Organic Geochemistry 156 (2021) 104223

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

Sources and seasonality of long-chain diols in a temperate lake (Lake Geneva)

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ARTICLE INFO

Article history:
Received 27 November 2020
Received in revised form 15 February 2021
Accepted 17 March 2021
Available online 23 March 2021

Keywords:
Long-chain diols
Lake Geneva
18S rRNA gene

ABSTRACT

Long-chain diols (LCDs) are lipids commonly found in freshwater environments. They are produced in lake waters and in low water-flow regions of rivers but their sources and the controls on their abundance are poorly constrained. To be able to use LCD as environmental proxy (e.g. for reconstructing lake temperature and as a freshwater indicator in marine systems) we need to understand the sources of these LCDs and the processes controlling their abundance and distribution. Therefore, we performed a seasonal study of suspended particulate matter in Lake Geneva, a temperate lake at the border of France and Switzerland in 2017–2018. LCDs were most abundant in lake surface water from late spring to early autumn, coinciding with the thermal stratification of the water column. Their distribution varied throughout the year, which points towards multiple producers. Incubation of lake water with ^{13}C-labelled bicarbonate only showed uptake of inorganic carbon in LCDs during their peak seasonal abundance. An 18S rRNA gene amplicon analysis revealed that eustigmatophytes, known producers of LCDs, are present in Lake Geneva and show the same seasonal trend in abundance as the LCDs, indicating that these algae are likely the most important producers of LCDs in this lake. In combination with previous studies our results suggest that LCDs show potential to trace changes in lake water-column stratification, and validate the use of the C_{32} 1,15-diol as a proxy for freshwater input from rivers and lakes in marine sediments.

1. Introduction

After their discovery in Black Sea sediments (de Leeuw et al., 1981), long-chain diols (LCDs) have commonly been found in marine environments (e.g., Versteegh et al., 1997, 2000; Schmidt et al., 2010; Rampen et al., 2012, 2014b; de Bar et al., 2016; Lattaud et al., 2017a; Ruan et al., 2017; Balzano et al., 2018; Zhu et al., 2018; Gal et al., 2018). They are composed of a long *n*-alkyl chain (with ≥ 26 number of C atoms) containing one alcohol group at C_1 and one at a mid-chain position (e.g., carbon number 12–17). In marine settings 1,13- and 1,15-diols often predominate. LCDs preserved in marine sediments can be used to reconstruct past sea surface temperatures with the application of the Long-chain Diol Index (LDI, Rampen et al., 2012), a proxy which has now been used in several regions worldwide (Naafs et al., 2012; Smith et al., 2013; Plancq et al., 2014; Becker et al., 2015; Rodrigo-Gámiz et al., 2015; de Bar et al., 2016; dos Santos et al., 2016; Jonas et al., 2017; Kotthoff et al., 2017; Warnock et al., 2018; Lattaud et al., 2018b). However, in coastal regions like the Kara Sea (Lattaud et al., 2017) or the Portuguese margin (de Bar et al., 2016), LDI-derived sea surface temperatures in surface sediments do not match measured temperatures. Versteegh et al. (1997) and Rampen et al. (2012) observed that in proximity of estuaries and river deltas, the distribution of LCDs tends to change towards a dominance of the C_{32} 1,15-diol, suggesting an influence of the nearby rivers on LCD distributions. Indeed, LCDs are also common in rivers (de Bar et al., 2016; Lattaud et al., 2017, 2018; Häggi et al., 2019) and lakes (Cranwell et al., 1987; Robinson et al., 1989; Xu et al., 2007; Shimokawara et al., 2010; Castaneda et al., 2011; Zhang et al., 2011; Romero-Viana et al., 2012; Atwood et al., 2014; Rampen et al., 2014b; Villanueva et al., 2014; Zhang et al., 2015; Zhu et al., 2019; Castañeda et al., 2009; van Bree et al., 2018; Häggi et al., 2019; Toney et al., 2020) with generally the 1,15-

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https://doi.org/10.1016/j.orggeochem.2021.104223
0146-6380/

diols dominating the LCD distribution. Lattaud et al. (2017) used this observation to propose a new riverine input proxy based on the fractional abundance of the C_{32} 1,15-diol ($F_{C32\ 1,15}$), which was found to increase at increasing influence of the river outflow. Lattaud et al. (2018) showed that LCDs are likely produced in stable, low flow freshwater areas such as alluvial lakes and ponds and not within flowing rivers or floodplain soils. However, the environmental controls on their production are unknown, which hinder the application of $F_{C32\ 1,15}$ in marine coastal environments.

The most likely producers of 1,13- and 1,15-diols in freshwater environments are eukaryotic algae belonging to the Eustigmatophyceae (eustigmatophytes) since the LCD distributions in suspended particulate matter (SPM) collected from rivers, lakes and their sediments fit with those of eustigmatophyte cultures, showing a prevalence of the C_{32} or C_{30} 1,15-diols (e.g., Rampen et al., 2014b). Indeed, eustigmatophyte sequences have been detected in Lake Tanganyika (De Wever, 2006), although no direct link between the presence of LCDs and eustigmatophyte was made in that study. Furthermore, in the water column of the East African Lake Chala, the water depth distribution of eustigmatophyte 18S rRNA gene copies matched that of the LCD concentrations, i.e., a maximum in abundance occurred at 9 m depth (Villanueva et al., 2014). This study also showed that the eustigmatophyte 18S rRNA gene sequences detected were not closely related to the known LCD-eustigmatophyte producers available in culture, suggesting uncultured eustigmatophytes might be an important LCD source. Following this study, van Bree et al. (2018) did a survey of LCDs in suspended particulate matter (SPM) from Lake Chala, collected at five depths from September 2013 to January 2015, and compared them to phytoplankton cell counts for the same depths. They showed that LCDs were most abundant during periods of water column stratification, but no direct link could be made with the phytoplankton cell counts, likely because this approach overlooked cells of the size range of eustigmatophyte algae (<3 μm). Hence, it remains unclear whether eustigmatophytes are a common source of LCDs in lakes, and if so which biological and environmental factors control LCD abundance and distributions.

Another group of LCDs are those who are dominated by members having the mid-chain hydroxy group attached to an even-membered carbon atom of their long *n*-alkyl chain (1,12- and 1,14-diols). A likely source of the commonly encountered 1,14-diols in open marine settings are diatoms of the genus *Proboscia* (Bacillariophyceae – diatoms) (Sinninghe Damsté et al., 2003; Rampen et al., 2007). The marine heterokont alga *Apedinella radians* (Dictyochophyceae), mainly occurring in estuarine and brackish areas, also produces C_{28}, $C_{30,}$ and C_{32} 1,14-diols. The 1,14-diols are also frequently encountered in freshwater systems (Rampen et al., 2014b; Lattaud et al., 2017, 2018), although their sources are unclear. Only *Pseudostaurastrum enorme*, a freshwater eustigmatophyte species that occurs in lakes, is known to produce C_{28} 1,14- and C_{30} 1,14-diols in culture, although 1,15-diols still predominate (Rampen et al., 2014b). Therefore, also for this group of LCDs biological sources remain poorly constrained.

To further constrain the sources of LCDs and environmental controls on their production in lakes, we investigated the abundance and distribution of LCDs in suspended particulate matter in a seasonally stratified lake in a temperate climate zone, i.e., Lake Geneva in Switzerland/France. Incubation of surface water with ^{13}C-labelled bicarbonate was applied to monitor in situ production of LCDs in the lake. Furthermore, we analyzed the eukaryotic community composition and abundance using 18S rRNA gene amplicon sequencing and quantitative PCR (qPCR) in order to constrain potential biological sources of LCDs.

