

**Climate changes and human impacts in the North Atlantic over the last 2,000
years inferred from organic geochemical proxies and lacustrine archives**

By

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A dissertation submitted in partial fulfillment of the requirements for the
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– CHAPTER 1 –

Introduction

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1. Climate change in the North Atlantic region and insights from paleoclimate archives

By the end of the century we will experience unprecedented changes in our climate system, including extreme changes in temperature and precipitation patterns in the North Atlantic region (Hoegh-Guldberg et al., 2018). For instance, drought frequency is predicted to increase in the Mediterranean and summer heat waves are expected to be more common in the mid-latitudes of the North Atlantic region (Hoegh-Guldberg et al., 2018; Li et al., 2018). However, there is still uncertainty as to how ocean circulation and the position and intensity of the North Atlantic jet stream will change under a warming world, and what implications this has for extreme events (e.g., hurricanes, floods/droughts, and heat waves) (Cohen et al., 2014; Caesar et al., 2018; Hoegh-Guldberg et al., 2018; Li et al., 2018). To improve the accuracy of future climate predictions, we need a better mechanistic understanding of both forced and unforced (e.g. North Atlantic Oscillation (NAO)) climate dynamics in the North Atlantic region. In this respect, paleoclimate records are essential for understanding long-term changes in the climate system.

On multi-decadal to centennial timescales, climate in the North Atlantic region is strongly modulated by internal climate dynamics in addition to external forcings (e.g. volcanic eruptions and total solar irradiance) (Hurrell, 1995; Otto-Bliesner et al., 2016; PAGES 2K Consortium, 2019). During the winter months the NAO, defined as the difference in sea level pressure between the Icelandic Low and Azores High, is the dominant mode of atmospheric variability that drives changes in temperature, precipitation, and storm tracks in the North Atlantic region (Figure 1) (Hurrell, 1995; Hurrell & Deser, 2010). However, multi-decadal to centennial predictions for the NAO are poorly constrained (Smith et al., 2016). During the cold season, temperature variability in Iceland and hydroclimate variability in the Azores are strongly

correlated to fluctuations in the NAO (Hurrell, 1995; Hanna et al., 2004; Hernández et al., 2016). Thus, paleoclimate reconstructions from Iceland and the Azores could provide valuable insights on the low frequency variability of the NAO.

Northern Hemisphere (NH) temperature reconstructions that span the last 2,000 years are typically characterized by a long-term cooling trend that is often attributed to decreasing summer insolation (Kaufman et al., 2009; PAGES 2K Consortium, 2013). Despite the importance of wintertime climate variability and the NAO to north-temperate regions, most existing temperature reconstructions from the NH are biased towards the warm season and might not capture the climate variability associated with the cold season (Liu et al., 2014). It is important to constrain the primary drivers of both short-term and long-term cold season variability, in addition to summer variability, particularly in the mid- to high latitudes of the North Atlantic where cold season dynamics are often amplified relative to the summer months (Cohen et al., 2014; Li et al., 2018). Thus, a principal goal of this thesis is to develop paleoclimate reconstructions that capture climate variability during the cold season in the North Atlantic region.

2. Humans in the high North Atlantic and Macaronesia over the last 2,000 years

In addition to ongoing climate change in the North Atlantic region, ecosystems are under pressure from increased land use for agriculture and urban areas, the overexploitation of resources, and continued loss of biodiversity (Arneth et al., 2019). Although the islands in the high North Atlantic (i.e. Iceland and the Faroe Islands) and Macaronesia were only settled in the last millennium (Figure 2), they are faced with severe land degradation from agricultural practices and a loss of native flora (e.g. Dugmore et al., 2005; Connor et al., 2012; Fernández-

Palacios et al., 2016; Castilla-Beltrán et al., 2019). This stems partly from the rapid introduction of agricultural and trade practices from Europe, including the release of livestock into previously “pristine” ecosystems (Dugmore et al., 2005; Schäfer, 2003; Smith, 2010). To develop effective adaptation and management strategies for these highly modified landscapes, we need to understand how these ecosystems changed since humans settled the islands, and the role of climate change and direct human impacts on both terrestrial and aquatic ecosystems (Willis & Birks, 2006).

The Norse settlement of the high North Atlantic (c. 800-1000 CE) is usually associated with a warmer/milder climate interval, or the Medieval Climate Anomaly (c. 950-1250 CE) (Figure 2) (Ogilvie et al., 2000). Early settlers in Iceland (c. 874 CE) cleared forests and introduced livestock, leading to the expansion of grasses and shrubs and increased soil erosion (Hallsdóttir 1987; Dugmore et al. 2005, 2009; Hallsdóttir & Caseldine 2005). Colder temperatures and/or an intensification of agricultural practices during the Little Ice Age (LIA, 1450-1850 CE) likely lead to a further increase in soil erosion (Dugmore et al., 2009; Geirsdóttir et al., 2009; Streeter et al., 2015). Soil erosion in Iceland is still considered to be a major environmental issue despite warmer temperatures in the 20th and 21st century and efforts to improve land management strategies (Arnalds, 2005; Dugmore et al., 2009).

In contrast, the Portuguese settlement of Macaronesia (c. 1400-1500 CE) coincides with the onset of a colder time period, the LIA (Figure 2) (Björck et al., 2006; Hernández et al., 2017; Rull et al., 2017). Early Portuguese settlers arrived on the islands of Madeira about 1418 CE, the Azores Archipelago in 1432 CE, and eventually Cabo Verde in 1456 CE (Smith, 2010; Fernández-Palacios et al., 2016). Harsher climate conditions were noted in early historic records from the Azores and most likely contributed to the slow and intermittent influx of settlers to the archipelago (Smith, 2010). Livestock were introduced to almost all of the islands in the Azores

Archipelago before any settlements were established (Schäfer, 2003; Smith, 2010). Later settlers began to clear the dense laurel forests for wood and to make space for crops and livestock (Connor et al., 2012; Rull et al., 2017). It remains unclear how the cumulative effects of land use change and harsh climate conditions influenced erosion rates and vegetation composition on the islands. Reforestation efforts are now being made to reduce land degradation; however, projected warming and changing precipitation patterns in the near future could increase the risk of landslides, floods, and coastal erosion in the Azores Archipelago (Frias, 2000; Santos et al., 2004; Rull et al., 2017).

