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Production and temperature sensitivity of long chain alkenones in the cultured haptophyte *Pseudoisochrysis paradoxa*

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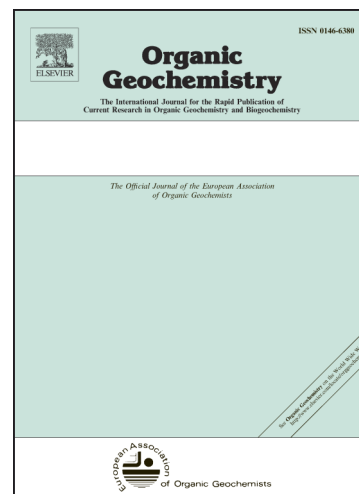
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2 Production and temperature sensitivity of long chain alkenones

3 in the cultured haptophyte *Pseudoisochrysis paradoxa*

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30 ABSTRACT

31 The alkenone unsaturation index (U_{37}^K or $U_{37}^{K'}$) serves as a critical tool for
32 reconstructing temperature in marine environments. Lacustrine haptophyte algae are
33 genetically distinct from their ubiquitous and well studied marine counterparts, and
34 the unknown species-specific genetic imprints on long chain alkenone production by
35 lacustrine species have hindered the widespread application of the U_{37}^K temperature
36 proxy to lake sediment records. The haptophyte *Pseudoisochrysis paradoxa* produces
37 alkenones but its U_{37}^K calibration has never been determined. It has an alkenone
38 fingerprint abundant in tetraunsaturated alkenones, a hallmark of lacustrine
39 environments. We present here the first calibration of the U_{37}^K index to temperature
40 for a culture of *P. paradoxa*. We found that the U_{37}^K index accurately captured the
41 alkenone response to temperature whereas the $U_{37}^{K'}$ index failed to do so, with $U_{37}^{K'}$
42 values below 0.08 projecting to two different temperature values. Our results add a
43 fifth species-specific U_{37}^K calibration and provide another line of evidence that
44 different haptophyte species require different U_{37}^K calibrations. The findings also
45 highlight the necessary inclusion of the $C_{37:4}$ alkenone when reconstructing
46 temperatures from *P. paradoxa*-derived alkenone records.

47

48 **Keywords:** Alkenones, haptophytes, paleolimnology, U_{37}^K

49

50 **1. Introduction**

51 For over two decades, long chain alkenones have been used to reconstruct sea
52 surface temperature from marine sediments. Haptophyte algae in the order
53 Isochrysidales are the exclusive producers of these lipids, an extremely specific
54 biomarker group found throughout open ocean, coastal, and lacustrine environments.
55 The paleotemperature proxy U_{37}^K incorporates the abundance of the di-($C_{37:2}$), tri-($C_{37:3}$)
56 and tetra-($C_{37:4}$) unsaturated alkenones as a proxy for water temperature (Brassell et
57 al., 1986), with greater proportions of the more unsaturated alkenones occurring at
58 lower temperature (Marlowe, 1984; Brassell et al., 1986). A modified proxy $U_{37}^{K'}$
59 (Prahl and Wakeham, 1987; Prahl et al., 1988) has been widely applied to marine
60 sediments where the tetraunsaturated alkenone is largely absent. The cosmopolitan
61 marine species *Emiliania huxleyi* and *Gephyrocapsa oceanica* are responsible for the
62 majority of alkenone production in marine waters (Conte et al., 1994), allowing the
63 universal application of the marine $U_{37}^{K'}$ calibration (Volkman et al., 1980, 1985,
64 1995; Prahl and Wakeham, 1987; Muller et al., 1988; Sikes and Volkman, 1993;
65 Sikes et al., 1997; Conte et al., 2006).

66 Alkenones are also found in lake sediments around the world (Cranwell, 1985;
67 Volkman et al., 1988; Fulford-Smith and Sikes, 1996; Li et al., 1996; Wang and
68 Zheng, 1998; Zink et al., 2001; Chu et al., 2005; D'Andrea and Huang, 2005; Pearson
69 et al., 2008; Theroux et al., 2010; Toney et al., 2010; 2011). Those in lakes have been
70 shown to reflect mean annual air temperature (Chu et al., 2005) and in situ lake water
71 temperature (Toney et al., 2010; D'Andrea et al., 2011). However, there is no
72 universal $U_{37}^{K'}$ calibration applicable to all lake sediment records, largely as the result

73 of the genetic diversity of lacustrine haptophytes and resulting differences in
74 alkenone biosynthetic pathways (Theroux et al., 2010). Identification of haptophyte
75 species via alkenone fingerprint has been difficult, as many haptophyte species have
76 similar alkenone profiles (Marlowe et al., 1984, Theroux et al., 2010). Using the ratio
77 of C_{37}/C_{38} alkenones for species differentiation (Prah1 et al., 1988) was found to be
78 inconsistent across haptophyte species (Theroux et al., 2010). DNA sequencing has
79 allowed more accurate identification of alkenone-producing haptophytes in lake
80 environments (Coolen et al., 2004; D'Andrea et al., 2005; Theroux et al., 2010) and a
81 close relative of the brackish water haptophyte *Pseudoisochrysis paradoxa* has been
82 identified in lakes in North America and the Tibetan Plateau in China (Theroux et al.,
83 2010). The definition of a *P. paradoxa* U_{37}^K calibration would therefore expand our
84 reference dataset of species-specific calibrations and allow temperature reconstruction
85 from sediments with alkenones derived from *P. paradoxa*.

