclusively, so far, for cells harvested in the nutrient-replete, exponential growth phase? Given the recognized dependence of $\alpha_{\mathrm{K37}s\mathrm{-water}}$ on growth conditions, one may expect that $\delta D_{\mathrm{K37}s}$ values would vary systematically from one biogeographical regime to another, as now seems to be the case

10 for $U_{37}^{K'}$ measurements (Prahl et al., 2009). If a significant difference in $\alpha_{\mathrm{K37}s\mathrm{-water}}$ exists between cells in the exponential and nutrient stress-imposed stationary growth phases, $\delta D_{\mathrm{K37}s}$ measurements could provide a physiological context within which $U_{37}^{K'}$ records may be interpreted more objectively from a biological-oceanographic perspective.

1.3 Analytical concerns

15 In addition to environmental and physiological effects on actual isotopic fractionation, the effects of growth conditions on the *molecular* fingerprint of alkenones also pose a potential problem for the interpretation of $\delta D_{\mathrm{K37}s}$ measurements from field samples. While the chromatographic peaks of C_{37} alkenones are generally baseline-resolved when analyzed for molecular composition by gas chromatography (GC),

20 the order-of-magnitude increase in sample size necessary for δD analysis by gas chromatography-pyrolysis-isotope ratio mass-spectrometry (GC-P-IRMS) overloads capillary GC columns and results in unresolved peaks (D'Andrea et al., 2007). Consequently, alkenone-specific δD measurements are now typically reported as an integrated signal (e.g. Englebrecht and Sachs, 2005; Schouten et al., 2006). Integrated

25 values for $\delta D_{\mathrm{K37}s}$ include contributions from di- (K37:2), tri- (K37:3), and, if present, tetra-unsaturated (K37:4) components. This analytical concern is moot if all three compounds are isotopically identical. However, if these compounds are isotopically

distinct, changes in $U_{37}^{K'}$, such as those that occur during the onset of nutrient-imposed stationary growth (Prahl et al., 2003) or when growth temperature changes, would complicate interpretation of a measured $\delta D_{\mathrm{K37}s}$ value. Fueled by just this premise, D'Andrea et al. (2007) developed a manual silver-silica gel chromatographic method
5 to separate alkenones according to their degree of unsaturation. They then measured the δD of the isolates and observed K37:3 to be significantly more deuterium-depleted than K37:2 in samples of *E. huxleyi*. The fractionation factor calculated for the two compounds ($\alpha_{\mathrm{K37:3-K37:2}}$) was 0.94.

Existence of an isotopic offset between these two compounds has ramifications for
10 down-core analyses. Given that 1) the measured δD value of a combined K37:3 and K37:2 GC-P-IRMS peak (see Fig. 2) is defined by:

$$\delta D_{\mathrm{K37}s} = \delta D_{\mathrm{K37:3}} f_{\mathrm{K37:3}} + \delta D_{\mathrm{K37:2}} f_{\mathrm{K37:2}} \quad (2)$$

where f is the fraction of K37:3 or K37:2 represented in the peak, 2) the isotopic contrast between these compounds is defined by:

15
$$\alpha_{\mathrm{K37:3-K37:2}} = \frac{\delta D_{\mathrm{K37:3}} + 1000}{\delta D_{\mathrm{K37:2}} + 1000} \quad (3)$$

and 3) $U_{37}^{K'}$ is defined as:

$$U_{37}^{K'} = \frac{[\mathrm{K37:2}]}{[\mathrm{K37:3}] + [\mathrm{K37:2}]} \quad (4)$$

the isotopic difference between two K37*s* samples synthesized at two different temperatures, for example, can be calculated using the expression:

20
$$\delta D_{\mathrm{tot2}} - \delta D_{\mathrm{tot1}} = \left((\alpha_{\mathrm{K37:3-K37:2}})(\delta D_{\mathrm{K37:2}}) + 1000(\alpha_{\mathrm{K37:3-K37:2}})\right)\left(U_{37\,1}^{K'} - U_{37\,2}^{K'}\right) + \ldots$$
$$\ldots(1000 + \delta D_{\mathrm{K37:2}})\left(U_{37\,2}^{K'} - U_{37\,1}^{K'}\right) \quad (5)$$

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For measured values of $\alpha_{\mathrm{K37:3-K37:2}}$ (0.94) and $\delta D_{\mathrm{K37:2}}$ (−188.1‰) (D'Andrea et al., 2007) and a growth-temperature difference of 11°C (the range studied by Schouten et al., 2006) translated by standard means (0.034/°C) into a $U_{37}^{K'}$ difference, an ∼+18‰ difference in $\delta D_{\mathrm{K37}s}$ is calculated. A change in $U_{37}^{K'}$ of −0.2, the magnitude associated with the onset of nutrient-depletion imposed senescence in *E. huxleyi* grown at 15°C (Prahl et al., 2003), would yield a $\delta D_{\mathrm{K37}s}$ difference of ∼+9‰. A difference of this magnitude is well outside the precision typical of $\delta D_{\mathrm{K37}s}$ measurements (e.g. ∼6‰, Englebrecht and Sachs, 2005) and, thus, would be interpreted as significant. Depending upon one's inclination, however, this difference could easily be construed, when measured down-core, as caused by a change through time either in 1) growth temperature, 2) the mean growth phase of cells contributing to alkenone export, or 3) water composition. Any interpretation would be ambiguous.

One solution to the issue of unsaturation-specific fractionation is to always separate alkenones prior to isotopic analysis and base interpretations on the δD of a single compound. This approach is now recommended by two groups of investigators (D'Andrea et al., 2007; Schwab and Sachs, 2009). It is not a practical strategy, though, if the research objective is to generate high-resolution down-core data sets. Additional chromatographic steps, even when automated, are labor and time intensive and raise concerns about fractionation effects associated with incomplete sample recovery (e.g. Smittenburg and Sachs, 2007). Measurement of $\delta D_{\mathrm{K37}s}$ and calculation of the δD of a single compound by an isotope mass balance approach (algebraic rearrangement of Eqs. 2, 3, and 4):

$$\delta D_{\mathrm{K37:3,calc}} = \frac{\left(\delta D_{\mathrm{K37}s} - \frac{1000 \cdot U_{37}^{K'}}{\alpha_{\mathrm{K37:3-K37:2}}} + 1000 \cdot U_{37}^{K'}\right)}{1 - U_{37}^{K'} + \frac{U_{37}^{K'}}{\alpha_{\mathrm{K37:2-K37:2}}}} \quad (6)$$

is a much more expedient approach and, hence, conceptually more attractive. However, this alternative approach does require that a well-constrained, constant value of

$\alpha_{K37:3-K37:2}$ exists. D'Andrea et al. (2007) have provided evidence that this condition may be true. Recent follow-up work by others (Schwab and Sachs, 2009) provides further support for this premise. Nonetheless, the available body of evidence is still quite limited and the actual value of $\alpha_{K37:3-K37:2}$ remains in question.

1.4 Objectives of this work

The background information presented in Sect. 1.1 shows that use of δD_{K37s} as an unequivocal salinity proxy is, at best, currently problematic. Even without *any* variation in assessed $\alpha_{K37s-water}$ values, the typical precision of δD_{K37s} measurements (±3–5‰) corresponds to an uncertainty in salinity reconstructions of ∼±1.5 units (1σ). This evaluation assumes a *perfect* meteoric $\delta^{18}O/\delta D$ relationship, a *perfect* "ocean main sequence" $\delta^{18}O/S$ relationship, and an $\alpha_{K37s-water}$ value of 0.790 (the mean value reported by Schouten et al., 2006) with *zero* uncertainty. This theoretical, "best case" scenario poses a serious impediment given the small salinity variations typically of interest in paleoceanographic studies (e.g. de Vernal et al., 2000).