2. Material and methods

2.1. Study site

Lake Geneva is a large sub-alpine lake located at an altitude of 372 m on the border between France and Switzerland. It is the largest lake of Western Europe with a surface area of 580 km^2, a volume of 89 km^3, and a maximum depth of 310 m (Fig. 1). The lake is meso-oligotrophic and monomictic with its principal mixing period from late fall to early spring, which can either be complete (the whole water column) or partial (the upper 100 m only) (Rimet et al., 2015). The last complete mixing of the water column occurred in winter 2012 (Rapport CIPEL 2017). The Rhône River discharges water to the eastern tip of the lake and flows southwestwards (Oesch et al., 2005). Winter (December to February) is characterized by low water temperatures (5–7 °C), high nutrient

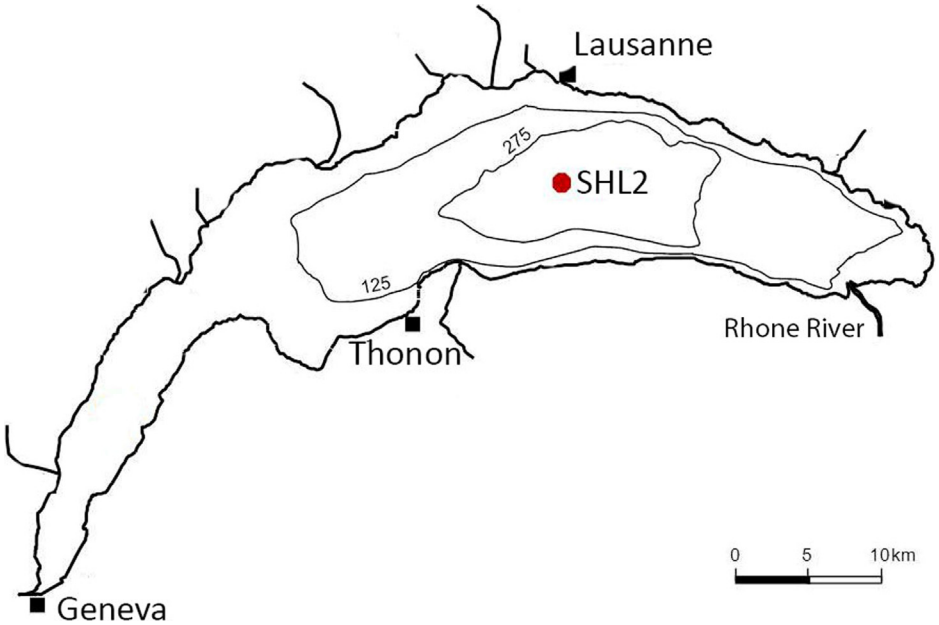

Fig. 1. Map showing the location of the sampling site (SHL 2) in Lake Geneva.

availability and low zoo- and phytoplankton biomass with mainly diatoms present, such as *Stephanodiscus neoastrae* (Anneville et al., 2002). Spring blooms typically occur between March and June, after the onset of thermal stratification, leading to a decrease in water transparency (Anneville et al., 2002). These spring blooms are dominated by phytoplankton with indicator species belonging to Bacillariophyta (diatoms) and Cryptophyceae (Anneville et al., 2002). A characteristic clear-water phase occurs in June as a result of zooplankton grazing on phytoplankton and depletion of dissolved silica (Si) in the upper water column hampering further diatom growth. Following further warming of the surface water, an early summer phytoplankton bloom develops and a shift to microplankton grazers occurs. Once the stratified upper water column becomes nutrient depleted, a late summer phytoplankton community develops, which is typically dominated by the dinoflagellate *Ceratium hirundinella* and the diatom *Fragilaria crotonensis*, both known to be adapted to low nutrient levels (Anneville et al., 2002). Once the onset of mixing ends summer stratification, an autumn phytoplankton community arises with the diatom *Stephanodiscus binderanus* and the cyanobacterium *Planktothrix rubescens* as indicator species. These species thrive during water column mixing and lower temperatures and benefit from renewed nutrient upwelling and low insolation of the surface water (Anneville et al., 2002).

2.2. Sampling and incubation

Ca. 60 L of surface water was collected with a bucket at the Observatoire des lac alpins (UMR CARRTEL) sampling station SHL 2 (Commission internationale pour la protection des eaux du Léman contre la pollution, 2017) at the deepest part of the lake (Fig. 1) on five sampling dates between August 2017 and June 2018 covering one annual cycle. SHL 2 is a long-term monitoring station for Lake Geneva for parameters such pH, chlorophyll and in situ lake temperature (Table 3, http://si-ola.inra.fr). In situ lake temperature profiles were measured with a CTD during sampling and used to determine the period of thermal stratification. At each sampling time, 20 L of the water was used to analyze LCDs and 40 L was used for labeling experiments. For these labelling experiments, 40 L of lake surface water were distributed over two 20 L Nalgene bottles and 100 mg ^{13}C-labelled bicarbonate (natural abundance at the Rhone River mouth inflow and at the outflow ~ 110 mg L^{-1}, Aucour et al., 1999; Cambridge Isotope Laboratories, Inc., USA) was added (which represent ca. 5% of the total natural bicarbonate) to each bottle. The bottles were incubated for 52 h (normal day/night cycle) following Lattaud et al. (2018) and Suominen et al. (2019). Bicarbonate was chosen for labelling, as the putative LCD producers are likely phototrophic organisms (eustimatophytes), and, as such, likely consume dissolved inorganic carbon (DIC) as their carbon source. SPM from the time-series experiment (unlabelled 20 L) and the incubation experiment (labelled 40 L) were filtered and analyzed separately. Half of the filter (from both incubation and the time-series) was used for lipid analysis and 1/8th of the filter was used for DNA extraction. All filters were kept frozen at −10 °C for 3 days and subsequently kept at −80 °C until extraction. The unlabelled filter (20 L) sampled in August was also analyzed for ^{13}C-signature of LCDs as it was the only one with sufficient LCDs to analyses their stable carbon isotopic composition.

2.3. Lipid extraction and ***LCD analysis***

Filters from the time series (direct filtering of 20 L bottles) and the incubation experiments from Lake Geneva were extracted as described in Lattaud et al. (2018). In short, the filters were base hydrolyzed using 1 N KOH/methanol (MeOH), then acid hydrolyzed using 1.5 N HCl/MeOH after which an internal standard was added (C_{22} 7,16-diol). The hydrolyzed extracts were subsequently separated into three fractions: apolar, ketone and polar fractions (containing the LCDs) using hexane : dichloromethane (DCM) (9 : 1, v/v), hexane : DCM (1 : 1, v/v) and DCM : methanol (1 : 1, v/v), respectively.

For detection and quantification of the LCDs, the polar fractions were analyzed by gas chromatography – mass spectrometry (GC–MS) as described in Lattaud et al. (2018). Briefly, the fraction was silylated with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (10 μL each) and LCDs were identified and quantified via Single Ion Monitoring (SIM) of fragment ions m/z 299.3 (C_{28} 1,14-diol, C_{30} 1,16-diol), 313.3 (C_{28} 1,13-diol, C_{30} 1,15-diol), 327.3 (C_{30} 1,14-diol), 339.3 ($C_{32:1}$ 1,15-diol) and 341.3 (C_{30} 1,13-diol, C_{32} 1,15-diol, C_{34} 1,17-diol) ions (Versteegh et al., 1997; Rampen et al., 2012). Absolute concentrations were calculated using the C_{22} 7,16-diol as internal standard (m/z = 187.2). SPM from the time-series experiment (unlabelled 20 L) and the incubation experiment (labelled 40 L) were filtered and analyzed separately.

The fractional abundance of each LCD was calculated as follows, where × represents a LCD isomer [Eq. (1)]:

$$F_{diolx} = \frac{[Diolx]}{\sum[alldiols]} \quad (1)$$

2.4. Stable carbon isotope analysis of LCDs

Before stable carbon isotope analysis, the different LCD isomers were isolated from the polar fraction of the incubation experiments as well as the LCDs collected during August in the unlablled bottle using semi-preparative normal phase High Performance Liquid Chromatography (HPLC) following the method of de Bar et al. (2016) and Lattaud et al. (2019). To this end, the polar fraction was dissolved in 150 μL hexane : isopropanol (99 : 1, v/v) and filtered over a PTFE filter (0.45 μm pore size) prior to injection on an 1260 infinity Liquid Chromatography (LC) system (Agilent) equipped with a thermostated auto-injector, and a Foxy R1 fraction collector (Teledyne Isco, Lincoln, NE, USA). The different diol isomers were separated over a Sunfire silica column (Waters, 250 mm × 10 mm; 5 μm) at room temperature. After 35 min at 82% A (hexane) and 18% B (hexane : isopropanol, 9 : 1, v/v) the mobile phase was adjusted to 100% B in 1 min and then held at 100% B from 35 to 61 min. Finally, the column was reconditioned with 82% A and 18% B at 3 mL min^{-1}. Fractions were collected every 30 s from 30 to 44 min and analyzed by GC–MS as described above. The LCDs of interest eluted between 33.5 and 37.5 min and were collected in three pools. Pool 1 from 33.5 to 35 min (containing 100% of the C_{32} 1,15-diol originally present), pool 2 from 35.5 to 36 min (containing 80% of the C_{30} 1,15-diol originally present) and pool 3 from 36.5 to 37.5 min (containing 100% of the C_{30} 1,13- and C_{28} 1,13-diols). De Bar et al. (2016) showed that if more than 80% of the compound was retrieved, carbon isotopic fractionation due to semi-preparative HPLC is < 0.5‰, i.e., within the analytical error of a typical gas chromatography–isotope ratio mass spectrometry (GC–irMS) analysis. The pooled fractions were analyzed using a ThermoFinningan Delta^{PLUS} isotope ratio monitoring mass spectrometer coupled to an Agilent 6890 GC via a Combustion III interface. The gas chromatograph was equipped with a fused silica capillary column (25 m × 320 μm) coated with CP Sil-5 (film thickness = 0.12 μm) with helium as carrier gas (2 mL min^{-1}). The LCDs for isotope analysis were silylated as described above using BSTFA with a known δ^{13}C value of –32.2 ± 0.5‰. Subsequently, the LCDs were injected splitless at an oven temperature of 70 °C (injector temperature was 250 °C), then the oven was programmed to 130 °C at 20 °C min^{-1}, and then at 20 °C min^{-1} to 320 °C min^{-1} at which it was held for isothermal

