Composition of long chain alkenones and alkenoates as a function of growth temperature in
marine haptophyte *Tisochrysis lutea*

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ABSTRACT

We investigated the compositions of long chain alkenones and alkenoates in cultured strains of the marine haptophyte *Tisochrysis lutea* CCMP463 and *T. lutea* NIES-2590 (formerly classified as *Isochrysis galbana*). Both *T. lutea* strains grown at various temperatures of 15–35 °C could be characterized by the lack of tetraunsaturated alkenones and alkenoates in comparison with strains in other genera *Isochrysis* and *Ruttnera*, which are classified in the same family Isochrysidaceae and order Isochrysidales that contain alkenone-producing haptophytes. We found that *T. lutea* has a distinct alkenone response to temperature, which is characterized by high U_{37}^K -temperature sensitivity at 15–30 °C and continuing sensitivity at warm-end temperatures over 30 °C, where the other species fail to adapt. Both strains showed similar trends in alkenone compositions and alkenone unsaturation index–temperature calibrations. In addition, both strains CCMP463 and NIES-2590 showed notably close phylogenetic relationships, even those that were collected from remote regions of the Atlantic Ocean, namely the Caribbean Sea and English Channel, where their habitats, particularly the sea surface temperature, are notably different. Considering previous published datasets, three genera, *Isochrysis*, *Ruttnera* and *Tisochrysis*, showed very different trends in the alkenone unsaturation index–temperature calibration. These results suggest that the lack of tetraunsaturated alkenones, warm-water oriented growth and the high sensitivity to growth temperature serve as distinct chemotaxonomic characteristics of *T. lutea* in the Isochrysidaceae family.

Keywords: Alkenones; Chemotaxonomy; *Tisochrysis*; *Isochrysis*; U_{37}^K

1. Introduction

Long chain alkenones and their derivatives such as long chain alkyl alkenoates are unique classes of compounds that are exclusively produced by haptophyte algae, particularly species of the order Isochrysidales. Those compounds are ubiquitously found in the marine environment, particularly sediments, and the alkenone unsaturation indices (U_{37}^K and $U_{37}^{K'}$) have been widely applied to reconstruct the sea surface temperature at which alkenones were synthesized (Prahl and Wakeham, 1987; Prahl et al., 1988, Brassell, 1993; Müller et al., 1998).

Alkenones are known to be produced by two cosmopolitan haptophyte algae, in the family Noëlaerhabdaceae (corresponding to Group III clade; Theroux et al., 2010) in marine environments: *Emiliania huxleyi* (Volkman et al., 1980) and *Gephyrocapsa oceanica* (Conte et al., 1994; Volkman et al., 1995; Sawada et al., 1996). The ubiquitous occurrence of marine alkenone producers enables a simple application of the global marine $U_{37}^{K'}$ -temperature calibration (Müller et al., 1998). On the other hand, non-calcifying haptophyte species in the family Isochrysidaceae, such as *Isochrysis galbana*, *Ruttnera lamellosa* (revised from *Chrysotila lamellosa*; Andersen et al., 2014) and related species, constitute another major clade of alkenone producers; the family Isochrysidaceae (Group II; Theroux et al., 2010). Isochrysidaceae strains are distributed in a wide range of environments including marine, brackish waters and saline lakes (Marlowe et al., 1984; Theroux et al., 2010). In addition, a family level clade of undescribed haptophytes was found in Greenland and Alaskan lakes (Group I; D'Andrea et al., 2006; Theroux et al., 2010; Longo et al., 2013) and demonstrated as a distinct clade distributed solely in freshwater environment (Longo et al., 2016 and references therein).

The distribution and abundance of Isochrysidaceae species in the marine environment have been virtually unknown, although most culture strains of Isochrysidaceae have been described from coastal areas (Marlowe et al., 1990; Theroux et al., 2010). These species have

been generally considered as minor contributors in open marine settings and are consequently not intensely investigated for alkenone compositions and U_{37}^K -temperature calibrations. However, alkenones are increasingly found in many saline and freshwater lakes including polar lakes and inland areas of major continents (e.g., Cranwell, 1985; Volkman et al., 1988; Li et al., 1996; Zink et al., 2001; Chu et al., 2005; D'Andrea and Huang, 2005; Theissen et al., 2005; Pearson et al., 2008; Theroux et al., 2010; Toney et al., 2010, 2012; Longo et al., 2013) and are used to reconstruct continental paleoclimates (e.g., D'Andrea et al., 2011; Chu et al., 2012; Hou et al., 2016).

The molecular phylogenetic analysis of environmental samples revealed that various Isochrysidaceae strains are distributed in saline lakes and brackish waters (Coolen et al., 2004, 2009; Theroux et al., 2010; Toney et al., 2012, Randlett et al., 2014). Different U_{37}^K -temperature calibrations obtained from different lakes as well as culture experiments with different species suggests a need for site-specific or species-specific calibrations for the paleotemperature reconstruction from non-marine settings. Further research is needed on the possible control of phylogeny on both biomarker fingerprint and the U_{37}^K -temperature sensitivities (e.g., Theroux et al., 2010; D'Andrea et al., 2016) in order to enable alkenone-inferred temperature reconstruction through geologic time. However, only a limited number of U_{37}^K -temperature calibrations have been reported for genetically distinct haptophyte algae compared to the phylogenetic diversity postulated in Isochrysidaceae strains distributed in lake environments (Theroux et al., 2010; Bendif et al., 2013). Therefore, defining the alkenone chemotaxonomic fingerprints and species-specific U_{37}^K -temperature calibrations for the less explored but widely distributed Isochrysidaceae haptophytes is the key for further understanding of the relationship between phylogeny and alkenone production. Furthermore, it may lead to the application of alkenone compositions for paleoecological investigation and

paleotemperature reconstruction, particularly where unusual distributions of alkenones are found: for example, Chesapeake Bay estuary (Schwab and Sachs, 2011), Black Sea (Coolen et al., 2009), Nordic sea (Bendle et al., 2005) and Baltic Sea (Schulz et al., 2000).

Recent genetic analysis revised the phylogeny of the family Isochrysidaceae and recognizes three genetically distinct genera: *Isochrysis*, *Ruttnera* (*Chrysotila*) and *Tisochrysis* gen. nov. (Bendif et al., 2013). The genera *Tisochrysis* currently consists of a single species *T. lutea*, which is represented by strains that were formerly called *Isochrysis* aff. *galbana* clone T-iso (also “Tahiti Isochrysis”). Some earlier studies noted the variation in lipid compositions among the *Isochrysis* strains (i.e., fatty acid profiles; Liu and Lin, 2001; sterols and alkenone compositions, Patterson et al., 1994), although both *I. galbana* Parke and *Isochrysis* aff. *galbana* clone T-iso, have been often referred to simply as *I. galbana*. Therefore, the biochemical character of the two distinct genera, *Tisochrysis* and *Isochrysis*, can be reassessed using genetically identified strains that are grown in the laboratory.

