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Exploring the theoretical upper temperature limit of alkenone unsaturation indices: Implications for paleotemperature reconstructions

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ABSTRACT

The structural specificity, exceptional diagenetic stability, and linear response of unsaturation to temperature have made alkenones an indispensable tool for reconstructing past sea surface temperatures, with the well known $U^{K'}_{37}$ proxy widely applied in the past 40 years. However, $U^{K'}_{37}$ approaches unity at around 28 °C in cultures of *Emiliania huxleyi* (*E. huxleyi*), the most widely distributed alkenone producer in global oceans. Recent studies using surface sediments suggest $U^{K'}_{38Me}$ has ~1.5 °C higher upper temperature limits than $U^{K'}_{37}$. However, *E. huxleyi* generally does not grow above 28 °C. *Gephyrocapsa oceanica* (*G. oceanica*), on the other hand, is the dominant alkenone producer when sea surface temperature is above 22 °C and thrives in ocean regions such as the Pacific Warm Pool. So far there have been no culture data for *G. oceanica* above 29 °C to evaluate the temperature response of alkenone distributions and the (theoretical) upper temperature limit of alkenone unsaturation indices. Here we performed the first culture experiments on two strains of *G. oceanica* isolated from warm ocean sites at up to 32 °C: RCC6484 from the Pacific Warm Pool and RCC3483 from the South China Sea. We show both strains display higher growth rates at higher temperature, with the highest growth rates at 32 °C (the highest temperature we tested), although alkenone production per cell decreases as temperature increases. Importantly, $U^{K'}_{37}$ and $U^{K'}_{38Me}$ values of RCC6484 and RCC3483 continue to respond to temperature changes beyond 28 °C, although temperature sensitivity decreases significantly above 28 °C. Above 30 °C, $U^{K'}_{37}$ and $U^{K'}_{38Me}$ responses to temperature further diminish, limiting their potential for paleo-SST applications using the currently available analytical technology. The ratio of $C_{38:3}$ over $C_{38:2}$ methyl ketone is on average 11 times higher than the ratio of $C_{37:3}$ over $C_{37:2}$ from 24 to 32 °C, suggesting a greater potential of using $U^{K'}_{38Me}$ for paleotemperature reconstruction in high temperature settings.

1. Introduction

Paleotemperature records, especially records from significantly warmer-than-present periods, are important for constraining global climate models and for obtaining more realistic future projections. Long chain alkenones produced by Isochrysidales represent one of the best lipid biomarkers ever developed for paleo-SST reconstructions because: 1) only Isochrysidales, an order of haptophyte algae, make these alkenones. The high specificity of producing organisms reduces potential noises for paleo-SST reconstructions; 2) Isochrysidales live in surface waters, hence alkenone paleothermometers record surface water temperatures that are climatically and ecologically important; 3) environ-

mental factors other than temperature do not significantly affect alkenone unsaturation ratios; and 4) alkenones are exceptionally stable during diagenesis, with the oldest known sediments containing alkenones dating back 120 Ma (de Leeuw et al., 1980; Brassell et al., 1986; Prahl and Wakeham, 1987; Sikes et al., 1991; Brassell and Dumitrescu, 2004). The unsaturation ratio of C_{37} methyl ketones derived from the relative percentage of tri- and di- unsaturated C_{37} methyl ketones, i. e., $U^{K'}_{37} = C_{37:2}/(C_{37:2} + C_{37:3})$ index displays strong linear correlation with SSTs in ocean sediments, except at extreme temperature ends (e.g., $< \sim 6$ and $> \sim 26$ °C) (Sikes and Volkman, 1993; Müller et al., 1998; Conte et al., 2006; Tierney and Tingley, 2018). While non-linear calibration may be used for SST reconstruction at temperature extremes

(Sikes and Volkman, 1993; Conte et al., 2006; Tierney and Tingley, 2018), the tri-unsaturated C_{37} alkenone ($C_{37:3}$) concentration often falls below the detection limit in conventional GC-FID analyses above 28℃. At this temperature, $U^{K'}_{37}$ index approaches unity in ocean sediments, hence is thought to be no longer practically viable for paleotemperature reconstructions (Tierney and Tingley, 2018; Novak et al., 2022). Nevertheless, alkenones are continuously found in sediment samples when temperatures are substantially above 28℃, for example in modern Pacific Warm Pool waters as warm as 32℃ (Novak et al., 2022) and during the Cretaceous middle latitude oceans (Brassell and Dumitrescu, 2004). Do Isochrysidales species capable of producing alkenones above 28℃ continue to regulate alkenone distributions via unsaturation ratios or any other mechanisms?

Extending the upper temperature limit of alkenone unsaturation ratios to 32 °C will help resolve the ongoing debate on the existence of Pliocene permanent El Niño-like state (Ravelo, et al., 2014; Zhang et al., 2014a, 2014b). The key arguments originate from the use of different proxies ($U^{K'}_{37}$, TEX$_{86}$, foraminifera Mg/Ca) to reconstruct temperature trends in the west and east Pacific Ocean (Wara et al., 2005; Zhang et al., 2014a). Lack of alkenone-based SST reconstructions in the Pacific Warm pool prior to 3 million years ago raises questions if the reconstructed SSTs (28 to 31 °C) based on TEX$_{86}$ are fully compatible with east Pacific temperature trend reconstructed using $U^{K'}_{37}$ (Ravelo, et al., 2014). This debate could be reconciled by reconstructing SSTs in the Pacific Warm Pool using $U^{K'}_{38Me}$ that is potentially extendable to 31 °C. While generating a new TEX$_{86}$ record in the eastern Pacific could also help alleviate this problem, TEX$_{86}$ is generally considered a proxy for sub-surface waters (Tierney et al., 2017; Zhang and Liu, 2018) and may not accurately represent temperature contrast in the east and west Pacific surface ocean.