for 10 min. The $\delta^{13}C$ values were calculated by integrating the masses 44, 45, and 46 ion currents of the peaks produced by combustion of the chromatographically separated compounds and compared with the CO_2 reference gas peaks with a known ^{13}C-content at the beginning and end of each analytical run. Prior to measurement of samples, an *n*-alkane mixture (Mix B, supplied by A. Schimmelmann, Indiana University) was measured. Sample analyses were only conducted when the average difference of the *n*-alkanes between online and given values was < 0.5‰. Duplicates were measured when possible.

The $\delta^{13}C$ value of the LCDs were corrected for BSTFA addition as follows [Eq. (2)]:

$$\delta^{13}C_{LCD} = \frac{(nC_{LCD} + 2 \times nC_{BSTFA}) \times \delta^{13}C_{LCD}measured - 2 \times nC_{BSTFA} \times \delta^{13}C_{BSTFA}}{nC_{LCD}} \quad (2)$$

With $nC_{LCD} = \text{numberofcarbonatomsofLCD}$, $nC_{BSTFA} = 3$ and $\delta^{13}C_{BSTFA} = -32.2 \pm 0.5‰$.

2.5. DNA extraction, PCR, and 18S rRNA gene amplicon sequencing

DNA was extracted from a 1/8th portion of the GF/F filters using the PowerSoil kit (QIAGEN, Valencia, CA) following manufacturer's instructions. We amplified a region of the 18S rRNA gene approximately corresponding to the initial 450 base pairs (bp), using the primers SSU_F04 (5′-GCTTGTCTCAAAGATTAAGCC-3) and SSU_R22mod (5′-CCTGCTGCCTTCCTTRGA-3′) as described previously (Sinniger et al., 2016). PCR reactions were performed on three replicates of each sample and each reaction included about 5 μL DNA template, 3 μL of each primer (10 μM), 10 μL of Phusion High-Fidelity PCR buffer (Thermo Scientific), 4 μL dNTP at 2.5 mM, 2 μL of BSA at 20 mg mL^{-1}, 0.5 μL of Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and 22.5 μL deionized nuclease-free water for a total volume of 50 μL. Specifically, the PCR consisted of an initial denaturation at 98 °C for 30 s, 27 × [98 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s] and 5 min at 72 °C. The PCR products were stained with SYBR® Safe (Life Technologies, the Netherlands) and visualized by electrophoresis on a 1% agarose gel at 75 V during 50 min. Bands were excised with a sterile scalpel and purified with Qiaquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Purified amplicons from Lake Geneva were combined in equimolar concentrations (20 ng of DNA per sample) and sequenced on Illumina NextSeq500 platform by USEQ, Utrecht as 2 × 150 bp paired end reads.

To estimate the total concentration of 18S rRNA gene copies per liter in lake water we carried out quantitative PCR (qPCR) using the same DNA extract, the same primers and the PCR conditions as described above. qPCR analysis was performed on a Biorad CFX96TM Real-Time System/C1000 Thermal cycler equipped with CFX ManagerTM Software. Each reaction contained 9.25 μL deionized nuclease-free water, 5 μL 5X Phusion HF Buffer, 2 μL dNTPs (2.5 mM), 1 μL BSA (20 mg mL^{-1}), 0,25 μL Phusion DNA Polymerase (2 U μL^{-1}), 1.5 μL from each primer (10 μM) and 0.5 μL Sybr green and 4 μL of 100 time diluted DNA template. Reactions were performed on an iCycler iQTM 96-well plate (Bio-Rad). All qPCR reactions were performed in triplicate. Specificity of the qPCR was verified with melting curve analyses (50 °C to 95 °C). The copy number per liter reported are based on the directly filtered 20 L bottles. Fig. S1 present the results for the incubation experiment which show similar trends.

2.6. Bioinformatic analyses

Data were analysed using the bioinformatic pipeline Cascabel (Abdala Asbun et al., 2020) that includes a combination of software for the analyses of amplicon sequencing data. Most steps of the pipeline used here are part of Quantitative Insight Into Microbial Ecology (QIIME) (Caporaso et al., 2010). A total of 51,862,159 raw Illumina forward reads and 57,186,747 reverse reads, with an average length of 150 bp, were obtained. Forward and reverse reads were paired using PEAR (Zhang et al., 2014) with a minimum length of 20 bp and a minimum overlap size of 7 bp. Paired reads were then extracted from the dataset based on the barcode sequences, demultiplexed into the different samples, and quality filtered using the QIIME scripts *extract_barcode.py* and *split_libraries_fastq.py*. Only reads with a phred (Ewing et al., 1998) quality ≥ 23 and a number of consecutive low quality base calls < 5 were retained for downstream analyses. The QIIME script *pick_otus.py* was used, based on the uclust algorithm (Edgar et al., 2010), to identify and remove singletons (sequences occurring only once). Chimeras were subsequently removed using the UCHIME algorithm (Edgar et al., 2011) within vsearch (https://github.com/torognes/vsearch) by comparison with the protist ribosomal database 2 (Guillou et al., 2013). Sequences were clustered into operational taxonomic units (OTUs) at 97% identity using the QIIME script *pick_otus.py*. OTUs were then identified using command-line blast (Camacho et al., 2009) by comparison with the protist ribosomal database 2 (Guillou et al., 2013). The final dataset consisted of 3,688,575 good quality reads representing 9,533 OTUs. Data were then converted to relative proportions and normalized by multiplying the contribution of each OTU in each sample to the number of gene copies calculated for that sample by qPCR.

A phylogenetic tree including of all the OTUs falling within the eustigmatophytes as well as reference sequences from freshwater eustigmatophytes was constructed. Specifically, we aligned the rRNA gene sequences to the 74 available 18S rRNA gene sequences from cultured freshwater Eustigmatophyceae downloaded from the Genbank and 20 uncultured eustigmatophytes from the ARB database (Ludwig, 2004). The sequences were aligned based on their secondary structure using ARB (version 6.0.2; Tamura et al., 2007) to find the best nucleotide substitution model (great time reversible, GTR + gamma). Phylogeny was inferred using FastTree (Price et al., 2009) from a final alignment consisting of 134 sequences.

3. Results

3.1. Concentration and distribution of LCDs in SPM

LCDs were detected in all five SPM samples collected from Lake Geneva, with summed concentrations ranging from 3.4 ng L^{-1} in January to 34.3 and 36.2 ng L^{-1} in August and October, respectively (Fig. 2, Table 1). LCD concentrations exhibited intermediate concentrations in April and June (15.7 and 14.0 ng L^{-1}, respectively). LCD distributions changed throughout the year (Fig. 2, Table 1), with the C_{30} 1,15-diol being the most abundant LCD in August (accounting for 68% of all LCDs), the C_{28} 1,13-diol in October (27%) and January (28%), and the C_{32} 1,15-diol in April (65%) and June (42%). The $C_{32:1}$ 1,15-diol was detected in all months except in April (Fig. 2), and reached its highest abundance in June (1.1 ng L^{-1}). The C_{28} and C_{30} 1,14-diols were presents in all months, albeit in relatively low concentrations ranging from 0.3 ng L^{-1} in January to 2.5 ng L^{-1} in October. The C_{30} 1,16-diol was only detected in August and October 2017 and January 2018 with concentrations ranging from 0.5 to 4.4 ng L^{-1} (Fig. 2).