Moreover, in terms of paleotemperature reconstruction using the alkenone unsaturation index-temperature calibration, systematic culture experiments have not been performed in this newly recognized species *T. lutea* for the U_{37}^K -temperature calibration. As a tropical strain, *T. lutea* strain T-iso favors higher temperature ($> 25\text{ }^{\circ}\text{C}$) than the other known alkenone producing haptophyte algae; e.g. T-iso’s optimum growth temperature was $27.5\text{ }^{\circ}\text{C}$, while that of *I. galbana* Parke was around $20\text{ }^{\circ}\text{C}$ and no growth was observed at $27.5\text{ }^{\circ}\text{C}$ (Ewart and Pruder, 1981). Meanwhile, *E. huxleyi* is known for a relatively wide temperature range and its optimum growth temperature is usually around $20\text{--}25\text{ }^{\circ}\text{C}$. One of the limitations of marine U_{37}^K -temperature calibration (*E. huxleyi*, Prahl et al., 1988; Global core-top, Müller et al., 1998) is the saturation temperature of ca. $28\text{ }^{\circ}\text{C}$. Therefore, current alkenone-based temperature reconstructions are not suitable for many tropical waters. Regarding the difference in the

growth-temperature norms, the questions are: (1) is the high temperature-oriented nature of T-iso a shared feature of *T. lutea*? and (2) does the species-specific U_{37}^K -temperature calibration exhibit sensitivity over 28 °C? If the species is capable of adjusting alkenone unsaturation in temperatures over 28 °C, it may shed light on the application of U_{37}^K in certain tropical locations harboring *T. lutea* or related high temperature species.

In the present study, we investigated two *T. lutea* strains, CCMP465 and NIES-2590, both in the Isochrysidaceae, to construct the first U_{37}^K -temperature calibrations and elucidate the chemotaxonomic fingerprints of *T. lutea* as a genetically distinct clade of alkenone-producing haptophytes.

2. Materials and methods

2.1. Cultures

The haptophyte strains were obtained from the National Center for Marine Algae and Microbiota (NCMA; <http://ncma.bigelow.org>; formerly CCMP, the Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and the Microbial Culture Collection at the National Institute of Environmental Sciences (NIES Collection; <http://www.shigen.nig.ac.jp/algae/>) (Table 1). The cells were grown in an MA-ESM medium (salinity; 30‰), which contains the synthetic seawater Marine Art SF (Osaka Yakken, Osaka, Japan) and additional micronutrients of the modified Erd-Schreiber medium (ESM). The ESM contained 10 nM disodium selenite instead of soil extracts according to our previous article (Danbara and Shiraiwa, 1999). The cells of *T. lutea* CCMP463 and *I. galbana* NIES-2590 were continuously illuminated at 100 $\mu\text{mol photon/m}^2/\text{s}^1$ with fluorescent lamps and bubbled with air at a rate of 100 ml/min. The temperature was maintained at 10, 15, 20, 25, 30, 35 or 40 °C.

Other haptophyte cells of *E. huxleyi* NIES-2697 and *G. oceanica* NIES-1000 were also grown under identical conditions, and the harvested cells were used for the genetic analysis to provide representative sequences of the family Noëlaerhabdaceae. The cells were harvested once between the late linear and early stationary phases by monitoring the chlorophyll fluorescence. Therefore, the culture period varied depending on the growth rate at each temperature.

2.2. DNA extraction, polymerase chain reaction (PCR) and sequencing

Cells of *T. lutea* CCMP463, *I. galbana* NIES-2590, *E. huxleyi* NIES-2697 and *G. oceanica* NIES-1000 were harvested by centrifugation. The total DNA was extracted from the harvested cells using a DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. The D1-D2 region of the LSU rRNA gene was amplified by the PCR reaction using primer set L1 (5'-ACCCGCTGAAYTTAAGCATAT-3'; Yabuki et al., 2010) and L5 (5'-CTTNAGCGYCATCCATTTTC-3'; this study). The amplifications consisted of 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 68 °C for 1 min. The amplified DNA fragments were purified after gel electrophoresis using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified DNA fragments were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy) with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Monza, Italy). We used the following primers for the sequencing reactions: Hapto_4 (5'-ATGGCGAATGAAGCGGGC-3'; Liu et al., 2009) and Euk_34r (5'-GCATCGCCAGTTCTGCTTACC-3' (Liu et al., 2009).

2.3. Sequence alignments and phylogenetic analysis

The newly obtained D1-D2 region of LSU rRNA gene sequences were added to a 36-taxa alignment set of the haptophyte LSU rRNA gene. The alignment set was automatically aligned with MAFFT (Kato and Toh, 2008) and manually edited with SeaView (Galtier et al., 1996). For the phylogenetic analyses, ambiguously aligned regions were manually deleted from each alignment. Finally, we prepared the LSU rDNA alignments (760 positions). The maximum-likelihood (ML) tree was heuristically searched using RAxML v8.1.15 (Stamatakis, 2014) under the GTR+ Γ model. Tree searches began with 20 randomized maximum-parsimony trees, and the highest log likelihood (lnL) was selected as the ML tree. A non-parametric bootstrap analysis with 1000 replicates was conducted under the GTR+ Γ model. A Bayesian analysis was run using MrBayes v. 3.2.2 (Ronquist and Huelsenbeck, 2003) with the GTR + Γ model for each dataset. One cold and three heated Markov chain Monte Carlo simulations (default chain temperature = 0.1) were run for 2,000,000 generations. The lnL values and trees were sampled every 100 generations. The convergence was assessed based on the average standard deviation of split frequencies, and the first 400,000 generations of each analysis were discarded as “burn-in”. Bayesian posterior probability (BPP) and branch lengths were calculated from the remaining trees.

2.4. Lipid extraction and separation

The lipids were extracted and separated according to our previous reports (Sawada et al., 1996; Nakamura et al., 2014). The lipids were successively extracted from whole cells with MeOH, CH₂Cl₂:MeOH(1:1, v/v) and CH₂Cl₂. After the addition of distilled H₂O, the combined extracts were vigorously shaken and subsequently centrifuged to separate the lipid and water-soluble fractions. The resulting organic solvent layer was siphoned off and passed through an

anhydrous Na₂SO₄ column. The lipid extract was dried in a rotary evaporator and subsequently re-dissolved in *n*-hexane. The lipid-containing hexane extracts were separated using a silica gel column into three fractions with *n*-hexane, *n*-hexane/ethyl acetate (9:1, v/v) and ethyl acetate/MeOH (1:1, v/v) to yield hydrocarbons, alkenones, and more polar lipids (e.g., sterols and fatty acids), respectively. After an internal standard (*n*-hexatriacontane) was added, those fractions were analyzed by GC and GC-MS to quantify the alkenones and other compounds. For 15 °C samples, portions of alkenone fractions were saponified by heating at 70 °C for 3h in 1 N MeOH/H₂O (95:5, v/v). After the reaction, lipids were extracted with hexane (3 × 1 ml) and analyzed by GC and GC-MS.