86 Originally isolated from the brackish York River Estuary in Chesapeake Bay,
87 Virginia, USA, *P. paradoxa* has never been formally described (*nomen nudum*;
88 Jordan et al., 2004). It was reported to have alkenones (Marlowe et al., 1984),
89 although its U_{37}^K temperature calibration was not determined. The 18S ribosomal
90 RNA (rRNA) gene sequence for *P. paradoxa* CCMP715 (CCAP 949/1, Genbank
91 AM490999; Medlin et al., 2008) is 99% identical to that of the coastal/lacustrine
92 alkenone-producing haptophytes *Isochrysis galbana* CCMP1323 (CCAP 927/1;
93 Genbank HM149540) and *Chrysolita lamellosa* ALGO HAP17 (CCAP 818/1;
94 Genbank AM490998). Similarly, marine species *E. huxleyi* and *G. oceanica* are
95 identical at the 18S rRNA level, highlighting the difficulty in distinguishing
96 haptophyte species through 18S rRNA gene sequences alone. Until a formal

97 description of *P. paradoxa* confirms otherwise, we will refer to culture CCMP715 by
98 its given name of *P. paradoxa*.

99 The global extent of *P. paradoxa* populations is unknown; if it ecologically
100 resembles its close relative *I. galbana*, it can survive in a wide range of fresh,
101 brackish and marine environments and contribute to their alkenone sediment records
102 (Volkman et al., 1980; Marlowe et al., 1990; Versteegh et al., 2001; Liu et al., 2009).
103 Only a few species of haptophytes have been grown in culture to determine their U_{37}^K
104 calibration: ubiquitous marine species *Emiliania huxleyi* (Prahl et al., 1988; Volkman
105 et al., 1995; Conte et al., 1998; Prahl et al., 2003) and *Gephyrocapsa oceanica* (Sikes
106 and Volkman, 1993; Volkman et al., 1995; Sawada et al., 1996; Conte et al., 1998)
107 and lacustrine/brackish species *I. galbana* (Versteegh et al., 2001) and *Chrysothila*
108 *lamellosa* (Sun et al., 2007). Given the diversity of haptophytes in brackish
109 environments (Theroux et al., 2010) and the need for a better understanding of
110 brackish haptophyte ecology, we grew *P. paradoxa* at a variety of temperatures to
111 determine its alkenone unsaturation-temperature relationship.

112

113 2. Methods

114

115 2.1. *Pseudoisochrysis paradoxa* cultures

116 Cultures of *Pseudoisochrysis paradoxa* (CCMP715, also known as CCAP
117 949/1, CCAPVA12, UTEX 1988) were from the Provasoli-Guillard National Center
118 for Marine Algae and Microbiota. We grew them in 0.2 μ m filter-sterilized seawater
119 amended with *f/2* nutrients (Guillard, 1975) in full spectrum light on a 24:0 h
120 light:dark cycle. We verified that the medium was alkenone-free. We grew the batch
121 cultures in triplicate volumes of 50 ml at 5, 10, 15, 21 and 24 °C. We initiated

122 cultures at an equal cell concentration of 8000 cell/ml using an inoculum from a
123 culture acclimatized to a given temperature for 2 weeks. We monitored cell
124 concentration for 3 weeks using haemocytometer counts to ensure that the cultures
125 remained in the exponential phase. After 3 weeks, the cultures were harvested. One of
126 the three 5 °C cultures was discarded because it failed to grow.

127

128 2.2. Lipid analysis

129 Culture material was filtered onto precombusted 47 mm glass fiber filters
130 (Whatman, Piscataway, NJ), immediately frozen at -20 °C and then freeze-dried
131 overnight (Labconco, Kansas City, MO). We extracted the filters using 3 x 20 minute
132 bursts of sonication in 50 ml dichloromethane (DCM) and ran the total lipid extracts
133 using an Agilent 6890 Plus gas chromatograph flame ionization detector (GC-FID)
134 instrument for detection and quantification of alkenones using an internal C₃₆ *n*-alkane
135 standard and an external alkenone standard of known U₃₇^K temperature value to ensure
136 analytical precision (< 0.1 °C proxy-derived temperature). A Varian VF200 60 m
137 fused silica GC column (60 m × 250 µm width × 0.10 µm film thickness) was used as
138 follows: 100 °C (1 min) to 200 °C (held 1 min) at 20 °C/min, then at 4 °C/min to 320
139 °C (held 5 min).