On the other hand, the physiological factors that may affect δD_{K37s} values are themselves valid targets for paleoceanographic inquiry. If growth rate does indeed have a control on fractionation (Schouten et al., 2006), δD_{K37s} values measured in sediments could provide information about the growth-phase status of the organisms contributing to the geologic record and, in particular, change in this status through time. Prior work suggests the physiological growth status of cells contributing to the export of alkenones from the euphotic zone plays an important role in modifying the quantitative nature of the sedimentary $U_{37}^{K'}$ record (Conte et al., 1995; Prahl et al., 2006). However, there is, as of yet, no defined method for objectively quantifying variation in stress effects on $U_{37}^{K'}$ values measured down-core. In light of results from Schouten et al. (2006), compound-specific hydrogen-isotopic data could provide such a tool, constraining some of the more significant non-thermal impacts on the $U_{37}^{K'}$-derived temperature record.

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A number of fundamental questions must be addressed before a hydrogen isotope-based proxy is developed to gauge unequivocally the magnitude of $U_{37}^{K'}$ variation due to non-thermal physiological changes in alkenone-producing haptophytes. Two important sets of questions which guided our laboratory-based experimental study are:

5

1. What is the actual value of the now well-recognized isotopic offset between K37:3 and K37:2? Is $\alpha_{\text{K37:3}-\text{K37:2}}$ a constant value in cells transitioning from an exponential to a nutrient-stress imposed stationary growth phase?

2. Does a difference in the hydrogen isotopic fractionation associated with alkenone biosynthesis exist between exponential and stationary growth phases? If so, what is the cause of the noted variation in $\alpha_{\text{K37}s-\text{water}}$?

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2 Methods

2.1 Algal cultures

Emiliania huxleyi (non-calcifying strain CCMP 1742) was batch cultured in duplicate isothermally (15°C) under cool-white light (~70 µEin/m^{2}s, 12 h light/12 h dark cycle) in $f/20$ media (~32 salinity). At the University of British Columbia (Canada) duplicate cultures from the same stock of *E. huxleyi* CCMP 1742 were grown isothermally (18°C) under constant, cool-white light (~80 µEin/m^{2}s) in enriched Ocean Station PAPA seawater. This medium contained ~300 µM nitrate, ~10 µM phosphate, ~32 salinity, and Aquil-standard additions of trace metals with 100 µM EDTA (Maldonado et al., 2006). *Gephyrocapsa oceanica* (calcifying strain PZ3-1) was batch cultured isothermally at three different temperatures (17, 21, and 25°C) in media (salinity ~32) equivalent to $K/8$ with a soil extract addition (https://ccmp.bigelow.org/node/81) at Biologique Roscoff (France). In each case, cool-white light was supplied at ~150 µEin/m^{2}s on a 14 h light/10 h dark cycle.

15

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For all experiments, cell counts were conducted daily to monitor the progression of growth and establish the average growth rate (Fig. 1). "Exponential" phase cells were sampled during the log-linear portion of each growth curve. "Stationary" phase cells were sampled after the cessation of cell division. Cell sampling was conducted in duplicate for analysis of the molecular and δD composition of alkenones. Duplicate samples of the culture media were also collected from the filtrate at each cell harvest for analysis of δD_{water} and nutrients. Sampling points for each experiment are identified on the growth curves shown in Fig. 1.

2.2 Sample preparation for molecular and isotopic characterization

2.2.1 Alkenone extraction and purification

Total lipids were extracted from the cell samples following a standard procedure (Prahl et al., 1989), modified for automated solvent extraction using a Dionex ASE-200. All samples from the *E. huxleyi* cultures were saponified in ethanolic KOH (Christie, 2003) to remove alkenoates. Since the alkenoate content of the *G. oceanica* samples was negligible, they were not saponified. However, all *G. oceanica* samples were adducted with urea (Christie, 2003) to remove a series of polysiloxane contaminants which were present and otherwise would have interfered with the alkenone analysis.

2.2.2 Unsaturation-specific separation

One of the duplicate alkenone samples from each harvest point of the *E. huxleyi* cultures was separated into di- and tri-unsaturated fractions using argentation chromatography (Nikolova-Damyanova, 1992). The tetra-unsaturated C_{37} ketone (K37:4) was a very minor component (<3% of the total C_{37} compounds) in all samples. Initially, the exact protocol described by D'Andrea et al. (2007) was used, but clean separation of K37:2 and K37:3 could not be obtained. A much smaller increase in solvent strength (100% dichloromethane (DCM) to 10% ethyl acetate (EtOAc) in DCM) than reported

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(10 to 30% EtOAc in DCM) was required to begin eluting the more unsaturated compounds and achieve complete separation. Consequently, a total volume of solvent 10× greater than that reported by D'Andrea et al. (2007) had to be run through the columns.

2.2.3 Cryodistillation of water

Prior to δD analysis using a thermal-conversion elemental analyzer coupled to an isotope ratio mass spectrometer (TCEA-IRMS), samples of culture media water were quantitatively cryodistilled in triplicate. This preparative step was taken to eliminate salt from the water samples and prevent fouling of the TCEA pyrolysis column. A published method (West et al., 2006) for quantitatively extracting water from leaf and soil samples using a vacuum manifold was modified for use with whole-water samples. The key modification was replacement of the straight extraction tubes with similar ones (14 cm by 1 cm i.d.) containing a bulb (2.5 cm i.d.) at the closed end. This modification eliminated a problem with sample processing caused by the buildup of pressure in the frozen sample when warmed under vacuum and distilled from one tube to the other. Distilled samples, stored in crimp-top autosampler vials until analysis, were shown to be isotopically identical to their undistilled counterparts to well within the ±2.5‰ internal precision of the TCEA-IRMS method (see Sect. 2.4.1).

2.3 Molecular characterization by GC-FID

All isolated alkenone fractions were characterized compositionally and quantified by gas chromatography with flame ionization detection (GC-FID, Prahl et al., 1989). Cellular alkenone concentrations are reported as values corrected for recovery (typically 80–90%) of hexatriacontan-2-one (K36:0). K36:0 is a reference standard added to each sample prior to extraction with the ASE-200. Percent recovery for the unsaturation-specific isolates is reported as percent of each compound (K37:2 or K37:3) relative to the amount quantified in the total C_{37} alkenone sample prior to separation by argentation chromatography.

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2.4 Isotopic analysis

δD analysis of C_{37} alkenones and water was performed using a ThermoQuest-Finnigan Delta Plus XL isotope ratio mass spectrometer operated in continuous flow mode. Using ISODAT software, "H_3^+" correction factors (Sessions et al., 1999) were
5 determined at the beginning of each day using eight sequential H_2 reference gas injections of increasing partial pressure. Values ranged between ~3 and ~7 ppm/nA. All δD values are reported vs. Standard Mean Ocean Water (SMOW).

