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Novel alkenone-producing strains of genus *Isochrysis* (Haptophyta) isolated from Canadian saline lakes show temperature sensitivity of alkenones and alkenoates

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Abbreviations: LCAs, long chain alkenones; LSU, Rubisco large subunit; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SST, sea surface temperature; SSU, Rubisco small subunit; $U_{37}^A$, $U_{38}^A$, RIA$_{38}$ and A$_{37}$/A$_{38}$, alkenoate indices; $U_{37}^K$, $U_{37}^{K'}$, $U_{38}^{K'}$, $U_{38}^K$ Et, $U_{41}^K$ Me and $U_{42}^K$ Et, alkenone unsaturation indices.
ABSTRACT

Alkenone-producing species have been recently found in diverse lacustrine environments, albeit with taxonomic information derived indirectly from environmental genomic techniques. In this study, we isolated alkenone-producing algal species from Canadian saline lakes and established unialgal cultures of individual strains to identify their taxonomical and molecular biological characteristics. Water and sediments collected from the lakes were first enriched in artificial seawater medium over a range of salinities (5–40 ppt) to cultivate taxa in vitro. Unialgal cultures of seven haptophyte strains were isolated and categorized in the *Isochrysis* clade using SSU and LSU rRNA gene analysis. The alkenone distributions within isolated strains were determined to be novel compared with other previously reported alkenone-producing haptophytes. While all strains produced the typical C$_{37}$ and C$_{38}$ range of isomers, one strain isolated from Canadian salt lakes also produced novel C$_{41}$ and C$_{42}$ alkenones that are temperature sensitive. In addition, we showed that all alkenone unsaturation indices (e.g., $U_{37}^K$ and $U_{37}^{K'}$) are temperature-dependent in culture experiments, and that alkenoate indices (e.g., $U_{37}^A$, $U_{38}^A$, RIA$_{36}$ and A$_{37}$/A$_{38}$) provide alternative options for temperature calibration based on these new lacustrine algal strains. Importantly, these indices show temperature dependence in culture experiments at temperatures below 10 °C, where traditional alkenone proxies were not as sensitive. We
hypothesize that this suite of calibrations may be used for
reconstructions of past water temperature in a broad range of lakes in
the Canadian prairies.

*Keywords: Alkenoates, Alkenones, Alkenone unsaturation index, Canadian salt lakes, Chemotaxonomy, Haptophytes, *Isochrysis*, Long-chain alkyl ketones, Paleothermometer, $U_3^{K}$*
1. Introduction

Long-chain alkenones (LCAs) were originally reported in marine sediments ca. 35 years ago (Boon et al., 1978; Brassell et al., 1980; de Leeuw et al., 1980; Volkman et al., 1980a, b; Marlowe et al., 1984) and in Quaternary lacustrine sediments a few years later (Cranwell, 1985; Volkman et al., 1988). Typically, these LCAs exhibit chain lengths from C$_{35}$ to C$_{40}$ and contain 2–4 trans double bonds, with C$_{37}$–C$_{39}$ LCAs appearing as the most common chain lengths in previous studies. The marine coccolithophore, _Emiliania huxleyi_, was the first haptophyte identified to have produced LCAs (Volkman et al., 1980a, b). These microalgae have been widely studied, in part because haptophytes change the proportion of alkenones having a different number of double bonds depending on growth temperature. Consequently, the ratio of LCAs with different unsaturation levels is now used to calculate indices, such as $U^{37}_{35}$ and $U^{39}_{37}$, that can be used as paleotemperature proxies to reconstruct past sea surface temperature (SST) (Brassell et al., 1986; Prahl and Wakeham, 1987; Brassell, 1993).

Only five species within the order Isochrysidales, phylum Haptophyta are reported to produce LCAs and analogous compounds such as alkyl alkenoates (Medlin et al., 2008). Although LCAs are now found frequently in saline and freshwater inland lakes (e.g., Pearson et al., 2008; Theroux et al., 2010; Toney et al., 2010, 2012; D’Andrea et al., 2011; Longo et al., 2013), it appears that these compounds are still
constrained to the phylum Haptophyta. At present, taxa known to produce alkenones fit into three taxonomical groups as defined by Theroux et al. (2010). Group III includes *E. huxleyi* and *Gephyrocapsa oceanica* (Family Noëlaerhabdaceae), which are characteristic of marine environments. Group II includes *Isochrysis galbana*, *Tisochrysis lutea* (Bendif et al., 2013), *Ruttnera lamellosa* (revised from *Chrysotila lamellosa*; Andersen et al., 2014), which belong to Family Isochrysidaceae and represent species found in a wide range of environments such as coastal regions, brackish waters and saline lakes. Group I is composed of haptophytes from which no living algal strains have been isolated, but for which putative haptophyte strains have been identified by using environmental genomics of Rubisco small subunit (SSU) rRNA from environmental samples collected in freshwater environments (D’Andrea et al., 2006; Theroux et al., 2010; Longo et al., 2013, 2016).

Freshwater alkenone-producing haptophytes have gained the interest of the paleoclimate community because of the potential for their LCAs as indices of past continental climates (Zink et al., 2001; Chu et al., 2005, 2012; Hou et al., 2016). However, the use of these compounds has been constrained to date because specific LCA-producing strains have not been isolated from the source lakes, and little is known of the environmental preferences of producer populations. Analysis of environmental SSU rRNA suggests that
several alkenone-producing haptophyte species closely related to *Isochrysis* and/or *Ruttnera* (family Isochrysidaceae) live in saline lakes and brackish waters where the LCAs are associated with organic matter suspended in water and sediments (Coolen, 2004, Coolen et al., 2009; Theroux et al., 2010; Toney et al., 2012, Randlett et al., 2014). The most recent investigation of northern Alaskan lakes reveals that those LCAs are characterized by abundant C37:4 homologues and a series of C37:3 alkenone isomers. Furthermore, Longo et al. (2016) used suspended particulate matter in Toolik Lake to determine an in situ $U_{37}^K$-temperature calibration for that freshwater site. However, despite these important advances, the absence of strain-specific information on environmental preferences makes it difficult to determine whether site-, habitat- and species-specific calibrations may be required for haptophytes from non-marine settings. While alkenone-derived, $U_{37}^K$-temperature calibrations have been developed for several genetically distinct strains of haptophytes (Versteegh et al., 2001; Rontani et al., 2004; Sun et al., 2007; Ono et al., 2012; Nakamura et al., 2014, 2016; Zheng et al., 2016), isolates from additional environments are still needed to determine the applicability of a universal calibration.

To date, existing $U_{37}^K$-temperature calibrations exhibit similar relationships (slopes) to environmental temperature, suggesting a similar dependence of unsaturation on temperature (Theroux et al., 2010; Bendif et al., 2013; Nakamura et al., 2016). However, the y-
intercepts of $U_{37}^{\alpha}$-temperature calibrations vary among indicator ratios and may reflect the influence of other physiological, taxonomic or environmental parameters. In addition, most LCA-based paleotemperature reconstructions have been performed in environments where diverse haptophyte species with distinctive LCA composition are expected to co-occur, including Chesapeake Bay (Schwab and Sachs, 2011), the Black Sea (Coolen et al., 2009), the Nordic Sea (Bendle et al., 2005) and the Baltic Sea (Schulz et al., 2000), making it difficult to determine whether reconstructions arise from temperature-related shifts in saturation level or environmentally controlled changes in species composition. Consequently, further detailed studies are needed on the thermal ecology of producing organisms and on how LCA compositions may change through time due to different control pathways (temperature, nutrient status, etc.) to fully understand the controls on unsaturation dependence on temperature.