Zheng et al. (2019) suggested unsaturation ratios of alkenones, especially C_{38} methyl ketones, might continue to respond to growth temperatures up to 32℃ and could significantly extend the upper temperature limit of the alkenone paleo-thermometer. The perceived upper temperature limit of ~ 28℃ for alkenone proxies in the literature (Sikes and Volkman, 1993; Sikes et al., 1997; Conte et al., 1998; Novak et al., 2022) might have largely originated from challenges in analytical methods. There were two problems. First, the conventional non-polar stationary phase GC columns (with 100 % polymethyl siloxane stationary phase) that have been commonly used for alkenone analysis since 1980s performed poorly in separating C_{38} alkenones due to partial coelution of C_{38} methyl and ethyl ketones (Longo et al., 2013). Chromatographic co-elution disproportionally increases the difficulty in accurate quantification of small peaks. Thus, it is generally impossible to obtain accurate measurements of unsaturation ratios of C_{38} alkenones such as $U^{K'}_{38Me}$, especially at relatively high temperatures when tri-unsaturated homologues are in very low abundance. This problem was largely overcome by using a mid-polarity poly(trifluoropropylmethylsiloxane) stationary phase GC column that allowed baseline resolution of C_{38} to C_{42} methyl and ethyl unsaturated alkenones (Longo et al., 2013; Zheng et al., 2017; Liao et al., 2020). Second, co-elution of alkenones, especially low concentration alkenones such as $C_{37:3}$ or $C_{38:3}$ at high temperatures, with steryl ethers or other unknown compounds, made accurate quantification difficult in sediment samples. Our ability to deal with this problem was enhanced considerably by the development of the silver thiolate chromatographic material facilitating sample cleanup (Aponte et al., 2012; Dillon et al., 2012; Liao et al., 2021b). This chromatographic material allows efficient separation and purification of unsaturated compounds (e.g., alkenones) from the sample matrix and can be used repeatedly as the silver ion is stabilized by a thiol group covalently bonded onto silica gel. The sample cleanup procedures using silver thiolate liquid chromatographic columns (Wang et al., 2019) led to, for the first time, the discovery of alkenones in the Unit III glacial lake sediments of the Black Sea (Huang et al., 2021). Alkenones in the Black Sea Unit III sediments are derived from variably

contributions from Group 1 and 2 isochrysidales (Theroux et al., 2010; Kaiser et al., 2019; Zheng et al., 2019), allowing a quantitative assessment of salinity changes during the last deglaciation in the Black Sea (Huang et al., 2021).

Applying these improved analytical methods for alkenones has permitted a global surface sediment calibration of $U^{K'}_{38Me}$ index, which demonstrates an extended upper temperature limit to ~ 30 C (Novak et al., 2022). It is still, however, unknown what the true theoretical upper temperature limit of the alkenone paleothermometer is. Modern ocean SSTs are relatively cool compared with past greenhouse worlds and do not permit accurate core-top calibration above 28℃. Analytically, it is necessary to completely remove all compounds coeluting with alkenones for accurate SST reconstructions. However, complete removal of coelution is difficult even with the most elaborate cleanup procedures. Any minor co-elution, especially when tri-unsaturated alkenones are of low abundance at high temperature settings, could result in significant errors in reconstructed temperatures. A better approach to determine the theoretical upper temperature limit is to analyze culture samples grown at high temperatures, as the possibility of coelution is minimal relative to sediment samples and large amounts of alkenones can be isolated from cultures for analysis. A thorough understanding of the true upper temperature limit of the alkenone paleothermometer could spur development of more sensitive and accurate analytical methods for alkenones and broaden the application of the paleothermometer in warm oceans.

Emiliania huxleyi (*E. huxleyi*) and *Gephyrocapsa oceanica* (*G. oceanica*) are the two dominant alkenone producers in the modern ocean (Theroux et al., 2010). While *E. huxleyi* and *G. oceanica* can share similar ecological niches, *G. oceanica* becomes increasingly dominant over *E. huxleyi* as SST rises above 22 °C and is widely found in warm ocean sites including the Pacific Warm Pool and other equatorial ocean regions (Gafar and Schulz, 2018). Therefore, assessing how alkenone distributions change at high temperatures (greater than 28 °C) would require growth of *G. oceanica* cultures isolated from warm ocean sites. So far, the only published *G. oceanica* culture experiment above 28 °C is the study of Volkman et al. (1995) who grew strain JB02 isolated from Jervis Bay in offshore eastern Australia at 29 °C. There are no culture data available for assessing alkenone distributions above 29 °C.

The main objectives of the present study are: 1) to grow two strains of *Gephyrocapsa oceanica*, RCC6484 and RCC3483 isolated from warm oceans at temperatures up to 32 °C, in order to explore the theoretical upper temperature limit of alkenone unsaturation ratios; 2) to further examine the differences in the upper saturation temperatures of $U^{K'}_{37}$ and $U^{K'}_{38Me}$ proxies; and 3) to determine the growth characteristics of the *G. oceanica* strains as temperatures are extended beyond 28 °C, including cell growth rates, alkenone content per cell, and changes in chain lengths and ratios of methyl over ethyl ketones for C_{38} alkenones. Our ultimate goal is to test the hypothesis that alkenone unsaturation ratios have a much higher (theoretical) saturation temperature than the conventionally believed 28 °C derived from ocean core-top sediment calibrations. We hypothesize that alkenone paleothermometers, especially $U^{K'}_{38Me}$, are of great potential for temperature reconstructions beyond 28 °C, especially when more sensitive and more quantitative methods than the traditional GC-FID approach are developed to quantify alkenones in the future.

2. Materials and methods

2.1. Culture experiments

G. oceanica strains RCC3483 and RCC6484 were purchased from the Roscoff Culture Collection. RCC6484 was isolated the Pacific Warm Pool on December 15, 2016 (141.75 °E, 2.04 °N) with *in-situ* temperature at 28 °C (mean annual SST at 29.4 °C based on WOA18 data, monthly temperature changes in Supplementary Fig. 2), while RCC3483 was

isolated from the South China Sea on February 18, 2009 (103.92 °E, 10.27 °N) with *in-situ* temperature at 22 °C (mean annual SST at 28.8 °C based on WOA18 data, monthly temperature changes in Supplementary Fig. 2) (Locarnini, et al., 2018). Culture growth conditions and harvest procedures followed those reported in Liao et al. (2020, 2021a, 2022, 2023). *G. oceanica* strains were acclimatized for two weeks before the start of corresponding culture experiments with k/2 medium, which was prepared from seawater collected from Vineyard Sound, Woods Hole, MA, USA at a salinity of 31 psu. The sea water was filtered through 0.2 μm Whatman nylon membrane filter and then autoclaved before use. *G. oceanica* RCC3483 and RCC6484 were cultured at 24, 26, 28, 30, 31 and 32 °C, with culture experiments at 28, 30, 31 and 32 °C performed in triplicate cultures. Cultures were grown under a light:dark cycle set at 16:8. The light intensity was 140 μE·m^{-2}·s^{-1}. All cultures experiments

were performed in 165 mL medium.