2.5. GC and GC-MS analyses

The alkenone fractions were analyzed using gas chromatography-mass spectrometry (GC-MS), which was equipped with an VF-200ms column (Agilent; 60 m × 0.25 mm × 0.10 μm), under the following GC-MS conditions: Agilent 6890N GC instrument is 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 50 °C (1 min) to 255 °C at 20 °C/min, to 300 °C at 3 °C/min, and subsequently to 320 °C (held 10 min) at 10 °C/min. The alkenones and alkenoates were quantified using a Shimadzu GC-2025 GC-FID instrument with the identical column and temperature program as for GC-MS. He was the carrier gas in both GC and GC-MS. The use of the VF-200ms column was recently demonstrated by Longo et al. (2013) to significantly improve the separation for long chain alkenones.

3. Results

3.1. *Tisochrysis lutea* —Molecular taxonomic identification

Fig. 1 shows a phylogenetic tree of haptophytes in the Isochrysidales. The topology of a phylogenetic tree is similar to the inferred ML tree from a concatenated sequence of three genes (SSU and LSU rDNA, *cox1*), as described by Bendif et al. (2013). The newly obtained sequencing data in this study show that both *T. lutea* CCMP463 and *I. galbana* NIES-2590 are classified in the *Tisochrysis* clade as shown in Fig. 1. According to the results, we propose to revise the taxonomic name of *I. galbana* NIES-2590 as *T. lutea* NIES-2590 (Table 1).

The distribution of *T. lutea* and its abundance in natural environments are poorly understood. All *T. lutea* strains that were identified by Bendif et al. (2013) originate from the marine environment, particularly coastal regions, and are considered to be coastal marine species. At least three *T. lutea* strains have originally been isolated from different regions: CCMP463 from Little Water Cay in Turks and Caicos Islands, Caribbean Sea, NIES-2590 (originally described as *Isochrysis galbana* but revised as *T. lutea* in this study (Fig. 1, Table 1)) from the Bretagne coast of the English Channel, and T-iso (originally described as *Isochrysis* aff. *galbana* T-iso but revised to *T. lutea* by Bendif et al., 2013) from Mataiva, Tahiti, South Pacific.

3.2. Cell growth

Both *T. lutea* strains CCMP463 and NIES-2590 show similar growth patterns against different growth temperatures at 10, 15, 20, 25, and 35 °C (Fig. 2; Supplementary Table S1). All cultures failed to grow at 40 °C (data not shown). The highest growth rate at the logarithmic phase was obtained at 35 °C, which subsequently decreased with the decrease in temperature. At 15 °C, the growth rate was not so seriously diminished, but the cells began to

grow after a long lag phase (ca. 70 h) in both tested strains (Fig. 2). At 10 °C, the cultures were maintained with either a slight increase or decrease in chlorophyll fluorescence up to 91 h, but subsequently stopped growing. Cells were motile and homogenous in size, shape and color suggesting healthy growth in all harvested temperatures (15, 20, 25, 30, 35 °C; Supplementary Fig. S1). Meanwhile, cells at 10 °C were aggregated, lacking in color and heterogeneous in size and shapes suggesting that cells may have been damaged and the temperature was not suitable for physiological adaptation (10 °C; Supplementary Fig. S1). Therefore, cells were not harvested at 10 °C and 40 °C.

3.3. Analysis of alkenones and alkenoates

Both *T. lutea* strains CCMP463 and NIES-2590 contained identical suites of alkenones and alkenoates, and their concentrations and compositions resemble each other as shown in the partial GC-FID chromatograms (Figs. 3 and 4), concentrations (Table 2) and parameters of alkenones and alkenoates (Table 4), which were calculated using the equations in Table 3.

The C₃₇–C₄₀ di- and tri-unsaturated alkenones are the main constituents of alkenone fractions under all experimental conditions (Fig. 3). High values (> 3.0) of alkenone chain-length ratio (K₃₇/K₃₈) indicate the predominance of C₃₇ alkenones (Table 4). C₄₀ alkenones were detected in both *T. lutea* strains that were grown at various temperatures, and the content (expressed as %K₄₀) increased at low temperature, although the values were mostly less than 0.5 (Table 4). When the growth temperature decreased, the content of triunsaturated alkenones increased, whereas that of diunsaturated alkenones decreased (Table 2), which decreased the alkenone unsaturation index (Table 4).

Interestingly, the present data clearly show that both *T. lutea* strains lack tetraunsaturated

alkenones, even the cells that were grown at variety of growth temperatures (15 to 35 °C). If present, tetraunsaturated alkenones should elute before triunsaturated alkenones, and C_{37:4}Me should elute very close to the C_{36:2}FAEE on a VF-200ms column (Fig. 2). The absence of C_{37:4}Me was confirmed by the analysis of saponified alkenone fractions of 15 °C experiments (Fig. 4; no peak left at the retention time where C_{36:2}FAEE eluted before saponification), as well as the lack of a molecular ion peak (M⁺; *m/z* 526.5) in the GC-MS analysis. The ratio of C₃₈ ethyl to C₃₈ methyl alkenones (K₃₈ Et/Me = 11.8–21.8), which was calculated to express the relative abundance of C₃₈ homologs (Conte et al., 1998), indicates the overall predominance of C₃₈ ethyl form in C₃₈ alkenones (Table 4).

Of note, the present data show that both strains contain minor proportions of C₃₈ methyl alkenoates and C₃₉ ethyl alkenoates (Fig. 3, Table 2), which are commonly detected in the strains of Noëlaerhabdaceae and marine sediments (e.g., Prahl and Wakeham, 1987) but rarely reported in Isochrysidaceae strains that are grown in culture (e.g., Rontani et al., 2004; Toney et al., 2012). Alkenoates, methyl or ethyl esters of C₃₆ unsaturated fatty acids (C₃₆ FAMES and C₃₆ FAEEs) are dominated by diunsaturated compounds in comparison with triunsaturated alkenoates. The proportions of triunsaturated alkenoates increased when the cells were grown at low temperatures (Fig. 3, Table 4), which shows a similar trend with the alkenones. The two strains have similar ratios of methyl to ethyl alkenoates (A₃₇/A₃₈), which are not correlated with the growth temperatures (Table 3). Similar to alkenones, tetraunsaturated alkenoates are also absent in both *T. lutea* strains.

3.4. Correlation of alkenone and alkenoate unsaturation indices with the growth temperature

The alkenone unsaturation indices for C₃₇ methyl alkenones (U_{37}^K ; Brassell et al., 1986; Prahl

and Wakeham, 1987; Prahl et al., 1988), C₃₈ methyl and ethyl alkenones ($U_{38}^K\text{Me}$ and $U_{38}^K\text{Et}$, respectively; Conte et al., 1998) in two *T. lutea* strains in this study are listed in Table 4. We also defined the analogous unsaturation indices U_{37}^A and U_{38}^A for C₃₆ fatty acid methyl esters (C₃₇ alkenoates) and C₃₆ fatty acid ethyl esters (C₃₈ alkenoates), respectively (Tables 3 and 4). Because of the lack of tetraunsaturated alkenones, the original and simplified pairs of unsaturation indices (e.g., U_{37}^K and $U_{37}^{K'}$) give identical values in all experiments. Hence, we only show the original forms in the text and tables for simplicity and use the identical values for comparison with previously reported simplified indices (e.g., Tables 3 and 4, and Fig. 6).