In this study, we isolated and identified seven alkenone-producing algae from Canadian saline lakes to better establish the relationship between environmental conditions and LCA production. Our new strains were grouped at unique phylogenetic positions within the genus *Isochrysis*, including the first report of alkenone-producing *I. galbana* from inland lakes. The LCA compositions of some strains were different from those of marine *I. galbana*, which was reported by
Although the unsaturation dependence of the LCAs on temperature for the newly isolated *Isochrysis* strains was similar to those from unidentified alkenone-producing sedimentary isolates in a previous study (Toney et al., 2012), the y-intercept of new LCA ratios was different, suggesting another control on the y-intercept of the $U^{K}_{37}$ calibration. The addition of the multiple, phylogenetically distinct *Isochrysis* strains enabled us to discuss the relationship between phylogeny and LCA composition, including that between the degree of compound unsaturation and environmental temperature for species within the genus *Isochrysis*.

2. Materials and methods

2.1. Location of Canadian lakes for isolating alkenone-producing microalgae

We selected ten Canadian saline lakes where LCAs were already detected by Toney et al. (2011) and environmental data were available from an earlier comprehensive survey (Pham et al. 2009). Lakes Antelope, Snakehole, Success, Fishing, Humboldt, Waldsea, Deadmoose, Charron, Rabbit, and Redberry are saline lakes located in Saskatchewan, Canada (Supplementary Fig. S1, Table 1). In the present study, temperature, pH and salinity profiles were measured at 1 m depth intervals using a YSI Pro Plus meter (YSI Inc., Yellow Springs, Ohio, USA).
2.2. Isolation and establishment of alkenone-producing microalgal strains as unialgal culture

Samples of lake water, lake sediments, shore sand and plankton were collected with a 4 L Van-Dorn water sampler, Ekman grab, and plankton net (mesh pore size 5 μm), respectively, in September 2014. The samples were transported to the laboratory of the University of Regina, Regina, Canada, for the isolation of microalgae. The sample types (water, sediment, and plankton) were individually combined with a fresh culture medium for microalgae using either AF6 modified (prepared according to NIES-Culture Collection Media List, NIES, Japan; originally from Watanabe et al., 2000) or an artificial seawater (Marine Art SF-1; Osaka Yakken, Osaka, Japan) enriched with a modified Erd-Schreiber’s medium containing 10 nM disodium selenite (MA-ESM as described in Danbara and Shiraiwa, 1999).

MA-ESM media was prepared with a range of salinities to match the salinity of each lake by adjusting the amount of Marine Art SF-1 powder to achieve salinities varying from 5 ppt to 40 ppt. Then the medium was diluted using techniques for isolating single species of microalgae, as described by Allen and Stanier (1968). The algal suspension diluted with the fresh medium was dispensed into wells of a transparent, plastic, 96 well microplate. The salinity of the culture medium was set at 40, 30 or 10 ppt, as shown in Table 2. For the
cultivation of microalgae, the plates were maintained in a plant growth chamber under illumination by 20 W fluorescent lamp with an intensity range of 28 to 43 μmol photons m$^{-2}$ s$^{-1}$ (range: 400–700 nm) with a 12 h light/12 h dark regime. The temperature in the chamber was kept constant at 10 °C during culture.

2.3. Culture of the established strains as unialgal culture for testing temperature effect

Algal strains isolated from Canadian lakes were individually established as unialgal cultures by using the dilution method, as described above. Those strains were grown in 50 mL plastic flasks containing the MA-ESM medium with various ranges of salinity from 10 to 40 ppt (Table 2). All cultures were maintained in the algal growth chamber where light intensity and temperature were controlled. The cultures were continuously illuminated by 20 W fluorescent lamps at the intensity of 100 μmol photons m$^{-2}$s$^{-1}$. For testing temperature effect, three representative strains were used. The temperature was separately set at 5, 10, 15, 20 and 25 °C. Cells were grown until the late linear or the early stationary growth phases when they were harvested. Monitoring was achieved using the optical cell density of the cell suspension. The culture periods were different among culture vessels depending on growth rate in each culture and ranged from 16 days to 23 days. The harvested cells were used for the lipid analysis of LCAs.
and alkenoates.

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

After algal cells were harvested by centrifugation, DNA was extracted from the cells using a DNeasy plant mini kit (Qiagen, Hilden, Germany). The D1–D2 region of the large subunit (LSU) rRNA gene was amplified by the PCR reaction using the haptophyte specific primer set Hapto_4 (5'-ATGGCGAATGAAGCGGGC-3') and Euk_34r (5'-GCATCGCCAGTTCTGCTTACC-3') (Liu et al., 2009). The amplifications consisted of 30 cycles of denaturing at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 1 min by using PrimeSTAR GXL DNA polymerase (Takara Bio, Ohtsu, Japan). The SSU rRNA gene of isolates was amplified by the PCR reaction using primer set 18F (5'-AACCTGGTTGATCCTGCCAG-3') and 18R (5'-CYGCAGGTTTACCTACGGAA-3') (Yabuki et al., 2010). The amplifications consisted of 30 cycles of denaturing at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 2 min by using PrimeSTAR GXL DNA polymerase (Takara Bio). These amplified DNA fragments were sub-cloned into *E. coli* strain JM109 and then sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).
2.5. Sequence alignments and phylogeny

For molecular phylogenetic analysis, we newly created two datasets of haptophytes; one for the D1–D2 region of LSU rRNA gene sequences, while the other was the whole region of SSU rRNA gene sequences. The datasets were automatically aligned with mafft-linsi (Katoh and Standley, 2013), and then edited manually with SeaView (Galtier et al., 1996). Ambiguously aligned regions were manually deleted from the alignments. Finally, we prepared a LSU rRNA gene alignment with 58 operational taxonomic units (OTUs) and 995 positions, and a SSU rRNA gene alignment with 100 OTUs and 1713 positions. The maximum likelihood (ML) tree was constructed using IQ-TREE (Nguyen et al., 2015) under the best-fit model (TIM2+G4 for LSU rRNA and TNe+G4 for SSU rRNA gene) determined by IQ-TREE. Non-parametric bootstrap analysis with 100 replicates was conducted under the best-fit models. The Bayesian analysis was performed on each alignment using MrBayes v. 3.2.2 (Ronquist et al., 2012) with the GTR+Γ model. Two separated Metropolis-coupled Markov chain Monte Carlo, each with one cold and three heated chains (default chain temperature = 0.1), were run for $5 \times 10^6$ generations. The lnL values and trees were sampled at every 100 generation intervals. The convergence was assessed based on the average standard deviation of split frequencies, and the first $2 \times 10^6$ generations of each run were discarded as “burn-in.” Bayesian posterior probability (BPP) and
branch lengths were calculated from the remaining of the trees.