2.4.1 δD analysis of water in culture media

Values for δD_{water} in all distilled samples were determined using a Thermo-Electron
10 TCEA-IRMS equipped with a Finnigan-MAT A200S liquid autosampler. The instrument was configured and operated as specified in the manual for liquid samples. Each vial was sampled using one "memory clearing" cleaning step as specified by a procedure from the Arizona State University Keck Foundation Laboratory for Environmental Biogeochemistry (http://kfleb.asu.edu), followed by three replicate 1 µL sample injec-
15 tions. δD_{water} values for each sample injection were calculated against the mean of two H_2 reference gas injections made prior to the elution of the analyte peak in each run. For each batch of samples analyzed, the reference gas was standardized against three working standards (HOTSW=−0.55‰; LROSS=−68.12‰; WAIS3=−262.91‰) that had been calibrated using three working standards provided by William Rugh (Na-
20 tional Health and Environmental Effects Research Laboratory, US-EPA, Corvallis, OR). The standards from the EPA laboratory had, in turn, been calibrated vs. the GISP, SLAP, and SMOW standards from National Institute of Standards and Technology (http://www.nist.gov). The internal precision of the analysis was ±2.48‰, reported as the mean of the standard deviations of the working standards (2 vials of each stan-
25 dard ×3 injections each). The mean standard deviation of 25 different triplicate sample distillations was ±1.64‰.

Water samples from the *G. oceanica* culture experiments were lost due to breakage

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during shipment from France. Fractionation factors for alkenone samples from these cultures are calculated assuming a δD_{water} of 0‰. This value is chosen as an estimate because the medium was prepared using water from the English Channel with a salinity of 32. The exact choice of the value is not critical, however, as variability in $\alpha_{\text{K37}s-\text{water}}$ determinations is driven largely by variability in $\delta D_{\text{K37}s}$ values. δD_{water} values ranging between 2 and −8‰ may be used in the calculation of $\alpha_{\text{K37}s-\text{water}}$ without shifting the results outside of the ±0.005 uncertainty of the reported values.

2.4.2 δD analysis of alkenones

The δD of the alkenones was analyzed by GC-P-IRMS. Just prior to analysis, samples were dissolved in a suitable volume of toluene to ensure that a 2 µL GC injection introduced ~500 ng of the minor C_{37} component. An exception was made for the *G. oceanica* samples harvested from the 25°C batch culture experiment because there was not enough of the minor component, K37:3, to make this approach practical. In this case, the dilution volume was chosen to ensure 500 ng injections of the major component, K37:2. Samples, coinjected with 1 µL of an *n*-alkane working standard (530 ng nC_{36}/µL, 330 ng nC_{37}/µL), were chromatographically separated using an HP6890 GC equipped with a cool on-column injector and a SGE BF5 (30 m×0.32 mm i.d., 1.0 µm film) column.

The GC was operated at a constant helium carrier gas flow rate (2.5 mL/min). Compound separations were achieved by temperature programming (80–270°C at 10 °C/min, 270–320°C at 5 °C/min, 43 min hold at 320°C). The alumina pyrolysis reactor (32 cm×0.5 mm i.d.), installed in the Thermo-Finnigan GC-TC interface, was maintained at 1450°C. Upon initial use, the reactor was pre-conditioned (graphitized) by passing several 1 µL injections of isooctane through it. If necessary, such preconditioning was repeated until measured standard values stabilized. Figure 2 shows an example chromatogram from GC-P-IRMS analysis of an alkenone sample.

Isotopic values for C_{37} alkenones were calculated vs. the coinjected nC_{36} and nC_{37} alkane and C_{36} ketone (K36:0) standards. "Known" values of these working stan-

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dards were determined by standardization through co-injection with "Mixture B", a 15 compound mixture of *n*-alkanes obtained from A. Schimmelmann (Indiana University). The average of 20 such standardization analyses performed over 10 months yielded the following "known" values: $nC_{36}=-264.5\pm4.5‰$, $nC_{37}=-217.2\pm4.6‰$, K36:0$=-185.5\pm4.8‰$. The internal precision of our compound specific δD measurements is taken as $\pm4.6‰$, the mean of these three standard deviations.

For each day of sample analysis, a linearity correction for the mass spectrometer was determined by linear regression of measured vs. known δD values for each *n*-alkane in a "Mixture B" standardization run. This correction was then applied to each sample measurement using the equation:

$$\delta D_{\text{corrected}} = \frac{\delta D_{\text{uncorrected}} - b}{m} \tag{7}$$

where m and b are the slope and intercept, respectively, of the linear regression. The magnitude of the applied correction averaged 1.3‰ and never exceeded 2.7‰. In Table 1, all δD values for alkenones are reported as the mean of two or three replicate injections of the same sample.

3 Results

Table 1 summarizes the results of the molecular and hydrogen isotopic analysis of samples from the *E. huxleyi* and *G. oceanica* batch culture experiments.

3.1 Molecular characterization

Total C_{37} alkenone abundance (K37s, pg/cell), alkenone unsaturation values ($U_{37}^{K'}$) and combined C_{37} as a percentage of total C_{37-39} alkenone abundance (%K37) measured in the *E. huxleyi* samples grown at 15°C followed trends expected for cells transitioning from the exponential to the stationary phase of growth (Table 1). Cellular K37s increased by a factor of three upon exposure to nutrient depletion, in keeping with their

purported use as an energy storage product (e.g. Elgroth et al., 2005). $U_{37}^{K'}$ values for the exponential-phase samples (∼0.4) were notably lower than expected for this strain when grown at 15°C (i.e. ∼0.55, Prahl and Wakeham, 1987). However, the ∼0.1 unit decrease in $U_{37}^{K'}$ observed for the stationary-phase samples compares well with the shift documented for exposure of this strain to isothermal nutrient stress at this growth temperature (Prahl et al., 2003). Likewise, while %K37 values for the exponential-phase samples (∼55%) were lower than those previously reported for nutrient-replete samples (∼65%), the ∼5% decrease observed in the stationary-phase samples is consistent with the previously-documented impact of nutrient depletion on this property.

The same pattern of *absolute* $U_{37}^{K'}$ values being lower than expected for the growth temperature (18°C), coincident with the expected *relative* shifts in the molecular fingerprint, was documented in the exponential and stationary-phase samples harvested from the grow-out experiment conducted at UBC using the same culture stock of *E. huxleyi* (Table 1). This similarity suggests that low values of this parameter are now an inherent property of our working stock of *E. huxleyi* and not an artifact of the culturing methods.

Results from analysis of the *G. oceanica* samples generally paralleled those just described for the *E. huxleyi* experiments (Table 1). Cellular concentrations of K37s did not increase as significantly, however, and an increase was only apparent in the 17°C and 21°C experiments. The decrease in $U_{37}^{K'}$ upon the transition of cells from the exponential to the stationary growth phase was also seen (−0.05, −0.06, −0.01 units for the 17, 21, 25°C treatments, respectively). The magnitude of the decrease, smaller than that observed for *E. huxleyi*, was likely due to harvesting the cells at the onset of stationary phase and not at a later point in time (Fig. 1). The apparent lack of change in $U_{37}^{K'}$ for cells from the 25°C experiment may reflect the fact that values for the alkenone unsaturation index at this growth temperature are very nearly unity, the maximum possible for this index. Finally, the magnitude of the decrease in %K37 values from exponential to stationary phase growth roughly paralleled the pattern expected for *E. huxleyi* (Prahl

et al., 2006).