Cultures were harvested at early stationary phase (monitored using hemocytometer counts (Hausser Scientific, PA, USA)) by filtering onto 0.7 μm glass fiber filters (Merck Millipore, MA, USA). All filters were wrapped with aluminum foils and immediately frozen at −20 °C for further extraction and analysis (Zheng et al., 2016; Liao et al., 2020, 2021a, 2022, 2023).

2.2. Analysis of alkenones

Analysis of culture samples followed the procedure reported in Liao et al. (2020, 2021a, 2022, 2023). Filters of culture samples were freeze-dried overnight and then sonicated three times with dichloromethane (DCM, 3 × 30 min, 20 mL each time) for lipid extractions. Total extracts
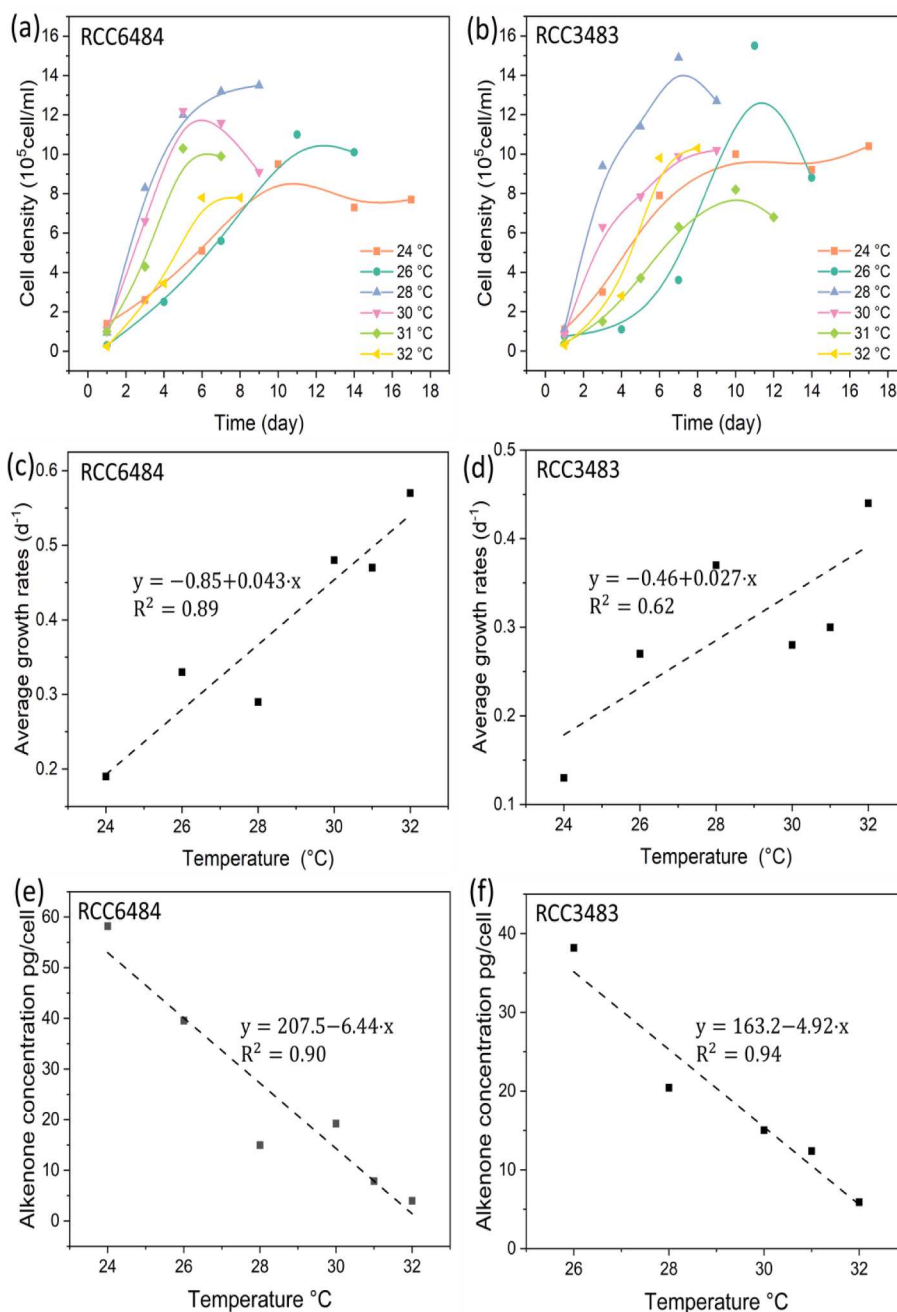
Fig. 1. Growth curves at 24, 26, 28, 30, 31 and 32 °C for *G. oceanica* RCC6484 (a) and RCC3483 (b). Correlation between temperature and average growth rates for RCC6484 (c) and RCC3483 (d). Effect of temperature on alkenone concentration per cell from 24 (26) to 32 °C for RCC6484 (e) and RCC3483 (f).

of culture samples were separated into three fractions using silica gel (230–400 mesh, 40–63 μm) in glass pipettes, and eluted with hexane, DCM and methanol. Alkenones were in the DCM fraction.