Terrestrial ecosystem changes, and declines in forest cover in particular, are well-known in these regions. Lake ecosystems are also changing in response to direct human impacts, such as through land use changes in the catchment area or the introduction of exotic species, and/or a changing climate (e.g. rising temperatures) (Antunes & Rodrigues, 2011; Dubois et al., 2018; Woolway & Merchant, 2019). However, physical and biological thresholds in lakes are very poorly constrained and vary widely depending on the buffering capacity of the system, lake depth, the cumulative effects of past impacts, and other environmental variables (Langdon et al., 2016; Dubois et al., 2018). Paleoecological records can provide insights to constrain the natural variability of these lake ecosystems and their sensitivity to land use changes and climate change (Dubois et al., 2018). There are few existing records from the high North Atlantic islands and Macaronesia that assess how lake ecosystems have changed since human settlement and what implications this has for future climate change.

3. Overview of thesis chapters

This thesis provides new insights into climate change in the North Atlantic region over the last 2,000 years and an ecological perspective on how previously pristine landscapes responded to human settlement and climate change. This thesis addresses three main objectives: (1) development of a new proxy that records temperatures during the cold season; (2) understanding the mechanisms that control cold season temperature variability in the Northern high latitudes and hydroclimate variability in the sub-tropical North Atlantic; and (3) assessing the role of humans and climate in shaping the terrestrial and lacustrine ecosystems in the high North Atlantic and Macaronesia.

As noted above, most paleoclimate proxies used in north temperate ecosystems are biased toward the summer months, when warmer temperatures stimulate biological productivity. Chapter 2 uses a combined organic geochemical and molecular biological approach to identify the major algal producers of alkenones, long-chain ketones that are commonly used as a proxy for temperature, in freshwater lakes from the NH. This chapter focuses on alkenone-producers with a distinct alkenone signature, marked by the production of a tri-unsaturated ketone and its isomer, also known as Group I Isochrysidales (D'Andrea & Huang, 2005; Theroux et al., 2010; Longo et al., 2013). We found that Group I Isochrysidales are a very diverse group of alkenone-producers, and even include a distinct subclade. Despite the diversity of these alkenone producers, the alkenone signatures and response to cold season temperatures are conserved in NH lakes. Thus, there is potential for these biomarkers to be more widely used as a proxy for cold season temperatures in the NH.

In Chapters 3 and 4, complementary paleoclimate and paleoecological records are developed from a lake in southwest Iceland, Vestra Gíslholtvatn (VGHV). Chapter 3 explores

the role of humans and climate in driving changes in terrestrial and aquatic ecosystems in Iceland during the last two millennia. Despite marked changes in temperature, the landscape is resilient to natural climate variability, whereas humans drive major shifts in vegetation and soil erosion in the catchment area of VGHV. Land use changes in the catchment area of VGHV lead to a temporary increase in primary productivity within the lake; however, only in the 20th century does primary productivity substantially increase. Chapter 4 develops a cold season climate record for VGHV using alkenones produced by Group I Isochrysidales, and explores how lake water temperatures in VGHV, and therefore Group I alkenones, are sensitive to air temperature perturbations and changes in radiative forcings. Our temperature reconstruction from VGHV shows that cold season temperatures gradually warmed over the last 2,000 years in response to increasing winter and spring insolation. On multi-decadal to centennial timescales, the cumulative effects of multiple radiative forcings and feedbacks (i.e. volcanic eruptions, solar minima, and sea ice feedbacks) correspond to colder temperatures in the VGHV record. However, an inconsistent temperature response to past volcanic eruptions and solar minima suggests that the NAO strongly modulates cold season temperatures in Iceland.

In Chapter 5 and Appendix 1, we develop hydroclimate and paleoecological records from Lake Funda on Flores island in the Azores Archipelago. Chapter 5 identifies early human impacts on the landscape, mainly from the release of livestock on the islands, which spurred increases in primary production in Lake Funda. Major changes in vegetation composition and further eutrophication in Lake Funda occur shortly after the establishment of settlements on Flores island. Despite reforestation efforts to reduce soil erosion in the 20th century, internal phosphorous loading continues to promote eutrophication in Lake Funda. Appendix 1 uses hydrogen isotopes from leaf waxes to reconstruct hydroclimate variations in the sub-tropical

North Atlantic over the last millennium. Opposing but coherent changes in precipitation between the Azores hydroclimate reconstruction and a speleothem record from Northern Scotland during the LIA, strongly resemble climate variability associated with the NAO. Strong negative radiative perturbations result in wetter conditions in the Azores and highlight the importance of both internal climate dynamics and external forcings in driving multi-decadal to centennial climate variability in the Azores.

4. Future work

We developed a qualitative cold season temperature reconstruction from a critical region – Iceland – over the last 2,000 years. Unfortunately, existing temperature calibrations provide unreasonable temperature estimates, highlighting the need for an Icelandic temperature calibration. It would be interesting to extend this temperature record back through the Holocene to test the role of seasonal orbital insolation on cold season temperatures. Additional cold season reconstructions using Group I alkenones from the NH, and even Iceland, would be useful to validate some of the mechanisms proposed in Chapter 4. A synthesis of cold season temperature reconstructions and hydroclimate records that are sensitive to the NAO, could provide valuable insights into the multi-decadal variability of the NAO over the last 2,000 years. In addition, I have access to samples that would allow us to develop a hydroclimate record from the Azores that spans the last 8,000 years (and potentially a record from the last glacial period), which would provide valuable insights into precipitation changes throughout the Holocene in the subtropical North Atlantic.