140

141 3. Results

142 3.1. Cultures

143 Triplicate cultures of *P. paradoxa* behaved similarly at each temperature
144 regime (Table 1), with growth rate fluctuating only by 0.01 division/day. Growth rate
145 and alkenone concentration per cell displayed an inverse relationship (Fig. 1), with

146 growth rate highest at 21 °C (1.01 division/day) and lowest at 5 °C (0.48
 147 division/day) and alkenone concentration highest at 5 °C (1.961 pg/cell) and lowest at
 148 21 °C (0.042 pg/cell). Final cell count was highest at 21 °C and lowest at 5 °C (Table
 149 1). The $C_{37:4}$ alkenone comprised almost 40% of the C_{37} alkenones in the 5 °C culture
 150 (Fig. 3), although at all temperatures $C_{37:3}$ was the dominant alkenone (Fig. 4).

151

152 3.2. U_{37}^K and $U_{37}^{K'}$ calibrations

153 We calculated both polynomial and linear U_{37}^K and $U_{37}^{K'}$ calibrations for the
 154 cultures and plotted them vs. growth temperature (Fig. 2). For both U_{37}^K and $U_{37}^{K'}$, the
 155 polynomial equation had a better fit to the alkenone unsaturation data. The U_{37}^K
 156 calibration ($U_{37}^K = 0.012T^2 - 0.0142T - 0.2935$, $R^2 0.98$, root mean squared error, RMSE
 157 2.26) was more robust than the $U_{37}^{K'}$ calibration ($U_{37}^{K'} = 0.001T^2 - 0.0256T + 0.1754$, R^2
 158 0.89, RMSE 2.38) and, most importantly, the $U_{37}^{K'}$ temperature calibration failed to
 159 reconstruct temperature < 15 °C (Fig. 2). Below 15 °C, the cultures had increasing
 160 $U_{37}^{K'}$ values with decreasing temperature, such that most $U_{37}^{K'}$ values afforded two
 161 temperature values. The linear U_{37}^K calibration ($U_{37}^K = 0.0226T - 0.5149$, $R^2 0.91$,
 162 RMSE 2.16) also had a better fit than the linear $U_{37}^{K'}$ calibration ($U_{37}^{K'} = 0.0047T -$
 163 0.0071 , $R^2 0.39$, RMSE 10.27).

164 The linear U_{37}^K calibration for had a similar slope to the in situ calibration
 165 from Lake George, ND (0.0226 vs. 0.0169; Fig. 5; Toney et al., 2012), and clustered
 166 with other lake-based calibrations, apart from the marine calibrations. The linear $U_{37}^{K'}$
 167 calibration was also distinct from reported $U_{37}^{K'}$ calibrations from other haptophyte

168 cultures and environmental samples (Fig. 6) and had a y intercept and slope (0.0047)
169 closest to that of *I. galbana* (0.009; Versteegh et al., 2001).

170

171 **4. Discussion**

172 *4.1. Growth stage and alkenone production*

173 The effect of growth rate on haptophyte U_{37}^K and $U_{37}^{K'}$ values is unclear (Conte
174 et al., 1995; Epstein et al., 1998; Popp et al., 1998). Studies have shown that
175 differences in alkenone indices can exist between batch methods and continuous
176 culture methods (Popp et al., 1998) although it is debated as to which of the two
177 methods more accurately replicates conditions in the natural environment. Growth
178 phase has also been shown to influence alkenone unsaturation in batch culture (Conte
179 et al., 1998; Epstein et al., 1998), although continuous culture, and therefore constant
180 growth state, imparted no change on U_{37}^K values (Popp et al., 1998). We harvested all
181 *our* culture samples during exponential growth phase to control this variation.

182 The cultures demonstrated higher alkenone concentration per cell at the
183 slowest growth rate in the 5 °C culture (Table 1, Fig. 1). This agrees with
184 observations in culture for *E. huxleyi* and *G. oceanica* (Conte et al., 1998). However,
185 low growth temperature often corresponds to low growth rate, so it is unclear wheter
186 or not low growth temperature alone would result in enhanced alkenone
187 accumulation. Alkenones are believed to serve as an energy storage molecule in
188 haptophytes (Epstein et al., 2001; Eltgroth et al., 2005) and the concentration per cell
189 increases during stationary growth phase and decreases after cultures are placed in the
190 dark (Epstein et al., 2001; Eltgroth et al., 2005). The accumulation of alkenones at
191 low temperature and low growth rate, as seen here for *P. paradoxa*, may be the result

192 of photosynthetic energy input exceeding cell capacity for growth and division
193 (Roessler, 1990).

194 In batch culture experiments, $U_{37}^{K'}$ decreases under nutrient stress and
195 increases under prolonged darkness (Versteegh et al., 2001; Prah et al., 2006), both
196 conditions that may result in slower growth rate. We used a 24:0 h light to dark
197 regime to eliminate alkenone metabolism during darkness. Cultures of various strains
198 of *E. huxleyi* grown in 12:12 or 0:24 light to dark regimes exhibited contrasting
199 increases or decreases in $U_{37}^{K'}$ values depending on light regime (Epstein et al., 2001,
200 Versteegh et al., 2001). The fluctuations in $U_{37}^{K'}$ were 0.013 to 0.029 units, lower than our
201 $U_{37}^{K'}$ standard deviation (Table 1), so we do not believe the 24 h light regime exerted a
202 significant change in U_{37}^{K} and $U_{37}^{K'}$ values.