All alkenone and alkenoate unsaturation indices such as U_{37}^K , $U_{38}^K\text{Et}$, $U_{38}^K\text{Me}$, U_{37}^A , and U_{38}^A in both *T. lutea* strains CCMP463 and NIES-2590 increased with the growth temperature from 15 °C to 35 °C (Fig. 5; Table 3). All fitted regressions of the unsaturation indices vs growth temperature are characterized by high correlation coefficients ($r^2 > 0.9$), which indicates that the growth temperatures tightly control the unsaturation of alkenones and alkenoates in the *T. lutea* strains (Table 5). The incremental changes of those unsaturation indices with temperature tend to decrease at higher temperature extremes. Hence, the second-order polynomial calibrations of unsaturation indices provide higher correlation coefficients than the linear calibrations (Table 5). For ease of comparison with previously reported linear calibrations, we also obtained the linear U_{37}^K and $U_{38}^K\text{Me}$ calibrations in the range of 15–30 °C, where the indices are characterized with a quasi-linear relation with temperature (Fig. 5). In addition, we set up linear U_{37}^K calibrations between 30 and 35 °C to examine if *T. lutea* strains were not only capable of growing, but also persist in adjusting the alkenone compositions under these unprecedented warm temperatures (Table 5).

303 4. Discussion

304 4.1. Characteristics of alkenone and alkenoate compositions

305 With the support of molecular phylogenetic analyses, the feature of the alkenone-producing
306 haptophyte *T. lutea* was characterized by: (1) the lack of tetraunsaturated alkenones and
307 alkenoates, (2) high K_{37}/K_{38} values (> 3), and (3) predominance of C_{38} ethyl and C_{39} methyl
308 alkenones over C_{38} methyl and C_{39} ethyl homologues, respectively. A previous report suggests
309 that tetraunsaturated compounds are produced as a biochemical response of cells to cold
310 stress (e.g., Conte et al., 1998). *T. lutea* strains CCMP465 and NIES-2590 showed similar
311 growth pattern against temperature. They grow well at high temperatures ($> 25\text{ }^{\circ}\text{C}$) as does
312 tropical strain T-iso (e.g., Ewart and Pruder 1981; Kaplan et al., 1986). On the contrary,
313 growth of *T. lutea* strains show significant decrease with decreasing temperature below $20\text{ }^{\circ}\text{C}$.
314 The photomicrographs of poorly growing $10\text{ }^{\circ}\text{C}$ cultures shows cells are aggregated, lack color
315 and are sometimes deformed (Supplementary Fig. S1), hence $10\text{ }^{\circ}\text{C}$ is probably the low-end
316 temperature limit for healthy growth of the studied strains. Although $15\text{ }^{\circ}\text{C}$ cultures adapted
317 to the temperature, lower growth rate observed as changes in chlorophyll fluorescence
318 probably attributed to cold-stress being cultured in much lower temperature from the
319 relatively high optimum temperature. Therefore, the lack of tetraunsaturated alkenone
320 production by both *T. lutea* strains CCMP465 and NIES-2590 even at $15\text{ }^{\circ}\text{C}$ suggests that *T.*
321 *lutea* cannot genetically produce tetraunsaturated alkenones under the studied conditions.
322 Our results do not preclude the possibility that *T. lutea* may produce minor amounts of
323 tetraunsaturated alkenones at the low-end of tolerated temperatures, nor that factors other
324 than species and temperature (i.e. nutrient quantity and quality, light, pH, salinity
325 characteristics) may stimulate the production of tetraunsaturated moieties under certain
326 conditions.

The capability of tetraunsaturated alkenone production has been considered a chemotaxonomic fingerprint, which is represented as intra-specific genetic variations to some extent in the marine alkenone-producing haptophyte *E. huxleyi*, although tetraunsaturated alkenones are generally a minor component if present (Conte et al., 1998; Marlowe et al., 1984; Prahl and Wakeham, 1987). Moreover, most species in the Isochrysidaceae, such as *I. galbana* (strains PLY 'I', PLY507 and CCMP715) and *R. lamellosa* (PLY353, PLY528 and CCMP1307), are known as significantly high producers of tetraunsaturated alkenones even when grown at 15–25 °C (ca. 10–40%, Marlowe et al., 1984; Rontani et al., 2004; Sun et al., 2007; Theroux et al., 2013; Nakamura et al., 2014).

Therefore, some strains in Isochrysidaceae that lack tetraunsaturated alkenones can be classified as *T. lutea*, although they were once classified as *Isochrysis* species. In fact, according to the genetic identification by Bendif et al. (2013) and this study, the three distinct strains lacking C_{37:4} alkenones were renamed as *T. lutea*: PLY506A (Marlowe et al., 1984), NIES-2590 (this study) and T-iso (synonym strains AC102, CCMP1324 and RCC1349 shown in Fig. 1) (e.g., Marlowe et al., 1984; Brown et al., 1993; Ono et al., 2012). *T. lutea* is the only species that was characterized by the lack of tetraunsaturated alkenones among the coastal–lacustrine alkenone producers (family Isochrysidaceae; Fig. 1).

The ratios of C₃₇/C₃₈ alkenone content (K₃₇/K₃₈) in the *T. lutea* strains, CCMP463 and NIES-2590, are characterized by high values (> 3) regardless of the growth temperatures (Table 4). According to the literature, the K₃₇/K₃₈ ratio tends to significantly vary between open oceanic species (< 2; *E. huxleyi* and *G. oceanica*) and coastal–lacustrine species (> 3; *I. galbana* and *R. lamellosa*) in alkenone-producing haptophytes. Therefore, marked high/low values of this ratio have been used as a parameter to presume alkenone-producing species from lake alkenone profiles (e.g., Chu et al., 2005; Pearson et al., 2008), and gradual changes between

endmember values have been interpreted to reflect changing contributions of different alkenone producers in coastal region (Schulz et al., 2000; Schwab and Sachs, 2011). However, Conte et al. (1998) reported that the K_{37}/K_{38} ratio decreased depending on the physiological status during growth at the late logarithmic and stationary growth phases in *E. huxleyi* and *G. oceanica*. This result suggests that the K_{37}/K_{38} ratio is affected by both genetic difference and physiological status. The same trend applies in the K_{37}/K_{38} ratio of *R. lamellosa* cells that were harvested at different growth phases (Marlowe et al., 1984; Nakamura et al., 2014; Rontani et al., 2004). The K_{37}/K_{38} ratios in *T. lutea* strains CCMP463 and NIES-2590 in this study were significantly lower than that in *T. lutea* PLY506A (T-iso) that was harvested at the exponential growth phase (Marlowe et al., 1984). A high content of C_{38} alkenones in the later growth stage (Conte et al., 1998) is a common trend at least in the families Noëlaerhabdaceae and Isochrysidaceae, nevertheless, the overall range of the K_{37}/K_{38} ratio remains significantly different between the two families. Hence, a high K_{37}/K_{38} ratio (> 3) is considered as a chemotaxonomic characteristic of *T. lutea*, and can also be useful to categorize alkenone-producing species that are isolated from a natural environment.