New paleoclimate and paleoecological records from the high North Atlantic and Macaronesia can provide additional insights on how climate influences the establishment,

resiliency, and growth of human settlements. For instance, it is still debated why settlements in Greenland were abandoned in the 15th century despite evidence of warming winter temperatures in the NH. Similarly, in Macaronesia there are few studies that have looked at how climatic changes influenced the establishment of major trading ports in Madeira and the Canary Islands, whereas the Azores and Cabo Verde never grew to the same economic levels of success.

More paleoecological studies of human impacts on lacustrine ecosystems are needed to understand how resilient and sensitive different lake ecosystems are to disturbances, particularly in areas where lakes are a valuable natural resource. There are also few constraints on tipping points in lakes, i.e. what drives a permanent shift in the lake ecosystem (Langdon et al., 2016). This crucial for informing lake management and adaptation strategies that have to account for both past and present human-induced changes (e.g., nutrient loading) and warming temperatures.

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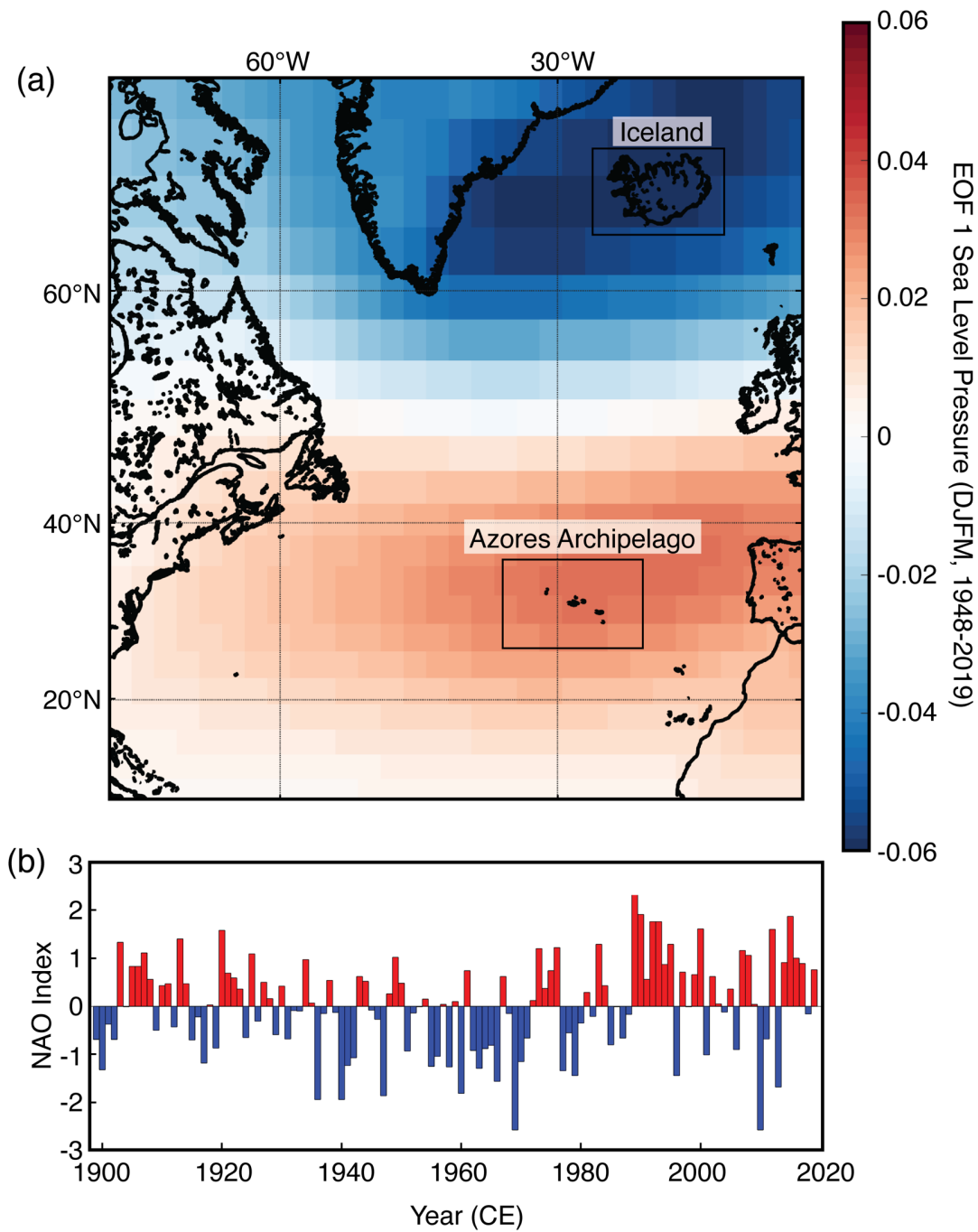


Figure 1. (a) A map of the North Atlantic region showing the leading empirical orthogonal function (EOF1) for sea level pressure over the North Atlantic sector (20°N-80°N, 90°W-40°E) for the winter months (DJFM) from 1948-2019 (Kalnay et al., 1996). **(b)** For comparison, the principal component-based North Atlantic Oscillation (NAO) index for the winter (DJFM) months is shown (National Center for Atmospheric Research, 2019). The map was rendered in Matlab using the Climate Data Toolbox (Greene et al., 2019).