203

204 4.2. Comparison with other species

205 *P. paradoxa* alkenones resembled other lacustrine haptophyte alkenone
206 profiles via a high abundance of the $C_{37:4}$ alkenone (Cranwell, 1985; Li et al., 1996;
207 Zink et al., 2001). Like its close relative *I. galbana* CCMP1323, *P. paradoxa* had a
208 predominant $C_{37:3}$ alkenone and absence of the C_{38} Me ketone (Fig. 3), as also
209 observed in the original Marlowe et al. (1984) alkenone description. In our *P.*
210 *paradoxa* 15 °C culture, $C_{37:3}$ alkenone comprised 70% of the total C_{37} alkenones, very
211 similar to the Marlowe et al. (1984) value of 68.4%.

212 Sediments and water samples from Lake George, North Dakota (Toney et al.,
213 2010), as well as Lake BrayaSø in Greenland (D'Andrea and Huang, 2005; D'Andrea
214 et al., 2011) and Ace Lake, Antarctica (Coolen et al., 2004) contain alkenone

215 signatures with dominant $C_{37:4}$. Previously, $C_{37:4}$ ($C_{37:4}/C_{37:4}+C_{37:3}+C_{37:2}$) as a % value
216 was proposed as a paleosalinity proxy (Roselle-Melé et al., 1994; 2002; Schulz et al.,
217 2000; Sikes and Sicre, 2002; Bendle and Rosell-Melé, 2004), although the lack of
218 correlation between salinity and $C_{37:4}$ alkenone % in a global array of lake systems
219 (Chu et al., 2005; Mercer et al., 2005; Theroux et al., 2010; Toney et al., 2010)
220 instead suggests that the relationship between $C_{37:4}$ and salinity is a result of
221 haptophyte community shifts along a salinity or temperature gradient (Harada et al.,
222 2003, 2008).

223 $C_{37:4}$ can range from 8-53% in *C. lamellosa* (Rontani et al., 2004) and 0-34%
224 in *I. galbana* (Marlowe et al., 1984), and up to 96% in a series of lakes in China (Chu
225 et al., 2005), with *P. paradoxa* salinity values fitting this range, with values between
226 6% at 24 °C and 40% at 5 °C (Table 1, Fig. 4). The alkenone concentration was also
227 within the range observed for *I. galbana* (0.0098-0.61 pg/cell; Versteegh et al., 2001).
228 The highest value for *P. paradoxa* (1.96 pg/cell) was close to the range observed in
229 an *Isochrysis* culture (1.8 pg/cell; Marlowe, 1984).

230 Although the haptophytes *P. paradoxa*, *I. galbana* and *C. lamellosa* found in
231 brackish waters have similar alkenone signatures and cellular concentration, they
232 have different U_{37}^K and $U_{37}^{K'}$ calibrations (Figs. 5, 6). Studies have shown that
233 geographically isolated strains of the same species of haptophyte, *E. huxleyi* and *G.*
234 *oceanica*, have different patterns of alkenone unsaturation with temperature (Conte et
235 al., 1995, 2006; Volkman et al., 2005). Therefore, it is no surprise that different but
236 closely related haptophytes will also possess different U_{37}^K calibrations. The slopes of
237 U_{37}^K calibrations across brackish haptophyte species are similar (Fig. 5), suggesting
238 that the temperature dependence of alkenone unsaturation is consistent, but the

239 determining factor for the U_{37}^K calibration y intercept is still unknown. Although the
240 *P. paradoxa* U_{37}^K and $U_{37}^{K'}$ calibrations were distinct from marine haptophyte
241 calibrations from *G. oceanica* and *E. huxleyi*, non-linear calibrations have been
242 suggested for cultures of *G. oceanica* (Volkman et al., 1995) and a global marine
243 surface water calibration (Conte et al., 2006).

244

245 4.3. Application to the natural environment

246 Lake George, ND, harbors two alkenone producing haptophytes (Theroux et
247 al., 2010; Toney et al., 2011), one closely related to *P. paradoxa* and one related to an
248 uncultured Ace Lake, Antarctica haptophyte (Volkman et al., 1988; Fulford-Smith
249 and Sikes, 1996; Coolen et al., 2004). The downcore alkenone distribution in Lake
250 George is dominated by $C_{37:4}$ (Toney et al., 2010) and the distribution in an
251 enrichment culture of Lake George haptophytes was also $C_{37:4}$ -dominant (Toney et al.,
252 2012). In contrast, *P. paradoxa* cultures had abundant $C_{37:4}$ but dominant $C_{37:3}$ (Fig. 4).
253 In cultures of *P. paradoxa* and Lake George haptophytes, the U_{37}^K calibration had a
254 more robust relationship to temperature than the $U_{37}^{K'}$ calibration (Toney et al., 2012),
255 a result of the abundant $C_{37:4}$ in both. The similarity in slope between the linear *P.*
256 *paradoxa* calibration and the Lake George in situ calibration (0.0226 and 0.0169,
257 respectively) suggests a significant alkenone contribution to Lake George alkenones
258 by *P. paradoxa*-like haptophyte (Fig. 5). The offset between the Lake George in situ
259 U_{37}^K calibration and the *P. paradoxa* culture-based calibration may be explained by a
260 missing contribution from the second alkenone-producer in Lake George, the subject
261 of a current study (Theroux, 2012). These results emphasize the utility of in situ

