Long chain alkenes, alkenones and alkenoates produced in the haptophyte alga *Chrysotila lamellosa* CCMP1307 isolated from a salt marsh

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ABSTRACT

Compositions of long chain alkenes, alkenones and alkenoates in a cultured strain of the haptophyte *Chrysotila lamellosa* CCMP1307, isolated from a salt marsh, were investigated. The biomarker patterns were distinctive and showed a high proportion of the tetraunsaturated alkenones and alkenoates, with a pronounced proportion of C₄₀ alkenones and a lack of C₃₈ methyl and C₃₉ ethyl alkenones. Linear regression of the alkenone unsaturation degree (U_{37}^K) with growth temperature (T) was obtained over the range of possible CCMP1307 growth temperatures (4-20 °C): $U_{37}^K = 0.045 \times T (\text{°C}) - 1.016$, ($n = 13$, $r^2 = 0.96$), while the $U_{37}^{K'}$ values were weakly correlated with T : $U_{37}^{K'} = 0.0035T (\text{°C}) + 0.0511$ ($n = 18$, $r^2 = 0.70$). The U_{37}^K calibration exhibited a low y-intercept in comparison with that of a Chinese inland lake strain reported previously. The data show significant intraspecific variation in U_{37}^K for *C. lamellosa* between strains from different geographic origins. The *C. lamellosa* CCMP1307 reproduced typical U_{37}^K values observed in C_{37:4} rich lakes, especially at lower temperatures (<15°C). In addition, we identified C_{29:2}, C_{31:1}, C_{31:2} and C_{31:3} alkenes, and found that the unsaturation degree of C₃₁ alkenes (U_{31}^{en}) calculated with C_{31:1}, C_{31:2} and C_{31:3}

alkenes may also be a useful index of growth temperature in the haptophyte *C.*

lamellosa.

1. Introduction

Long chain alkenones are ubiquitous in the marine environment (e.g. de Leeuw et al., 1980) and their extent of unsaturation (U_{37}^K and $U_{37}^{K'}$) are established proxies for sea surface temperature estimation (Brassell et al., 1986; Prahl and Wakeham, 1987; Prahl et al., 1988). These indices have been widely applied for reconstructing paleotemperature variation in marine systems (e.g. Rostek et al., 1993; Bard et al., 1997; Sawada and Handa, 1998; Sachs and Lehman, 1999; Kienast et al., 2001; Seki et al., 2004; Haug et al., 2005). Alkenones and related compounds such as long chain alkyl alkenoates are mainly produced by two cosmopolitan haptophycean algae in the family Noëlaerhabdaceae, *Emiliania huxleyi* (Volkman et al., 1980; Prahl et al., 1988) and *Gephyrocapsa oceanica* (Conte et al., 1995**b**; Volkman et al., 1995; Sawada et al., 1996), in marine environments. The $U_{37}^{K'}$ unsaturation index (Müller et al., 1998) showed a good fit with data obtained from culture experiments with *E. huxleyi* 55a (Prahl et al.,

1988).

In addition to these two haptophyte species, coastal haptophyte algae in the family Isocrysidaceae such as *Isochrysis galbana* and *Chrysotila lamellosa* are known to synthesize alkenones and related compounds (Marlowe et al., 1984; Versteegh et al., 2001; Rontani et al., 2004; Sun et al., 2007; Ono et al., 2012). Moreover, the alkenones and related compounds have been found in inland freshwater, brackish and hypersaline waters, highlighting the potential use of the alkenone unsaturation index for the paleotemperature assessment of continental environments (e.g. Cranwell, 1985; Zink et al., 2001; Coolen et al., 2004; Chu et al., 2005; D'Andrea and Huang, 2005; Theissen et al., 2005; Pearson et al., 2008; Theroux et al., 2010; Liu et al., 2011; Toney et al., 2011). Zink et al. (2001) reported that the $U^{K'}_{37}$ and U^K_{37} correlated with average summer surface water temperature for German lake sediments. Chu et al. (2005) found a good correlation between $U^{K'}_{37}$ and mean annual air temperature for Chinese lakes, suggesting different calibrations depending on salinity and environment, viz. fresh/brackish lakes and saline lakes. Investigating suspended particles obtained by filtering lacustrine water from Lake George, USA, Toney et al. (2010) indicated that the

U_{37}^K values correlated linearly with temperature within the water column, but the $U_{37}^{K'}$ values were not well correlated.

More recently, D'Andrea et al. (2011) also developed a U_{37}^K temperature calibration, established by combining water filter samples from Lake Braya SØ, Greenland, with previous lacustrine datasets (Zink et al., 2001; D'Andrea and Huang, 2005). From previous studies, it is likely that the alkenone unsaturation ratio values in lakes generally reflect surface water temperature and mean annual air temperature, although there is a wide variation in the U_{37}^K and $U_{37}^{K'}$ calibrations between individual lakes. Recent molecular phylogenetic investigations suggest that individual lakes could host phylogenetically different alkenone producing species (strains) of Isochrysidales, resulting in the large variations in alkenone composition and calibrations for lakes (Coolen et al., 2004; D'Andrea et al., 2006; Theroux et al., 2010).