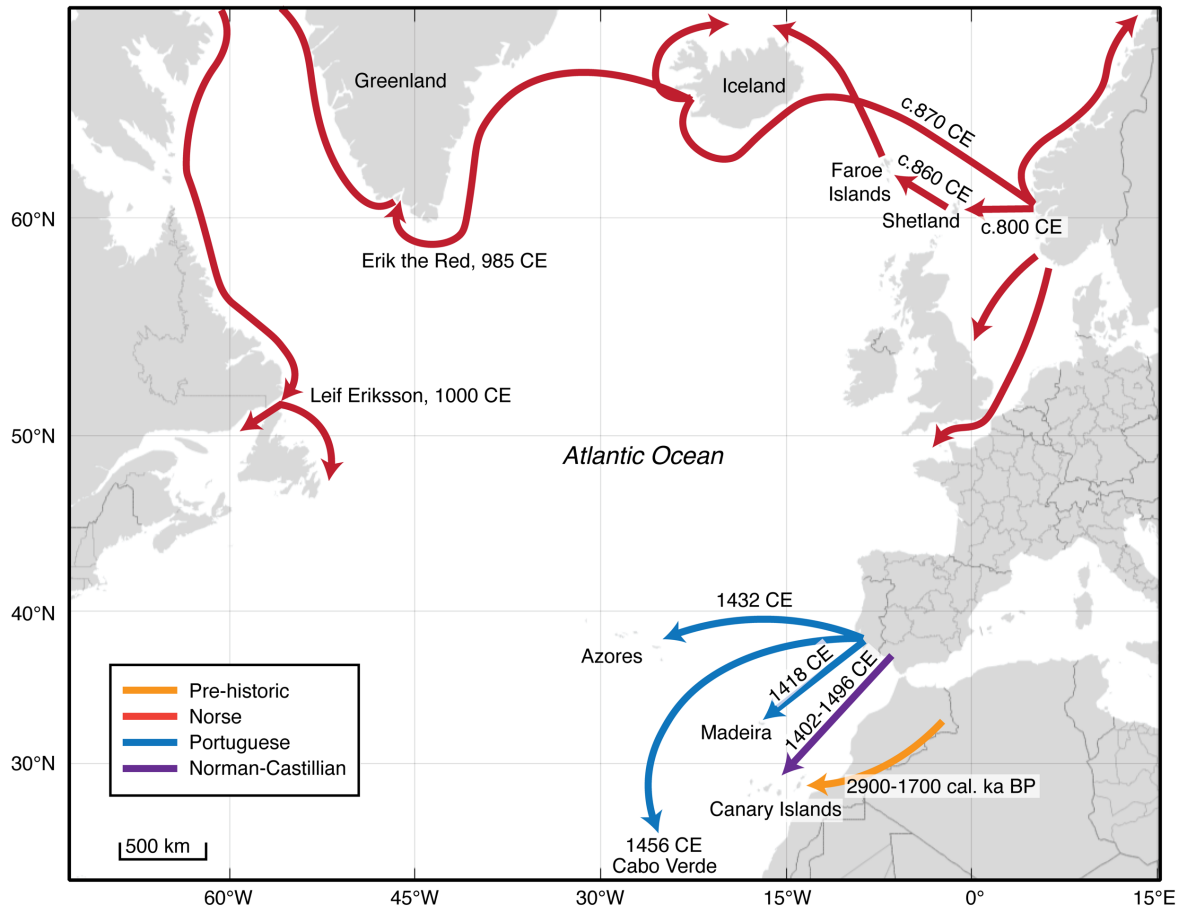


Figure 2. This map highlights major explorations and establishment of human settlements in North Atlantic lands over the last 2,000 years. The red arrows correspond to early Norse explorations and the timing of settlements in the high North Atlantic. The settlement of Macaronesia includes the pre-historic settlement of the Canary Islands by the Guanches from Northern Africa (yellow) and more recently the conquest of the Canary Islands by Norman-Castilians in the 15th century (purple). The Portuguese settlement of the Azores, Madeira, and Cabo Verde also began in the 15th century as indicated by the blue arrows (adapted from Fernández-Palacios et al. (2016)).

– CHAPTER 2 –

**Phylogenetic diversity in freshwater-dwelling Isochrysidales haptophytes with implications
for alkenone production**

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Abstract

Members of the order Isochrysidales are unique among haptophyte lineages in being the exclusive producers of alkenones, long-chain ketones that are commonly used for paleotemperature reconstructions. Alkenone-producing haptophytes are divided into three major groups based largely on molecular ecological data: Group I is found in freshwater lakes, Group II commonly occurs in brackish and coastal marine environments, and Group III consists of open ocean species. Each group has distinct alkenone distributions; however, only Groups II and III Isochrysidales currently have cultured representatives. The uncultured Group I Isochrysidales are distinguished geochemically by the presence of tri-unsaturated alkenone isomers ($C_{37:3b}$ Me, $C_{38:3b}$ Et, $C_{38:3b}$ Me, $C_{39:3b}$ Et) present in water column and sediment samples, yet their genetic diversity, morphology, and environmental controls are largely unknown. Using small-subunit (SSU) ribosomal RNA (rRNA) marker gene amplicon high-throughput sequencing of environmental water column and sediment samples, we show that Group I is monophyletic with high phylogenetic diversity and contains a well-supported clade separating the previously described “EV” clade from the “Greenland” clade. We infer the first partial large-subunit (LSU) rRNA gene Group I sequence phylogeny, which uncovered additional well-supported clades embedded within Group I. Relative to Group II, Group I revealed higher levels of genetic diversity despite conservation of alkenone signatures and a closer evolutionary relationship with Group III. In Group I, the presence of the tri-unsaturated alkenone isomers appears to be conserved, which is not the case for Group II. This suggests differing environmental influences on Group I and II and perhaps uncovers evolutionary constraints on alkenone biosynthesis.

1. Introduction

Quantitative estimates of past terrestrial temperatures are essential for testing and developing climate models that extend past the historical record to assess regional temperature variations (Otto-Bliesner et al., 2016). Lake sediment archives are sensitive to continental-scale, local, and regional climate variations and are often ideal for the preservation of organic proxies as recorders of terrestrial temperature changes (Castañeda & Schouten, 2011). Alkenones, for instance, are long-chain methyl and ethyl ketones (C_{35} – C_{42}) that show potential as lake surface temperature proxies (Zink et al., 2001; Chu et al., 2005; Sun et al., 2007; Castañeda & Schouten, 2011; Toney et al., 2012; Theroux et al., 2013; Longo et al., 2016). These compounds were first identified in ocean sediments (de Leeuw et al., 1980), and it was later noted that varying degrees of alkenone unsaturation were correlated with fluctuations in sea surface temperatures (Brassell et al., 1986; Prahl & Wakeham, 1987). Haptophyte alkenone producers belong to the order Isochrysidales and are divided into three major groups: Group I freshwater species, Group II brackish/estuarine species, and Group III open ocean species (Theroux et al., 2010; D’Andrea et al., 2016).