The relative abundance of C_{38} methyl and C_{39} ethyl alkenones is considered partly affected by genetic variation among alkenone producers. Open ocean species *E. huxleyi* and *G. oceanica* constantly produce both ethyl and methyl homologues (e.g., $C_{38}Et/Me$ was ca. 1–4 in the mid-log phase), whereas coastal–lacustrine species usually lack C_{38} methyl alkenones (Conte et al., 1998). In the present study, the separation technique for ethyl and methyl alkenones was improved using a VF-200ms column in GC-FID, which enabled us to unambiguously identify and quantify minor amounts of $C_{38}Me$ produced by the *T. lutea* strains (11.8–21.8 in $C_{38}Et/Me$). The present confirmation of the production of C_{38} methyl and C_{39} ethyl in *T. lutea* strains reinforces the suggestion of Theroux et al. (2010) that the presence of C_{38} methyl

alkenones cannot be directly attributed to the contribution of open ocean species. Nevertheless, a significant variation in C₃₈ methyl alkenone production has been reported in lacustrine samples and cultivated cells in Isochrysidaceae species (Theroux et al., 2010; Toney et al., 2012). Therefore, the relative abundance of C₃₈ methyl and C₃₉ ethyl alkenones (ex. C₃₈Et/Me) can be evaluated as a complementary genetic fingerprint for certain taxa of alkenone producers in Isochrysidaceae by further studying a wide variety of strains using the VF-200ms column.

4.2. Comparison of U_{37}^K calibrations with other species

The $U_{37}^{K'}$ and U_{37}^K calibrations of genetically identified Isochrysidaceae strains are shown in Fig. 6, where *E. huxleyi* 55a represents a typical open ocean producer and serves as a global marine SST calibration. Previous $U_{37}^{K'}$ values of *T. lutea* strains PLY506A (Marlowe et al., 1984) and T-iso clones (Brown et al., 1993; Ono et al., 2012) are plotted and are consistent with the present data. This result suggests that the obtained U_{37}^K calibrations in the present study well represent the temperature response of the alkenone biosynthesis in *T. lutea*. The U_{37}^K calibrations of *T. lutea* strains CCMP463 and NIES-2590 (Fig. 6A) significantly deviate from those of the other Isochrysidaceae species: *I. galbana* strains CCMP1323 and CCMP715 (Versteegh et al., 2001; Theroux et al., 2013) and *R. lamellosa* strain CCMP1307 (Nakamura et al., 2014). *T. lutea* calibrations are more similar to those of *E. huxleyi* 55a in terms of the range of unsaturation indices (ca. 0.2–0.9), whereas it has the highest sensitivity to the growth temperature among Isochrysidaceae strains and *E. huxleyi* 55a, as shown by the calibration slope. The low slope values in the $U_{37}^{K'}$ calibrations of *I. galbana* and *R. lamellosa* do not imply insensitivity of the alkenone composition to temperature. Studies have demonstrated that

these species and lacustrine alkenone producers produce a significant amount of tetraunsaturated alkenones. Hence, U_{37}^K calibrations usually show higher sensitivity and better correlation to temperature (Toney et al., 2012; Theroux et al., 2013; Nakamura et al., 2014). The temperature sensitivity of the U_{37}^K calibrations is comparable among different species, except *I. galbana* CCMP715 which showed a relatively lower slope (Fig. 6B). Nevertheless, the calibrations of *T. lutea* are significantly deviated from those of the other two genetically distant species. The uniqueness of the temperature response of the alkenone unsaturation of *T. lutea* among Isochrysidaceae strains highlights that the obtained relationships serve as species-specific U_{37}^K ($U_{37}^{K'}$) calibrations for *T. lutea*.

4.3. Implications for environmental reconstructions

The response of growth against temperature clearly indicates that *T. lutea* is a warm temperature species among known alkenone producers, and to our knowledge, our result is the first to show the alkenone composition for haptophyte algae grown over 30 °C. Although the incremental change in U_{37}^K with increasing temperature obviously diminished at higher temperature extremes, both strains still adjusted alkenone unsaturation over 30 °C. The U_{37}^K -temperature sensitivity, expressed as the slope value of the linear regression, were 0.0045 and 0.017 for CCMP463 and NIES-2590, respectively. These values are lower than that of *E. huxleyi* over its temperature range (0.033; Prahl and Wakeham, 1987) and even lower than that of U_{37}^K calibration for *I. galbana* CCMP715 (0.0226; Theroux et al., 2013) (Fig. 6). Because the lower U_{37}^K -temperature sensitivity of CCMP463 is linked with less diminished growth at 10 °C compared to NIES-2590 (Figs. 1A and 5), CCMP463 seems slightly less thermophilic compared with NIES-2590, despite CCMP463 derived from tropical region. Although specific reasons are

currently unknown, such intra-specific variations in the temperature tolerance and adjustment of alkenone compositions are also observed in *E. huxleyi* (Conte et al., 1998). Nevertheless, the species-specific U_{37}^K -temperature calibration of *T. lutea* extends the ceiling of the maximum inferable temperature up to 35 °C. According to the curvature of the calibrations on the warm-end temperatures, full range (15–35 °C) second order polynomial calibrations would be more appropriate for *T. lutea* strains (Table 5; Fig. 5).

The predominance of tetraunsaturated alkenones is a frequently observed characteristic of lacustrine alkenones (i.e. Theroux et al., 2010) and has been considered as collateral evidence of the contribution of Isochrysidaceae species in such environments. Interestingly, the alkenone profiles of carbonate-dominated lakes in the interior of the United States are characterized by the lack of tetraunsaturated alkenones (NE Sandhills Lakes; Toney et al., 2010). The observed alkenone unsaturation indices (U_{37}^K and $U_{37}^{K'}$) for NE Sandhills Lakes only slightly differed from the *T. lutea* calibrations (Fig. 6). Because of this result and because *T. lutea* is hitherto the only known species that completely lacks tetraunsaturated alkenones among the coastal–lacustrine alkenone producers, we may expect that *T. lutea* or its uncultured close relative can occur in the lakes that produce C_{37:4}-lacking alkenones. Indeed, the phylogenetic trees that were inferred from partial 18s rDNA sequences from lacustrine samples and culture haptophytes indicate that the occurrence of lacustrine sequences is related to *T. lutea* (shown as *Isochrysis* sp. CCAP927/14 in Fig. 2 within Theroux et al., 2010; Toney et al., 2012) instead of *I. galbana* (shown as *Pseudoisochrysis paradoxa*) and *R. lamellosa* (shown as *Chrysotila lamellosa*). The non-marine distribution of *T. lutea* is obscure; hence, upcoming environmental DNA analysis and ultimate identification of the actual producer may find a further unknown alkenone producer that is characterized by the lack of C_{37:4} and similar U_{37}^K -calibration. In either case, the observed link between biomarker

compositions and temperature responses in morphologically indistinguishable Isochrysidaceae species encourages further effort in applying the lipid biomarker chemotaxonomy and establishing species-specific calibrations, which include uncultured relatives in natural environments.