Relatively high proportions of tetraunsaturated alkenones, especially a $C_{37:4}$ homologue, have been reported as a characteristic feature of alkenone distributions in lacustrine environments (e.g. Cranwell, 1985; Volkman et al., 1988; Chu et al., 2005; Toney et al., 2010). The $C_{37:4}$ alkenone is usually absent from marine environments, or

present in small amounts in polar regions. Several studies have confirmed that *C. lamellosa* is a significant producer of the tetraunsaturated alkenone (Marlowe et al., 1984; Rontani et al., 2004; Sun et al., 2007). Culture experiments have emphasized that alkenone composition and alkenone-based temperature calibrations vary depending on species and population (e.g. Volkman et al., 1995; Sawada et al., 1996; Conte et al., 1998; Yamamoto et al., 2000; Versteegh et al., 2001; Sun et al., 2007).

There have been only a few investigations on the physiological adaptations of *C. lamellosa* (Rontani et al., 2004; Sun et al., 2007) and *I. galbana* (Versteegh et al., 2001; Ono et al., 2012), which highlight the variation in alkenone profiles of these coastal/lacustrine species. Alkenone profiles have been obtained from cultured *C. lamellosa* strains PML 353 (isolated from coastal water, the estuary of the River Avon in south Devon, England; Green and Parke, 1975) and PML 528 (isolated from Lake Neusiedlersee near Vienna, Austria; Green and Course, 1983; Marlowe et al., 1984) and another coastal water strain, HAP 17 (Rontani et al., 2004).

Sun et al. (2007) examined the $U_{37}^{K'}$ and U_{37}^K calibrations vs. growth temperature (10-22 °C) using a strain of *C. lamellosa* from Lake Xiarinur, although the culture did

not dominantly contain C_{37:4}. There are therefore no reports of culture strains that produce adequate amounts of tetraunsaturated alkenones among lacustrine alkenone producers (Zink et al., 2001; Toney et al., 2010; D'Andrea et al., 2011; Toney et al., 2012). Such a gap between experimental data from the cultured haptophytes and field data in the lacustrine alkenone profiles makes the application of alkenone proxies for reconstructing paleotemperature difficult. To fill the gap, further exploration of calibrations for different strains of *C. lamellosa* is a promising approach.

Here, we report the variation in alkenones and alkenes in the haptophyte *C. lamellosa* CCMP1307, isolated from a salt marsh, with varying growth temperature between 4 °C and 25 °C. The objectives were to establish a relationship between alkenone unsaturation indices and growth temperature for this strain, and to assess intra- and interspecific differences in alkenone and alkene compositions when physiological conditions are changed.

2. Materials and methods

2.1. Cultures

The haptophyte *Chrysotila lamellosa* Anand CCMP1307 (originally isolated from Keystone Salt Marsh on Whidbey Island, Washington, USA) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, <http://ccmp.bigelow.org>). It was grown in synthetic seawater, MA-ESM medium containing Marine Art SF (Osaka Yakken, Osaka, Japan) enriched with additional micronutrients of the modified Erd-Schreiber medium (ESM) by replacing soil extracts with 10 nM disodium selenite according to the method of Danbara and Shiraiwa (1999). The cultures were continuously illuminated at $100 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ with fluorescent light and maintained at 4, 5, 10, 15, 20 and 25 °C. Cells were harvested at the late linear to early stationary phase by monitoring the optical density at 750 nm (OD_{750}). The culture period varied depending on the growth at each temperature.

2.2. Chemical analysis

Extraction and separation of lipids were performed according to Sawada et al. (1996) and Sawada and Shiraiwa (2004). After extraction, the lipids were separated

using a silica gel column and fractions 1 and 2 (F1 and F2; hexane and hexane/EtOAc (9/1, v/v), respectively) were analyzed using gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-MS conditions were: Agilent 6890N GC instrument; 30 m × 0.25 mm i.d; DB-5HT fused silica column (J&W scientific) directly coupled to an Agilent 5975 inert XL MSD quadrupole mass spectrometer (electron ionization, 70 eV; emission current, 350 µA; m/z 50-650). The GC-MS temperature program was: 80 °C (3 min) to 180 °C at 15 °C/min, then to 310 °C (held 20 min) at 10 °C/min. The alkenones, alkenoates and other compounds were quantified using a Shimadzu GC-2025 GC-FID instrument equipped with 50 m × 0.32 mm i.d. CPSil5CB fused silica column (Chrompack), with the same temperature program as for the GC-MS.