Previous studies identified two main alkenone producers in the open ocean: *Gephyrocapsa oceanica* (Conte et al., 1995; Volkman et al., 1995) and *Emiliania huxleyi* (Volkman et al., 1980; Conte et al., 1995). A combination of ocean surface sediment and sediment trap calibration studies (Brassell et al., 1986; Prahl & Wakeham, 1987; Prahl et al., 1988) and culture studies (Conte et al., 1995; Conte et al., 1998) were used to demonstrate that alkenone production by Group III Isochrysidales corresponds to sea surface temperature changes. Understanding how alkenone production in lakes relates to temperature changes, however, is more complex for three main reasons: (a) lake environments tend to be more chemically diverse

and are more susceptible to varying regional environmental and climatic factors (Castañeda & Schouten, 2011), this can drive differences in haptophyte productivity, alkenone production, and potentially, species variability; (b) species mixing between Group I and II phylotypes (Theroux et al., 2010) and even within the Group II phylotypes, is a known problem in many lakes and can influence temperature reconstructions (Toney et al., 2010, 2012; Randlett et al., 2014); and lastly, (c) while there are cultures available for Group II Isochrysidales (Sun et al., 2007; Toney et al., 2012; Theroux et al., 2013; Zheng et al., 2016), there are no available cultures for Group I haptophytes, complicating our ability to isolate and determine environmental controls on Group I alkenone temperature responses.

Recent environmental studies show that we can distinguish Group I alkenone profiles from Group II and III by the identification of tri-unsaturated isomeric alkenones (Longo et al., 2013, 2016). Group I alkenones are present in numerous freshwater, alkaline lakes across the Northern Hemisphere (Longo et al., 2018); however, little is known about the haptophyte producers since they have never been physically described or isolated in pure culture.

The phylum Haptophyta and the order Isochrysidales are very diverse and occur in a range of environments (Liu et al., 2009; Bendif et al., 2013; Egge et al., 2013; Edvardsen et al., 2016; Gran-Stadniczeňko et al., 2017); however, few studies have focused on the diversity of Group I Isochrysidales.

Previous studies confirmed that multiple Group I operational taxonomic units (OTUs) occur in the same lake (D'Andrea et al., 2006, 2016; Theroux et al., 2010), and a later study further recommended the establishment of a new clade within Isochrysidales called Group “EV” (Simon et al., 2013). Here, we show that the shared recent common ancestry of the “Greenland phylotype” and “EV” is strongly supported, and we also provide evidence for

additional well-supported branching patterns within the Group I. We focus on this diversity in a suite of Alaskan lakes, but then extend this comparison to lakes in Germany and Iceland to demonstrate the broader applications of our findings (Supporting Information, Figure S1). These findings describe the genetic diversity of the monophyletic Group I haptophytes, thereby guiding future interpretations of their distinct biomarker distributions in lacustrine sedimentary records.

2. Methods

2.1 Study sites and sampling

We selected study sites containing the Group I signature (Longo et al., 2016, 2018) from lakes distributed throughout the Northern Hemisphere. These sites included lakes in the U.S. (Alaska), Iceland, and Germany (see Figure S-1 and Table S-1a, b). In addition, we analyzed surface sediments from brackish Lake Fryxell, Antarctica as an external control due to good preservation of DNA and the presence of Group II Isochrysidales alkenone signatures (sediment sample from Rachael Morgan-Kiss). Prior to this study, Group II was shown to be the most closely related to Group I haptophytes (Edwardsen et al., 2016), thus we selected a site where Group II was previously sequenced and alkenone signatures were studied. In addition, we wanted to contrast the diversity of Group I Isochrysidales with Group II and Group III with respect to both small subunit (SSU) and large subunit (LSU) ribosomal RNA (rRNA) sequences. However, taxon sampling for LSU is limited (Gran-Stadniczeňko et al., 2017), although it provides better branching support for certain parts of the tree. Additional sequences for Group II LSU sequences were generated in this study, alongside Group I, to address this issue.

We collected lake surface sediment and suspended particulate matter samples on the North Slope of Alaska in June 2016 from five lakes containing Group I alkenone signatures: E1, E5, Fog2, S6, and Toolik (Table S-1a, b). Unfortunately, some of our alkenone samples were compromised, and we are only presenting the data we were able to analyze. These lakes were selected for the wide range of environmental and chemical characteristics (e.g., pH, lake depth, water chemistry) and relatively high alkenone concentrations (Longo et al. 2016). We focused our sampling efforts around the spring transitional season, which was previously established as a period of heightened alkenone production in Alaska (Longo et al., 2016). We used a Van Dorn sampler to collect water samples at various depths, and a HachTM Hydrolab water column sensor array (temperature, pH, conductivity, dissolved oxygen content, and chlorophyll fluorescence) for concurrent water column measurements. We collected water samples for alkenones (4 L) and kept them in the cold and dark (for up to 2 hours) until the samples were vacuum filtered using combusted (450°C, 5 hrs) Whatman GF/F filters (0.7 µm). The filters were immediately frozen until analysis. Similarly, water samples (1 L from the same water column samples used for alkenones) were collected for DNA extraction, and remained in the dark and cold until we filtered them through Sterivex filters (0.22 µm; Millipore, Bedford, MA, USA), and immediately placed them at -20°C. Filters with 0.22µm pore size were used because Group I Isochrysidales were previously detected in the 0.22µm-5µm size fraction (Simon et al., 2013) and to ensure comparability with previous studies (e.g, D'Andrea et al., 2006; Theroux et al., 2010). We took surface sediments with a gravity corer and subsampled the top 1 cm for alkenones and DNA. We collected all of our samples in Whirl-PakTM bags and immediately froze them (-20°C) and kept them in the dark until analysis.