5. Conclusions

T. lutea is an authentic species of the third genera *Tisochrysis* in the family Isochrysidaceae, and it is genetically distant from the more closely related *I. galbana* and *R. lamellosa*. Although *T. lutea* is morphologically indistinguishable from *I. galbana*, its alkenone composition is characterized by the lack of tetraunsaturated alkenone and alkenoates, whereas these compounds are common constituents of alkenones produced by *I. galbana* and *R. lamellosa*. The linear and nonlinear regressions of the alkenone unsaturation degrees (U_{37}^K and $U_{37}^{K'}$) with growth temperatures for *T. lutea* are also significantly different from the previously reported regressions in the strains of other genera in Isochrysidaceae. Combined with the previous dataset, the lack of tetraunsaturated alkenones, which are associated with high K_{37}/K_{38} values, and the predominance of C_{38} methyl alkenones are proposed as distinct chemotaxonomic fingerprints of *T. lutea*. The defined U_{37}^K calibrations serve as species-specific calibrations for *T. lutea* strains, and are the first calibrations which empirically defined up to 35 °C. The application of the alkenone paleotemperature proxy in the coastal–lacustrine environments depends on the concomitant establishment of reliable U_{37}^K – T calibrations and chemotaxonomic identification of alkenone producers. The current result from *T. lutea* showcases a new suite of specific calibrations that are tied to the chemotaxonomically distinct taxa of alkenone producers. It is important to broaden the target species from the established Isochrysidaceae

cultures (whose alkenone compositions and DNA sequences have not been examined) to the uncultured/undescribed alkenone producers that are actually isolated from lakes and coastal regions.

Acknowledgments

This study was supported by a fund of Core Research for Evolutional Science and Technology in Japan Science and Technology Agency (CREST/JST), Japan. We thank T. Ando, C. Kanou and R. Abe for laboratory support; Prof. N. Suzuki for discussions; Prof. Yongsong Huang, Prof. Stuart Wakeham and an anonymous reviewer for constructive comments.

Associate Editor–**Stuart Wakeham**

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643

644 **Captions**

645 Table 1. List and sequence accession numbers of the haptophyte strains in this study.

646 (footnote) ^a The original name of *I. galbana* NIES-2590 was revised as *T. lutea* NIES-2590

647 according to the phylogenic analysis in this study. See Fig. 1.

648

649 Table 2. Compositions and concentrations of alkenones and alkenoates in *Tisochrysis lutea*

650 strains CCMP463 and NIES-2590 cells, which were grown at different temperatures.

651

652 Table 3. Formulae for unsaturation indices and the other compositional parameters for the

653 alkenones and alkenoates. Me = methyl alkenone; Et = ethyl alkenone; FAME = fatty acid

654 ethyl ether; FAEE = fatty acid ethyl ether. All U_{37}^K indices and $U_{37}^{K'}$ indices were calculated with

655 and without tetraunsaturated moieties, respectively.

656

657 Table 4. Parameters obtained by calculating the data on alkenone and alkenoate

658 compositions in *Tisochrysis lutea* strains CCMP463 and NIES-259, which were grown at

659 different temperatures. See Table 2 for the original data on alkenone and alkenoate

660 compositions.

661

662 Table 5. Data of linear and second-order polynomial regressions on the relationship

663 between the alkenone and alkenoate unsaturation indices and the growth temperature.

664

665

666 **Fig. 1.** Maximum likelihood tree of 36 strains in Isochrysidales and 4 strains in Coccolithales
 667 based on DNA sequences using 760 position of the D1-D2 region of LSU rRNA gene. The values
 668 on each node are bootstrap percentage (BP, left) and Bayesian posterior probability (BPP,
 669 right). Only BP $\geq 50\%$ and BPP ≥ 0.5 are shown. Symbols denote presence (■)/absence (●)
 670 of tetraunsaturated alkenones in the references: 1, Marlowe et al. (1984); 2, Rontani et al.
 671 (2004); 3, This study. Symbols show synonymous (equivalent) strains. See Table 1 in Bendif et
 672 al. (2013) for the list of equivalent strains.

673

674 **Fig. 2.** Growth curves (A) and total contents of alkenones (B) ($\mu\text{g/ml}$) in *T. lutea* strains
 675 CCMP463 and NIES-2590 grown at various temperatures in the range of 10–35 °C. The data in
 676 (B) denote the average values of triplicate experiments. The vertical bars extending from the
 677 circles indicate $\pm\sigma$.

678

679 **Fig. 3.** Partial gas chromatograms showing the distribution of long chain alkenones and
 680 alkenoates in the *T. lutea* strains CCMP463 (left) and NIES-2590 (right), which were grown at
 681 various temperatures (15, 20, 25, 30 and 35 °C from bottom to top).

682

683 **Fig. 4.** Partial gas chromatograms showing the removal of methyl and ethyl alkenoates by
 684 saponification of the alkenone fraction of *T. lutes* strains CCMP463 (left) and NIES-2590
 685 (right) grown at 15 °C. Chromatograms with a dashed orange line and a blue line are derived

686 from alkenone fractions before (pre) and after saponification (sap), respectively.

687

688 **Fig. 5.** Relationship between the unsaturation index from C₃₇ alkenones (U_{37}^K) in *T. lutea* strains
 689 CCMP463 (left) and NIES-2590 (right). The linear and polynomial calibrations are shown with
 690 solid and dashed lines, respectively.

691

692 **Fig. 6.** Comparison of the culture-based $U_{37}^{K'}$ - (A) and U_{37}^K -temperature calibrations (B) in
 693 various species of the family Isochrysidaceae, Haptophyta. The data for *E. huxleyi* 55a
 694 (Noëlaerhabdaceae) are taken as representative of open marine species (Prahl and Wakeham,
 695 1987). Symbols: a–f: alkenone unsaturation index in various haptophytes. Other symbols: the
 696 reported alkenone unsaturation index in the literature for various strains of *T. lutea*: □,
 697 average of 5 experiments in different light intensities at 25 °C (Brown et al., 2004); ○, average
 698 of 5 (15 °C) and 4 (20 °C) experiments in different salinity (Ono et al., 2012); ▲, Marlowe et al.
 699 (1984); ●, data of C_{37:4}-lacking lakes in NE Sandhills lakes are plotted in purple (Toney et al.,
 700 2010). The vertical bars extending from the circles indicate $\pm 1\sigma$.

701

Table 1.