2.3. Alkenone and alkene unsaturation indices

Alkenone unsaturation indices (U_{37}^K and $U_{37}^{K'}$) were calculated with the equations of Brassell et al. (1986) and Prahl et al. (1988):

$$U_{37}^K = ([37:2] - [37:4]) / ([37:2] + [37:3] + [37:4])$$

$$U_{37}^K = [37:2] / ([37:2] + [37:3])$$

In addition, the alkenone chain length ratio (the ratio of total C₃₇ alkenones to total C₃₈ alkenones; K₃₇/K₃₈) and the ratio of alkyl alkenoates to alkenones (the ratio of fatty acid ethyl esters (FAEEs) to total C₃₇ alkenones; EE/K₃₇) were calculated as follows (Prahl et al., 1988):

$$K_{37}/K_{38} = ([37:2] + [37:3]) / ([38:2Et] + [38:2Me] + [38:3Et] + [38:3Me])$$

$$EE/K_{37} = [36:2FAEE] / ([37:2] + [37:3])$$

3. Results

3.1. Cell growth and change in total alkenone content

Growth curves were made using the OD₇₅₀ at 4, 10, 15, 20 and 25 °C (Fig. 1A, Table 1). The most significant growth as measured by change in turbidity (OD₇₅₀) was observed at 15 °C. The final cell density (OD₇₅₀) at 4 °C to 20 °C was reasonably comparable (0.41–0.70) while the 25 °C (0.13) cell density was relatively lower. The growth curve at 25 °C showed that growth was limited beyond 100 h and that cells may

have been damaged prior to harvesting at 264 h, suggesting that the temperature was not suitable for physiological adaptation. Therefore, data at 25 °C are not discussed below.

Total concentrations of alkenones (Σ alkenone), alkenoates (Σ alkenoate) and alkenes (Σ alkene) calculated using the total abundance of each component relative to the OD₇₅₀ value, tended to increase with decreasing growth temperature (Table 1, Fig. 1B). This suggested that the intracellular concentration of these compounds significantly increased in response to decrease in growth temperature. Relatively large deviations in concentrations presumably reflect heterogeneity of the culture samples, and also due to the errors induced during harvesting due to cellular aggregation in a part of these culture samples.

3.2. Analysis of alkenones and alkenoates

Fig. 2 shows partial GC-FID chromatograms from the alkenone fraction (F2).

Tetraunsaturated alkenones (C_{37:4} methyl alkenone, C_{38:4} ethyl alkenone) and alkenoate (C_{36:4} FAEE) were abundant and C₄₀ alkenones were clearly present. The proportions of tetraunsaturated alkenones and C_{36:4} FAEE were higher in cells grown at low

temperature (10 °C). Interestingly, the strain consistently synthesized C₄₀ alkenones over a wide range of growth temperature, although the proportion varied. The C₄₀ alkenones to total alkenones ratio (K₄₀%) decreased with temperature from 0.75% at 20 °C to 3.68% at 4 °C (Table 2). Other significant features were the high C₃₇/C₃₈ alkenone ratio (K₃₇/K₃₈) and lack of C₃₈ methyl alkenones and C₃₉ ethyl alkenones (Table 2, Fig. 2).

3.3. Correlation of alkenone unsaturation indices with growth temperature

The U^K₃₇ values linearly increased with growth temperature from 4 °C to 20 °C (Table 2, Fig. 3A). The linear regression showed weak correlation, as follows: $U^{K}_{37} = 0.0035T (^{\circ}C) + 0.0511$ (n = 18, r² = 0.70). On the other hand, U^K₃₇ gave a strong correlation with growth temperature: $U^{K}_{37} = 0.045T (^{\circ}C) - 1.016$ (n = 13, r² = 0.96) (Fig. 3B). These results clearly indicate that growth temperature principally controlled the production of C_{37:4} alkenones in the strain.

3.4. Assignment of long chain alkenes and the alkene unsaturation index

The C₃₁ mono- (C_{31:1}) and diunsaturated (C_{31:2}) alkenes were major components of the apolar lipid fraction (Fig. 4). C₃₁ alkene isomers (viz. C_{31:2} and C_{31:3}), C_{29:2} alkene and C₃₃ homologues were also present but mostly as minor components (<1% of total alkenes). Relative abundances of two major C_{31:1} and C_{31:2} alkenes also seemed to be influenced by association with growth temperature (Fig. 4). The C₃₁ alkene unsaturation ratio (U^{en}₃₁), calculated as

$$U_{31}^{en} = [C_{31:1}] / ([C_{31:2}] + [C_{31:1}]),$$

showed significant correlation with growth temperature (Fig. 5). The second order polynomial regression of the index vs. growth temperature was expressed as $U_{31}^{en} = -0.0019T^2 + 0.078T - 0.112$ (n = 18, r² = 0.95). The linear regression was expressed as $U_{31}^{en} = 0.034T - 0.072$ (n = 18, r² = 0.90). The U^{en}₃₁ values for *C. lamellosa* HAP 17 at 10 °C and 20 °C, calculated from data of Rontani et al. (2004), were very similar to our data for *C. lamellosa* CCMP1307.