3.2. Labeling experiment with Lake Geneva surface waters

Incubation with ^{13}C-labelled bicarbonate of surface waters from Lake Geneva revealed substantial ^{13}C enrichment in the LCDs

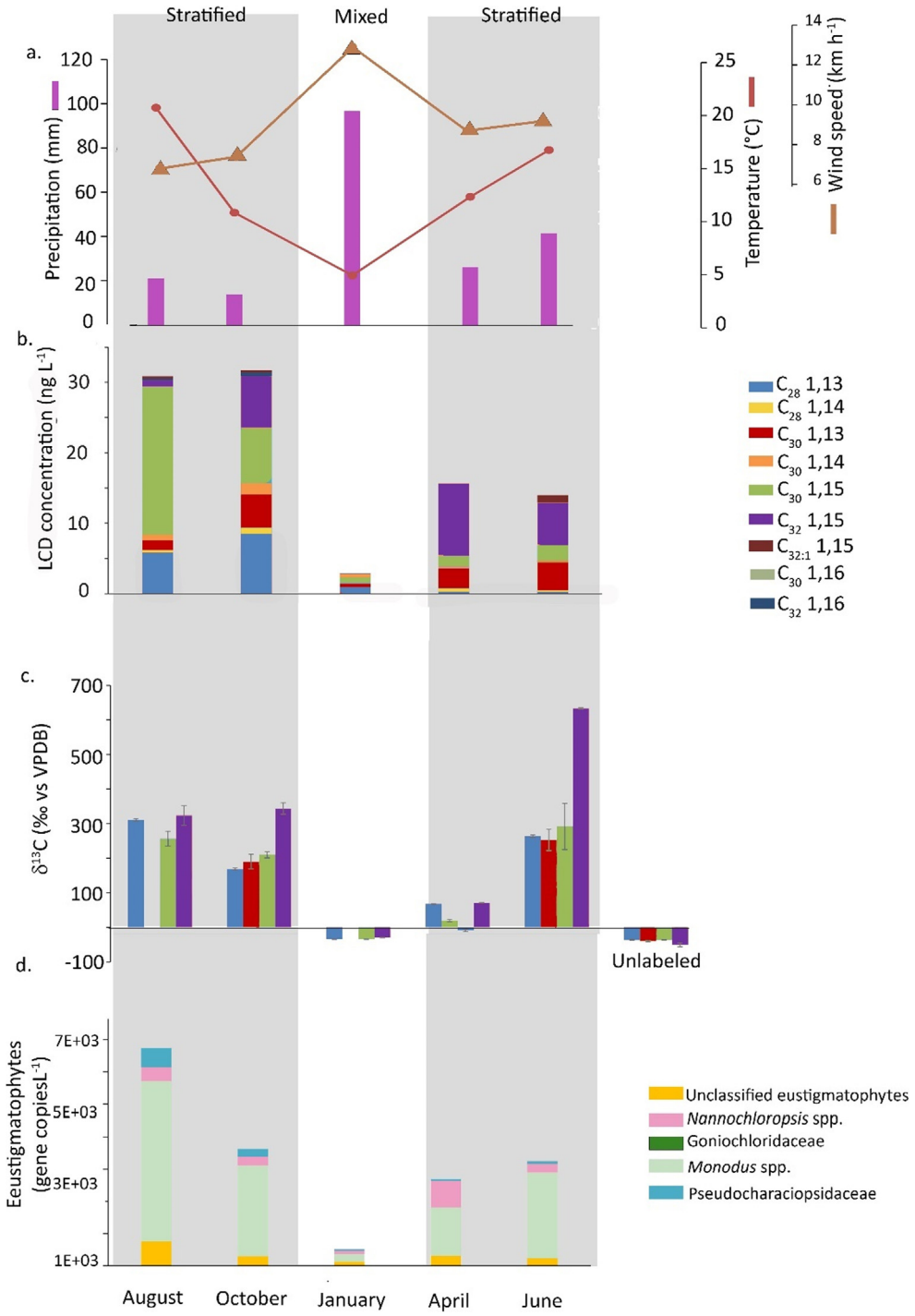

Fig. 2. Seasonal variation in Lake Geneva of (a) temperature, wind speed, and precipitation, (b) the concentration and distribution of LCDs, (c) the degree of ^{13}C label incorporation in the different LCDs (standard deviation between duplicates is indicated where applicable), and (d) the abundance of 18S rRNA gene reads affiliated to eustigmatophytes over the total 18S rRNA gene copies L^{-1}. The data shown on panel (a) is based on average values of 20 years of data from the weather station at Thonon-les-Bains (https://www.meteoblue.com/fr/meteo/historyclimate/weatherarchive/thonon-les-bains_france). Unlabelled in (c) indicate the natural stable carbon isotopic composition of the LCDs measured in August 2017, without the addition of ^{13}C-labelled bicarbonate. Grey bars indicate periods of thermal stratification of the water column.

Table 1
Dates of sampling at station SHL 2 in Lake Geneva and physical water properties and biological parameters.

Sampling date	Air temperature (°C)	Surface water temperature (°C)	pH	Chlorophyll concentration (μg L^{-1})	Water column stratification
07/08/2017	20.5	23	8.56	2.44	Yes
09/10/2017	10.6	16	8.17	2.42	Yes
08/01/2018	4.7	7	8.08	1.90	No
23/04/2018	18.0	16	8.60	2.34	Onset
25/06/2018	22.0	22	8.60	2.92	Yes

relative to the natural ^{13}C abundance of LCDs as measured in August when sufficient amounts could be obtained ($\delta^{13}C_{C28\ 1,13}$ = −36 ± 2‰, $\delta^{13}C_{C30\ 1,13}$ = −40 ± 2‰, $\delta^{13}C_{C30\ 1,15}$ = −36 ± 1‰, $\delta^{13}C_{C32\ 1,15}$ = −50 ± 6‰, Table 2). Only in January (Fig. 2) no ^{13}C label incorporation was detected in any of the measured diols ($\delta^{13}C_{C32\ 1,15}$ = −30‰, $\delta^{13}C_{C30\ 1,15}$ = −34‰, $\delta^{13}C_{C28\ 1,13}$ = −34‰). In April, the degree of labelling was substantially less than in August, October, and June. The C_{32} 1,15-diol had the highest $\delta^{13}C$ values in these months, ranging from 71 ± 0‰ to 633 ± 2‰. Lower values were found for the C_{30} 1,15-diol (−9 ± 4‰ to 256 ± 22‰), the C_{30} 1,13-diol (20 ± 4‰ to 253 ± 30‰) and the C_{28} 1,13-diol (67 ± 1‰ to 310 ± 3‰) (Fig. 2). The $\delta^{13}C$ values of the C_{28} and C_{30} 1,14-diols and $C_{32:1}$ 1,15-diol could not be measured because of their low abundance in Lake Geneva water.

3.3. Genetic analysis of the eukaryotic plankton community

The total concentration of eukaryotic 18S rRNA gene sequences retrieved from the SPM of the surface water ranged between 1.1 ± 0.1 to 2.8 ± 0.2 × 10^6 gene copies L^{-1} (Fig. 3). The lowest concentration was noted in January and the highest in August. The 18S rRNA gene amplicon sequences were dominated by sequences affiliated to Opisthokonta (varying from 2 to 71%), Hacrobia (15–61%), Stramenopiles (6–34%; n = 5), Alveolata (2–20%) and Archaeplastida (5–17%) (Fig. 3).

Reads attributed to Stramenopiles, which include all known LCD producers (Balzano et al., 2018), comprised 23 OTUs (out of 9532) associated with eustigmatophytes, which only represents 0.04–0.32% of all 18S rRNA gene reads. However, it should be noted that eustigmatophytes could be underrepresented with 18S rRNA sequencing due to their low gene copies per cell (i.e., 1–2 gene copies for *Nannochloropsis salina*, while larger phytoplankton can have 100–1000 gene copies; Zhu et al., 2005).

Genetic reads associated with different eustigmatophycean taxa were found throughout the period of study and the abundance of 18S rRNA gene copies L^{-1} assigned to eustigmatophytes was highest in August and lowest in January (Fig. 2d). Phylogenetic analyses (Fig. 4) indicate that of these 25 OTUs, 10 OTUs fall within distinct eustigmatophyte families and 15 OTUs are closely related to eustigmatophytes. Of the 4,751 reads attributed by blast analysis

Table 2
Concentration of the long-chain diols in Lake Geneva during the period of October 2017 and June 2018. ND: not detected.