2.6. Lipid extraction and fractionation by organic solvents

The total lipid content of cells were extracted and separated into different compound classes for analysis using methods of Sawada et al. (1996) and Nakamura et al. (2014). Briefly the lipids were successively extracted from whole algal cells with methanol (MeOH), dichloromethane (DCM):MeOH (1:1, v:v) and DCM. The combined extracts were vigorously shaken after the addition of distilled water, and subsequently centrifuged to separate into two layers: lipid and water-soluble fractions. The resulting organic solvent layer was passed through an anhydrous Na$_2$SO$_4$ column to remove water. The lipid extract was dried in a rotary evaporator and subsequently re-dissolved with $n$-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, LCAs, and polar lipids (e.g., sterols and fatty acids), respectively. After adding an internal standard ($n$-hexatriacontane), those three fractions were analyzed by GC and GC–MS to quantify the various compounds they contained.

The LCA fraction was further cleaned up by saponification to reduce contamination from non-LCA compounds. Here a portion of the alkenone fraction was saponified by heating at 70 °C for 3 h in 1 N
KOH MeOH:H₂O (95:5, v:v). After saponification, resultant products were extracted three times with 1 ml each of n-hexane and subsequently brought to the analysis of components using both GC and GC–MS.

LCAs were also extracted from the lake sediments collected at the Canadian sites. Briefly, freeze-dried sediments were homogenized and extracted with DCM:MeOH (9:1, v:v) using a Dionex model ASE350 accelerated solvent extractor. Following evaporation of the solvent, the total lipid extracts were separated into neutral and acid fractions by elution through a LC-NH₂ SPE column using DCM:isopropyl alcohol (1:1, v:v) followed by ether with 4% acetic acid (v:v) as eluents, respectively. The neutral fractions were further separated into four fractions of increasing polarity by chromatography over a silica gel column packed with 35–70 μm particles using hexane, DCM, ethyl acetate:hexane (1:3, v:v) and MeOH as eluents. The second fractions (DCM fraction) containing LCAs were saponified using the same procedure as described above.

2.7. GC and GC–MS analyses

GC was conducted using a Shimadzu GC-2025 instrument equipped with FID for quantification of alkenone fractions. Two methods were applied with different columns and respective temperature programs: Agilent VF-200ms column (60 m × 0.25 mm ×
0.10 µm), 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 at 10 °C/min (held 10 min); Agilent CPSil5CB column (50 m × 0.32 mm × 0.12 µm), temperature program was 90 °C (2 min) to 255 °C at 40 °C/min, to 300 °C at 1 °C/min, and subsequently to 320 °C at 10 °C/min (held 10 min).

The use of the VF-200ms column was demonstrated by Longo et al. (2013) to significantly improve the separation for long-chain LCAs. However, C\textsubscript{37:4} alkenone and C\textsubscript{36:2} FAEE alkenoate co-eluted under these conditions, therefore the CPSil5CB column was used to separate both compounds clearly, quantify the ratio of C\textsubscript{37:4} to C\textsubscript{37:2} compounds, and calculate the abundances of C\textsubscript{37:4} and C\textsubscript{36:2} FAEE (Nakamura et al., 2014).

The LCAs and alkenoates were identified by Agilent 6890N GC instrument coupled to an Agilent 5975 inert XL MSD quadruple mass spectrometer (electron ionization: 70 eV; emission current: 350 µA; m/z 50–650). The VF-200ms column and the identical temperature program as for GC analysis were used for GC–MS analysis. Helium was the carrier gas in both GC and GC–MS.

The LCAs and alkenoates consist of di-, tri- and tetra-unsaturated homologues. Generally, di-unsaturated LCAs possess double bonds at Δ\textsuperscript{14} and Δ\textsuperscript{21} positions (Δ\textsuperscript{14,21}), tri-unsaturated LCAs have a third double bond at the Δ\textsuperscript{7} position (Δ\textsuperscript{7,14,21}) and the tetra-unsaturated alkenone has a fourth double bond at Δ\textsuperscript{28} (Δ\textsuperscript{7,14,21,28}) (Dillon et al., 2016;
Longo et al., 2016; Zheng et al., 2017). By using a GC column lined with
a VF-200ms column, isomers of tri-unsaturated LCAs and alkenoates
exhibit doublet-like peaks consisting of a left-hand peak and a right-
hand peak correspond to $\Delta^{7,14,21}$ (Delta isomer; isomer a) at $\Delta^{14,21,28}$ (Delta isomer; isomer b), respectively.

2.8. Temperature indices

The alkenone unsaturation indices ($U_{37}^K$, $U_{37}^{K'}$, $U_{38}^K$ Et, $U_{41}^K$ Me
and $U_{42}^K$ Et), alkenoate unsaturation indices ($U_{38}^A$ and $U_{38}^A$), methyl and
ethyl alkenoate ratio ($A_{37}/A_{38}$), and the isomeric ratio of alkenoates
(RIA$_{38}$) were all calculated based on biochemical profiles of Canadian
algal samples raised under diverse temperature regimes. Refer to
Nakamura et al. (2016) for original references for these indices. Finally,
RIA$_{38}$ was determined as the isomeric ratio of alkenoates (C$_{36:3}$FAEE)
according to the definition of RIK indices proposed by Longo et al.
(2016), calculated by the following equation; RIA$_{38}$ = C$_{36:3}$FAEE /
(C$_{36:3}$FAEE + C$_{36:2}$FAEE), where C$_{36:3}$FAEE and C$_{36:2}$FAEE are isomers.

3. Results

3.1. Haptophyte strains isolated from Canadian lakes

Haptophyte strains were isolated successfully from nearshore
sand or sediments of three central Canadian lakes, specifically lakes
Snakehole, Success and Deadmoose (Tables 1 and 2A). All basins were
saline lakes, and two exhibited a clearly defined thermocline accompanied by changes in pH and salinity (Supplementary Fig. S2). In contrast, algal isolation was unsuccessful for samples obtained from lake water and plankton net samples, even though marker genes were identified in some living algal samples and whole-water environmental samples of lakes Snakehole, Success and Waldsea (Table 1).

Strains isolated from Lake Snakehole were named as Sh 1 and 2 (Fig. 1A and B), two from Lake Success were named as Sc 1 and 2 (Fig. 1C and D), and three from Lake Deadmoose named as Dm 1, 2 and 3 (Fig. 1E–G). DNA sequences of SSU and LSU rRNA were amplified from seven isolated haptophytes and algal mixtures from environmental samples such as water, plankton net and sediment by using haptophyte-specific primers. The results of successful amplification are as shown in Table 2 with the GenBank accession numbers. DNA sequences obtained from environmental samples, the prefix E was added to the strain number (e.g., Sh E1). The LSU rRNA sequence data of the environment DNA sample from Lake Waldsea was coded Ws E1.

The phylogenetic analysis showed that DNA sequences of the Canadian lake isolates and algal mixture from environmental samples were composed of four groups: namely Group A including Sh E2, Sh 2, Sh 1 and Sh E1, Group B including Sc E2, Sc 1, Sc 2, Group C including Dm 2, Dm 3, Dm 1, Sc E1, Sc E3 and Group D including Ws E1. Group
A from Lake Snakehole occupied a unique phylogenetic position in the *I. galbana* clade. This phylogenetic position was suggested by both maximum likelihood tree analysis of SSU and LSU rRNA sequences of the Canadian haptophyte strains (Figs. 2 and 3). Groups B and C were included in the *I. galbana* clade. Ws E1 obtained from Lake Waldsea suggested the existence of *R. lamellosa* (Fig. 3). Overall, unialgal strains isolated from lakes Success and Deadmoose showed a swimming ability driven by flagella under microscopic analysis; whereas, that from Lake Snakehole did not (Supplementary Fig. S3). This observation is also supported by determinations of the phylogenetic positions of Groups A, B and C.