3.2 Isotopic characterization

The δD_{water} for the *E. huxleyi* cultures was essentially invariant between the exponential and stationary sampling points, averaging −5.9±2.2‰ (Table 1). $\delta D_{\text{K37}s}$ values measured on unseparated samples of *E. huxleyi* from the exponential growth phase averaged −188.7±0.5‰, corresponding to an $\alpha_{\text{K37}s-\text{water}}$ of 0.817±0.005. Note that uncertainty in α calculations is based on the generally more-conservative internal precision estimates for measurements of δD_{water}, ±2.48‰, and $\delta D_{\text{K37}s}$, ±4.6‰, rather than the standard deviations associated with individual samples. The average $\delta D_{\text{K37}s}$ values measured in stationary-phase samples was −214.1±2.8‰, corresponding to an $\alpha_{\text{K37}s-\text{water}}$ of 0.790±0.005. The magnitude and consistency of the difference in $\alpha_{\text{K37}s-\text{water}}$ between exponential and stationary phase cells for replicate culture experiments is illustrated graphically in Fig. 3.

Isolated K37:2 and K37:3 exhibited similar shifts in δD composition between the exponential and stationary growth phase samples. Furthermore, K37:3 was, in all cases, the more deuterium-depleted of the two compounds. For *E. huxleyi* sampled in the exponential growth phase, the mean $\delta D_{\text{K37:2}}$ (−175.0±0.4‰) and $\delta D_{\text{K37:3}}$ (−187.8±5.0‰) values yielded an average $\alpha_{\text{K37:3}-\text{K37:2}}$ of 0.985±0.008. In the stationary growth phase samples, inter-compound differences in isotopic fractionation appeared to increase ($\alpha_{\text{K37:3}-\text{K37:2}}$=0.973±0.008) given the measurements of $\delta D_{\text{K37:2}}$ (−199.9±2.4‰) and $\delta D_{\text{K37:3}}$ (−221.7±1.6‰). Figure 3 has been annotated to illustrate the differences in assessed values of $\alpha_{\text{K37:3}-\text{K37:2}}$ for exponential and stationary growth phase samples.

An isotopic mass balance approach was used to reconstruct the unseparated $\delta D_{\text{K37}s}$ value in the four argentation chromatography experiments (Table 1). This exercise was possible given measured values of $U_{37}^{K'}$, $\delta D_{\text{K37:2}}$, $\delta D_{\text{K37:3}}$ and the equation:

$$\delta D_{\text{K37}s} = \delta D_{\text{K37:3}} \left(1 - U_{37}^{K'}\right) + \delta D_{\text{K37:2}} U_{37}^{K'} \quad (8)$$

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Reconstructions based on results for both sets of exponential-phase measurements yielded an average value for $\alpha_{K37s-water}$ (0.823±0.005) that is statistically different (two-sided student's t-test p value=0.016) from the measured value (0.817±0.005). On the other hand, reconstructions for both sets of stationary-phase measurements yielded an average $\alpha_{K37s-water}$ value (0.789±0.005) which is identical (two-sided p=0.85) to the actual measured value (0.790±0.005). Again, these findings are summarized in Fig. 3.

In the *E. huxleyi* batch culture grow-out experiment conducted at UBC, a comparable shift was seen in the mean δD_{K37s} value between exponential (−185.4±0.4‰) and stationary (−202.8±3.4‰) growth phase samples (Table 1). This finding substantiates that there is indeed a phase-dependant fractionation process affecting alkenone biosynthesis in this organism. Furthermore, the phenomenon is not restricted to this species of alkenone-producing haptophytes. A similar trend in deuterium depletion is apparent in δD_{K37s} measurements for *G. oceanica* when the exponential and stationary growth phase samples are compared. This feature was characteristic of results from all three temperature treatments. The decrease in δD_{K37s} between growth phases yielded shifts in $\alpha_{K37-water}$ from 0.811 to 0.788, 0.798 to 0.768, and 0.772 to 0.740 for the 17°C, 21°C, and 25°C cultures, respectively. A scatter plot of these data suggests the decline in $\alpha_{K37s-water}$ with increasing temperature is systematic (Fig. 4). Notably, results obtained from the experiments with *E. huxleyi* appear to lie along the trends defined by the *G. oceanica* samples.

4 Discussion

4.1 Analysis of unsaturation-specific results

Results from our argentation chromatography work confirm the original finding of D'Andrea et al. (2007) that a significant isotopic offset exists between K37:3 and K37:2, with the tri-unsaturated compound being the more deuterium-depleted of the

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two compounds. In addition, an *initial* reading of the results suggest that the fractionation between the two compounds ($\alpha_{K37:3-K37:2}$) is greater in the stationary (~0.973) than in exponential (~0.985) growth phase (Fig. 3). While both compounds are more deuterium-depleted in the stationary growth phase, the shift appears greater for K37:3. The apparent difference in the degree of fractionation between these two compounds with growth phase, however, may be an analytical artifact.

The likelihood of this artifact is evidenced by Fig. 3. Results for the separated, exponential growth phase samples do not bracket the corresponding δD_{K37s} values, as expected based on isotopic mass-balance requirements, while the stationary growth phase samples do. Closer examination of results of the argentation chromatography separations suggests a cause for the discrepancy. The isotopic difference between both K37:2 and K37:3 and their associated, unseparated K37s samples were calculated ($\Delta\delta D$) to normalize against the effect of changing overall isotopic composition with growth phase. The $\Delta\delta D$ results are plotted in Fig. 5 vs. percent recovery for each compound from the argentation chromatography procedure. Given that unseparated δD_{K37s} measurements are comprised only of contributions from K37:2 and K37:3, isotopic mass balance requires that these two compounds plot on opposite sides of the zero-difference line. If the tight vertical clustering of all $\Delta\delta D_{K37:2}$ values (Fig. 5) indicates that these data are accurate, then the $\Delta\delta D_{K37:3}$ values for the exponential growth phase samples *should* plot below zero. This apparent deviation of only one compound may reflect an isotopic fractionation effect imparted by low recovery from the argentation chromatography procedure (Schwab and Sachs, 2009). Regardless of the cause, however, the observation justifies a revised analysis of the $\delta D_{K37:3}$ results from both exponential-phase samples.

In our revised analysis, all $\delta D_{K37:2}$ values are assumed to be accurate, based on the consistent $\Delta\delta D$ values and uniformly high recovery (80 to 90%, Fig. 5). Given this condition, the $\delta D_{K37:3}$ value required to properly close the isotopic mass balance for the two experiments where K37:3 displayed conspicuously low recovery (~70%) can

be calculated using an algebraic rearrangement of Eq. (8):

$$\delta D_{\text{K37:3,calc}} = \frac{\delta D_{\text{K37}s} - \delta D_{\text{K37:2}} U_{37}^{K'}}{1 - U_{37}^{K'}} \quad (9)$$

The calculated $\delta D_{\text{K37:3}}$ values average −198.1±0.9‰ (Fig. 5). The average $\alpha_{\text{K37:3-K37:2}}$ value obtained for the exponential growth phase samples when these "corrected" $\delta D_{\text{K37:3}}$ values are used (0.972±0.012) is statistically identical (two-sided p=0.85) to that assessed for stationary growth phase samples (0.973±0.008). The issue of growth phase-dependence on the differential fractionation of K37:2 and K37:3 appears resolved – there is no evidence that it occurs.

While the *direction* of the unsaturation-specific offset seen in our work agrees with the findings of D'Andrea et al. (2007), the magnitude of the fractionation effect does not. Our work shows an $\alpha_{\text{K37:3-K37:2}}$ value of 0.973±0.008 while their result (0.94), albeit without mass-balance constraints, is significantly lower. It is possible that this difference is due to changes in $\alpha_{\text{K37:3-K37:2}}$ with growth conditions. However, D'Andrea et al. (2007) observed the same α value between K37:4 and K37:3 in two polar lacustrine sediments as between K37:3 and K37:2 in their *E. huxleyi* laboratory culture. This finding, coupled with the consistency of $\alpha_{\text{K37:3-K37:2}}$ across extremes in growth conditions that our work now documents, suggests that $\alpha_{\text{K37:}n\text{-K37:}(n-1)}$ is a constant value independent of the species of alkenone producer or its physiological state.