262 calibrations for reconstructing relative temperature fluctuations, but highlight the fact
263 that in situ calibrations are a composite of alkenone contributions that may be derived
264 from multiple haptophytes. Accurate absolute temperature reconstruction requires not
265 only an accurate U_{37}^K calibration, but also knowledge of the contributing haptophyte
266 species and their fluctuation in abundance back through time, something that can be
267 discerned using preserved paleoDNA (Coolen et al., 2009).

268

269 5. Conclusions

270 The application of the U_{37}^K paleotemperature proxy to lake sediments depends
271 upon a robust U_{37}^K calibration. As evidenced by studies to date, lakes are not uniform
272 in their alkenone-producing haptophyte populations, thereby complicating the use of
273 a universal lacustrine U_{37}^K proxy calibration. Our study is one of few to cultivate an
274 individual haptophyte species at various temperatures to calibrate the U_{37}^K -
275 temperature relationship. *P. paradoxa* has proved to be distinct in its alkenone
276 distribution vs. temperature, with a calibration equation significantly different from
277 its close relative *I. galbana* CCMP1323 and other brackish haptophytes. The
278 similarity between the Lake George in situ calibration and the *P. paradoxa* calibration
279 reported in this study gives credence to the applicability of in situ U_{37}^K calibrations.

280 The failure of the *P. paradoxa* U_{37}^K calibration to reconstruct temperature < 15 °C
281 highlights the importance of incorporating $C_{37:4}$ alkenones for temperature
282 reconstruction when *P. paradoxa* is the likely alkenone contributor, and such a rubric
283 may extend to all $C_{37:4}$ -abundant alkenone producers. As with all alkenone-based
284 temperature reconstructions, it is important to verify the continuity of alkenone

285 distributions throughout downcore sediments in order to apply a single calibration to
286 the temperature reconstruction. We anticipate future studies comparing both in situ
287 and culture-based U_{37}^K calibrations to further resolve species-specific modes of
288 alkenone production in lake environments.

289

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296

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468

469 **Table Captions**

470

471 **Table 1**

472 Average growth rate, cell concentration and alkenone concentration for cultures of *P.*

473 *paradoxa*.

474 **Fig. Captions**

475 **Fig. 1.** Alkenone cell concentration vs. growth rate for *P. paradoxa* cultures (error
476 bars represent standard deviation).

477 **Fig. 2.** (Left) *P. paradoxa* U_{37}^K calibration; (Right) *P. paradoxa* $U_{37}^{K'}$ calibration. Both
478 linear and polynomial calibrations are provided.

479 **Fig. 3.** Gas chromatogram of *P. paradoxa* grown at 10 °C. Inset: Photomicrograph of
480 *P. paradoxa* culture. Scale bar 5 μ m.

481 **Fig. 4.** Average alkenone concentration for *P. paradoxa* cultures at different
482 temperatures. Error bars represent standard deviation.

483 **Fig. 5.** Comparison of *P. paradoxa* U_{37}^K calibration with other species and lake-based
484 calibrations. References: German Lakes, Zink et al. (2001); Lake George in situ,
485 Toney et al. (2010); Lake BrayaSø, D'Andrea et al. (2011); marine *E. huxleyi*, Prahl et
486 al. (1988); *P. paradoxa*, this paper; polar marine waters, Sikes and Volkman (1993);

487 Lake George in situ 2008, Toney et al., (2012); *C. lamellosa*, Sun et al. (2007);

488 marine *G. oceanica* JB02, Volkman et al., (1995).

489 **Fig. 6.** Comparison of *P. paradoxa* U_{37}^K calibration with other species and lake-based

490 calibrations. References: *P. paradoxa*, this paper; German lakes, Zink et al. (2001);

491 Chinese lakes, Chu et al. (2005); Chinese freshwater/brackish lakes, Chu et al.

492 (2005); Chinese saline lakes, Chu et al. (2005); *C. lamellosa*, Sun et al. (2007);