### 3.2. Growth characteristics of the Canadian lake haptophyte strains

Strains Sh 1, Sc 2 and Dm 2 showed similar temperature-dependent growth patterns when cultured over the range 5–25 °C (Fig. 4). In all strains, the logarithmic growth phase ended within 100 h and then proceeded to the linear growth phase above 15 °C up to 25 °C. The optimum growth temperature was 25 °C for Sc 2 and 20 °C for Sh 1 and Dm 2. Below 20 °C, the growth pattern of those three strains was similar. Algal growth also showed a clear lag phase slowly followed by the logarithmic growth phase with a low rate below 10 °C.

The strain Sh 1 showed the greatest preference for cold waters, with a high growth rate at 5 °C and suppressed growth at 25 °C (Fig. 4).
The strain Sc 2 showed an opposite trend to Sh 1, with low growth at low temperatures and increased growth rates at high temperatures. The strain Dm 2 showed intermediate properties. In addition to growth parameters, some strains exhibited physiological or morphological responses to changes in temperature. For example, the number of cells swimming decreased below 10 °C in strains Sc 2 and Dm 2 (data not shown), whereas the shape of some cells became more round than oblong (Supplementary Fig. S3).

3.3. LCAs and alkenoates in newly established Canadian lake haptophyte strains

All haptophyte strains (Table 2A) were analyzed for LCAs using a GC–FID approach (Supplementary Fig. S4). As there was minimal variation in GC–FID profiles for samples obtained from individual lakes, only one strain from each lake was presented as representative of the composition of LCAs and alkenoates including derivatives (Fig. 5, Supplementary Table S1).

The distributions of LCAs and alkenoates of the Canadian lake strains were characterized by the occurrence of major components such as C₃₁ methyl alkenones, C₃₈ ethyl alkenones, and relatively minor components such as C₃₉ and C₃₀ alkenones. Overall, these patterns were similar to those already published from other Isochrysidaceae strains (Rontani et al., 2004; Sun et al., 2007; Theroux et al., 2013; Nakamura
et al., 2014, 2016; Zheng et al., 2016). Additionally, C\textsubscript{38} methyl and C\textsubscript{39} ethyl LCAs were also detected as minor alkenone components. Furthermore, along with LCAs, C\textsubscript{38} methyl alkenoates (C\textsubscript{38} FAMEs) and C\textsubscript{36} ethyl alkenoates (C\textsubscript{36} FAEEs) were also detected as significant components in the non-saponified alkenone fractions (Fig. 5C).

Isomers of tri-unsaturated LCAs and alkenoates exhibited doublet-like peaks consisting of a left-hand peak and a right-hand peak that may correspond to $\Delta^{7,14,21}$ (isomer a) at $\Delta^{14,21,28}$ (isomer b), respectively (Fig. 5). Among the previously reported tri-unsaturated isomers of LCAs, only minor amounts of the $\Delta^{28}$ isomer of C\textsubscript{38:3} alkenone (C\textsubscript{38:3}Et in Fig. 5C; Supplementary Table S1) were identified in Canadian lake samples. However, the double bond positions are still considered tentative in our analysis, even though the identification of isomeric alkenones and alkenoates using DMDS treatment in Dillon et al. (2016) and Zheng et al. (2017) is a robust protocol.

There were also lake-specific characteristics in LCAs among strains grown at 20 °C. Specifically, Sh 1 was characterized by the production of extended LCAs that eluted after C\textsubscript{40} LCAs in GC–FID analysis (peaks 23–26 in Fig. 5). Previously, similar peaks have been reported as C\textsubscript{41} methyl and C\textsubscript{42} ethyl LCAs with two and three double bonds in samples isolated from Chinese inland saline lakes (Zhao et al., 2014). Peaks 23–26 were assigned as C\textsubscript{41} methyl- and C\textsubscript{42} ethyl-
alkenones by comparing elution patterns and mass spectra reported by Zhao et al. (2014). C_{41:3}^{Me} (peak 23) and C_{41:2}^{Me} (peak 24) were characterized by M^+ at m/z 584 and 586, respectively. Both compounds exhibited [M–18]^+ ion, indicating a methyl ketone. C_{42:3}^{Et} (peak 25) and C_{42:2}^{Et} (peak 26) exhibited [M^+] (m/z 598 and 600, respectively) and [M–29]^+ ion, indicating an ethyl ketone.

The sedimentary LCAs from lakes Snakehole, Success and Deadmoose showed similar LCA profiles to the cultured isolates with some notable differences. For instance, only the Snakehole sedimentary LCAs were characterized by the presence of C_{41:3}^{Et}. Meanwhile, the sedimentary LCA profiles showed consistently fewer numbers of LCAs compared to the culture isolates, lacking C_{36:3}^{FAME}, C_{36:2}^{FAME}, C_{36:3}^{FAEE}, C_{38:3}^{Et}, C_{38:3}^{Me}, C_{38:2}^{Me}, C_{39:3}^{Et}, C_{41:2}^{Me}, C_{42:3}^{Et}, and C_{42:2}^{Et}. The Snakehole sedimentary LCA profiles also lacked C_{36:4}^{FAME} and C_{36:4}^{FAEE} that were present in the culture samples. The Success sedimentary LCA profile also lacked C_{39:2}^{Me}, C_{40:3}^{Et}, and C_{40:2}^{Et} that were present in the culture samples. The Deadmoose sedimentary LCA profiles also lacked C_{40:3}^{Et}, C_{40:2}^{Et} that were present in the culture samples. Of those “culture-only” LCAs, C_{38} methyl- , C_{39} ethyl- , and above C_{40} alkenones were minor compounds in culture isolates, so their lack in sedimentary LCA profiles might be due to small amount of these compounds.
3.4. Growth temperature-dependent changes in alkenone and alkenoate compositions and the alkenone unsaturation index

The unsaturation indices of C\textsubscript{37}–C\textsubscript{42} LCAs were established using Canadian lake haptophyte strains of Sh 1, Sc 2 and Dm 2 (Table 3, Fig. 6, Supplementary Table S2). Importantly, \( U^K_{41} \) Me and \( U^K_{42} \) Et were available only for Sh 1 strain, because only Sh 1 produces C\textsubscript{41} and C\textsubscript{42} LCAs. Overall, all alkenone unsaturation indices increased above 10 °C, but were relatively invariant below that temperature. Such a low-temperature plateau in alkenone unsaturation index values between 5 and 10 °C is seen in other isolates (Conte et al., 1998; Versteegh et al., 2001; Nakamura et al., 2014).

Similar to findings with the LCAs, alkenoate unsaturation indices based on both methyl and ethyl alkenoates (\( U^A_{37} \) and \( U^A_{38} \), respectively) increased linearly with incubation temperature, whereas, RIA\textsubscript{38} and A\textsubscript{37}/A\textsubscript{38} decreased with growth temperature from 10 °C to 25 °C (Table 3, Fig. 7, Supplementary Table S2). Comparison of linear and second-order polynomial regressions of the alkenone and alkenoate indices (10 °C to 25 °C) revealed that the second-order polynomial regressions gave a slightly better fit due to a slight curvature of thermal relationships, for example, \( r^2 = 0.99 \) vs \( r^2 = 0.93 \), respectively (\( U^A_{37} \) of Sh 1).