Work just recently published by Schwab and Sachs (2009) reports values of $\alpha_{\text{K}n\text{:3-K}n\text{:2}}$ identical to ours (~0.97) in both sediment and suspended-particulate samples from Chesapeake Bay for both C_{37} and C_{38} alkenones. Furthermore, the complete lack of C_{38} methyl components in the alkenone signature preserved in their sediment samples is inconsistent with production by *E. huxleyi* (Volkman et al., 1980). The GC-illustrated molecular fingerprint documented in their publication is more indicative of alkenone-producing haptophytes such as *Chrysotila lamellose* (Rontani et al., 2004). This finding suggests that an $\alpha_{\text{K}n\text{:3-K}n\text{:2}}$ of ~0.97 is indeed applicable to various species of alkenone producers as well as across disparate growth conditions.

It appears that the relative difference in hydrogen isotopic fractionation between K37:3 and K37:2 is somewhat less than initially reported. Furthermore, with certain educated qualifications applied to our results to correct for the alteration of samples during preparation, the value of $\alpha_{K37:3-K37:2}$ appears to remain constant despite changes in cell growth-phase.

4.2 Analysis of growth-phase results

The fractionation that takes place when alkenones are synthesized in stationary phase is distinctly greater than that exhibited by exponential-phase cells when these values are measured on the combined C_{37} alkenone signal (Fig. 3). In light of the unsaturation-specific fractionation discussed above, a shift in the *total* signal between exponential- and stationary-phase samples could be interpreted as solely due to the $U_{37}^{K'}$ change that occurs at the onset of senescence. However, since both the K37:2 and K37:3 isolates exhibit an increase in hydrogen isotopic fractionation relative to water in the stationary growth phase samples (Fig. 3), the changing fractional abundance of these compounds cannot be the exclusive cause of the observed isotopic shift from the exponential to the stationary growth phase.

The following exercise demonstrates quantitatively that the change in relative compound abundance makes only a minor contribution to the growth phase shift in measured $\alpha_{K37s-\text{water}}$ values. The average exponential-phase values of $\delta D_{K37:2}$ (−175.0‰) and $\delta D_{K37:3}$ (−198.1‰, "corrected" value), would correspond, given a $U_{37}^{K'}$ value of 0.4 (observed here for exponential-phase samples) and Eq. (8), to a δD_{K37s} value of −186‰ and an $\alpha_{K37-\text{water}}$ of ~0.819. Were $U_{37}^{K'}$ shifted to 0.3 (observed here for stationary-phase samples), these same compositions would yield a δD_{K37s} value of −189‰ and an $\alpha_{K37-\text{water}}$ of 0.816. This pair of hypothetical exponential- and stationary-phase samples would have an $\alpha_{K37-\text{water}}$ difference of 0.003. This difference is within the uncertainty of our measurements and is much smaller than the 0.027 difference with growth phase that we observe for actual K37*s* samples. A real biosyn-

thetic change has taken place, independent of the changing relative contributions of K37:3 and K37:2 to the "weighted average" δD_{K37s} measurements.

Results from analysis of the *G. oceanica* samples show a similar offset between exponential- and stationary-phase samples (Fig. 4), indicating that the growth-phase effect on $\alpha_{K37s-water}$ is not species-specific. The shift in $\alpha_{K37s-water}$ with growth phase for *G. oceanica* also appears to be independent of the decrease in $U_{37}^{K'}$ which marks the onset of nutrient-imposed stationary-phase growth at some temperatures. At 25°C, there is only a minor decrease in $U_{37}^{K'}$ associated with this transition in cell physiology (Fig. 4), but a large shift in $\alpha_{K37s-water}$ is still apparent. This observation further demonstrates there is real growth phase-dependence in $\alpha_{K37s-water}$, independent of the changing proportion of K37:3 and K37:2. Although not statistically verifiable given our limited data set, the apparent "stress" contours in the $\alpha_{K37-water}$ vs. growth-temperature data from the *G. oceanica* samples appear to extrapolate smoothly to the data from the *E. huxleyi* cultures. This observation suggests that both the growth-phase offset in isotopic fractionation and the absolute fractionation at each phase is general to different species of alkenone-producing haptophytes (Fig. 4).

Furthermore, the apparent trend for stationary-phase *G. oceanica* measurements, which were obtained from cells collected at the onset of senescence, seemingly extrapolate to a position somewhat above the stationary-phase data for *E. huxleyi*. The latter samples were harvested several days after the onset of nutrient-limited stationary-phase growth (Fig. 1). Additionally, the *G. oceanica* stationary-phase data interpolates to a point slightly *below* the stationary-phase points from the UBC *E. huxleyi* cultures, which entered senescence without depleting the available nutrients. The high values of nitrate and phosphate present at stationary phase (Table 1) suggest that crowding, self-shading, CO_2 or trace metal limitation, rather than macronutrient depletion, induced senescence in this particular experiment. This finding suggests that $\alpha_{K37s-water}$ responds not in a *stepwise* manner to growth phase changes, but rather in a smooth, progressive manner that could be calibrated and may ultimately provide a way to quantify the magnitude of stress. More work is warranted to determine whether this is

indeed a valid prospect and, if so, how effectively this potential paleostress index (see Sect. 4.3) can be calibrated.

The apparent variation in $\alpha_{K37s-water}$ with growth temperature cannot be due to changes in $U_{37}^{K'}$ with temperature, as increased temperature leads to decreased relative abundance of K37:3, the more deuterium-depleted of the component compounds, which would increase δD_{K37s}. An increase in δD_{K37s} would, in turn, increase the value of $\alpha_{K37s-water}$, a trend opposite to the observation illustrated in Fig. 4. The prospect of a temperature effect on $\alpha_{K37s-water}$ in the *G. oceanica* data set is a significant result. Employing a multivariate experimental approach, Schouten et al. (2006) concluded that there is no temperature dependence in $\alpha_{K37s-water}$. Additionally, they reported consistently smaller $\alpha_{K37s-water}$ values for *G. oceanica* than for *E. huxleyi* when grown isothermally under a range of other environmental conditions. This result conflicts with the trend inferred from our yet-limited data set for these two organisms (Fig. 4). A more thorough side-by-side study of *G. oceanica* and *E. huxleyi* is now warranted to test the veracity of the apparent trends with nutrient stress and temperature that our work now documents.

4.3 Implications for paleoceanographic research

Finding a value of $\alpha_{K37:3-K37:2}$ significantly less than unity raises a valid practical concern for any potential paleoceanographic application involving δD_{K37s} measurements. Our results, combined with those of others (D'Andrea et al., 2007; Schwab and Sachs, 2009), confirm that a significant isotopic offset between K37:3 and K37:2 does exist. For gradual changes in sedimentary $U_{37}^{K'}$ through time, unsaturation-specific fractionation would not undermine the utility of δD_{K37s} measurements as a *qualitative* tool. However, for *quantitative* interpretations of paleoceanographic conditions, the impacts of changing $U_{37}^{K'}$, discussed in Sect. 1.3, must be taken into account. The most computationally sound method for avoiding the "changing weighted average" effect on δD_{K37s} values is to separate every sample and base interpretations on δD analysis of pure,

single compounds. However, this approach would be time and labor intensive and, therefore, limit the acquisition of high-resolution data sets. If the value for $\alpha_{K37:3-K37:2}$ is a constant, as all current evidence now suggests, a more attractive alternative exists – measure the integrated δD_{K37s} and derive component δD values by isotopic mass balance (Eq. 6) using $U_{37}^{K'}$ data and an accepted value of $\alpha_{K37:3-K37:2}$. Despite the unexplained difference between the $\alpha_{K37:3-K37:2}$ values reported here (echoed by Schwab and Sachs, 2009) and by D'Andrea et al. (2007), the sparse existing evidence shows that this alternative strategy holds promise and should be possible using an $\alpha_{K37:3-K37:2}$ value of ∼0.97.