4. Discussion

4.1. Characteristics of alkenone composition

The alkenone composition was characterized by three factors: (i) high abundance of C_{37} and C_{38} tetraunsaturated alkenones, (ii) presence of C_{40} alkenones and (iii) absence of C_{38} methyl alkenones and C_{39} ethyl alkenones (Fig. 2). In particular, a distinctive feature was a high proportion of tetraunsaturated alkenones and relatively minor proportion of diunsaturated alkenones.

A comparatively high abundance of $C_{37:4}$ alkenone has been commonly reported to occur in lacustrine sediments (Cranwell, 1985; Volkman et al., 1988; Thiel et al., 1997; Wang and Zheng, 1997; Zink et al., 2001; Chu et al., 2005; D'Andrea and Huang, 2005; Jaraula et al., 2010; Theroux et al., 2010; Toney et al., 2010; 2011) and in hypersaline and marine coastal microbial mats (Lopez et al., 2005; Rontani and Volkman, 2005). In culture experiments of *C. lamellosa* strains PML 353 and PML 528 at 15 °C (Marlowe et al., 1984), as well as the strain HAP 17 at 10 °C (Rontani et al., 2004), a significant production of the $C_{37:4}$ alkenone was also reported. On the other hand, *C. lamellosa* isolated from Lake Xiarinur did not show a predominance of the $C_{37:4}$ alkenone (up to 24.9% of total C_{37} alkenones at 10 °C; Sun et al., 2007). The present study reveals that *C. lamellosa* CCMP1307 is one of the most $C_{37:4}$ -rich strains of *C. lamellosa* and the

other alkenone-producing species.

The occurrence of tetraunsaturated alkenoates such as C_{36:4} FAEE was associated with tetraunsaturated alkenones (Fig. 2). A similar trend showing the predominance of the C_{36:4} FAEE was reported for another strain of *C. lamellosa*, HAP 17 (Rontani et al., 2004). The production of tetraunsaturated alkenoates may be metabolically related to the tetraunsaturated alkenones.

The C₄₀ alkenone proportion of total alkenones (0.75-3.68%, K₄₀%) in *C. lamellosa* CCMP1307 (Table 2) is higher than that in cultures of *C. lamellosa* HAP 17 (0.4–0.7 %; Rontani et al., 2004) and *C. lamellosa* isolated from Lake Xiarinur (quantitative values not reported; Sun et al., 2007). However, no C₄₀ alkenones were detected in a culture of *I. galbana* (Marlowe et al., 1984; Ono et al., 2012). In field studies, trace amount of the C₄₀ alkenones have been detected in many lacustrine sediments (Cranwell, 1985; Li et al., 1996; Theroux et al., 2010) and hypersaline and marine coastal microbial mats (Lopez et al., 2005). They are considered to be a possible indicator of *C. lamellosa* in freshwater, brackish and hypersaline environments (Theroux et al., 2010).

The lack of C₃₈ methyl and C₃₉ ethyl alkenones is considered to be a distinctive

feature of *C. lamellosa* strain CCMP1307, although significant amount of C₃₈ ethyl and C₃₉ methyl alkenones were produced (Table. 1, Fig. 2). Previously, both *C. lamellosa* and *I. galbana* were shown to contain no, or only trace amounts of, C₃₈ methyl alkenones, while the ubiquitous open oceanic species *E. huxleyi* and *G. oceanica* contain a mixture of C₃₈–C₃₉ methyl and ethyl alkenones (Marlowe et al., 1984; Versteegh et al., 2001; Rontani et al., 2004; Sun et al., 2007; Ono et al., 2012). Rontani et al. (2006) proposed biosynthetic pathways for alkenone production that start from acetyl-SCoA or propionyl-SCoA, and attributed the lack of C₃₈ methyl and C₃₉ ethyl alkenones in coastal strains to their inability to synthesize alkenones from propionyl-SCoA or due to a lack of enzymes involved in the α -oxidation-decarboxylation sequence. The presence of C₃₈ methyl alkenones in the environment has been considered as a contribution from open ocean haptophytes (Schulz, 2000). From recent studies, some lacustrine haptophyte populations closely related to *C. lamellosa* and *I. galbana* were also suggested to produce C₃₈ methyl alkenones, although the particular species was not identified (Theroux et al., 2010; Toney et al., 2012).

These features, such as a high abundance of tetraunsaturated alkenone and presence of C₄₀ alkenones, are seemingly interlinked for many lakes and coastal marine environments. This suggests that combinations of these alkenone profiles have the potential for distinguishing the alkenone-producing species in the lacustrine environment, leading to better applications in paleotemperature reconstruction, particularly for geologic samples where molecular genetic techniques are not applicable.