4. Discussion
4.1. Isolation and establishment of new strains of alkenone-producing haptophytes from Canadian lakes

This study succeeded in the isolation and establishment of seven new strains of alkenone-producing haptophytes from Canadian saline lakes. Study basins (lakes Snakehole, Success and Deadmoose) are located immediately north of the North American freshwater and saline lakes where alkenone-producing microalgae were first identified based on SSU rRNA analysis (Toney et al. 2010, 2012). Interestingly, both Lakes Success and Deadmoose exhibited strong vertical stratification of both temperature and salinity, whereas shallow, but saline, Lake Snakehole seemed to be well-mixed due to vigorous wind at the time of sampling (Supplementary Fig. S2). These differences are consistent with the recent environmental survey of 106 lakes in this region that shows that salinity is the primary control on alkenone presence and concentration, with a secondary influence of stratification (Plancq et al., 2018).

Alkenones were most commonly present in deep lakes that ranged in salinity from 2.4 g/L to 44.4 g/L. Although there is no direct experimental evidence on the relationship between such environmental characteristics and the presence of alkenone-producers, the fact that isolation of haptophytes was only successful at sites with hypersaline deep waters suggest that chemical characteristics may be important at some phase of the haptophyte life cycle. In particular, high salinity is
often associated with strong anoxia in deep waters (stratified) or sediments (all lakes), consistent with the prior observation that LCAs are associated in lakes with anoxic bottom waters (e.g., Toney et al. 2010; Plancq et al., 2018).

The presence of flagella and swimming ability in isolates from deep, meromictic lakes Success and Deadmoose, but not isolates from shallower Lake Snakehole may suggest that access to anoxic water depends in part on the motility of the haptophytes present (Supplementary Fig. S3). On the other hand, non-motile strains might have a tolerance for dry conditions, since it was isolated from shore sand. However, as the isolation process employed in this study was just performed once in late September 2014 when the ambient temperature was very low, it is possible that cell growth rates were depressed and inadequate to establish high populations of alkenone-producing microalgae at some sites. Further, as there should be pronounced seasonal variation in haptophyte abundance, we speculate that it may be necessary to collect samples throughout a year from diverse habitats in each lake to fully characterize the presence of alkenone-producing taxa. Thus, the presence of alkenones in sites where viable populations were not isolated most likely reflects differences in the timing of haptophyte growth and alkenone deposition in sediments.

The phylogenetic analysis showed that the Canadian lake-isolates can be grouped into three groups, namely Group A including
the Lake Snakehole strains closely related to *I. litoralis* and *I. nuda*,

Group B including the Lake Success strains, and Group C including the Lake Deadmoose strains. The LSU rRNA sequences from Lake Snakehole occupy a unique phylogenetic position in the *I. galbana* clade (Fig. 3). Similar relationships were confirmed by the analysis of SSU rRNA sequencing data (Fig. 2). Here, Lake Snakehole strains are positioned near to *Dicrateria* sp. ALGO HAP49 although the *Dicrateria* sp. was renamed to *Isochrysis nuda* after Bendif et al. (2013). The microscopic observations also supported the phylogenetical analyses and showed that the Lake Snakehole strains are different species from *I. galbana*; whereas, the strains from Lake Success and Deadmoose are *I. galbana*.

4.2 Characteristics of LCA and alkenoate compositions

4.2.1. Tetra-unsaturated LCAs and alkenoates

Production of abundant tetra-unsaturated LCAs is considered a common feature of non-calcifying, LCA-producing haptophyte algae that are classified into Groups I and II (Theroux et al., 2010). This pattern appears to also hold for alkenone distributions obtained from either unialgal isolates or environmental samples from a diverse range of lake water salinity, ranging from highly saline brackish and saline inland waters to relatively dilute oligotrophic freshwater systems (Theroux et al., 2010; Longo et al., 2016) (Fig. 2). Among the
Isochrysidaceae, both genera *Ruttnera* and *Isochrysis* are known to produce relatively high amounts of tetra-unsaturated LCAs, whereas *Tisochrysis* produces only di- and tri-unsaturated LCAs (Nakamura et al., 2016). Accordingly, the newly isolated Canadian haptophytes strains, such as Sh 1, Sc 2 and Dm 2, were characterized by production of tetra-unsaturated LCAs, especially at lower temperatures. We infer that these results suggest that Sh 1, Sc 2 and Dm 2 are closely related to genus *Isochrysis* and note that this hypothesis is also consistent with phylogenetic trees based on SSU and LSU rRNA sequences (Fig. 2, Fig. 3).

4.2.2. Double bond position of tri-unsaturated LCA and alkenoate isomers

A small amount of the tri-unsaturated isomer of alkenone $C_{38:3\text{a}}$ Et was detected from newly isolated *Isochrysis* strains Sh 1, Sc 2 and Dm 2, while incomplete chromatographic separation with closely eluting major peak of $C_{38:3\text{a}}$ Et precludes accurate quantification of $C_{38:3\text{b}}$ Et (Fig. 5, Supplementary Table S1). Patterns of presence/absence of tri-unsaturated LCAs and alkenoates are considered to serve as chemotaxonomic characteristics for taxonomic assessments of alkenone producers. For example, in general, the LCA profiles of the Group I haptophytes are different from those of the Group II and III haptophytes, including the production of tri-unsaturated alkenone
isomers throughout both ethyl and methyl LCAs of the whole range of chain-lengths (Longo et al., 2016). Zheng et al. (2017) further observed *R. lamellosa* LG strain (Group II) and *E. huxleyi* Van 556 (Group III) do not produce tri-unsaturated LCAs, other than a relatively minor amount of C$_{38:3}$Et. In addition, confirmation of similar patterns (i.e., the sole occurrence of C$_{38:3}$Et isomer among known tri-unsaturated LCA isomers) in three genetically different strains of *Isochrysis* reinforces the idea that this pattern is a shared feature for representatives of both *Isochrysis* and *Ruttnera* genera within the Family Isochrysidaceae (Fig. 3).

Relative (%) abundance of C$_{36:3}$FAEE isomers differed between strains closely related to both *I. litoralis* and *I. nuda* (Sh 1) and those more closely related to *I. galbana* (Sc 2, Dm 2). In particular, Sh 1 exhibited a dominant α-type isomer of C$_{36:3}$FAEE, while the β-type isomer was recorded in both Sc 2 and Dm 2 strains (Supplementary Table S1). Interestingly, tri-unsaturated isomers were detected only in C$_{36:3}$FAEE, but not in C$_{36:3}$FAME. This result is in accordance with the sole occurrence of C$_{38:3}$Et isomers in LCAs. These data provide insights on the synthesis of LCAs, and alkenoates in particular, as unsaturation may be specific to a specific chain-length or methyl and ethyl group in the unsaturation at the position of Δ$^{28}$ in the Group II haptophytes.