The existence of distinct differences in the hydrogen isotopic fractionation in alkenones associated with nutrient stress-induced changes in growth phase, however, has implications at the most basic level for use of δD_{K37s} as a paleoproxy. When δD_{K37s} data for surface sediments from the Emerald Basin (Englebrecht and Sachs, 2005) are paired with δD_{water} values for the overlying surface ocean to estimate a value for $\alpha_{K37-water}$, the result (∼0.79) is similar to what Fig. 4 would lead us to expect for stationary-phase cells grown at temperatures <20°C (Emerald Basin maSST ≈8°C, World Ocean Atlas 2005). All else being equal, if $\alpha_{K37-water}$ really *does* serve as an indicator of growth phase, then these results serve to strengthen the argument that the vectors of export for the $U_{37}^{K'}$ signal preserved in sediments sculpt a fossil record that is biased towards the biochemical signature of physiologically stressed organisms (Prahl et al., 2006). It is possible, then, that δD_{K37s} measurements could indeed provide a means to identify and quantify stress, a prospect which now warrants careful examination. $U_{37}^{K'}$ measurements may yield estimates of maSST with a well-characterized statistical constraint (i.e. ±1.4°C, Herbert, 2003), but this degree of uncertainty is climatically significant. Non-thermal physiological effects may account for some of the variability about this statistical calibration (Prahl et al., 2003), which recent evidence suggests displays a significant degree of biogeographical coherence (Prahl et al., 2009). Judging from the trends apparent in Fig. 4, δD_{K37s} measurements and their use in deriving $\alpha_{K37s-water}$ estimates may provide an objective means to assess the

degree to which non-thermal physiological growth factors act to fine-tune the primary temperature signal encoded in sedimentary $U_{37}^{K'}$ records.

The paleoceanographic literature now exhibits many studies where data from $U_{37}^{K'}$ and other temperature proxies disagree (e.g. Chapman et al., 1996). In these situations, it is easy to invoke deficiencies in one proxy in favor of another. Studies are published, however, in which $U_{37}^{K'}$ and another temperature proxy agree in *some* parts of the record and disagree in others (e.g. Mix, 2006). This situation is a more difficult one to resolve, as it begs questions for which objective answers are often unavailable: what changed oceanographically to alter the relative signals of two measurements which *do* agree under other circumstances, and which temperature proxy is "correct" when divergence is apparent? Mix (2006), for example, describes the time-dependant agreement and disagreement of two nearby Mg/Ca and $U_{37}^{K'}$ SST records from the eastern tropical Pacific. The cause for apparent disagreement during the glacial-interglacial transition period was left as an open question. Mix proposed either depth-habitat changes in the proxy-producing organisms or changes in the nutrient regime as two viable biological – oceanographic explanations. δD_{K37} measurements may provide a tool with which to test these options. In open ocean settings, such as the equatorial Pacific example presented by Mix (2006), changes in $\delta D_{\mathrm{water}}$ would have little impact on $\delta D_{\mathrm{K37}s}$ (see Sect. 1.1) relative to the variability caused by the effects that nutrient stress appears to impose on $\alpha_{\mathrm{K37}s\mathrm{-water}}$ (Fig. 4). Thus, $\delta D_{\mathrm{K37}s}$ measurements could indicate whether changes in the nutrient regime, and the associated changes in $U_{37}^{K'}$ imparted by variable nutrient stress, were a cause for time-variable discrepancy of the two paleotemperature proxies.

Additionally, if $\alpha_{\mathrm{K37}s\mathrm{-water}}$ indeed responds in a progressive rather than a stepwise manner to stress (Sect. 4.2), as is now a well-documented response for $U_{37}^{K'}$ (Prahl et al., 2003), then $\delta D_{\mathrm{K37}s}$ could be used to correct, at least semi-quantitatively, for nutrient-stress biasing of $U_{37}^{K'}$ temperature records. The associated shifts in both $\alpha_{\mathrm{K37}s\mathrm{-water}}$ and $U_{37}^{K'}$ with growth phase (Fig. 4) suggest that the calibration of $U_{37}^{K'}$ as

a function of growth temperature could be extended into a third dimension ($\alpha_{\text{K37-water}}$) using $\delta D_{\text{K37}s}$ measurements. This calibration "surface", once adequately defined with considerably more data, would have the potential to collapse much of the vertical scatter in environmental data into real variation in a third dimension, i.e. define a continuum of unique $U_{37}^{K'}$ vs. temperature relationships for contours of constant stress. To apply a calibration of this sort down-core, estimates of the isotopic composition of the surface water would be necessary in order to calculate $\alpha_{\text{K37-water}}$. δD_{water} estimates could be derived, potentially, from δ^{18}O measurements of planktonic-foraminiferal calcite and the use of regional relationships between δD and δ^{18}O (e.g. Dansgaard, 1964).

The relationship between $\alpha_{\text{K37}s\text{-water}}$, $U_{37}^{K'}$, and temperature needs to be thoroughly evaluated by conducting more batch culture experiments, together with field testing in modern oceanographic settings, before the type of paleoproxy approach just discussed could be fruitfully realized. Knowledge gained from further study of stress-dependent behavior of $\alpha_{\text{K37}s\text{-water}}$ in alkenone-producing haptophytes could lend valuable insight into ambiguous paleotemperature data sets. For this reason, it would seem a most worthwhile investment of future research effort.

5 Conclusions

Results from our batch culture experiments have shown:

1. K37:3 synthesized by *E. huxleyi* is fractionated, relative to the hydrogen isotopic composition of water in the growth medium, to a higher degree than K37:2. Furthermore, this difference appears constant across growth phases and, in light of the results of Schwab and Sachs (2009), between different species of alkenone-producers. If true, an accepted $\alpha_{\text{K37:3-K37:2}}$ value (~0.97, given current evidence) could be used in a simple isotopic mass balance to account for the effect of changing $U_{37}^{K'}$ on $\delta D_{\text{K37}s}$ when measuring environmental or down-core alkenone samples. This approach would alleviate the need to perform time-consuming, analyt-

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ically risky chromatographic separations on every sample, and thereby increase the research community's ability to make high resolution, precise compound-specific δD measurements on alkenones down-core.

2. A distinct increase in fractionation (decrease in $\alpha_{\mathrm{K37}s-\mathrm{water}}$) for C_{37} alkenones vs. the growth medium occurs in both *E. huxleyi* and *G. oceanica* when cells shift from an exponential to a nutrient-imposed stationary growth phase. This effect cannot be explained by the associated growth phase-dependent changes in $U_{37}^{K'}$ values altering the isotopic mass balance of the two component compounds. It is a true biological phenomenon, related to the incompletely understood mechanism of alkenone biosynthesis (Rontani et al., 2005), which warrants further investigation.