4.2. Correlation of the alkenone unsaturation index with growth temperature

The $U_{37}^{K'}$ temperature calibration calculated without C_{37:4} content showed a weak correlation (Fig. 3A). On the other hand, the U_{37}^K temperature calibration calculated by including the C_{37:4} content showed a strong correlation (Fig. 3B). The U_{37}^K values showed a good fit (Fig. 3B) with data for *C. lamellosa* strains PML 353 and PML 528 at 15 °C (Marlowe et al., 1984) and *C. lamellosa* HAP 17 at 10 °C, but not at 20 °C (Rontani et al., 2004).

The U_{37}^K growth temperature calibration of the brackish water strain *C. lamellosa* isolated from Lake Xiarinur ($U_{37}^K = 0.038T (°C) - 0.599$; Sun et al., 2007) is obviously

different from that of the salt marsh haptophyte strain *C. lamellosa* CCMP1307. The slope is similar but the y intercept is significantly different, reflecting the high proportion of C_{37:4} alkenone in CCMP1307. The difference may be due to species-specific variation in physiological response to temperature. Notably, previous studies have demonstrated that there is genotypic variation in U^K₃₇ temperature calibrations for open oceanic species such as *E. huxleyi* and *G. oceanica* (e.g. Conte et al., 1995; 1998; Yamamoto et al., 2000).

In general, the entire suite of alkenone homologues, as well as alkenoates, is engaged in the adjustment of alkenone unsaturation (e.g. Prahl et al., 1988; Conte et al., 1998). The U^K₃₇ index is known to have a tighter fit with growth temperature for certain strains of *E. huxleyi* containing significant amount of tetraunsaturated alkenones (up to 6% of total alkenones; Conte et al., 1994a). In the case of C_{37:4} alkenone-abundant species, the U^K₃₇ index can serve as a better proxy for estimating temperature, irrespective of habitat.

The U^K₃₇ temperature calibrations among C_{37:4} alkenone-abundant samples from lakes, surface sediments from several German lakes (Zink et al., 2001) and particles

filtered from Lake George water (Toney et al., 2010), as well as several culture strains, are compared in Fig. 6. Simply put, none of the samples shows a good fit with the others. Notably, *I. galbana* UTEX LB2307 (clone T-ISO) did not contain tetraunsaturated alkenones (Ono et al., 2012). On the other hand, *E. huxleyi* 55a and *C. lamellosa* strains contain higher proportions of tetraunsaturated alkenones. The *C. lamellosa* strain from Lake Xiarinur has the least content of $C_{37:4}$ alkenones among *C. lamellosa* strains (Sun et al., 2007). The new U_{37}^K temperature calibration for *C. lamellosa* CCMP1307 gives a more reliable temperature estimate for $C_{37:4}$ alkenone-abundant species.

The U_{37}^K values for the *C. lamellosa* CCMP1307, particularly below 15 °C, can only serve as typical values for a possible alkenone producer in the extremely $C_{37:4}$ alkenone-abundant lakes such as Lake George and several German lakes (Fig. 6). Therefore, the new U_{37}^K temperature calibration for *C. lamellosa* CCMP1307 might represent an authentic physiological adaptation of a yet unidentified alkenone producer in such lakes. However, the discrepancy between experimental data for *C. lamellosa* CCMP1307 and field data for these lakes becomes obvious at higher temperatures. This

might be explained by that the field samples comprising mixed contributions from various types of *C. lamellosa* like our CCMP1307 strain and Hap-A-type strains being characterized by a high proportion of $C_{37:4}$ alkenone at higher temperatures (Fig. 6).

The occurrence of multiple alkenone producers in a single lake has been proposed in previous studies of many lakes, including Lake George, with two different phylotypes (e.g. Theroux et al., 2010). Toney et al. (2012) reported two phylotypes of haptophytes Hap-A and Hap-B, and found that only the enrichment of Hap-A produces $C_{37:4}$ alkenone-abundant profiles. It must be noted that their enrichment samples were also a mixture of Hap-A and other haptophyte phylotypes, possibly Hap-B. Therefore, the U_{37}^K temperature calibration from Hap-A may not represent the authentic physiological adaptation of a single haptophyte species.