493 Global marine, Prahl and Wakeham (1987); *I. galbana*; Versteegh et al. (2001);

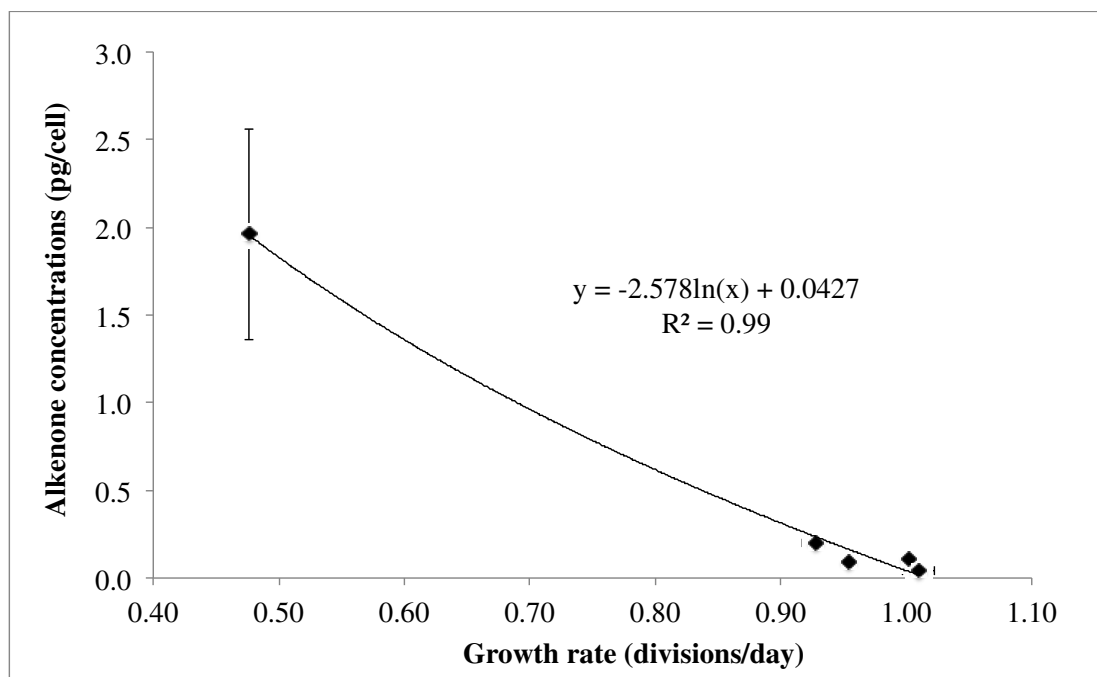
494 marine *G. oceanica* GO1, Sawada et al. (1996); marine *G. oceanica* JB01, Volkman

495 et al. (1995).

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513 Editor's note+ the titles on the x & y axes should not be in bold

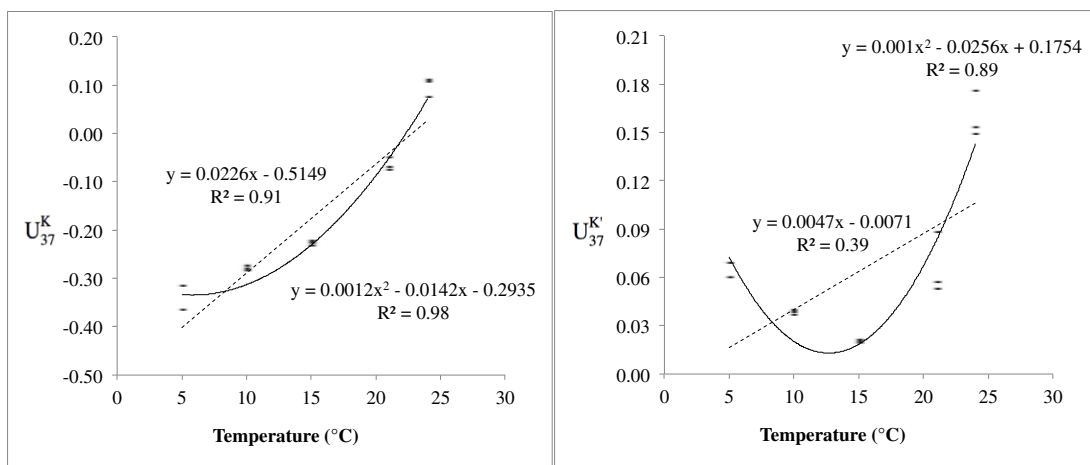
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515 **Fig. 1.**

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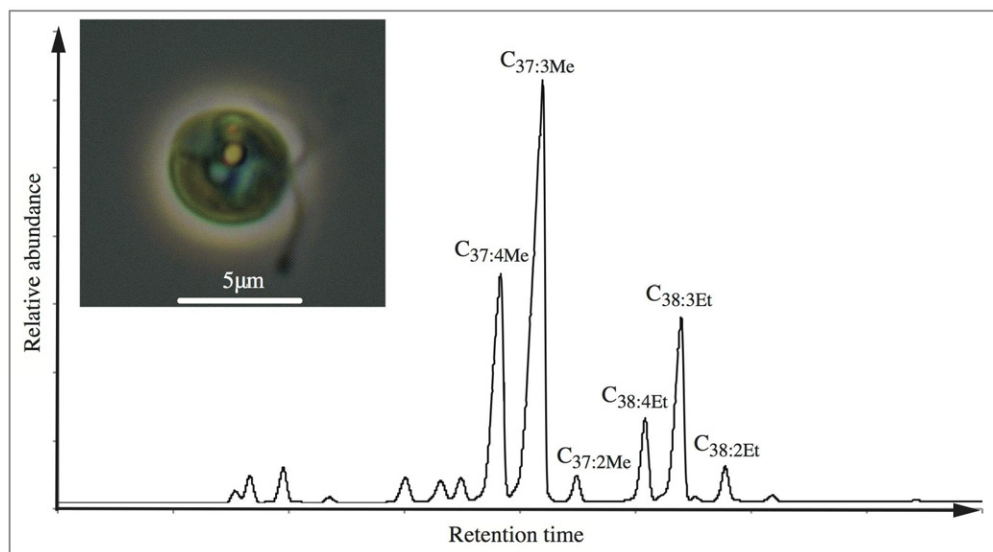