These two conclusions suggest that δD measurements of sedimentary alkenones have utility as an indicator of stress impacts on the primary temperature signal encoded in $U_{37}^{K'}$ values. Further development and future application of this stress index may help to improve the interpretation of $U_{37}^{K'}$ temperature records and resolve discrepancies between proxies that are currently problematic for paleoceanographic/paleoclimatic research.

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Table 1. Results from growth-phase experiments with *E. huxleyi* CCMP 1742 and *G. oceanica* PZ3-1.

Sampling phase	Replicate culture	Growth temperature (°C)	Growth rate (day^{-1})	$[NO_3^-]+[NO_2^-]$ (µmol/L)	$[PO_4^{3-}]$ (µmol/L)	% Recovery**	Uk'37	%K37	K37s/cell (pg/cell)	δD_{K37} (‰ vs. SMOW)	±(1σ)	n	δD_{water} (‰ vs. SMOW)	±(1σ) (n=9)	$\alpha_{K37-water}$*** (±0.005)	$\alpha_{K37:3-K37:2}$ (±0.008)
E. huxleyi CCMP 1742 Cultures																
Exponential	Culture 1	15	0.58	74.4	2.9	68	0.41	55	0.44	−186.4	0.9	3	−6.35	1.77	0.819	
	Replicate*		0.58			72	0.40	54	0.44							
	K37:2					89				−171.2	0.5	2	" "		0.834	
	K37:3					72				−184.7	7.8	3	" "		0.821	0.984
	Reconstructed Total K37									−179.3	4.7		" "		0.826	
	Culture 2	15	0.56	66.2	2.3	76	0.41	55	0.46	−191.0	0.1	2	−6.85	2.68	0.815	
	Replicate*		0.56			77	0.41	55	0.45							
	K37:2					91				−178.8	0.3	2	" "		0.827	
	K37:3					67				−190.9	2.1	3	" "		0.815	0.985
	Reconstructed Total K37									−185.9	1.3		" "		0.820	
Stationary	Culture 1	15		0.3	0.31	59	0.30	52	1.3	−215.3	3.5	2	−5.28	3.40	0.789	
	Replicate*					75	0.29	50	1.5							
	K37:2					94				−200.9	3.2	2	" "		0.803	
	K37:3					85				−221.4	2.8	2	" "		0.783	0.974
	Reconstructed Total K37									−215.5	2.2		" "		0.789	
	Culture 2	15		0.6	0.28	78	0.30	52	1.4	−212.9	2.1	2	−5.23	0.79	0.791	
	Replicate*					72	0.29	50	1.6							
	K37:2					91				−198.9	1.6	2	" "		0.805	
	K37:3					86				−222.1	0.3	2	" "		0.782	0.971
	Reconstructed Total K37									−215.3	0.5		" "		0.789	
UBC *E. huxleyi* CCMP 1742 Cultures																
Exponential	A	18	0.61				0.36	64	1.3	−182.6	0.3	2			0.822	
	B	18	0.62	308	9.7		0.37	64	3.0	−188.3	0.4	2			0.816	
Stationary	A	18					0.30	63	4.5	−204.3	6.4	3			0.800	
	B	18		244	5.8		0.30	62	2.8	−201.3	0.3	2			0.803	
G. oceanica PZ3-1 Cultures																
Exponential	Culture 1	17	0.62				0.44	55	0.60	−190.9	1.3	2			0.811	
	Culture 2	21	0.92				0.74	56	0.82	−204.6	0.2	2			0.798	
	Culture 3	25	0.93				0.99	59	2.0	−230.0	2.0	2			0.772	
Stationary	Culture 1	17					0.38	48	0.78	−213.2	7.6	2			0.788	
	Culture 2	21					0.68	41	1.2	−235.4	0.3	2			0.768	
	Culture 3	25					0.98	53	1.1	−262.6	0.5	2			0.740	

* Values measured on samples prior to separation by argentation chromatography.
** Values for total K37*s* samples are calculated as described in Sect. 2.3. Percent recovery for the K37:2 and K37:3 isolates is specific to the argentation chromatographic procedure.
*** Assuming δD_{water}=−5.7‰ for the UBC *E. huxleyi* cultures (average value from previous experiments using the same medium); assuming δD_{water}=0‰ for *G. oceanica* samples due to loss of water samples (see Sect. 2.4.1).

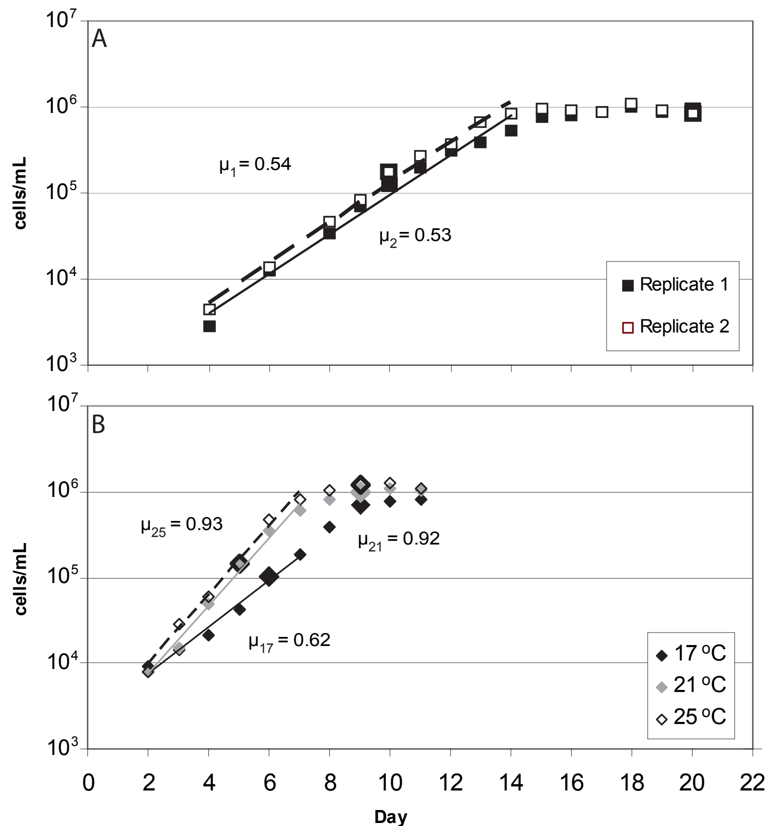

Fig. 1. Growth curves for batch cultures of **(A)** *E. huxleyi* (15°C cultures) and **(B)** *G. oceanica*. Growth rates (μ, day^{-1}), derived from linear fits for the exponential portion of each curve, are denoted on the plots. The time points when cell and water samples were collected are identified by the enlarged symbols.