Surface sediments from Germany were collected from the top 0-1 cm or 0-2 cm via sediment cores and were then freeze-dried and shipped to Brown University for processing as described by Longo et al. (2018). Sediment core samples from Iceland were obtained from the University of Minnesota National Lacustrine Core Facility (LacCore), Minneapolis, Minnesota, USA and shipped to Brown University for processing as described by Longo et al. (2018).

2.2 Lipid analyses

We freeze-dried and extracted the samples using established methods (e.g., Longo et al., 2016, 2018). In brief, we freeze-dried all of the filters and extracted them via sonication with dichloromethane:methanol (9:1 v/v), while we freeze-dried and extracted the surface sediments with a DionexTM accelerated solvent extraction (ASE) system at 120°C and 1200 psi. We separated all of the extracts by flash chromatography with silica gel (40-63 µm, 60 Å) columns to obtain alkane, ketone, and polar fractions. We then saponified the ketone fractions and purified them by silica gel column chromatography prior to analyzing them using gas chromatography-flame ionization detection (GC-FID). Alkenone concentrations were then quantified using an internal standard, 18-pentatriacontanone.

2.3 DNA extraction and analysis

We kept all of the samples either freeze-dried or frozen prior to analysis. The sediment samples (0.25 g) were then extracted using the MoBio PowerSoil[®] DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. We extracted all of the Sterivex filters using the Puregene Tissue Kit (Qiagen, Carlsbad, CA, USA) protocol. This was followed by quantification of DNA yields using a NanoDrop nucleic acid spectrophotometer

(Thermo Scientific, Wilmington, DE). If needed, we performed additional clean-up using a DNeasy PowerClean Pro Kit (Qiagen, Carlsbad, CA, USA). Samples were then amplified using: (1) haptophyte-specific primers targeting the V4 hypervariable region of the small-subunit rRNA gene (hereon designated HSSU; Egge et al., 2013); (2) haptophyte-specific primers targeting the D1/D2 hypervariable region of the large-subunit rRNA gene (hereon designated HLSU; Bittner et al., 2013); and, (3) eukaryotic-specific primers targeting the V4 hypervariable region of the SSU rRNA gene in eukaryotes (hereon designated Ev4; Balzano et al., 2015; Stoeck et al., 2010; see Table S-2).

For Ev4, we used fusion primers for PCR using the conserved regions of the Eukaryotic V4 sequence based on Balzano et al., (2015) and Stoeck et al., (2010) (see Table S-2). Illumina adaptors, consisting of 24 unique inline barcodes and 8 indices, enabled us to sequence 192 samples with unique barcode-index combinations in a single Illumina MiSeq run. We carried out all polymerase chain reactions (PCRs) in triplicate with 33 μ L using a combination of 1.0 U Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) with x1 Phusion High-Fidelity buffer, 200 μ M dNTP mix, 1.5 mM MgCl₂, DMSO, and 0.2 μ M of each primer. Approximately, 5-20 ng of template DNA was added to each PCR and a no-template control was run. We conducted PCR reactions using an Eppendorf Gradient Thermocycler (Hamburg, Germany) with the following PCR conditions for Ev4: initial denaturing for 30 s at 98°C, denaturing for 10 s at 98°C, followed by 30 s of annealing at 53°C, primer extension for 30 s at 72°C, repetition from step 2 for 10 cycles, followed by 98°C for 10 s, 48°C for 30 s, 72°C for 30 s with 15 cycles, and a final extension for 2 min at 72°C. We verified the resulting PCR products using the Caliper LabChip GX (Caliper Life Sciences, Hopkinton, MA). The amplicons were cleaned with Agencourt AMPure XP magnetic beads (Agencourt Bioscience

Corporation/Beckman Coulter, Beverly, MA) and quantified with Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Inc., Eugene, OR). The amplicons were equimolarly pooled and we verified the size using the Agilent Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). The concentration of the amplicon pool was further analyzed with qPCR prior to sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA) 500-cycle paired-end run at the Marine Biological Laboratory W. M. Keck Ecological and Evolutionary Genetics Facility.

Fusion primer sequences consisted of 528F_long_HAP_454 and PRYM01+7_HAP454 for HSSU (Table S-2) and HAP_LSU_F and LHapto20R_bis for HLSU (Table S-2). Similarly, Illumina-specific adaptors, with 6 unique inline barcodes and 8 indices, enabled us to distinguish and sequence 48 samples for HSSU and HLSU. We carried out triplicate PCR reactions for each genomic DNA sample with a 33 μ L cocktail of 1.0 U Platinum High-Fidelity DNA Polymerase (Life Technologies, Carlsbad, CA): with x1 Phusion High-Fidelity buffer, 200 μ M dNTP mix, 1.5 mM MgSO₄, and 0.2 μ M of each primer. We added 5-20 ng of template DNA to each PCR and included a no-template control for the run. The PCR conditions for HSSU was as follows: initial denaturing for 30 s at 94°C, denaturing for 15 s at 94°C, followed by 30 s of annealing at 55°C, primer extension for 60 s at 68°C, with repetition at step 2 for 30 cycles, and final extension for 5 min at 68°C. We used the following HLSU PCR conditions: initial denaturing for 2 min at 94°C, denaturing for 30 s at 94°C, followed by 30 s of annealing at 55°C, primer extension for 3 min at 68°C, with repetition from step 2 for 35 cycles, and final extension for 5 min at 68°C. The resulting PCR triplicate reactions were pooled and the PCR products were verified using a Caliper LabChip GX. We cleaned the amplicons with Agencourt AMPure XP magnetic beads and quantified the amplicons with Quant-iT PicoGreen dsDNA Assay Kit. The amplicons were equimolarly pooled and the size was verified using the Agilent Bioanalyzer

DNA 1000 chip. In addition, we checked the concentration of the amplicon pool with qPCR prior to sequencing on an Illumina MiSeq 500-cycle paired-end run. The pool of amplicon libraries was diluted to 13 pM prior to clustering while PhiX was added as a diversity control for the MiSeq sequencing run. All sequences are available through Visualization and Analysis of Microbial Population Structures (<https://vamps2.mbl.edu/>) and were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with the following numbers: SAMN09475369 - SAMN09475400 for HSSU sequences and SAMN09475416 - SAMN09475436 for HLSU sequences. A minimum information about a marker gene sequence (MIMARKS) table is included in the supplements of the published version of this paper.