Overall, the isolates from lakes Snakehole, Success and Deadmoose were from Group II haptophytes. However, while the LCA
profile of Sh 1 and Dm 2 showed the C_{38:3b} Et isomer, we detected no C_{38:3b} Et isomer from the sediment of Lake Snakehole. These results suggest that the presence/absence of tri-unsaturated isomers of alkenoates (Zheng et al., 2016), as well as their relative abundance and response to environmental temperatures, might serve as a chemotaxonomic feature of alkenone-producing haptophyte algae.

4.2.3. Unique occurrence of LCAs >C_{40} in the Sh 1 strain

The carbon chain length of LCAs in ocean sediments commonly ranges from C_{37} to C_{39}, whereas isolates from lake sediments sometimes contain C_{40} LCAs as minor components. The occurrence of LCAs longer than C_{40} are exceptionally rare as C_{41} and C_{42} LCAs have only been reported from two recent hypersaline lakes in the arid northwestern China (Zhao et al., 2014) and from the Cretaceous marine sediment (Cenomanian black shale, ca. 95 Ma) from western North Atlantic (Deep Sea Drilling Project (DSDP) Site 534; Farrimond et al., 1986).

In this context, the Sh 1 strain established in this study is the first cultured strain that is characterized as a producer of C_{41} and C_{42} LCAs (Fig. 5, Supplementary Table S1). The molecular phylogenetic tree of LSU and SSU rRNA gene sequences indicate that the Sh 1 strain is most closely related to both *I. litoralis* and *I. nuda* (Figs. 2 and 3). Further investigation on the alkenone profiles of *I. litoralis* and *I. nuda* will be necessary to clarify whether such chemotaxonomic
characteristic of $>C_{30}$ LCAs is specific to the Snakehole Lake strain, or whether it is also representative of *I. litoralis and I. nuda*. The present results also suggest that the Sh 1 strain might be a candidate taxon responsible for the production of $C_{41}$ and $C_{42}$ LCAs in the hypersaline lakes in the northwestern China reported by Zhao et al. (2014).

4.3. Changes in alkenone and alkenoate compositions with growth temperature

Similar to previous culture studies, our results show that the entire suite of alkenone homologues, as well as alkenoates, is engaged in the adjustment of alkenone unsaturation and greatly affected by growth temperature of alkenone-producing species (e.g., Prahl et al., 1988; Conte et al., 1998). However, the consistency in the alkenone and alkenoate unsaturation dependence on temperature in the lacustrine isolates is unusual relative to studies from marine isolates, in which a number of other factors have been cited as potentially interfering with the temperature dependence in cultures (e.g., Epstein et al. 1998, 2001; Conte et al., 1995, 1998; Popp et al., 1998; Laws et al., 2001). For example, previous reports suggested that alkenone unsaturation degree in some strains of *E. huxleyi* may also be affected by the difference in physiological conditions/status of cells. For example, the alkenone unsaturation degree increases (i.e. $U_{37}^{N}$ decreases) more in the stationary growth phase in comparison with the exponential growth
phase (Conte et al., 1995, 1998).

In this study, isolate cultures were all grown at the same time, under the same conditions, with an experimental design that controlled for temperature as the only variable (i.e., not nutrients, light, water chemistry, etc.). It is worth noting that similar haptophyte species in other lacustrine environments (e.g., Lake George, USA) have a similar temperature dependence, based on slopes (Fig. 8) with the isolate cultures (i.e. L. galbana CCMP715 and Dm 2). However, in this instance, the y-intercepts observed here differ from that of culture experiments, which suggests that similar biotic and abiotic conditions likely influence the y-intercept of this correlation as the marine findings, but not the temperature dependency (Toney et al. 2010, 2012).

The effects of other biotic and abiotic controls on the y-intercept would benefit from further research. Previous studies (Toney et al. 2010) show that an in situ temperature calibration could be derived for a given lake despite sampling over multiple years, seasons and water depths and that the temperature calibration was valid from 2 °C in more saline bottom waters to 25 °C in surface water. It is possible that the lacustrine algal taxa are better adapted to a variable environment, whereas marine taxa are used to a relatively constant environment. On an annual basis, lakes undergo more extreme variations than oceans, including pH (several units), photon flux (near-surface irradiance to zero at depth), and oxygen (over-saturation to microaerobic conditions
over 10–20 m); whereas such variables are more stable in both time and space in many marine systems. While these differences in observed environmental conditions may hold due to the difference between reported marine haptophyte cultures versus lacustrine haptophyte cultures, our data suggest that the temperature dependency of the alkenone and alkenoate unsaturation is consistent in both culture and environmental settings for the lake haptophytes.

4.4. Comparison of $\Delta^3\text{SST}$ calibrations with other species and the lacustrine alkenone profiles: Implications for environmental reconstruction

The $\Delta^3\text{SST}$-temperature calibrations obtained for the three newly isolated Canadian haptophyte strains (Sh 1, Sc 2, Dm 2) can be compared with those known previously for other strains of Isochrysidales (Fig. 8). *E. huxleyi* 55a represents a typical planktonic marine producer and serves as a global marine SST calibration (Prahl and Wakeham, 1987). Calibrations of our three Canadian haptophyte cultures most closely resemble that of *I. galbana* CCMP715 (Theroux et al., 2013) rather than *R. lamellosa* (Nakamura et al., 2014) and *T. lutea* (Nakamura et al., 2016). While similar calibrations were observed at the genus and species-level, variations within the *Isochrysis* clade become obvious with the comparison of multiple strains enabled by addition of our new cultures. Consequently, future work on *I. litoralis*
and *I. nuda* is critical to reinforce the potential species-specific characteristics of strains in the *Isochrysis* clade (i.e. higher temperature sensitivity of Sh 1 strain and production of chain lengths >C₄₀).

Application of Sh 1 and Sc 2 calibrations to the $U_{37}^K$ index inferred from the sediments of lakes Snakehole and Success gives modern temperature values of 11.2 °C and 10.3 °C, respectively (Fig. 8). This reconstructed temperature corresponds to a water column temperature above the thermocline in Lake Success in autumn (Supplementary Fig. S2). However, all $U_{37}^K$ values of strain Dm 2 (> – 0.35) were higher than that of environmental samples obtained from Lake Deadmoose (–0.41 as shown by a horizontal line), which suggests that those values are within range of expected environmental temperatures (Fig. 8). To understand these results, we speculate that other strains which possess a high ability to produce C₃₇:₄ LCAs may have contributed to the C₃₇:₄ alkenone pool in sediments. Alternately, we suggest that although the slope of the calibration is defined by temperature dependency, other lake properties, such as nutrients, salinity, etc., may regulate the exact y-intercept. These findings suggest that an in situ calibration (e.g., Toney et al., 2010; 2012) may still be the best approach to capture natural variations in LCA distributions and temperature sensitivity among alkenone producers.
5. Conclusions

Seven algal strains were successfully isolated from three salt lakes in Saskatchewan, Canada, and used to establish unialgal cultures. Those strains were classified into the genus *Isochrysis* clade of haptophytes according to SSU and LSU rRNA sequence analysis. One of strains, Sh 1 from Lake Snakehole, was closely related to both *I. litoralis* and *I. nuda* according to its phylogenetic position and morphological characteristics (i.e., mucoid polysaccharide sheath). The Sh 1 strain exhibited a unique alkenone composition with presence of C_{41} and C_{42} LCAs. This is the first finding of C_{41} and C_{42} LCAs in living haptophytes and the identification of alkenone producer from Canadian saline lakes.