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Growth phase dependent hydrogen isotopic fractionation

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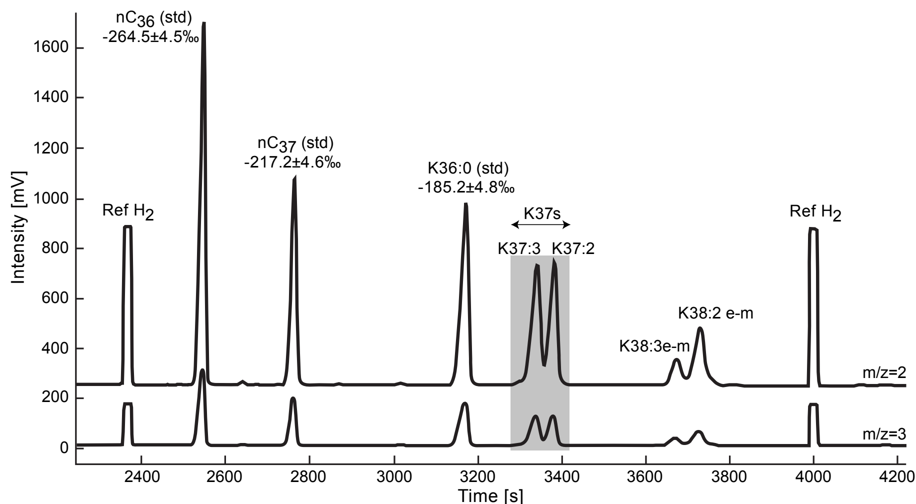

Fig. 2. Example chromatogram from compound-specific δD analysis of alkenones by GC-P-IRMS. For total C_{37} alkenone (K37s) measurements, an isotope ratio was determined by integrating across the unresolved K37:3 and K37:2 peaks (shaded area). "K38:3e-m" and "K38:2e-m" labels denote peaks comprised of the unresolved ethyl and methyl C_{38} ketones.

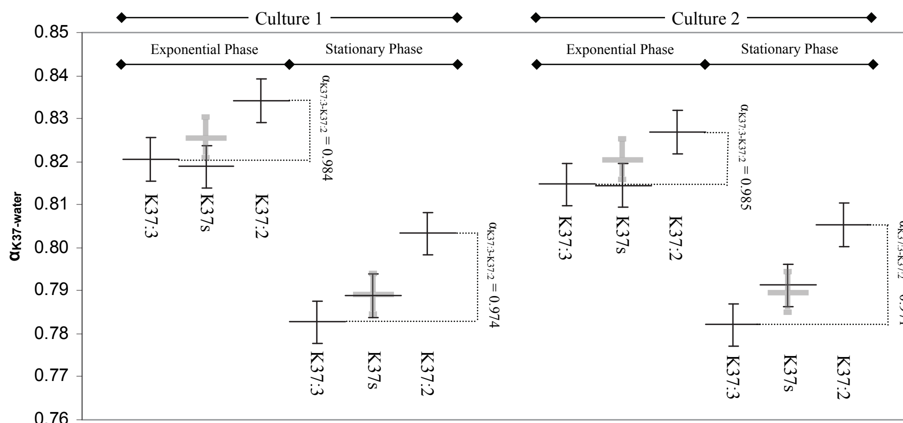

Fig. 3. Fractionation factors (α) relative to growth-water for the 15°C *E. huxleyi* growth-phase and unsaturation-specific fractionation experiments. Culture replicates 1 and 2 are shown on the left and right, respectively. Unseparated K37*s* samples for each culture replicate and growth phase are shown with values of the associated K37:3 and K37:2 isolates on either side. $\alpha_{K37s\text{-water}}$ values reconstructed via mass-balance for each pair of K37:2 and K37:3 measurements (Eq. 8, see Sect. 3.2) are shown by the shaded bars. Values of $\alpha_{K37:3\text{-}K37:2}$ are provided for each set of isolate measurements (see dashed lines).

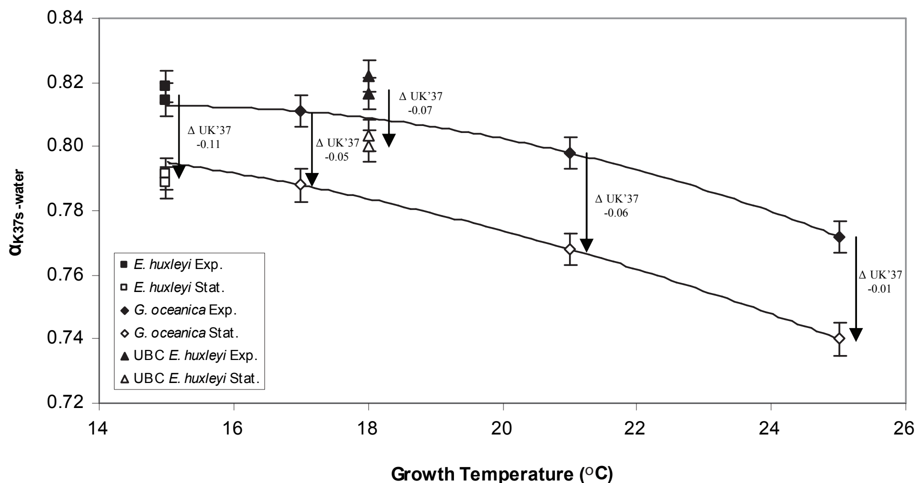

Fig. 4. Fractionation factors relative to growth-water for the *G. oceanica* cultures. As a visual aide, lines are drawn to show the apparently smooth trend in the data for *G. oceanica* sampled in the exponential growth phase and at the onset of nutrient-depleted senescence. Values for the $U_{37}^{K'}$ shift associated with transition between the exponential to stationary growth phases $\left(U_{37\,\mathrm{exp}}^{K'} - U_{37\,\mathrm{stat}}^{K'}\right)$ for each treatment are shown as an annotation. $\delta D_{\mathrm{K37}s}$ values for exponential and stationary growth phase samples of *E. huxleyi* from both the primary experiment and the experiment run at UBC are plotted for comparison.

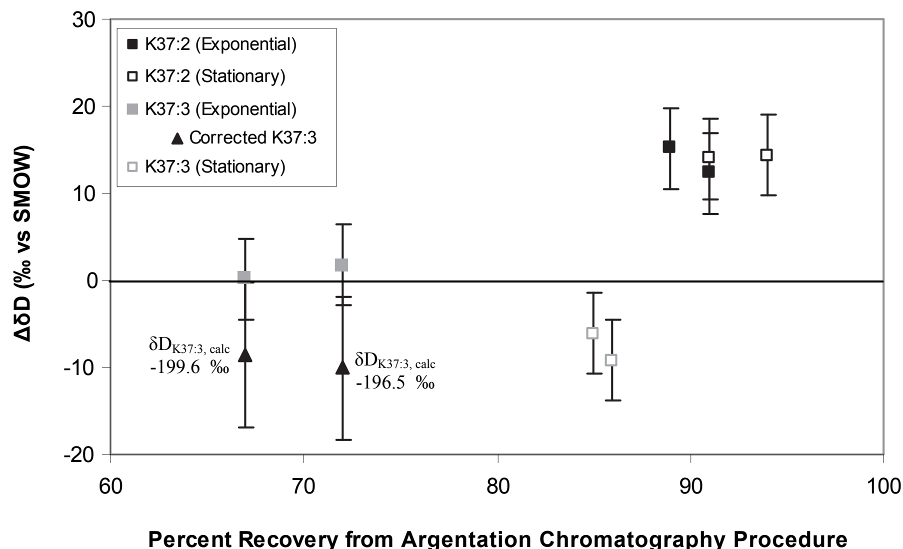

Fig. 5. Plot of differences between the δD of the unsaturation-specific isolates and their associated integrated K37s values ($\Delta\delta D = \delta D_{K37:n} - \delta D_{K37s}$) from the *E. huxleyi* growth-phase experiments (15°C cultures) vs. percent recovery from the argentation chromatographic procedure. Isotopic mass balance predictions (Eq. 9, see Sect. 4.1) for $\Delta\delta D$ values (absolute δD values shown by annotation) for the exponential growth phase K37:3 isolates (triangles) are also shown.