Sampling	Concentration (ng L^{-1})								
date	$C_{28:0}$ 1,13	$C_{28:0}$ 1,14	$C_{30:0}$ 1,13	$C_{30:0}$ 1,14	$C_{30:0}$ 1,15	$C_{30:0}$ 1,16	$C_{32:1}$ 1,15	$C_{32:0}$ 1,15	Sum
01/08/2017	5.8	0.4	1.4	0.8	21.0	3.5	0.3	1.0	34.2
01/10/2017	8.5	0.9	4.7	1.6	7.8	4.4	0.3	7.4	35.6
01/01/2018	0.8	0.2	0.5	0.1	0.8	0.5	0.0	0.5	3.4
01/04/2018	0.3	0.5	2.9	0.3	1.5	ND	ND	10.3	15.8
01/06/2018	0.2	0.2	4.0	0.2	2.2	ND	1.1	6.0	13.9

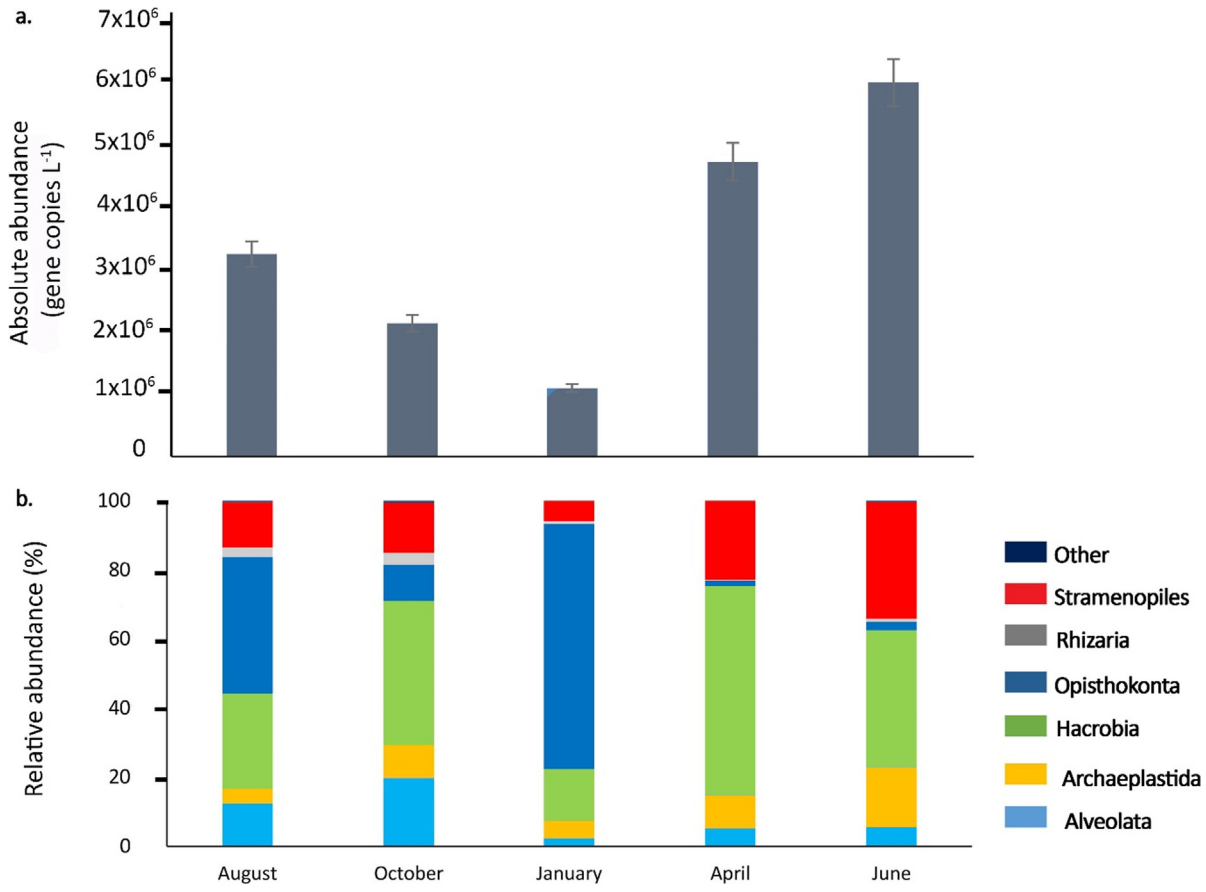

Fig. 3. Relative distribution of the major classes of eukaryotes in the surface waters of Lake Geneva in the five sampled months in 2017–2018 based on the 18S rRNA gene sequencing of SPM, (a) estimated absolute abundance of 18S rRNA gene reads and (b) relative distribution of the major eukaryotic classes.