DCM fractions were then analyzed on GC-FID (Gas Chromatography-Flame Ionization Detection; Agilent 7890B) and GC-EI-MS (Gas Chromatography-Electron Ionization-Mass Spectrometry; Agilent 7890B interfaced to 5977 inert plus MSD) equipped with a poly(tri-fluoropropylmethylsiloxane) RTX-200 column (105 m × 250 μm × 0.25 μm) (Zheng et al., 2017). GC columns with a poly(tri-fluoropropylmethylsiloxane) stationary phase provide much more efficient separation of di- and tri- unsaturated C_{38} methyl and ethyl alkenones than the conventional GC columns with non-polar (100% poly (dimethyl Siloxane) or 5% diphenyl/95% dimethyl Siloxane) stationary phases (Longo et al., 2013; Zheng et al., 2017). For the analysis on GC-FID, the carrier gas was hydrogen. Samples were injected under pulsed splitless mode at 320 °C. The initial pulse pressure was 35 psi for the first 1 min. Then the purge flow to split vent was 35.0 mL/min at 1.1 min. The flow rate (constant flow mode) was 1.5 mL/min. The initial oven temperature was 50 °C for 2 min, then increased to 255 °C at 20 °C/min, then increased to 320 °C at 3 °C/min and held for 35 min. For the analysis on GC-EI-MS, samples were injected under pulsed splitless mode at 320 °C. The initial pulse pressure was 35 psi for the first 0.5 min. The purge flow to split vent was 50 mL/min at 1.1 min. The flow rate (constant flow mode) was 1.6 mL/min. The initial oven temperature was 40 °C for 1 min, then increased to 255 °C at 20 °C/min, then increased to 315 °C at 3 °C/min and held for 35 min. Samples were analyzed under full-scan mode. The source temperature was 230 °C. The electron ionization energy was 70 eV. The mass range was from m/z 50 to 650.

3. Results and discussion

3.1. Effect of temperature on growth rates and alkenone production

Both RCC6484 and RCC3483 show increasing growth rates as temperature increases, with the highest (0.57 d^{-1} for RCC6484, 0.44 d^{-1} for RCC3483) and lowest (0.19 d^{-1} for RCC6484, 0.13 d^{-1} for RCC3483) growth rates at 32 and 24 °C, respectively (Fig. 1). The data suggest that these *G. oceanica* strains may be well adapted to high growth temperatures. The culture data are also consistent with the geographic distribution of *G. oceanica* that is dominant over *E. huxleyi* at > 22 °C (Gafar and Schulz, 2018).

We note that RCC6484 shows on average 34% higher growth rates than RCC3483. For example, at 30 °C, growth rate was at 0.48 d^{-1} for RCC6484 but 0.28 d^{-1} for RCC3483. Our experimental growth temperatures for RCC6484 and RCC3483 extend to a higher range (up to 32 °C) than the published maximum growth temperatures for *G. oceanica* strains (27 °C for AB1 in Conte et al., 1998, and 29 °C for JB02 in Volkman et al., 1995). Based on WOA18 data, strain AB1 was isolated from the southwest Pacific with a modern annual mean SST of 16.9 °C, whereas JB02 from Jervis Bay in offshore eastern Australia with modern annual mean SST of 19.3 °C. In contrast, the modern annual mean SSTs for the isolation sites of RCC6484 and RCC3483 are 29.3 and 28.4 °C, respectively. Our experimental results indicate *G. oceanica* strains isolated from hotter ocean waters may be better adapted to higher growth temperatures. Notably however, we used k/2 culture medium to grow RCC6484 and RCC3483, instead of f/2 used for AB1 and JB02 (Volkman et al., 1995; Conte et al., 1998). f/2 medium has 90 % higher N and 600 % higher P than k/2 medium: it is unclear if nutrient level might have also affected maximal growth temperatures. A similar phenomenon (i.e., Isochrysidales strains isolated from warmer oceans are better adapted to higher temperature in culture than strains isolated from cooler regions) has previously been observed in culture experiments of different *E. huxleyi* strains (Conte et al., 1998; Liao et al., 2023).

Alkenone production per cell decreases with increasing temperature for both strains (Fig. 1e-f). For RCC6484, alkenone concentration decreased by more than 10 times (58.2 pg/cell to 4.0 pg/cell) from 24 to

32 °C. For RCC3483, alkenone concentration decreased by ~ 6 times (38.2 to 5.9 pg/cell) from 26 to 32 °C. Such reduction in alkenone production per cell with rising temperature has previously been observed in the culture experiments of other Isochrysidales species (e.g., *E. huxleyi*, *G. oceanica*, *Isochrysis galbana*, *Isochrysis nuda* and *Isochrysis litoralis*) (Volkman et al., 1995; Versteegh et al., 2001; Bakku et al., 2018; Liao et al., 2020). This observation may be related to the proposed biofunction of alkenones as the energy storage lipids (Bakku et al., 2018), with greater amounts of alkenones per cell needed at lower temperatures.

3.2. Effect of temperature on alkenone unsaturation

For the first time, we have shown that the degrees of alkenone unsaturation of *G. oceanica* strains, especially RCC6484, continue to respond to temperature changes at up to 32 °C (Fig. 2). At 28 °C, both strains still contain significant amounts of tri-unsaturated alkenones: for RCC6484, 7.7% $C_{37:3}$ relative to total C_{37} alkenones and 8.9% $C_{38:3}Me$ relative to total $C_{38}Me$ alkenones; for RCC3483, 1.8% $C_{37:3}$ and 3.3% $C_{38:3}Me$. Even at 32 °C, RCC6484 and RCC3483 still contain GC-FID quantifiable $C_{37:3}$ (for RCC6484, 0.06% $C_{37:3}$ relative to total C_{37} alkenones and 1.8% $C_{38:3}Me$ relative to total $C_{38}Me$ alkenones, for RCC3483, 0.03% $C_{37:3}$ and 1.1% $C_{38:3}Me$) (Supplementary Table S1). Importantly, from 28 to 32 °C, $C_{37:3}$% and $C_{38:3}Me$% in both strains decrease gradually and continuously, indicating that the algae continue to regulate the relative proportion of tri- and di-unsaturated alkenones in response to temperature changes. Biosynthetically speaking, tri-unsaturated alkenones might never completely disappear even at higher temperatures. More sensitive and selective analytical techniques for alkenones would allow a more thorough examination of the ultimate maximal temperature for the unsaturation paleothermometer.