4.3. Ratio of C_{37} to C_{38} alkenone content (K_{37}/K_{38}) and alkenoate/alkenone content (EE/K_{37})

The ratio of C_{37} alkenone content to C_{38} alkenone content (K_{37}/K_{38}) in *C. lamellosa* CCMP1307 (Table 2) was compared with that of other *C. lamellosa* strains (Fig. 7). The

value of K_{37}/K_{38} has been considered to vary depending on taxonomic differences in haptophyte species such as open oceanic species (*E. huxleyi* and *G. oceanica*) and coastal/lacustrine species (e.g. Chu et al., 2005, Pearson et al., 2008), so the value has been used to distinguish alkenone-producing species in the environment (Pearson et al., 2008; Toney et al., 2010). The range of K_{37}/K_{38} values in *C. lamellosa* CCMP1307 (1.36–4.80) is similar (Fig. 7) to that of *C. lamellosa* HAP 17 (Rontani et al., 2004), but higher than that of open oceanic species *E. huxleyi* and *G. oceanica* and much lower than that of *C. lamellosa* strains PML 358 and PML 528 (Marlowe et al., 1984). The values for *C. lamellosa* strains PML 358 and PML 528 were remarkably high (Marlowe et al., 1984). The differences may also be attributed to the growth phase at which strains were analysed, as well as the genetic variability among strains. Cells were harvested during exponential growth phase for *C. lamellosa* strains PML 358 and PML 528 (Marlowe et al., 1984), but late linear to early stationary growth phase for strain Hap 17 (Rontani et al., 2004) and strain CCMP1307 (this study). Conte et al. (1998) reported that the K_{37}/K_{38} values decreased during late logarithmic and stationary phase in cultures of *E. huxleyi* and *G. oceanica*.

The relative abundance of alkenoates to alkenones (EE/K₃₇) in *C. lamellosa* CCMP1307 correlated moderately with growth temperature from 4 °C to 20 °C (n = 18, $r^2=0.79$; Table 2, Fig. 7). A similar negative correlation was described for *E. huxleyi* and *G. oceanica* (e.g. Prahl et al., 1988; Sawada et al., 1996; Conte et al., 1998). Conte et al. (1998) suggested that the increase in alkenoate abundance was due to physiological stress, since the proportion of alkenoates increased when cells were physiologically depressed by temperature and older growth stage. As the optimum temperature for growth of *C. lamellosa* CCMP 1307 was around 15 °C (Fig. 1), the negative correlation of EE/K₃₇ with growth temperature may be attributed to a combined effect of several factors in addition to temperature.

4.4 Long chain alkene composition and alkene unsaturation index

Two C₃₁ alkenes (C_{31:1} and C_{31:2}) were detected as a predominant component in the apolar lipid fraction of *C. lamellosa* CCMP1307 (Fig. 4). They have been found before in *C. lamellosa* (Marlowe et al., 1984; Rontani et al., 2004), *I. galbana* (Rieley et al., 1998), *E. huxleyi* (Grossi et al., 2000), *G. oceanica* (Conte et al., 1995) and the

chlorophyte *Botryococcus braunii* (Gelpi et al., 1968). The *C. lamellosa* CCMP1307 C₃₁ alkene isomer content in this study was comparable to that of HAP 17 (Rontani et al., 2004), but not to that of *C. lamellosa* strains PML 353 and PML 528 (Marlowe et al., 1984) which produce only one C₃₁ diene (Fig. 4).

The proportion of C₃₃ to total alkenes was quite different even between *C. lamellosa* strains CCMP1307 and HAP 17; C₃₃ alkenes were minor in CCMP1307 but significant in HAP 17 (11.6–12.1% of total alkenes; Rontani et al., 2004). Such a large variation might reflect strain-specific genetic differences, as described for *E. huxleyi* and *G. oceanica* (Marlowe et al., 1984; Conte et al., 1995; Rieley et al., 1998; Grossi et al., 2000) and *C. lamellosa* (Rontani et al., 2004). Among haptophyte species, long chain alkenes (> C₂₉) have exclusively been detected in alkenone-producing species, implying the possible presence of a metabolic relationship between alkene and alkenone production (Marlowe et al., 1984; Conte et al., 1995; Grossi et al., 2000) although their biosynthesis and physiological functions are unknown.

The possible effect of physiological adaptation by haptophyte species on alkene compositions has been investigated by Sikes et al. (1997) and Grossi et al. (2000). They

reported a non-linear or limited relationship between the unsaturation ratio for C₃₇–C₃₈ alkenes and temperature in *E. huxleyi*. Our data clearly show that the alkene unsaturation ratio correlates with growth temperature in *C. lamellosa* CCMP1307 (Fig. 5). This reinforces a suggestion by Rontani et al. (2004) concerning *C. lamellosa* HAP17 that a relationship exists between the unsaturation ratio of C₃₁ alkenes and temperature. The U^{en}₃₁ temperature relationship may be useful as another biomarker signature supplement for alkenone profiles in haptophytes.