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521 **Fig. 2.**

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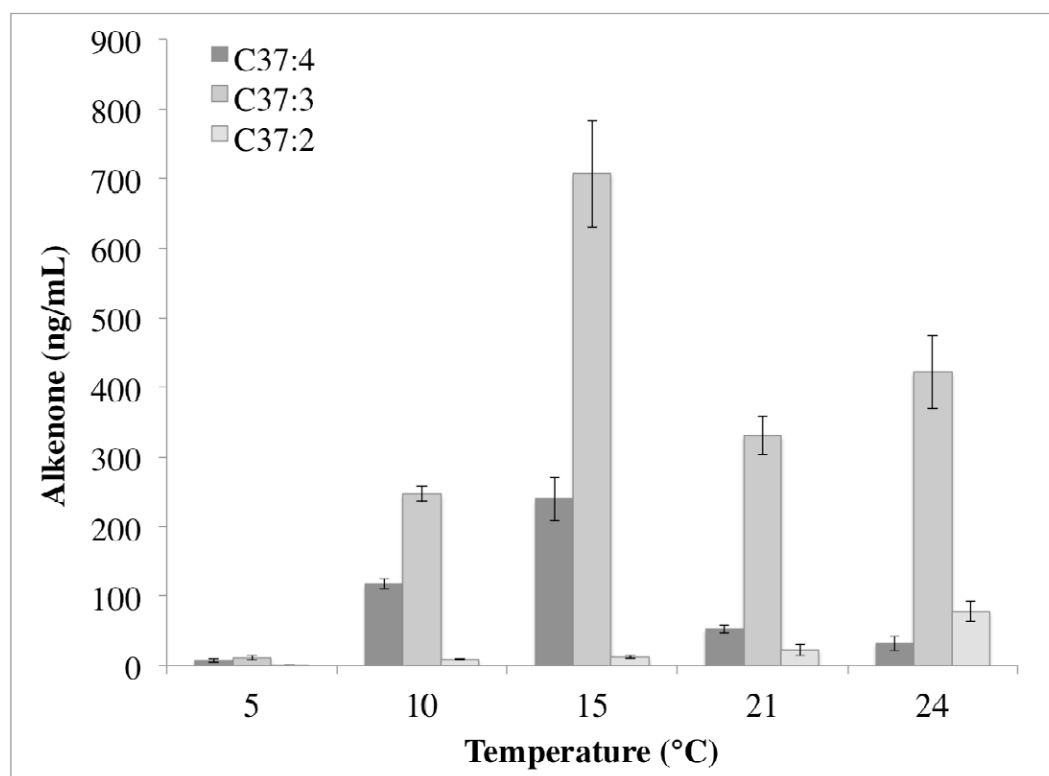
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525 **Fig. 3.**

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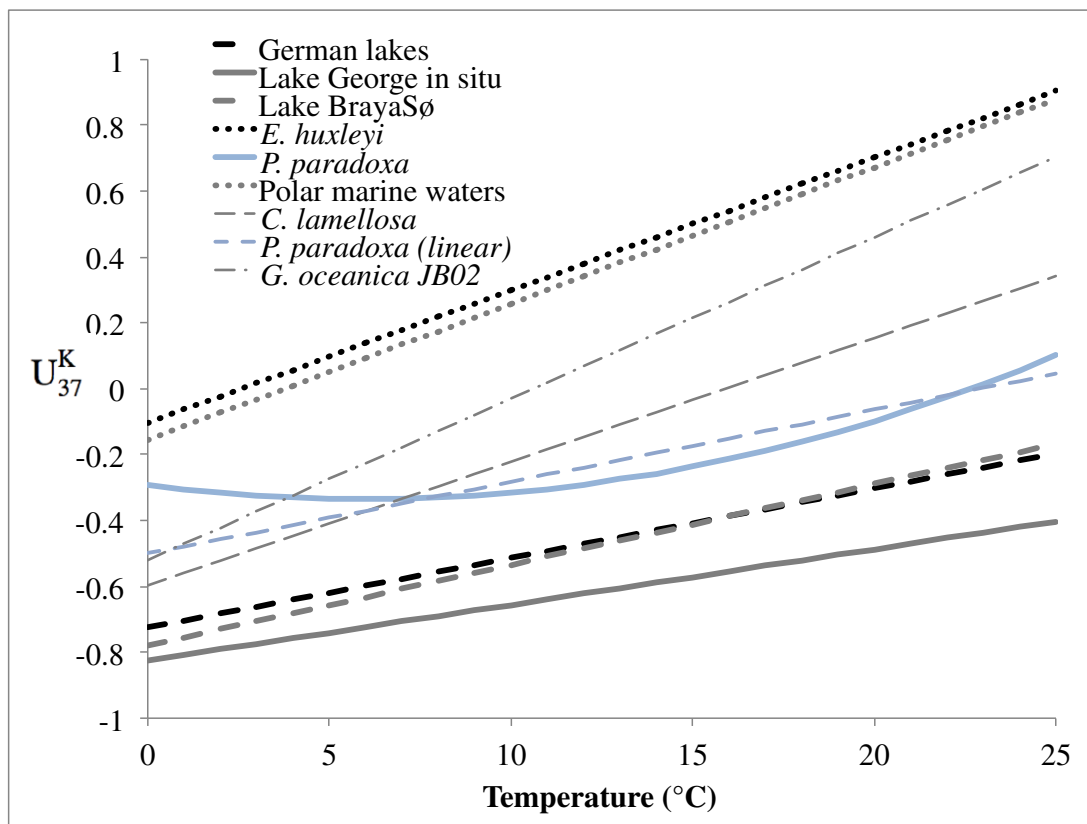