To best illustrate the relationship between temperature and unsaturation indices for RCC6484 and RCC3483 at different temperature ranges, we performed step-wise linear (Fig. 2) as well as second-order polynomial regressions from 24 to 32 °C (Supplementary Fig. S3). The polynomial regression results in a slight decrease in unsaturation from 30 to 32 °C, which is likely unrealistic (Supplementary Fig. S3). Thus, step-wise linear regression may provide a better mechanistic view of alkenone unsaturation ratios at the extreme high temperature range. Importantly, we note that $U^{K'}_{37}$ and $U^{K'}_{38Me}$ for RCC6484 show a slope of 0.031 and 0.029 respectively against the growth temperature from 28 to 30 °C, which is only slightly lower than that of the global ocean core-top calibration at 0.033 (Müller et al., 1998). From 30 to 31 °C, the slopes of $U^{K'}_{37}$ and $U^{K'}_{38Me}$ for RCC6484 are 0.013 and 0.012, respectively, which would still permit paleotemperature reconstructions. However, the error of the reconstruction would be larger: assuming a measurement error for $U^{K'}_{37}$ and $U^{K'}_{38Me}$ at 0.01 (Prahl et al., 1988; Dubois et al., 2009), the potential error for the reconstruction would be as large as ~ 0.8 °C. For RCC3483, the slopes of $U^{K'}_{37}$ and $U^{K'}_{38Me}$ index values vs. temperature are even lower (~0.0075) from 28 to 32 °C (Fig. 2b, R^2 = 0.96), which would preclude accurate temperature reconstructions using currently available analytical technologies. However, we hypothesize that behavior of RCC6484 might better represent the index changes at extremely warm ocean sites, as RCC6484 was isolated from the warmer Pacific Warm Pool. In addition to different annual mean temperatures at the locations of strain isolation (29.4 °C for RCC6484 vs. 28.8 °C for RCC3483), the *in-situ* temperatures may also be important to alkenone production by different *G. oceanica* strains. The South China Sea strain RCC3483 was isolated at 22 °C *in-situ* SST, whereas the Pacific Warm Pool strain RCC6484 at 28 °C (note that different strains may grow at the same location across the seasonal cycle). Thus, RCC6484 may be a strain more adapted to growth at higher temperatures than RCC3483 and, hypothetically, may display a greater dynamic range of alkenone unsaturation ratios at high temperatures. This is analogous to our recent study on the Arctic strains of *E. huxleyi*, where the strain isolated from
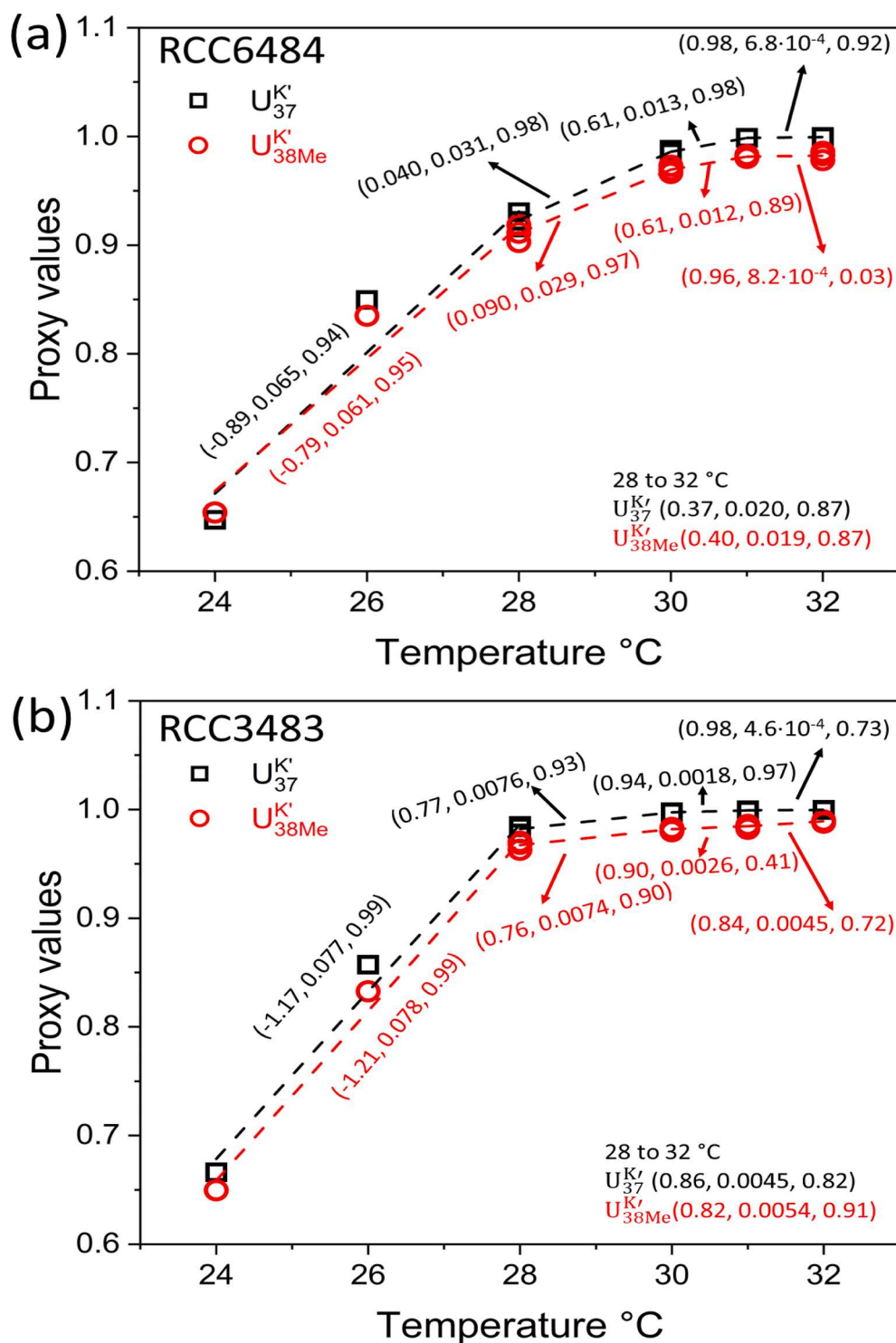
Fig. 2. Temperature calibrations of $U_{37}^{K'}$ and $U_{38Me}^{K'}$ for RCC6484 (a) and RCC3483 (b). The step-wise linear regressions were performed from 24 to 28 (N = 5), 28 to 32 (N = 12), 28 to 30 (N = 6), 30 to 31 (N = 6) and 31 to 32 °C (N = 6) to demonstrate the gradually reduced sensitivity of alkenone unsaturation degrees towards temperature. Numbers in parentheses (a, b, c) adjacent to regression lines represent intercept (a), slope (b) and correlation coefficient (R^2) for calibration equations for individual steps.