5. Conclusions

The biomarker patterns of *C. lamellosa* CCMP1307 isolated from salt marsh were characterized by a high proportion of tetraunsaturated alkenones and alkenoates, pronounced amounts of C₄₀ alkenones and a lack of C₃₈ methyl and C₃₉ ethyl alkenones. These features can distinguish and estimate the contribution of *C. lamellosa* in the water column and sediments from coastal and lacustrine environments. We propose a new linear calibration for the U^K₃₇ index with growth temperature: $U^K_{37} = 0.045 T (^\circ\text{C}) -$

1.016 ($n = 13$, $r^2 = 0.96$, $4 \leq T \leq 20$). *C. lamellosa* CCMP1307 reproduced typical U_{37}^K values observed in $C_{37:4}$ rich lakes, especially at lower temperatures ($<15^\circ\text{C}$). We propose that the unsaturation degree of C_{31} alkenes (U_{31}^{en}) is primarily controlled by growth temperature and that the U_{31}^{en} -temperature relationship may be useful as a new biomarker signature supplementing alkenone profiles.

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Captions

Table 1

Compositions of alkenones, alkenoates and alkenes, and optical density (OD₇₅₀) of *Chrysotila lamellosa* CCMP1307 grown at different temperatures.

Table 2

Parameters calculated from alkenone, alkenoate and alkene compositions in *Chrysotila lamellosa* CCMP1307 grown at different temperatures (See Section 2 for abbreviation of parameters).

Fig. 1. Growth curves (A) and total contents of alkenones, alkenoates and alkenes relative to cell density ($\mu\text{g/ml/OD}_{750}$), (B) for *C. lamellosa* CCMP1307 grown at different temperatures. Open circles in (B) denote average values of duplicate experiments. Vertical bars extending from the circles indicate $\pm 1\sigma$

Fig. 2. Partial gas chromatograms showing distribution of long chain alkenones and alkenoates in the *Chrysotila lamellosa* CCMP1307 grown at 10 °C (lower) and 20 °C (upper).

Fig. 3. U_{37}^K (A) and U_{37}^K (B) of *C. lamellosa* at different growth temperature. Open circles, CCMP1307 (this study). Previous data shown: Hap17, Rontani et al. (2004); PML 353 and PML 528 by Marlow et al. (1984); strain isolated from Lake Xiarinur, Sun et al. (2007). Calibration equations for the strain analysed by Sun et al. (2007) are $U_{37}^K = 0.0257 \times T(^{\circ}\text{C}) - 0.2609$ and $U_{37}^K = 0.0377 \times T(^{\circ}\text{C}) - 0.5992$.

Fig. 4. Partial gas chromatograms showing distribution of long chain alkenes in *C. lamellosa* CCMP1307 grown at 10 °C (lower) and 20 °C (upper); *, polysiloxanes.

Fig. 5. Relationship between unsaturation index from C₃₁ alkenes (U_{31}^{cn}) in *C. lamellosa* and growth temperature. Open circles, CCMP1307 (this study); filled diamonds (HAP 17), data from Rontani et al. (2004).

Fig. 6. Comparison of U_{37}^K temperature calibrations for different culture species of haptophyte algae and lakes. Culture calibration: closed circles with standard deviation ($\pm 1\sigma$), this study; a, *E. huxleyi* 55a (Prahl et al., 1987); b, *I. galbana* UTEX LB2307 clone T-ISO (Ono et al., 2012); c, *C. lamellosa* from Lake Xiarinur (Sun et al., 2007); d, Hap-A (Toney et al., 2012). Lake calibrations: e, German lake compilation (Zink et al., 2001); f, Lake George (Toney et al., 2010).

Fig. 7. Relationships between K_{37}/K_{38} (A) and EE/K_{37} (B) and growth temperature for *C. lamellosa* (for symbols, see Fig. 3).

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5

Figure 6

Fig. 6

Figure 7

Fig. 7

Table(s)