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530 **Fig. 4.**

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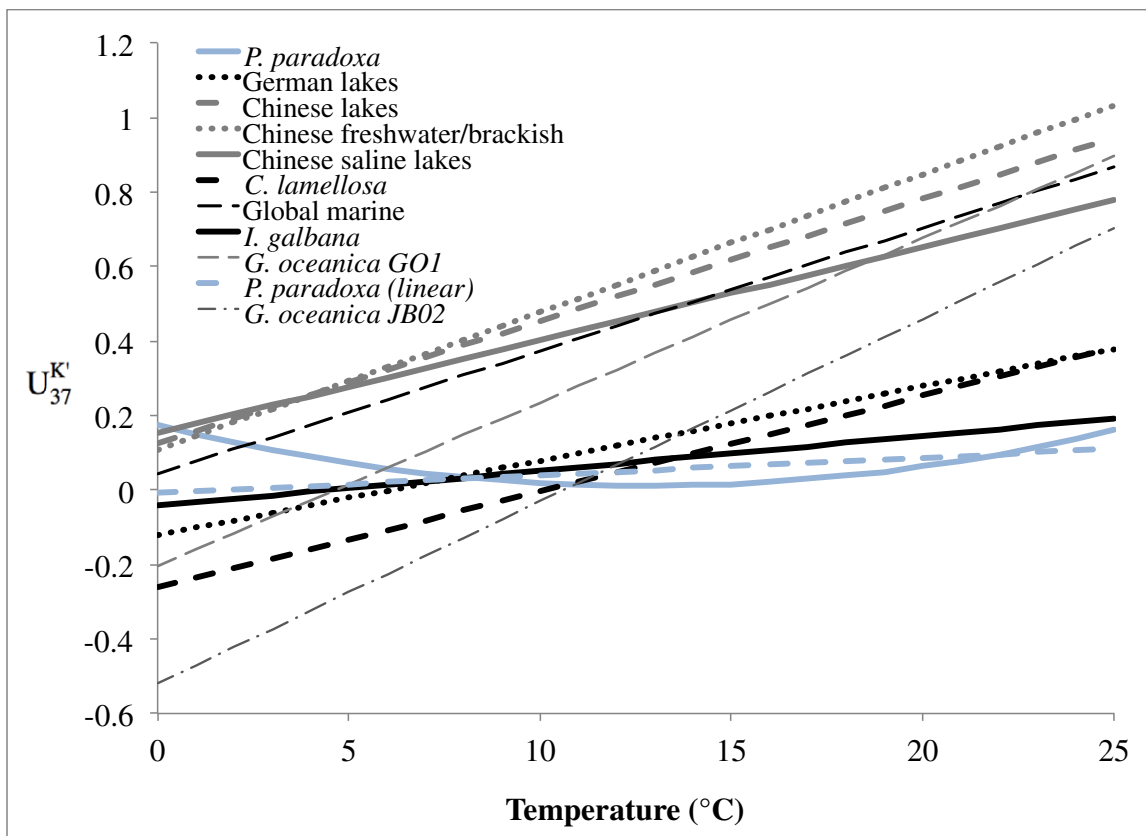


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534 **Fig. 5.**

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540 **Fig. 6.**

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Highlights

> Cultured *Pseudoisochrysis paradoxa* contained an abundant, but not dominant, $C_{37:4}$ alkenone.

> *P. paradoxa* U_{37}^K calibration matched lake U_{37}^K calibrations and was distinct from marine calibrations.

> The U_{37}^K calibration had a superior fit than the $U_{37}^{K'}$ calibration.

> $C_{37:4}$ should be incorporated for reconstructing temperature from *P. paradoxa* alkenone records.

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