colder ocean setting made greater amounts of tetra-unsaturated alkenones and the relationship between U_{37}^K and temperature extends to lower growth temperatures (Liao et al., 2023). More data are needed to test our hypothesis. Further reduction in analytical uncertainties of unsaturation ratios (e.g., through the development of more sensitive and more accurate analytical techniques) is also important for paleotemperature reconstruction at high temperature range.

For both RCC6484 and RCC3483 strains, $U_{38Me}^{K'}$ yields consistently lower proxy values (0.014 on average) than $U_{37}^{K'}$ at ≥ 26 °C (e.g., for RCC6484 at 30 °C, $U_{37}^{K'} = 0.99$, $U_{38Me}^{K'} = 0.97$). This indicates that $U_{38Me}^{K'}$

is more distanced from the saturation value (i.e., 1) than $U_{37}^{K'}$ at ≥ 26 °C. In other words, saturation of the $U_{38Me}^{K'}$ index occurs more slowly than the $U_{37}^{K'}$ index at high temperatures (Supplementary Fig. S5). $U_{38Me}^{K'}$ thus has a greater potential to extend the upper temperature limit of the alkenone paleothermometer than $U_{37}^{K'}$. This point was demonstrated recently in a global surface sediment calibration (Novak et al., 2022). Importantly, the slopes of $U_{37}^{K'}$ and $U_{38Me}^{K'}$ vs temperature for RCC6484 are very similar between 26 and 28 °C and 28 to 30 °C, supporting an extended linear dynamic range to 30 °C as suggested in our previous

study (Novak et al., 2022). Analytically using GC-FID, accurate measurements for $U_{38Me}^{K'}$ may be easier than for $U_{37}^{K'}$ for sediment samples when coelutions are often present, because the ratio of $C_{38:3}$ over $C_{38:2}$ methyl ketone is on average 11 times higher than the ratio of $C_{37:3}$ over $C_{37:2}$ from 24 to 32 °C in RCC6484 and RCC3483 (Fig. 2, Supplementary Table S1).

However, the response of $U_{38Me}^{K'}$ towards temperature varies between different samples (Fig. 3). For example, $y = -0.093 + 0.035x$ for RCC6484, $y = 0.35 + 0.021 \times$ for global core-top samples at ≥ 24 °C (single linear fitting through the whole temperature range was applied for easy comparison). The differences in calibration curves may relate

to: 1) the genetic difference of *G. oceanica* associated with isolated regions and: 2) the difference between culture and natural growth conditions.

We also note that the slope turning points of unsaturation ratio vs temperatures for RCC6484 and RCC3483 differ by more than 2°. RCC3483 displays an abrupt turning (or shallowing, slope of $U_{37}^{K'}$ decreases from 0.077 to 0.0045) of slope at ~ 28 °C, whereas RCC6484 shows little change in slope until 30 °C (Fig. 2). Even after 30 °C, RCC6484 shows only a gradual decrease in slope, rather than an abrupt decrease at 28 °C for RCC3483. Once again, we hypothesize that strains adapted to living in hot waters may downward regulate $U_{37}^{K'}$ and $U_{38Me}^{K'}$
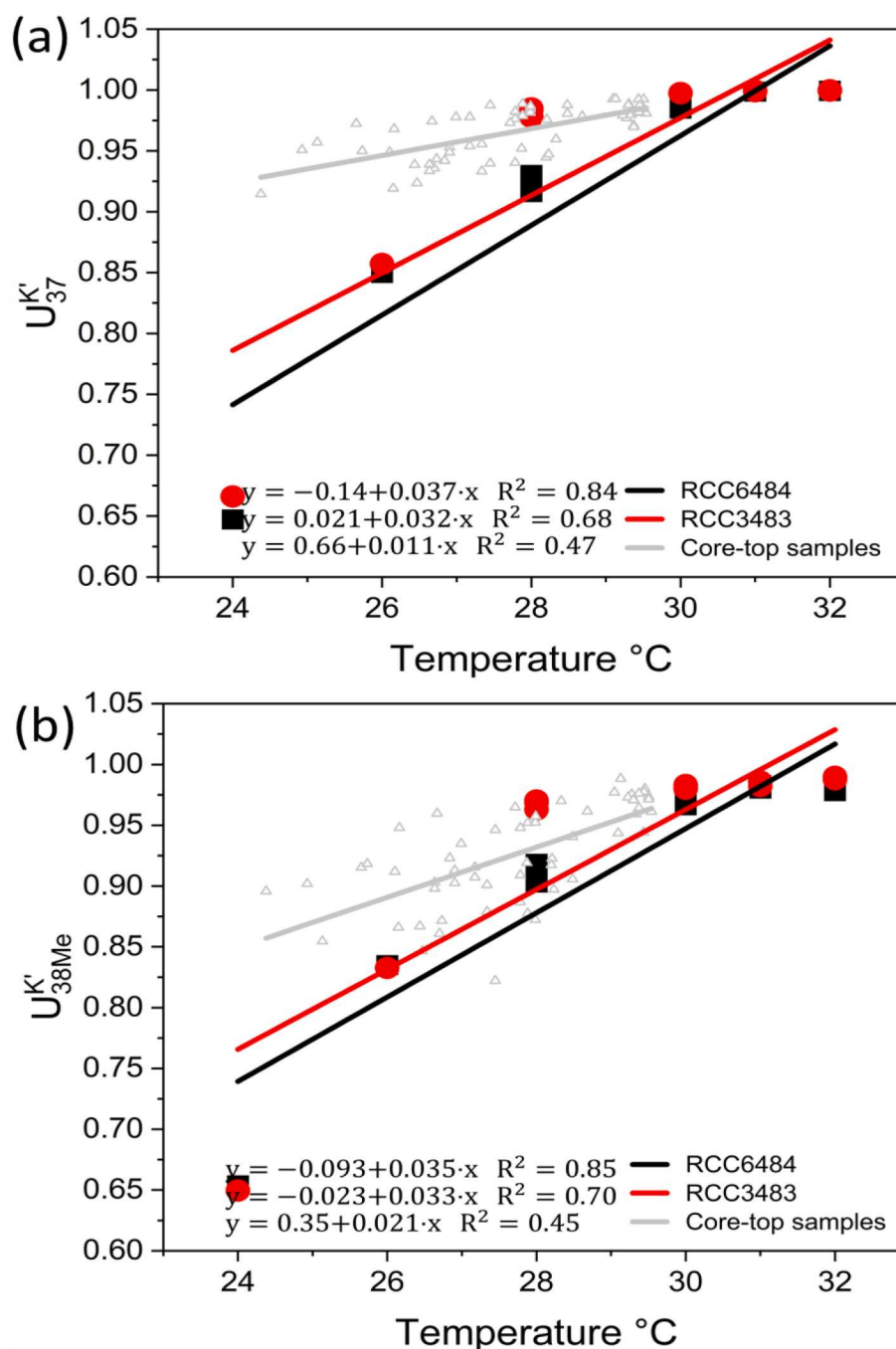
Fig. 3. Comparison of temperature calibrations of $U_{37}^{K'}$ (a), $U_{38Me}^{K'}$ (b) between culture samples of *G. oceanica* RCC6484 (N = 14, 24 to 32 °C), RCC3483 (N = 14, 24 to 32 °C), and global core-top samples (N = 59, 24.4 to 29.5 °C) at ≥ 24 °C (Novak et al., 2022). Such calibrations couldn't be performed for *G. oceanica* AB1, JB02 and North Atlantic seawater samples due to few data points available at ≥ 24 °C (Conte and Eglinton, 1993; Volkman et al., 1995; Conte et al., 1998).