The alkenone and alkenoate distributions that characterize the newly isolated haptophyte strains are in good agreement with their taxonomic position as Group II haptophytes and relative other known species. For example, the production of tetra-unsaturated alkenone and alkenoates are common features of the family Isochrysidaeae (except for *T. lutea*), while Sh 1 strain (closely related to both *I. litoralis* and *I. nuda*) has a unique alkenone/alkenoate distribution (i.e. >C_{40} LCAs, C_{36:3} FAEE compositions) compared to the Sc 2 and Dm 2 strains, which are closely related to *I. galbana*. Comparison of the $U^{K}_{37}$-growth temperature calibrations among various species also showed that Sh 1, Sc 2 and Dm 2 strains isolated from Lakes Snakehole, Success and
Deadmoose were similar to those from _I. galbana_ and _R. lamellosa_ of the _Isochrysis_ clade. Overall, it is hoped that the findings of this study will promote further study on the reconstruction of paleotemperature using the alkenone paleothermometer in inland lakes, a task which is substantially less developed in comparison to marine alkenone-paleothermometer studies. Future studies should also include a more comprehensive lake sampling program, to better characterize variability in the timing and spatial extent of alkenone-producing haptophyte populations.

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Geochimica et Cosmochimica Acta 64, 469–477.


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Table captions:

Table 1. List of Canadian lakes where the isolations of microalgae and LSU rRNA were performed from lake water and sediments with corresponding environmental data for the lakes.

Footnote: "Y and N indicate yes (positive) and no (negative), respectively."

"The results of alkenone analysis are also listed according to literature (Toney et al. 2010).

Table 2. List of seven algal strains established as unialgal cultures (A) and six environmental samples of which haptophyte marker genes were successfully amplified (B) accompanied with the names of lakes, materials used for isolation, salinity, culture medium used for algal isolation, and the GenBank accession numbers of SSU and LSU rRNAs registered in GenBank obtained by amplification.

Footnote: "Salinity of MA-ESM medium used for establishing unialgal strains and cultivation of algal mixture from environmental samples, respectively.

Table 3. Correlation equations between the LCAs and alkenoate unsaturation indices and the growth temperature.
Footnote: a These data were obtained from laboratory cultured alkenone-producing haptophyte strains Sh 1, Sc 2 and Dm 2 which had been isolated from three Canadian salt lakes of Snakehole, Success and Deadmoose, respectively.

b The correlation equations were obtained by calculation of the linear and second-order polynomial regressions with correlation coefficient (R2). For graphs, see Figs. 6 and 7 on the alkenone and alkenoate unsaturation indices including related parameters, respectively.

Figure legends:

Fig. 1. Light micrographs of seven algal strains established as unialgal cultures after the isolation of algae from three Canadian saline lakes. A and B, two strains isolated from Lake Snakehole (Sh), named as Sh 1 and Sh 2; C and D, two strains isolated from Lake Success (Sc), named as Sc 1 and Sc 2; E–G, three strains isolated from Lake Deadmoose (Dm), named as Dm 1, Dm 2 and Dm 3. Scale bar in each photo is 50 μm.

Fig. 2. Maximum likelihood tree of the three Canadian lakes haptophyte strains constructed by the analysis of SSU rRNA gene sequences. Six samples analyzed are prepared from strains Sh 1, Sh 2, Sc 1, Sc 2 and Dm 2, Dm 3 which were isolated from Lake
Snakehole, Success and Deadmoose, respectively. The values of bootstrap percentage (BP) and Bayesian posterior probability (BPP) are expressed as BP/BPP on each note by selecting values with only BP >P50% and BPP>P0.5.

**Fig. 3.** Maximum likelihood tree of the three Canadian lakes haptophyte strains constructed by the analysis of D1–D2 region of LSU rRNA gene sequences. For the values of BP, see Fig. 2. Symbols: ●, samples prepared from the seven isolated haptophyte strains of Sh 1 and Sh 2, Sc 1 and Sc 2, Dm 1, Dm 2 and Dm 3 isolated from Lakes Snakehole (Sh), Success (Sc) and Deadmoose (Dm), respectively; ▲, six environmental samples expressed as Sh E1 and Sh E2, Sc E1, Sc E2 and Sc E3, and Ws E1 extracted from the algal mixture cultivated from environmental samples of three Canadian high salt lakes of Snakehole (Sh), Success (Sc) and Waldsea (Ws), respectively.

**Fig. 4.** Growth curves of three haptophyte strains established as unialgal cultures of Sh 1 (isolated from Lake Snakehole), Sc 2 (isolated from Lake Success) and Dm 2 (isolated from Lake Deadmoose) grown under different temperatures. Comparison of growth curves among various temperatures at 5 (×), 10 (◆), 15 (■),
20 (▲) and 25 °C (●) in the strains Sh 1 (A), Sc 2 (B) and Dm 2 (C).

Comparison of growth curves among the strains of Sh 1 (○), Sc 2 (△) and Dm 2 (□) grown at 5 (D), 10 (E), 15 (F), 20 (G) and 25 °C (H).

The y-axes of graphs are logarithmic scales. Error bars denote the standard deviation of triplicate cultures.

**Fig. 5.** Partial GC-FID chromatograms of LCAs and alkenoates extracted from the lake sediments (rows A and B) and the isolated haptophyte strains of Sh 1, Sc 2 and Dm 2 (rows C and D) from the three Canadian saline lakes of Lake Snakehole, Success and Deadmoose, respectively. Samples B and D were prepared by removing alkyl alkenoates after saponification of alkenone fractions A and C, respectively. Peak assignments: 1. C\textsubscript{36:4} FAME, 2. C\textsubscript{36:3} FAME, 3. C\textsubscript{36:2} FAME, 4. C\textsubscript{36:4} FAEE, 5. C\textsubscript{36:3a} FAEE, 6. C\textsubscript{36:3b} FAEE, 7. C\textsubscript{36:2} FAEE, 8. C\textsubscript{37:4} Me, 9. C\textsubscript{37:3} Me, 10. C\textsubscript{37:2} Me, 11. C\textsubscript{38:4} Et, 12. C\textsubscript{38:3a} Et, 13. C\textsubscript{38:3b} Et, 14. C\textsubscript{38:2} Et, 15. C\textsubscript{38:3} Me, 16. C\textsubscript{39:3} Me, 17. C\textsubscript{39:2} Et, 18. C\textsubscript{39:3} Et, 19. C\textsubscript{40:3} Et, 20. C\textsubscript{40:2} Et, 21. C\textsubscript{40:3} Me, 22. C\textsubscript{40:2} Me, 23. C\textsubscript{41:3} Me, 24. C\textsubscript{41:2} Me, 25. C\textsubscript{42:3} Et, 26. C\textsubscript{42:2} Et. *: unidentified peaks.

**Fig. 6.** Relationship between growth temperature and the alkenone unsaturation indices in the three haptophyte strains isolated from Canadian saline lakes, namely Sh 1, Sc 2 and Dm 2 isolated from
lakes Snakehole, Success, and Deadmoose, respectively. Correlation equations (y) are shown in each figure with correlation coefficients ($R^2$). The definitions of various alkenone and alkenoate unsaturation indices are shown in the panel.