2.4 Bioinformatics

All sequences were demultiplexed using the unique barcodes, and then merged for quality control. Unique sequences were then identified and checked for chimeras. The initial taxonomy was assigned using a Global Alignment for Sequence Taxonomy (GAST) pipeline (Huse et al., 2008). A search using SILVA 119 taxonomy (Pruesse et al., 2007; Quast et al., 2013) did not produce reasonable hits for either HSSU or HLSU, thus a new reference database was created using SSU and LSU sequences from Edwardsen et al. (2016). The results were checked with the NCBI Basic Local Alignment Tool (NCBI BLAST; Altschul et al., 1997). All Isochyridales sequences were subsequently uploaded into ARB (Ludwig et al., 2004) for initial comparison with the existing SILVA 119 database (Pruesse et al., 2007; Quast et al., 2013). Overlap between the HSSU and Ev4 sequences (minimum 340 bp) was used to align the sequences to reference SSU rRNA sequences from Edwardsen et al., (2016) in PyNAST (Caporaso et al., 2010), and then checked manually. Similarly, HLSU sequences were aligned to

LSU reference sequences from Edvardsen et al., (2016) in PyNAST (Caporaso et al., 2010) and checked manually. We employed entropy as implemented in Oligotyping to analyze the resulting reads and generate Exact Sequence Variants ("oligotypes"; Callahan et al., 2017; Eren et al., 2013, 2014), which are independent of the reference database and will allow for comparability in future studies. For our analysis, we required that each oligotype occur at least 100 times in one or more sample to be considered a valid oligotype, this was based on the average number of reads per sample and was used to reduce noise from oligotypes with nominal abundance (Eren et al. 2013). A conservative estimate of 100 was used. The resulting oligotypes were then combined into one file with all available Group I Isochrysidales sequences (D'Andrea et al., 2006; Edvardsen et al., 2016; Gran-Stadniczeňko et al., 2017; Theroux et al., 2010) and compared against previously defined OTUs by Theroux et al. (2010) and D'Andrea et al. (2006) to check for overlap. PAUP* (v4.0b10; Swofford, 2003) and ModelTest (3.06; Posada & Crandall, 1998) were used to select a substitution model that best described the DNA data. For both HSSU (340 bp) and HLSU (370 bp) a general time reversible (GTR) model with gamma substitution rates was chosen. These models were used to infer phylogenetic trees in MrBayes (v3.2.6; Ronquist et al., 2012) for HSSU and HLSU. All phylogenetic trees were inferred using two simultaneous runs with 3 heated chains (chain temperature = 0.1) and one cold chain that consisted of a 25% burn in frequency and sampling frequency of 100. Analyses were stopped when the standard deviations of the split frequencies reached values below 0.01, such that the HSSU tree was run for 3.0×10^6 generations, and both the HLSU tree with all oligotypes and the simplified HLSU tree were run for 3.0×10^6 generations. The resulting trees were visualized in FigTree (v1.4.3; Rambaut, 2007).

2.5 Alkenone proxy calculations

To test for concurrent changes in alkenones and Group I haptophytes, we extracted and measured the fractional abundances and concentrations of 16 alkenones in our suite of Alaskan lakes. The ratio of isomeric ketones, RIK₃₇ index, was determined using changes in fractional abundance of C_{37:3a}Me compared to C_{37:3b}Me alkenones to test for Group I and II mixing (Longo et al., 2016):

$$\text{RIK}_{37} = \frac{\text{C}_{37:3a}\text{Me}}{\text{C}_{37:3a}\text{Me} + \text{C}_{37:3b}\text{Me}} \quad (1)$$

A RIK₃₇ value of 1.0 indicates that only C_{37:3a}Me is present, suggesting alkenone production by Group II, and values from 0.48-0.63 indicates the presence of both C_{37:3}Me ketone isomers and alkenone production by Group I (Longo et al., 2018). Temperature calibrations are commonly determined using the U₃₇^K index (Brassell et al., 1986), which was later modified and calibrated for sea surface temperatures (Müller et al., 1998; Prah & Wakeham, 1987) and further modified (Longo et al. 2016) to incorporate the C_{37:3b}Me isomer for lacustrine calibrations.

$$\text{U}_{37}^{\text{K}} = \frac{\text{C}_{37:2}\text{Me} - \text{C}_{37:4}\text{Me}}{\text{C}_{37:2}\text{Me} + \text{C}_{37:3a}\text{Me} + \text{C}_{37:3b}\text{Me} + \text{C}_{37:4}\text{Me}} \quad (2)$$

For Lake E1, Alaska we regressed calculated U₃₇^K index values against *in situ* water column temperatures to assess the similarity between Group I temperature responses in neighboring, but physically and chemically distinct lakes. To assess whether differences in Group I diversity corresponded with significant variations in alkenones, we compiled previously published alkenone data (Longo et al., 2016, 2018) for comparison with DNA analyses from this study. We

expressed alkenone quantifications as fractional abundances, in which each alkenone concentration is divided by the summed concentration data for all 16 alkenones in a given sample. The resulting data was graphically visualized using a heatmap (Figure S-3) with the ggplot2 package (Wickham, 2009) in R (v. 3.4.1).