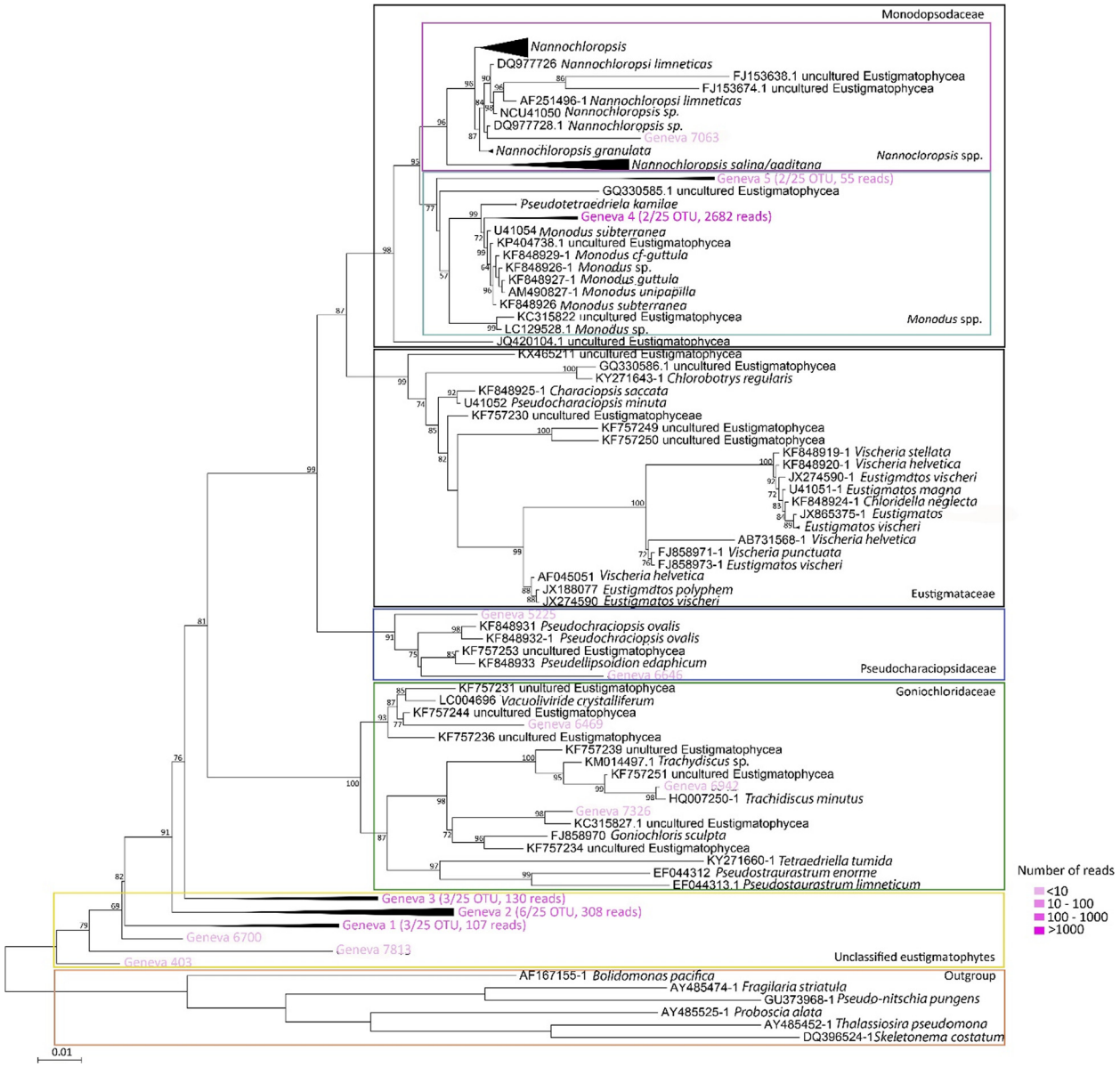

Fig. 4. Phylogenetic tree comparing the 18S rRNA gene sequences retrieved from Lake Geneva (based on the 25 most abundant OTUs) with sequences available of cultured eustigmatophyte freshwater species and 20 sequences of uncultured eustigmatophytes from the ARB database. The tree was inferred by maximum likelihood. Numbers next to the branches indicate bootstrap support (based on 1,000 replicates), and only values >50% are shown. NCBI accession numbers are indicated for the sequences of known eustigmatophytes. The classification of the eustigmatophytes is according to Fawley et al. (2014), with the main groups indicated by coloured rectangles. The pink colour-scale indicates the number of reads obtained for each branch of the tree. Note the large amount of OTUs related to uncultured eustigmatophytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to eustigmatophytes, 66% (3,133 reads) belong to 5 OTUs in the Monodopsidaceae family, 10% (478 reads) to 3 OTUs in the Goniochloridaceae and 5% (251 reads) to 2 OTUs in the Pseudocharaciopsidaceae. Reads belonging to the Eustigmataceae family were not detected and the remaining 19% (889 reads) belong to 15 unclassified OTUs closely related to eustigmatophytes (Fig. 4). *Monodus* spp. (4.0×10^3 gene copies L^{-1}) and Pseudocharaciopsidaceae (0.7×10^3 gene copies L^{-1}) dominated genetic reads from eustigmatophytes in August. OTUs closely related to *Monodus* spp. (Monodopsidaceae) were dominant in all five months (>50% of all eustigmatophyte 18S rRNA gene copies). The third taxa dominating the eustigmatophyte community was related to *Nannochloropsis* spp. (Monodopsidaceae) in June and April (31% and 8% of all eustigmatophyte 18S rRNA gene, respectively), Pseudocharaciopsidaceae in January, August and October (23%, 11% and 8% of all eustigmatophyte 18S rRNA gene) (Fig. 2).

No sequences related to the known LCD-producer *Proboscia* were detected. Since 1,14 diols might also be produced by some species genetically related to *Proboscia* spp., we investigated the contribution of all the radial centric diatoms in the Lake Geneva SPM. 18S rRNA gene sequences related to radial centric diatoms ranged from 5.4×10^4 gene copies L^{-1} in June to 4.5×10^6 gene copies L^{-1} in April (Fig. 5).

4. Discussion

4.1. Eustigmatophyte algae as a source of 1,13- and 1,15-diols

The LCD distribution in the SPM of Lake Geneva (Table 2) is similar to the LCD distribution reported for surface sediments from a large set of lakes from around the world (Robinson et al., 1984;

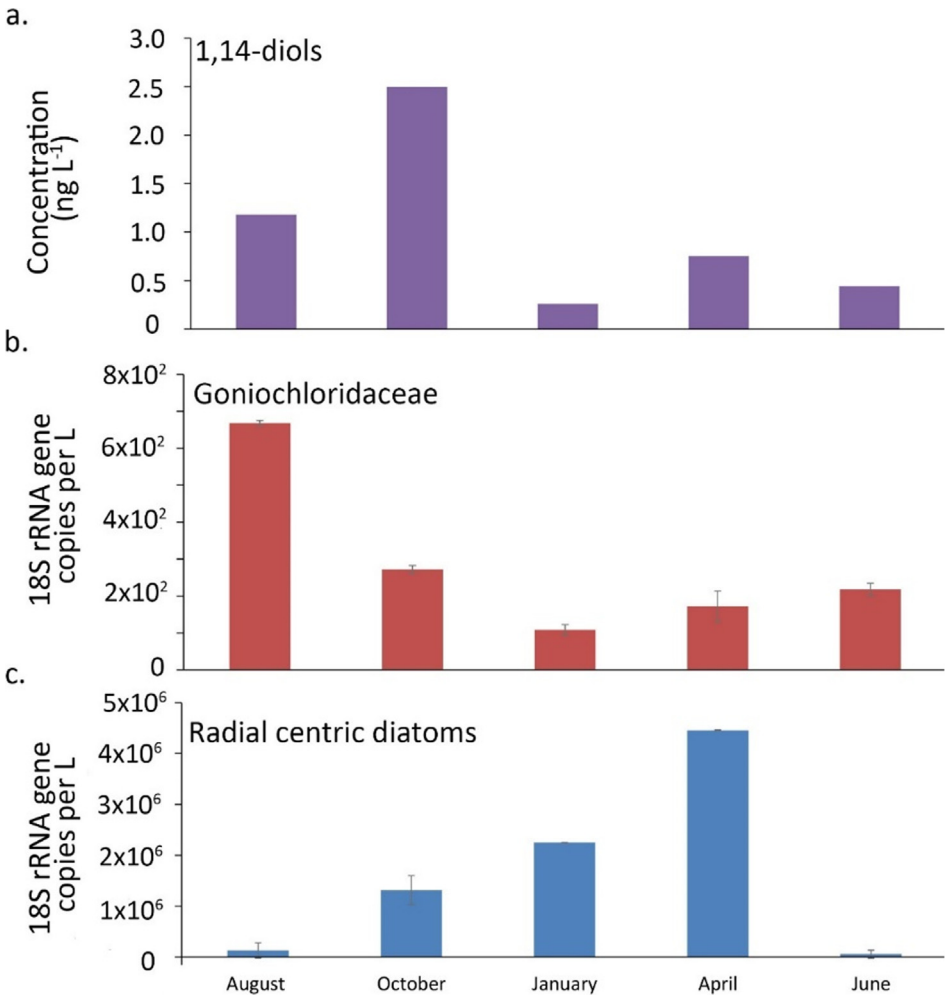

Fig. 5. Seasonal variation in Lake Geneva of (a) Concentration of 1,14-diols, (b) 18S rRNA gene copies affiliated to radial centric diatoms and (c) 18S rRNA gene copies affiliated to Goniochloridaceae.

Xu et al., 2007; Shimokawara et al., 2010; Zhang et al., 2011, 2015; Romero-Viana et al., 2012; Atwood et al., 2014; Rampen et al., 2014a; Toney et al., 2020): C_{32} and C_{30} 1,15-diols are the most abundant LCDs and C_{28} and C_{30} 1,13-diols are the second most abundant group of LCDs (Fig. 2). Also in Lake Chala, the LCD distribution of SPM of the surface waters was dominated by C_{32} and C_{30} 1,15-diols, although C_{34} 1,17-diols were also reported (Villanueva et al., 2014; van Bree et al., 2018). These previous studies concluded, on basis of comparison of LCD distributions with those of cultivated eustigmatophytes (Rampen et al., 2014b) and comparison with genetic data (Villanueva et al., 2014), that uncultured eustigmatophyte algae are the most likely source for the LCDs in lakes.

In Lake Geneva, the phytoplankton succession is relatively similar between years (Rapport CIPEL 2017). In 2016, diatoms, specifically *S. neoastraea*, dominated the lake water from January until March. They were then outcompeted by Cryptophyceae (especially *Plagioselmis nannoplanktica*), which were abundant until June (with a shift to *Plagioselmis lacustris*). In June and August, diatoms bloomed again during a short period of time, with an abundance of *Diatoma elongatum* and *Ulnaria acus*, respectively. The end of the year was characterized by no particular dominant species, i.e., a mix of Cyanobacteria, Cryptophyceae and Chrysophyceae (Rapport CIPEL 2017). Hence, this description of the taxonomical composition of Lake Geneva surface water does not indicate any known potential LCD producers (Volkman et al., 1992; Rampen et al., 2014b; Villanueva et al., 2014). However, because of their small size (<3 μm; cf. van Bree et al., 2018) eustigmatophytes can be hardly identified by light microscopy and are usually classified, along with other smaller-sized phytoplankters, as nanoflagellates or picoflagellates in microscopy counts. Instead, DNA sequencing of taxonomically diagnostic markers such as the 18S rRNA gene does allow for a more in-depth identification of the eukaryotic phytoplankton. Freshwater eustigmatophytes were indeed successfully identified and quantified in Lake Chala by sequencing and qPCR of their 18S rRNA gene, respectively (Villanueva et al., 2014). Since the number of rRNA gene copies per cell is weakly correlated with cell size (Zhu et al., 2005), the abundance of the rRNA gene from species within a restricted size range, such as eustigmatophytes (<3 μm, Andersen et al., 1998; Fawley et al., 2015), might reflect, with some degree of error, the number of eustigmatophyte cells in water. Accordingly, we sequenced the 18S rRNA genes present in the SPM of the surface water of Lake Geneva to identify eustigmatophyte algae and to constrain variations in their abundance. Gene copies from eustigmatophytes were detected in all SPM samples and their absolute abundance peaked in April, June, August and October ($3.1–6.3 \times 10^3$ 18S rRNA gene copies L^{-1}), and decreased by one order of magnitude in January (0.5×10^3 18S rRNA gene copies L^{-1}) (Fig. 2). The low abundance of eustigmatophytes based on 18S rRNA gene copies in January is in agreement with the lowest LCD concentration in this month (Fig. 2), whereas both the LCD concentration (Fig. 2) and eustig-