Strain name	Strain No.	Strain synonyms	Origin	Isolator	Isolation date	accession number
Isochrysidaceae						
<i>Tisochrysis lutea</i>	CCMP463	CISO, RCC1350	Atlantic Ocean, Caribbean Sea; coastal	Glazer, R.	1984	LC102346
<i>Isochrysis galbana</i> / <i>Tisochrysis lutea</i>	NIES-2590	RCC178, Cean	Atlantic Ocean, English Channel; coastal	unkown	unknown	LC102347
Noëlaerhabdaceae						
<i>Emiliana huxleyi</i>	NIES-2697	AC472, TQ26, RCC1216, CCMP3266	Pacific Ocean, Tasman Sea	Probert, I.	1998	LC102344
<i>Gephyrocapsa oceanica</i>	NIES-1000	RCC1562, CCMP2054	Pacific Ocean, Japanese coast; tide pool	Kawachi, M.	1999	LC102345

CCMP, National Center for Marine Algae and Microbiota (formerly Provasoli-Guillard Center for Culture of Marine Phytoplankton); NIES, National Institute for Environmental Studies; RCC, Roscof Culture Collection. A original name of *I. galbana* NIES2590 was revised as *T. lutea* NIES2590 according to phylogenetic analysis performed in this study. See Fig. 1.

Table 2.

Strain No.	T (°C)	Alkenone (%)												Σalkenones (mg/L)	Alkenoate (%)				Σalkenoates (mg/L)
		C _{37:3} Me	C _{37:2} Me	C _{38:3} Et	C _{38:2} Et	C _{38:3} Me	C _{38:2} Me	C _{39:3} Et	C _{39:2} Et	C _{39:3} Me	C _{39:2} Me	C _{40:3} Et	C _{40:2} Et		C _{36:3} FAME	C _{36:2} FAME	C _{36:3} FAEE	C _{36:2} FAEE	
CCMP463	35	5.8	69.0	0.2	20.8	tr	1.2	tr	0.3	0.2	2.1	tr	0.3	5.79	0.8	25.4	0.4	73.5	0.57
	35	3.9	70.5	tr	21.3	tr	1.2	-	0.3	0.2	2.0	tr	0.3	5.73	0.5	25.2	0.3	74.0	0.54
	35	6.5	69.1	0.3	19.5	0.2	1.3	tr	0.3	0.2	2.2	tr	0.5	5.15	0.8	26.5	0.4	72.3	0.59
	30	7.1	66.8	0.3	20.4	tr	1.4	-	0.3	0.2	2.9	tr	0.4	11.5	0.4	27.3	0.4	71.9	1.14
	30	6.9	67.2	0.2	20.2	tr	1.4	-	0.3	0.2	2.9	tr	0.4	12.6	0.5	26.9	0.5	72.1	1.32
	30	7.0	67.5	0.2	19.9	tr	1.4	-	0.3	0.2	2.9	tr	0.4	12.3	0.5	26.8	0.7	72.0	1.31
	25	21.0	53.3	0.6	19.0	0.3	1.1	-	0.3	0.7	3.1	tr	0.5	16.1	1.8	28.1	1.1	69.0	1.71
	25	16.4	57.5	0.5	19.2	0.4	1.2	-	0.3	0.6	3.4	tr	0.5	16.1	1.3	28.3	1.1	69.4	1.73
	25	16.0	58.2	0.6	19.1	0.4	1.3	-	0.3	0.6	3.2	tr	0.5	16.1	1.3	27.9	1.3	69.5	1.73
	20	44.7	29.8	3.0	16.6	0.6	0.6	tr	0.2	2.0	2.1	tr	0.4	11.5	4.9	22.0	3.7	69.4	1.26
	20	39.6	35.3	1.9	17.1	0.6	0.7	tr	0.3	1.7	2.5	tr	0.5	14.4	3.9	24.4	2.3	69.4	1.67
	20	39.3	36.1	2.0	16.8	0.6	0.7	tr	0.3	1.6	2.3	tr	0.4	14.6	4.1	24.4	2.8	68.7	1.73
	15	68.2	7.52	10.6	7.10	0.7	0.4	tr	tr	4.0	0.8	0.33	0.3	4.01	17.3	6.6	19.5	56.6	0.57
	15	65.6	10.7	9.1	8.40	0.9	0.2	tr	tr	3.4	0.9	0.27	0.3	5.31	15.0	9.1	17.1	58.8	0.73
	15	66.3	11.6	7.9	8.40	0.6	0.1	tr	tr	3.4	1.0	0.21	0.3	7.24	14.4	10.7	14.8	60.1	1.00
NIES-2590	35	3.9	68.9	tr	22.4	0.2	1.6	tr	0.4	0.2	2.0	tr	0.3	7.29	0.5	24.1	0.4	75.0	0.56
	35	3.1	68.3	0.3	23.5	0.3	1.5	tr	0.4	0.2	2.1	tr	0.3	5.90	-	22.6	-	77.4	0.44
	30	10.9	63.5	0.4	21.2	0.3	1.1	-	0.3	0.3	2.0	tr	0.3	14.7	1.1	25.6	0.3	73.0	1.47
	30	8.9	65.7	0.2	21.4	tr	0.9	-	0.3	0.3	1.8	tr	0.2	11.1	1.0	25.3	0.5	73.2	1.12
	30	9.6	65.3	0.3	21.0	tr	1.0	-	0.2	0.4	1.9	tr	0.3	9.21	0.9	25.2	-	73.9	0.92
	25	23.0	51.4	0.5	21.1	0.4	0.8	-	0.3	0.5	1.7	tr	0.3	11.6	2.6	26.7	0.4	70.4	1.47
	25	21.3	54.2	0.4	20.0	0.3	0.9	-	0.3	0.5	1.8	tr	0.3	14.7	2.1	26.7	0.5	70.7	1.71
	25	21.0	54.5	0.4	19.9	0.3	0.9	-	0.2	0.5	1.9	tr	0.3	15.0	2.0	26.7	0.5	70.8	1.73
	20	39.8	35.0	1.6	18.8	0.6	0.6	tr	0.2	1.2	1.8	tr	0.3	15.4	4.7	25.1	2.2	68.1	1.56
	20	37.9	37.1	1.4	18.8	0.6	0.7	tr	0.2	1.2	1.8	tr	0.4	17.7	4.1	26.0	1.7	68.2	2.00
	20	37.8	37.9	1.2	18.4	0.5	0.6	tr	0.2	1.1	1.8	tr	0.4	15.8	4.1	26.8	1.4	67.7	1.95
	15	56.8	16.7	7.4	14.1	1.0	0.3	tr	tr	2.1	1.0	tr	0.3	7.62	11.6	15.6	10.8	62.0	0.62
	15	58.8	15.5	7.4	13.0	0.9	0.3	tr	tr	2.4	1.1	tr	0.3	12.5	11.7	16.1	9.5	62.7	0.95
	15	58.1	16.1	7.3	13.4	1.0	0.4	tr	tr	2.1	1.1	tr	0.3	11.8	11.4	16.5	9.6	62.4	1.01

T: Growth temperature
tr: Trace amount (< 0.2 %)
-: not detected

Table 3.