index values so that linear responses could extend to a higher temperature range.

### 3.3. Effect of temperature on chain length and carbonyl position

In addition to changes in alkenone unsaturation degrees, strong effects of growth temperature on alkenone chain lengths and carbonyl positions were also observed. From 24 to 32 °C, both *G. oceanica* RCC6484 and RCC3483 display shorter chain length with increasing temperatures (Fig. 4a). The decreased chain lengths at higher temperature were also observed in other *G. oceanica* strains (AB1 from 9 to 27 °C, *G. oceanica* JB02 from 11.1 to 29 °C, Supplementary Fig. S6) (Volkman et al., 1995; Conte et al., 1998; Nakamura et al., 2016). We also observe a strong linear relationship between C_{38}/C_{39} alkenone ratios and growth temperature (higher values as temperature increases), with RCC6884 and RCC3483 showing very similar relationships (Fig. 4b). Interestingly, a similar relationship has been found in culture experiments for *T. lutea* from 15 to 35 °C (Supplementary Fig. S6b, $R^2 = 0.81$ for NIES2590, 0.89 for CCMP463) (Nakamura et al., 2016). Shorter chain length alkenones would contain less energy (or require less energy to produce) per molecule than longer chain length homologues. Hypothetically, the reduction in chain length with increasing temperature may reflect an overall reduced cell energy storage in the form of alkenones.

We also observe a systematic linear response of the relative percentage of C_{38} ethyl and methyl ketones as growth temperature increases – with more ethyl ketones at higher temperatures (Fig. 4c). These compounds were rarely measured and/or reported in previous studies because the traditional non-polar phase GC columns (e.g., DB-1) resolve them poorly (Longo et al., 2013), especially for sediment samples. For both RCC6484 and RCC3483, the relationships between temperature and $C_{38}Et/C_{38}Me$ ratios are strong and similar from 24 to 30 °C (y = $-10.65 + 0.54x$ for RCC6484, y = $-11.52 + 0.56x$ for RCC3483). Notably, the linear response of $C_{38}Et/C_{38}Me$ towards temperature extends to 32 °C for RCC6484. This further suggests for strains like RCC6484 which was isolated from warmer regions than RCC3483, the alkenone profiles show more consistent and larger amplitude variations than strains from cooler regions at high temperature ranges.

However, poor correlations between $C_{38}Et/C_{38}Me$ (as well as average chain lengths and C_{38}/C_{39} in Fig. 4) and temperature, were observed for seawater and core-top samples (e.g., $R^2 = 0.11$ for $C_{38}Et/C_{38}Me$ in core-top sediments, Fig. 4c, Supplementary Fig. S6). Factors other than temperature, such as nutrients, light etc. may significantly influence the relative carbonyl positions and chain lengths. For example, from f/2 to f/20 medium with 10 times diluted nutrient concentrations, C_{38}/C_{39} decreased from 4.5 to 2.0 for *Isochrysis litoralis* AC18 (Liao et al., 2020), $C_{38}Et/C_{38}Me$ decreased from 3.1 to 1.4 for *E. huxleyi* NIES3366 (Liao et al., 2023). More field studies and the establishment of *in-situ* calibrations may be needed to further examine the applicability of proxies based on chain lengths and carbonyl positions for paleotemperature reconstructions.