matophyte 18S rRNA gene copy values were substantially higher in all other months (Fig. 2). The LCD concentration in *Nannochloropsis* spp. is estimated to be 20 fg cell^{-1} (Balzano et al., 2017). Hence, assuming one 18S rRNA gene copy per cell for *Nannochloropsis* spp. (cf. Zhu et al., 2005), 5×10^3 gene copies L^{-1} (average abundance of OTUs related to LCD producers) would translate to a LCD concentration of ca. 10 ng L^{-1}, which is roughly comparable to what is detected in all months (15–30 ng L^{-1}; Fig. 2), except from January when it is considerably lower.

To further evaluate potential in situ production of LCDs (i.e., representing living, active organisms), Lake Geneva surface waters were incubated with ^{13}C-labelled bicarbonate. We observed that the ^{13}C label was incorporated in the LCDs in all targeted months except in January (Fig. 2), suggesting that LCDs were actively produced in the surface waters of the lake during these periods. The degree of labelling in April was lower than in the other months (except January) even though the LCD concentration and eustigmatophyte 18S rRNA gene copy values were relatively high (Fig. 2). The relatively high degree of labelling (as shown by the high ^{13}C values) of the C_{32} 1,15-diol compared to that of the other LCDs, indicates either a higher production rate of the C_{32} 1,15-diol, or the presence of a faster-growing organism that predominantly produces the C_{32} 1,15-diol. We calculated the uptake of labelled carbon using the following equations (Boschker et al., 1998):

$$^{13}F_{control} = \left(\frac{\frac{^{13}C}{^{12}C}unlabelled}{\frac{^{13}C}{^{12}C}unlabelled + 1}\right) \times 100 \quad (3)$$

$$\text{With } \frac{^{13}C}{^{12}C}unlabelled = \left(\frac{\delta^{13}C_{unlabelled}}{1000} + 1\right) \times 0.0112372$$

$$^{13}F = \left(\frac{\frac{^{13}C}{^{12}C}}{\frac{^{13}C}{^{12}C} + 1}\right) \times 100 \quad (4)$$

$$\text{With } \frac{^{13}C}{^{12}C} = \left(\frac{\delta^{13}C_{measured}}{1000} + 1\right) \times 0.0112372$$

$$^{13}\text{Cuptakerate} = \frac{[LCD] \times (\ ^{13}F - \ ^{13}F_{unlabelled}}{Incubationtime} \quad (5)$$

With [LCD] = the concentration of the LCD in the incubation vessels, and incubation time is 52 h.

The calculated uptake of labelled carbon is different between the sampled months and the isomers (Table 4): in August the C_{30} 1,15-diol is showing the highest uptake, while in October, April and June, the C_{32} 1,15-diol is showing the highest uptake, likely indicating two LCD-producing communities blooming under different environmental conditions. Overall, the comparison of gene with lipid abundances and the labelling results indicate that most LCDs are produced in situ by eustigmatophytes in Lake Geneva except in January. However, it should be noted that most of the OTUs retrieved from Lake Geneva are related to yet-uncultured eustigmatophytes, which may reflect unknown LCD producers.

4.2. Potential biological sources of 1,14-diols

Currently the producers of the 1,14-diols in lakes remain unknown. While *Proboscia* spp. are known to be the main producers of 1,14-diols in marine environments (Sinninghe Damste et al., 2003; Rampen et al., 2007), these diatoms are unlikely to be the source of the 1,14-diols detected in Lake Geneva, since this genus does not comprise freshwater species (Moita et al., 2003; Lassiter et al., 2006) and no sequences related to this species were detected. *Apedinella radians* (class Dictyochophyceae, order Pedinellales) also produces C_{28} and C_{30} 1,14-diols, although genetic reads associated to this species were only recovered in trace amounts in January 2018 (data not shown). Furthermore, this species is only known to occur in brackish environments such as estuaries (Throndsen, 1971; Rampen et al., 2011) and the C_{32} 1,14-diol, specific for this species (Rampen et al., 2011), is not detected in the SPM of Lake Geneva, making it an unlikely producer of the other 1,14-diols. Additionally, we do not observe clear relations between the abundance of sequences related to radial centric diatoms and the concentration of 1,14-diols in Lake Geneva (Fig. 5), suggesting that diatom species closely related to *Proboscia* spp. are unlikely sources of 1,14 diols in the lake waters. Although we cannot rule out that specific species of diatoms (not following the general diatom abundance trend) could still be producing the 1,14-diols, the screening of more than 100 species of diatoms (Rampen et al., 2010) indicated that *Proboscia* spp. are likely the only diatoms producing them (Schouten and Sinninghe Damsté, unpublished results).

Alternatively, species falling in the Goniochloridaceae family (class Eustigmatophyceae) could be the source of the 1,14-diols in our lakes, since one species of this family has previously been shown to produce these compounds, albeit in low relative abundances (Rampen et al., 2014b). However, there is no clear match between the abundance of sequences related Goniochloridaceae and the concentration of 1,14-diols in Lake Geneva (Fig. 5). Never-

Table 3
δ^{13}C values of LCDs in ^{13}C-labeled bicarbonate incubation experiments with Lake Geneva water. Standard deviation of duplicates measurements is indicated when applicable. ND = not determined due to low abundance.

Sampling	δ^{13}C (‰)			
date	$C_{28:0}$ 1,13-diol	$C_{30:0}$ 1,13-diol	$C_{30:0}$ 1,14-diol	$C_{32:0}$ 1,15-diol
01/08/2017	311 ± 3	ND	256 ± 22	324 ± 29
01/10/2017	169 ± 3	190 ± 21	210 ± 9	344 ± 16
01/01/2018	−34 ± 0	ND	−34 ± 0	−30 ± 0
01/04/2018	68 ± 1	20 ± 4	−9 ± 4	71 ± 0
01/06/2018	264 ± 4	253 ± 31	290 ± 67	634 ± 2
Unlabelled[a]	−36 ± 2	−40 ± 2	−36 ± 1	−50 ± 6

[a] The natural abundance value was obtained from water sampled on 01/08/2017.

Table 4
Uptake of ^{13}C by LCDs during the ^{13}C-labelled bicarbonate incubation experiments with Lake Geneva water. The propagated error from the δ^{13}C measurements is indicated.

Sampling	Uptake (ngC h^{-1})			
date	$C_{28:0}$ 1,13-diol	$C_{30:0}$ 1,13-diol	$C_{30:0}$ 1,15-diol	$C_{32:0}$ 1,15-diol
01/08/2017	42 ± 0.4	ND	129 ± 2	8 ± 3
01/10/2017	37 ± 0.4	23 ± 2	41 ± 1	61 ± 2
01/01/2018	0	ND	0	0
01/04/2018	1 ± 0.2	4 ± 0.5	1 ± 0.5	26 ± 0.7
01/06/2018	1 ± 0.4	25 ± 2	15 ± 1	86 ± 2

theless, the 1,14-diols in the SPM of Lake Geneva, which only occur in relatively low abundances (Fig. 2; Table 1) might be attributed to other eustigmatophyte species found here in Lake Geneva for which the LCD composition is still unknown. For example, gene copies from OTUs affiliated to Pseudocharaciopsidaceae (class Eustigmatophyceae) were found in all samples (Fig. 3). As Pseudocharaciopsidaceae are genetically different from the eustigmatophyte species for which the LCD composition is known (Fawley et al., 2014), they could be a source of 1,14-diols in lakes.