Parameters	Equation	Ref. ^a
<i>Unsaturation indices</i>		
U_{37}^K	$([C_{37:2}Me] - [C_{37:4}Me]) / ([C_{37:2}Me] + [C_{37:3}Me] + [C_{37:4}Me])$	1
U_{38Me}^K	$([C_{37:2}Me] - [C_{37:4}Me]) / ([C_{37:2}Me] + [C_{37:3}Me] + [C_{37:4}Me])$	2*
U_{38Et}^K	$([C_{38:2}Et] - [C_{38:4}Et]) / ([C_{38:2}Et] + [C_{37:3}Et] + [C_{37:4}Et])$	3*
U_{37}^A	$([C_{36:2}FAME] - [C_{36:4}FAME]) / ([C_{36:2}FAME] + [C_{36:3}FAME] + [C_{36:4}FAME])$	7
U_{38}^A	$([C_{36:2}FAEE] - [C_{36:4}FAEE]) / ([C_{36:2}FAEE] + [C_{36:3}FAEE] + [C_{36:4}FAEE])$	7
<i>Other ratios</i>		
$K_{38Et/Me}$	$([C_{38:2}Et] + [C_{38:3}Et] + [C_{38:4}Et]) / ([C_{38:2}Me] + [C_{38:3}Me] + [C_{38:4}Me])$	4
%K ₄₀	$100 \times (\Sigma C_{40} \text{ alkenones}) / (\Sigma C_{37-40} \text{ alkenones})$	
K_{37}/K_{38}	$(\Sigma C_{37} \text{ alkenones}) / (\Sigma C_{38} \text{ alkenones})$	5
A_{37}/A_{38}	$([C_{36:2}FAME] + [C_{36:3}FAME] + [C_{36:4}FAME]) / ([C_{36:2}FAEE] + [C_{36:3}FAEE] + [C_{36:4}FAEE])$	7
EE/K_{37}	$([C_{36:2}FAEE] + [C_{36:3}FAEE] + [C_{36:4}FAEE]) / ([C_{37:2}Me] + [C_{37:3}Me] + [C_{37:4}Me])$	6

^a 1: Brassell et al. (1986), 2: Conte and Eglinton (1993), 3&4: Conte et al. (1998), 5: Rosell-Melé et al. (1994), 6: Prahl et al. (1988). 7, This paper

* equations were modified after the original definition in the references; All UK indices and UK' indices are calculated with and without tetraunsaturated moieties, respectively.

Table 4.

Strain No.	<i>T</i> (°C)	Alkenone						Alkenoate			
		U ^K ₃₇	U ^K ₃₈ Et	U ^K ₃₈ Me	K ₃₈ Et/Me	%K ₄₀	K ₃₇ /K ₃₈	U ^A ₃₇	U ^A ₃₈	A ₃₇ /A ₃₈	EE/K ₃₇
CCMP463	35	0.92	0.99	0.88	15.2	0.29	3.33	0.97	1.00	0.35	0.10
	35	0.95	0.99	0.87	15.1	0.28	3.25	0.98	1.00	0.35	0.09
	35	0.91	0.99	0.87	12.8	0.50	3.55	0.97	1.00	0.38	0.11
	30	0.90	0.99	0.91	13.1	0.44	3.32	0.99	0.99	0.38	0.10
	30	0.91	0.99	0.92	13.3	0.41	3.37	0.98	0.99	0.38	0.10
	30	0.91	0.99	0.90	13.0	0.41	3.43	0.98	0.99	0.38	0.10
	25	0.72	0.97	0.76	14.0	0.49	3.53	0.94	0.98	0.43	0.10
	25	0.78	0.98	0.77	12.3	0.50	3.47	0.96	0.98	0.42	0.10
	25	0.78	0.97	0.77	11.8	0.48	3.47	0.95	0.98	0.41	0.10
	20	0.40	0.85	0.48	17.3	0.50	3.60	0.82	0.95	0.37	0.11
	20	0.47	0.90	0.53	15.1	0.51	3.71	0.86	0.97	0.40	0.11
	20	0.48	0.90	0.55	14.9	0.47	3.76	0.86	0.96	0.40	0.11
	15	0.10	0.40	0.34	16.2	0.59	4.05	0.28	0.74	0.31	0.14
	15	0.14	0.48	0.20	16.5	0.56	4.13	0.38	0.77	0.32	0.14
	15	0.15	0.52	0.19	21.8	0.47	4.56	0.43	0.80	0.34	0.13
NIES-2590	35	0.95	0.99	0.89	12.9	0.29	2.99	0.98	1.00	0.33	0.08
	35	0.96	0.99	0.86	13.3	0.31	2.79	1.00	1.00	0.29	0.08
	30	0.85	0.98	0.80	16.1	0.27	3.25	0.96	1.00	0.36	0.10
	30	0.88	0.99	0.86	20.1	0.26	3.29	0.96	0.99	0.36	0.10
	30	0.87	0.99	0.87	19.3	0.29	3.34	0.97	1.00	0.35	0.10
	25	0.69	0.98	0.69	17.6	0.31	3.27	0.91	0.99	0.41	0.12
	25	0.72	0.98	0.73	16.8	0.30	3.50	0.93	0.99	0.40	0.11
	25	0.72	0.98	0.72	16.9	0.31	3.52	0.93	0.99	0.40	0.11
	20	0.47	0.92	0.49	16.8	0.38	3.46	0.84	0.97	0.42	0.10
	20	0.50	0.93	0.52	15.9	0.38	3.51	0.86	0.98	0.43	0.11
	20	0.50	0.94	0.55	17.0	0.39	3.65	0.87	0.98	0.45	0.11
	15	0.23	0.66	0.24	16.8	0.37	3.23	0.57	0.85	0.37	0.08
	15	0.21	0.64	0.26	16.6	0.40	3.44	0.58	0.87	0.39	0.07
	15	0.22	0.65	0.26	15.0	0.40	3.37	0.59	0.87	0.39	0.08

T: Growth temperature

Table 5.

Unsaturation indices	Temperature range and type of fit	Regressions vs. temperature (T)		
		CCMP463	r^2	NIES-2590
U_{37}^K	15–35; polynom.	$-0.0022T^2 + 0.15T - 1.64$	0.99	$-0.0012T^2 + 0.099T - 0.99$
	15–30; linear	$0.053T - 0.63$	0.97	$0.044T - 0.41$
	30–35; linear	$0.0045T + 0.77$	0.55	$0.017T + 0.37$
$U_{38}^K\text{Me}$	15–35; polynom.	$-0.0021T^2 + 0.14T - 1.38$	0.98	$-0.0015T^2 + 0.11T - 1.03$
	15–30; linear	$0.045T - 0.40$	0.96	$0.0394T - 0.30$
$U_{38}^K\text{Et}$	15–35; polynom.	$-0.004T^2 + 0.21T - 1.82$	0.97	$-0.0028T^2 + 0.15T - 0.91$
U_{37}^A	15–35; polynom.	$-0.003T^2 + 0.18T - 1.61$	0.94	$-0.0015T^2 + 0.095T - 0.48$
U_{38}^A	15–35; polynom.	$-0.011T^2 + 0.064T + 0.078$	0.91	$-0.0007T^2 + 0.041T + 0.42$

Fig. 1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6