Table 1

<i>T</i> ^a (°C)	Alkene (%)				SAlkene (ng/ml)	Alkenone (%)								SAlkenone (ng/ml)	Alkenoate (%)			SAlkenoate (ng/ml)	OD ₇₅₀ ^d		
	C _{29:2}	C _{31:1}	C _{31:2}	C _{31:3}		37:4Me	37:3Me	37:2Me	38:4Et	38:3Et	38:2Et	39:3Me	39:2Me		40:3Et	40:2Et	36:4FAEE			36:3FAEE	36:2FAEE
20	0.69	76.1	22.1	1.08	14.5	15.9	37.3	5.0	9.4	19.7	9.9	1.9	tr ^c	0.5	0.2	172.5	27.2	46.5	26.3	14.7	0.70
20	0.92	67.9	30.6	0.60	41.5	10.6	38.8	6.3	4.3	18.7	18.0	2.5	-	0.4	0.4	1814.5	11.6	42.0	46.4	163.7	0.61
20	0.92	59.2	39.6	0.35	37.5	10.7	38.6	6.0	4.9	18.8	17.0	2.2	tr	1.1	0.5	2960.6	15.8	45.0	39.2	253.8	0.68
15	0.39	65.3	34.1	0.22	63.9	24.3	35.0	4.0	7.9	13.1	10.1	3.5	0.3	1.2	0.5	1823.6	26.4	48.0	25.6	280.8	0.89
15	0.51	61.5	37.7	0.37	144.9	28.4	33.9	2.8	8.5	11.9	7.2	4.7	0.3	1.8	0.5	6031.2	30.7	47.0	22.3	777.4	0.82
15	0.47	57.8	41.4	0.30	198.2	24.4	35.4	3.4	7.9	12.9	8.8	4.5	0.2	1.7	0.6	7548.7	27.2	47.6	25.2	844.8	0.87
10	0.45	43.1	55.2	1.26	111.5	45.1	24.5	2.2	8.9	6.9	3.6	5.9	0.6	1.9	0.4	1758.7	43.8	44.4	11.7	279.3	0.45
10	0.34	56.4	42.9	0.34	158.0	39.2	29.4	3.6	7.6	8.4	5.2	4.7	tr	1.5	0.3	2096.7	41.8	44.8	13.4	562.8	0.61
10	0.44	47.4	51.8	0.42	104.2	48.3	23.4	1.8	9.5	6.6	2.6	5.7	-	2.2	-	2850.7	50.5	39.3	10.2	662.4	0.65
10	0.49	47.2	51.1	1.15	90.8	51.2	23.5	1.6	8.1	5.8	2.0	5.8	-	1.9	-	2568.1	51.1	38.2	10.7	708.9	0.58
5	0.29	19.9	78.5	1.24	176.1	56.1	14.1	1.2	14.1	5.2	1.8	5.3	-	2.2	tr	2411.0	68.5	31.5	-	467.4	-
5	0.28	17.3	81.3	1.06	222.4	55.3	14.5	1.0	14.4	5.2	1.7	5.1	tr	2.6	tr	6129.5	69.5	30.5	-	1489.2	-
5	0.32	22.5	75.9	1.23	215.6	55.9	13.5	0.9	15.4	5.1	1.6	5.2	0.3	2.3	tr	7490.5	71.7	28.3	-	1896.6	-
4	0.30	19.3	79.7	0.65	18.7	51.2	12.7	0.8	17.8	6.3	1.8	6.0	-	3.2	tr	992.6	68.6	31.4	-	224.8	0.67
4	0.32	21.3	77.2	1.10	84.5	53.2	11.9	0.8	17.9	6.0	1.6	5.7	-	2.9	tr	3025.1	70.1	29.9	-	791.4	0.58
4	0.27	21.4	77.4	0.87	103.7	49.4	13.8	1.0	18.5	7.5	2.1	4.8	-	2.6	tr	3029.6	71.7	28.3	-	751.1	0.41
4	0.25	11.8	87.1	0.81	186.9	45.5	15.7	1.3	17.6	8.8	2.9	4.6	tr	3.4	tr	4316.4	62.7	37.3	-	973.2	0.47
4	0.26	16.3	82.6	0.75	134.3	48.3	14.8	1.2	17.5	8.1	2.3	4.8	tr	2.9	tr	4138.0	65.2	34.8	-	1014.1	0.45

^a *G*_{rowth temperature};

^b *g*_{rowth period};

^ctrace (< 0.2%);

^dat harvesting.

Table 2

Temp. (°C)	Alkenone						Alkene
	U_{37}^K	$U_{37}^{K'}$	K_{37}/K_{38}	EE/K_{37}	$K_{40}\%$	$K_{37:4}\%$	U_{31}^{en}
20	-0.19	0.12	1.49	0.15	0.75	27.35	0.77
20	-0.08	0.14	1.36	0.16	0.76	18.94	0.69
20	-0.08	0.14	1.36	0.15	1.66	19.32	0.60
15	-0.32	0.10	2.03	0.24	1.72	38.35	0.66
15	-0.39	0.08	2.35	0.20	2.37	43.59	0.62
15	-0.33	0.09	2.13	0.18	2.42	38.57	0.58
10	-0.60	0.08	3.71	0.22	2.32	62.75	0.44
10	-0.49	0.11	3.42	0.37	1.86	54.30	0.57
10	-0.63	0.07	3.93	0.32	2.27	65.74	0.48
10	-0.65	0.06	4.80	0.36	1.93	67.08	0.48
5	-0.77	0.08	3.38	0.27	2.33	78.67	0.20
5	-0.77	0.06	3.32	0.34	2.74	78.19	0.18
5	-0.78	0.06	3.19	0.36	2.36	79.53	0.23
4	-0.78	0.06	2.49	0.35	3.47	79.12	0.20
4	-0.79	0.06	2.58	0.40	3.01	80.73	0.22
4	-0.75	0.07	2.26	0.39	2.77	76.84	0.22
4	-0.71	0.08	2.12	0.36	3.68	72.76	0.12
4	-0.73	0.07	2.31	0.38	3.10	75.12	0.17