**Fig. 7.** Relationship between growth temperature and the alkenoate unsaturation indices $U^A_{37}$ and $U^A_{38}$, the ratio of isomeric alkenoates (RIA$_{38}$) and the ratio of methyl to ethyl alkenoates ($A_{37}/A_{38}$) in the three established haptophyte strains isolated from Canadian saline lakes, namely Sh 1, Sc 2 and Dm 2. Correlation equations (y) are shown in each figure with correlation coefficients ($R^2$). For the definitions of the unsaturation indices and the other parameters, see the inset (in the right bottom) of Fig. 6.

**Fig. 8.** Comparison of the culture-based $U^K_{37}$-temperature calibrations among the newly obtained strains Sh 1, Sc 2 and Dm 2 (classified into Isochrysidaceae, Haptophyta) and those of the other species reported in literatures. For reference, $U^K_{37}$-values in environmental samples extracted from sediments of Lakes Snakehole, Success and Deadmoose are indicated by colored horizontal lines at the values of $-0.38$, $-0.41$ and $-0.30$, respectively. Symbols: Sh 1 (▲ with a red line), strain Sh 1 isolated from Lake Snakehole; Sc 2 (□ with a
dashed line), strain Sc 2 isolated from Lake Success; Dm 2 (○ with a
dotted line), strain Dm 2 isolated Lake Deadmoose; a, *E. huxleyi* 55a
(Noëlaerhabdaceae), representative of typical planktonic marine
species (Prahl and Wakeham, 1987); b, *T. lutea* CCMP 463
(Nakamura et al., 2016); c, *T. lutea* NIES-2590 (Nakamura et al.
2016); d, *I. galbana* CCMP 715 (Nakamura et al., 2014). e, *R.
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Table 1.

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### Table 3.

| Unsaturation indices<sup>a</sup> | Temperature range and type of fit |  |  |  |  |  |  |
|----------------------------------|----------------------------------|---|---|---|---|---|
|                                  |                                  | Sh 1 | R² | Sc 2 | R² | Dm 2 | R² |
| **Alkenone indices**             |                                  |      |    |      |    |      |    |
| £<sub>37</sub>                   | 10–25; polynom.                  | -0.001<sup>2</sup>T + 0.085T - 1.21 | 1.00 | 0.001<sup>2</sup>T + 0.0046T - 0.43 | 1.00 | 0.0006<sup>2</sup>T + 0.0086T - 0.48 | 0.97 |
|                                  |                                  | 10–25; linear                        | 0.049T - 0.93 | 0.99 | 0.039T - 0.70 | 0.99 | 0.028T - 0.63 | 0.96 |
| £<sub>37</sub>                   | 10–25; polynom.                  | 0.0009<sup>2</sup>T - 0.016T + 0.17 | 0.99 | 0.0017<sup>2</sup>T - 0.043T + 0.35 | 0.99 | 0.0004<sup>2</sup>T - 0.0068T + 0.11 | 0.93 |
|                                  |                                  | 10–25; linear                        | 0.014T - 0.073 | 0.92 | 0.017T - 0.11 | 0.82 | 0.066T - 0.0005 | 0.87 |
| £<sub>37</sub>                   | 10–25; polynom.                  | -0.001<sup>2</sup>T + 0.094T - 1.30 | 1.00 | -0.0007<sup>2</sup>T + 0.046T - 0.76 | 1.00 | 0.002<sup>2</sup>T + 0.0145T - 0.57 | 0.96 |
|                                  |                                  | 10–25; linear                        | 0.033T - 0.82 | 0.95 | 0.022T - 0.57 | 0.98 | 0.021T - 0.61 | 0.96 |
| £<sub>38</sub>Et                 | 10–25; polynom.                  | -0.001<sup>2</sup>T + 0.082T - 0.86 | 1.00 | 0.0021<sup>2</sup>T - 0.022T - 0.21 | 1.00 | 0.002<sup>2</sup>T - 0.038T + 0.06 | 0.94 |
|                                  |                                  | 10–25; linear                        | 0.048T - 0.59 | 0.99 | 0.051T - 0.78 | 0.96 | 0.0342T - 0.50 | 0.87 |
| £<sub>41</sub>Me                 | 10–25; polynom.                  | 0.0011<sup>2</sup>T - 0.026T + 0.25 | 0.91 |      |      |      |      |
|                                  |                                  | 10–25; linear                        | 0.012T - 0.041 | 0.79 |      |      |      |      |
| £<sub>42</sub>Et                 | 10–25; polynom.                  | 0.0002<sup>2</sup>T + 0.014T - 0.047 | 0.98 |      |      |      |      |      |
|                                  |                                  | 10–25; linear                        | 0.023T - 0.11 | 0.98 |      |      |      |      |
| **Alkenoate indices**            |                                  |      |    |      |    |      |    |
| £<sub>37</sub>                   | 10–25; polynom.                  | -0.0029<sup>2</sup>T + 0.15T - 1.85 | 0.99 | -0.0003<sup>2</sup>T + 0.091T - 1.33 | 1.00 | 0.0006<sup>2</sup>T + 0.053T - 1.23 | 0.99 |
|                                  |                                  | 10–25; linear                        | 0.053T - 1.07 | 0.93 | 0.081T - 1.25 | 1.00 | 0.075T - 1.41 | 0.99 |
| £<sub>38</sub>                   | 10–25; polynom.                  | -0.0024<sup>2</sup>T + 0.14T - 1.32 | 1.00 | -0.0012<sup>2</sup>T + 0.099T - 0.85 | 1.00 | 0.0006<sup>2</sup>T + 0.030T - 0.36 | 0.98 |
|                                  |                                  | 10–25; linear                        | 0.053T - 0.67 | 0.96 | 0.057T - 0.52 | 0.99 | 0.052T - 0.53 | 0.98 |
| RIA<sub>38</sub>                 | 10–25; polynom.                  | 0.0013<sup>2</sup>T - 0.064T + 0.90 | 0.98 | -0.0002<sup>2</sup>T - 0.0026T + 0.83 | 0.82 | -0.0003<sup>2</sup>T + 0.0004T + 0.83 | 0.92 |
|                                  |                                  | 10–25; linear                        | -0.019T + 0.54 | 0.90 | -0.0096T + 0.88 | 0.81 | -0.011T + 0.92 | 0.91 |
| A<sub>37</sub>/A<sub>38</sub>    | 10–25; polynom.                  | 4.3×10<sup>-5</sup><sup>2</sup>T + 0.0081T + 0.22 | 0.98 | 0.0006<sup>2</sup>T - 0.036T + 0.75 | 0.99 | -0.0003<sup>2</sup>T - 0.0054T + 0.52 | 0.98 |
|                                  |                                  | 10–25; linear                        | -0.0066T + 0.21 | 0.98 | -0.016T + 0.59 | 0.97 | -0.017T + 0.61 | 0.97 |
Highlights

Novel LCA-producing strains from Canadian lakes established as unialgal cultures.

Seven strains were categorized in the *Isochrysis* clade using genomic analysis.

One strain produces C$_{41}$ and C$_{42}$ alkenones that are sensitive to temperature changes.

New alkenoate-based indices from isolated strains for temperature reconstructions.
Graphical Abstract

Max Depth = 60m