4. Conclusions

Our culture experiments demonstrate that *G. oceanica* strains isolated from warm ocean sites grow efficiently at high temperatures ranging from 24 to 32 °C. Both RCC6484 strain isolated from Pacific Warm Pool and RCC3483 strain isolated from South China Sea show increasingly higher growth rates at higher temperatures from 24 to 32 °C. RCC6484 is even more adapted to growth in warm conditions than RCC3824, as demonstrated by higher growth rates, as well as faster increase in growth rates, as temperature increases. The result suggests that strains living in warmer ocean waters grow more efficiently in warmer conditions. Alkenone content per cell decreases linearly as temperature increases. This observation is consistent with alkenones being energy storage lipids: less energy storage compounds at higher
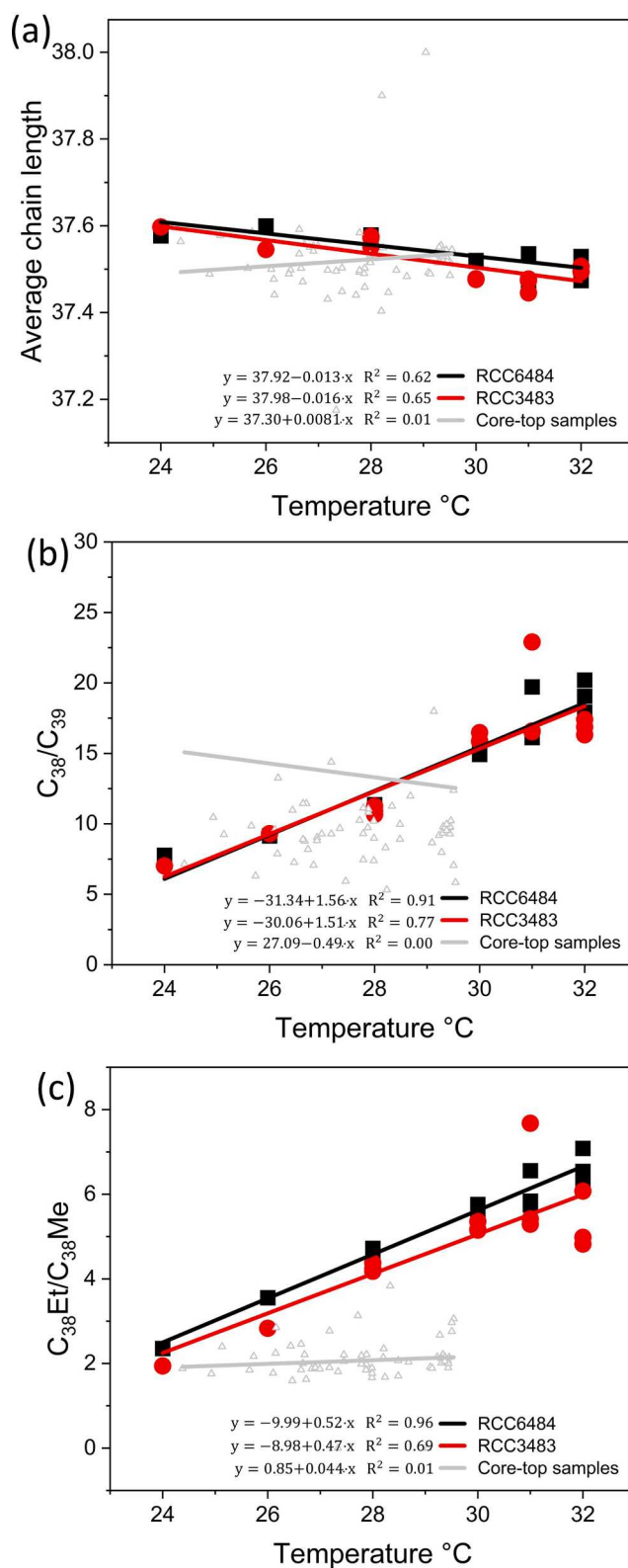
Fig. 4. Relationships between average chain length (a), C_{38}/C_{39} (b) and $C_{38}Et/C_{38}Me$ (c) and temperatures for culture samples of *G. oceanica* RCC6484 (N = 14, 24 to 32 °C) and RCC3483 (N = 14, 24 to 32 °C), as well as global core-top samples (N = 59, 24.4 to 29.5 °C) at ≥ 24 °C (Novak et al., 2022).

temperatures.

Importantly, both *G. oceanica* strains continue to regulate alkenone unsaturation ratios at temperatures ranging from 28 to 32 °C, which is up to 4 °C above the previously perceived saturation temperature for the $U_{37}^{K'}$ index. Tri-unsaturated C_{37} and C_{38} methyl alkenones are still present in measurable amounts even at 32 °C. $U_{38Me}^{K'}$ displays lower index values than $U_{37}^{K'}$ at nearly all temperatures and in both strains (the ratio of $C_{38:3}Me/C_{38:2}Me$ is ~ 11 times higher than the ratio of $C_{37:3}/C_{37:2}$), indicating $U_{38Me}^{K'}$ has a greater potential for paleotemperature reconstructions at extremely warm conditions. This is consistent with our recent global core-top calibration and comparison of sediment $U_{37}^{K'}$ and $U_{38Me}^{K'}$ indices at up to 30 °C (Novak et al., 2022). However, chromatographic coelutions in global core-top sediments and the limit of modern sea surface temperatures did not allow us to explore thoroughly the true upper temperature limit of alkenone paleo-thermometers. Our new culture data, which benefitted from the absence of chromatographic coelution, however, fill this important gap and suggest that the theoretical upper temperature limit of alkenone paleothermometer extends beyond 30 °C. Our results also call for the development of more sensitive, selective and quantitative analytical techniques to accurately measure alkenone unsaturation ratios at high temperatures (specifically, to accurately quantify trace levels of tri-unsaturated alkenones).

In addition to changes in the degrees of unsaturation, we find alkenone chain length and carbonyl position (ratio of C_{38} ethyl/methyl ketones) also respond linearly to growth temperatures. In fact, both strains display strong and similar linear relationships with growth temperatures in average chain length, C_{38}/C_{39} and $C_{38}Et/C_{38}Me$ ratios up to 32 °C. While a simple linear relationship between the alkenone chain length and SST does not appear to exist in global core-top sediments, alkenone chain length may still be valuable for reconstructing SST changes. At a given sediment coring site, especially in the warm low latitude oceans, environmental variables other than temperature that could affect alkenone chain lengths may be relatively stable and sea surface temperatures may become the dominant factor affecting the alkenone chain lengths. In such circumstances, alkenone chain lengths, combined with unsaturation ratios of C_{38} methyl ketones, may allow significant extension of the upper temperature limit for paleo-SST reconstructions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data have been provided in the paper

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2023.104606.

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