4.3. Environmental controls on LCD production

Interestingly, the LCD distribution and concentration varied seasonally (Fig. 2). This might be due to a shift in the biological producers of LCDs or the influence of seasonally changing environmental parameters. In addition, differences in LCD distribution might also be caused by an ex-situ origin of LCDs (e.g., by river transport) and differences in degradation rates since they can persist in aquatic environments for long periods (Grossi et al., 2001; Reiche et al., 2018) and can thus derive from fossil material (Balzano et al., 2018; Lattaud et al., 2018) and do not always reflect production by living organisms (as DNA degrade faster). In January no ^{13}C label uptake was measured in LCDs (Fig. 2), suggesting that the LCDs present at this time likely originated from fossil material (cf. Balzano et al., 2018). However, in the other months in situ production of LCDs was clearly evident from the high degree of labelling (Fig. 2).

In the marine environment, the LCD distribution is regulated, in part, by temperature (Rampen et al., 2012; de Bar et al., 2020), with relatively more C_{30} 1,15-diols in warmer water. In contrast, inconsistent reports have been made on the impact of lake water temperature on LCDs. Rampen et al. (2014b) showed that the distributions of LCDs produced in freshwater environments and preserved in lake surface sediments are not correlated with annual mean lake temperature. In contrast, Toney et al. (2020) reconstructed lake temperature in a Spanish alpine lake during the Holocene using LCDs. Here, the LDI, which is used to reconstruct seawater temperature, based on the relative distribution of LCDs in marine sediments, does not show any correlation with lake water or air temperature in Lake Geneva (data not shown). Rampen et al. (2014b) showed that certain eustigmatophyte species (*Nannochlorospis gaditana* and *Goniochloris sculpta*) increase the relative abundance of $C_{32:1}$ 1,15-diol and decrease that of C_{32} 1,15-diol at higher growth temperatures. However, in Lake Geneva we observe an opposite trend as the fractional abundance of the C_{32} 1,15-diol was lower in August (Fig. 2), which was the warmest month (Fig. 2). Furthermore, the fractional abundance of the $C_{32:1}$ 1,15-diol was highest in June (0.08) but remains constant at ca. 0.01 for all other months (Table 1). Thus, temperature alone cannot fully explain the seasonal change in LCD distributions in Lake Geneva.

The change in the LCD distribution might also be the result of changes in the LCD producing community following changes in nutrient availability (delivered via rivers or erosion) or inter/intra-species competition. The variations in the distribution of LCDs between April/June and August/October in Lake Geneva (higher fractional abundance of C_{32} 1,15-diol in April/June and of C_{30} 1,15-diol in August/October, Table 1) are in agreement with this hypothesis, as no major environmental change occurred during this period. In August and October, the number of 18S rRNA gene copies related to the eustigmatophycean families Goniochloridaceae and Pseudocharaciopsidaceae increased (from 1.7×10^2 in April to 6.7×10^2 in August and from 1.9×10^2 in April to 5.1×10^2 in August 18S rRNA gene copies L^{-1}, respectively, Fig. 2). This implies that Goniochloridaceae and/or Pseudocharaciopsidaceae could be responsible for the increased C_{30} 1,15-diol production at this time. However, although Rampen et al. (2014b) found that freshwater species belonging to the Goniochloridaceae (*G. sculpta*, *Microtalis aquatica* and *P. enorme*) contain the C_{30} 1,15-diol, they do so in only low relative abundance (5–20%). Furthermore, species belonging to the Pseudocharaciopsidaceae family have not yet been analysed for their LCD composition, and thus we cannot assess if they are responsible for the high contribution of the C_{30} 1,15-diol found in Lake Geneva. As such, both hypotheses still stand, i.e., an adaptation within one of the producers to environmental changes or a change in the LCD-producing community.

4.4. Implication for LCD proxies

By combining the results of this study and those of Lake Chala (Villanueva et al., 2014; van Bree et al., 2018), it seems that LCD abundances predominantly increase during stratification of the lake water-column. This potentially suggests that, in the geological record, a relative increase of LCDs might be a proxy for water-column stratification. However, it should be noted that more lakes need to be investigated to test this observation. The increased abundances of LCDs at this time might be caused by intra-species competition, possibly with diatoms. Indeed, in Lake Chala diatom biomarkers and LCDs do not maximize in abundances at the same time (van Bree et al., 2018), while in Lake Geneva, diatoms dominate in spring and during some short periods in summer while eustigmatophyte are mainly present during summer and fall.

A previous study has shown that the hydrogen isotope composition (δ^2H values) of LCDs is correlated with the δ^2H values of the water and salinity in the marine environment (Lattaud et al., 2019). If this is the case in lake environments than the δ^2H values might be a tool for reconstructing $\delta^2H_{lake\ water}$, in turn a proxy for the precipitation-evaporation balance of a lake (Sachse et al., 2004 and references cited therein). Our results suggests that LCDs will then mainly reflect the $\delta^2H_{lake\ water}$ at times of water-column stratification.

The use of LCDs as a proxy for lake temperature still needs more study as it seems to work in a Spanish alpine lake (Toney et al., 2020) but not on a global scale (Rampen et al., 2014a). Our results do not show a relation between LCD distribution and lake temperature either; neither for what has been observed in cultures (Rampen et al., 2014a) or what has been observed in the marine environment.

Finally, in the marine environment, the amount of C_{32} 1,15-diol relative to other LCDs is used to trace riverine input (Lattaud et al., 2017b). Research has shown that LCDs is mainly produced in slowly flowing water such as lakes and ponds where rivers will then transport the C_{32} 1,15-diol to the river mouth and into the marine environment. The relatively high abundance of the C_{32} 1,15-diol in the SPM in Lake Geneva (Fig. 2) is consistent with this interpretation. As LCDs in lake seems to be mainly produced during period of water-column stratification, the amount of C_{32} 1,15-diol carried by rivers may vary over time depending on stratification changes but also depending on the lake drainage and river outflow. However, as sediments often integrate relatively long (at least annual) signals, the use of this proxy in geological records still likely is valid.

5. Conclusions

In Lake Geneva, LCDs are produced in situ from spring to late summer. Their distribution is dominated by the C_{32} 1,15-diol in April/June and C_{30} 1,15-diol in August/October. 18S rRNA gene analysis revealed that the estimated abundance of eustigmatophytes, the presumed main LCD-producers in freshwater environ-

ments, covaries with LCD concentrations. The potential role of eustigmatophytes as main producers of LCDs (at least for the 1,13- and 1,15-diols) in Lake Geneva supports previous observations. Seasonal changes in LCD distributions are most likely due to changes in the eustigmatophyte community composition possibly caused by changes in environmental conditions. Overall, LCDs in Lake Geneva, and potentially in other lakes, seem to be mainly produced at times of thermal stratification of the water column when the relatively high surface water temperature or low interspecies competitivity favours the blooming of LCD producers. These results show that changes in LCD abundances may be used as an indicator for water-column stratification in lakes. In addition, the use of the C_{32} 1,15-diol as proxy for freshwater input in coastal sediment remains possible although a thorough understanding if the hydrology of the catchment is needed, such as the onset of water-column stratification in the catchment's lakes.

Declaration of Competing Interest

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

Acknowledgments

We thank Jort Ossebaar and Monique Verweij for analytical help, Maartje Brouwer for help during DNA analysis, Laura Schreuder, Jean-Christophe Hustache and the UMR CARRTEL for assistance during sampling of Lake Geneva, and Alejandro Abdala for assistance in bioinformatic analyses. The environmental data from Lake Geneva have been obtained thanks to OLA (Observatory on Lakes), © OLA-IS, AnaEE-France, INRA Thonon-les-Bains, CIPEL 7-12-18, developed by Eco-Informatics ORE INRA Team. We thank the Utrecht Sequencing Facility for providing sequencing service. We thank three anonymous reviewers and Associate Editor Isla Castaneda for their constructive comments which improved the manuscript. This research has been funded by the European Research Council (ERC) under the European Union's Seventh Framework Program (FP7/2007-2013), ERC grant agreement [339206] to S.S. The work was further supported by funding from the Netherlands Earth System Science Center (NESSC) through a gravitation grant (NWO 024.002.001) from the Dutch Ministry for Education, Culture and Science to J.S.S.D. and S.S. JL is supported by a Rubicon grant from NWO (EN. 019.183EN.002).

Data availability

A representative set of sequences from the OTUs used here, as well as the OTU table reporting the abundance of each OTU in each sample, have been submitted to the GenBank under the bioproject ID PRJNA545457. Data from this study are available from the PANGEA repository (submitted).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2021.104223.

Associate Editor–Isla S. Castaneda

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