3. Results

3.1 *Haptophyte small-subunit (HSSU) rRNA gene oligotypes*

Haptophyte-specific primers targeting the small-subunit rRNA gene (hereon designated haptophyte small-subunit [HSSU]; Egge et al., 2013) followed by oligotyping revealed 31 out of 50 distinct oligotypes for Group I Isochrysidales (designated as S-Oligotypes from hereon; Figure 1) that we further divided into S-Oligotypes Ia and Ib. We recovered relatively high read counts for Group I even though the HSSU primers we employed also amplified non-haptophyte species including a large number of fungi in each of our samples. The number of non-haptophyte sequences recovered was higher in the sediment samples ($91.0 \pm 25.5\%$) than the water column samples ($77.7 \pm 11.8\%$). The greatest number of Isochrysidales identified oligotypes was present in the water column (0.1%-27.8% Isochrysidales sequences) and sediment samples (0.5%-1.5% Isochrysidales sequences) from Alaska (mainly Lakes E1 and E5; Figure 2a). We also recovered Group I oligotypes from surface sediment samples from German and Icelandic lakes (0.1%-1.0% Isochrysidales sequences; Figure 3a), despite these samples being collected for alkenone characterization and not molecular ecology work. In almost all of our samples, we note a predominance of S-O ligotypes Ia (making up 43.2%-100.0% of the Isochrysidales sequences recovered) over S-Oligotypes Ib (ranging from 0.0%-56.8% for all the Isochrysidales sequences recovered). In the Alaskan water column samples (i.e., E1, E5, Fog2, and Toolik; Figure 2), we

observed a seasonal succession of different oligotypes during the month of June as lake ice-out, isothermal mixing, and incipient stratification occurred. We also observed a change in Isochrysidales community composition with water column depth (i.e., E1, E5, and Fog2).

Phylogenetic inference revealed significant branch support (posterior probability, PP = 1.0) for the monophyly of Group I, Group II (PP = 1.0), and Group III (PP = 1.0) Isochrysidales clades (Figure 1), independently. We noted that S-Oligotypes Ia were closely related to previously defined OTUs in freshwater and brackish lakes from Canada, China, France, Greenland, and the United States (D’Andrea et al., 2006; Simon et al., 2013; Theroux et al., 2010). In particular, S-Oligotype 2 in the S-Oligotype Ia group was closely related to previously defined OTU 1 (Theroux et al., 2010; Figure 1) and was present in all of the HSSU-amplified samples (except the Lake Fryxell sample, which contained only Group II sequences; Figures 1 & 3a). In addition, within Group I, we noted the presence of a distinct clade (PP = 1.0) that consisted of oligotypes from this study and previous sequences from Lakes Etang des Valles (“EV”) and Annecy in France (Simon et al., 2013). Our HSSU tree also showed robust support for recent shared ancestry of Groups I and III, which differs from previous studies that suggest more recent common ancestry between Groups I and II (Gran-Stadniczeňko et al., 2017; Simon et al., 2013; Theroux et al., 2010).

3.2 Haptophyte large-subunit rRNA gene oligotypes

We report the first haptophyte-specific large-subunit rRNA gene (hereon designated haptophyte large-subunit [HLSU]; Egge et al., 2013) sequences for the Group I clade (Figure 4). We detected 50 out of 72 oligotypes for the LSU rRNA Group I clade (referred to as L-Oligotypes from hereon) with several subgroupings, which we define here as L-Oligotypes Ia, Ib,

Ic, and Id. Despite low total sequence counts, our trees demonstrated significant support for the monophyly of the Group I clade (PP = 1.0). We noted a general predominance of L-Oligotypes Ia (relative recovery of 43.4%–100% of the total Isochrysidales sequences) over L-Oligotypes Ib, Ic, and Id (0%–54.6% out of the total Isochrysidales sequences; Figures 2b & 3b).

3.3 Group I alkenones

We selected sampling locations based on the previous detection of Group I alkenone signatures. All alkenone profiles analyzed for this study contained the distinct $C_{37:3b}$ Me alkenone that is the hall- mark of Group I Isochrysidales (Longo et al., 2016, 2018). The ratio of isomeric ketones, RIK_{37} index, which is based on changes in the fractional abundance of the $C_{37:3a}$ Me compared to the $C_{37:3b}$ Me alkenones was used to test for Group I and II mixing (Longo et al., 2016). In our study, RIK_{37} values ranged from 0.48 to 0.64, corroborating the HSSU and HLSU datasets that identified Group I as the dominant Isochrysidales haptophyte in all of these systems. The U_{37}^K index for the Lake E1 water column samples showed a linear correlation with temperature ($U_{37}^K = 0.015T - 0.64$, $r^2 = 0.84$, $p < 0.05$; Supporting Information, Figure S2). Alkenone fractional abundances were visualized using a heatmap (Supporting Information, Figure S3). Overall, alkenone distributions were consistent among samples. C_{37} Me alkenone distributions were highly conserved with slightly greater variation among the C_{38} Et, C_{38} Me, and C_{39} Et alkenones.

4. Discussion

4.1 Group I diversity inferred from SSU and LSU rRNA gene oligotyping and phylogenetic tree reconstructions

We found Group I oligotypes (HSSU and HLSU) in samples from freshwater lakes with Group I alkenone signatures in Alaska, Germany, and Iceland (Figure 1). We demonstrated robust support for the monophyly of the Group I assemblage that includes members of the “Greenland phylotypes” and previously described Group “EV” by Simon et al. (2013). Because no LSU data existed for this clade, we were unable to determine its phylogenetic position in our HLSU phylogenetic trees (Edwardsen et al., 2016). During our alignments of all existing Group I SSU rRNA gene sequences, however, we determined that Group “EV” sequences were likely not identified in previous haptophyte molecular diversity studies that targeted the region between 427–889 bp (D’Andrea et al., 2006, 2016; Theroux et al., 2010; Toney et al., 2010) because the primers used in those studies (Coolen et al., 2009) are not compatible with Group “EV” amplification due to primer mismatch. In addition, our high-throughput amplicon sequencing in combination with oligotyping allowed us to amplify sequences present in relatively low abundance and identify exact nucleotide variants in our sequences (Callahan et al., 2017; Eren et al., 2013, 2014). This was not possible using earlier techniques that were based on clustering approaches in combination with clone library and second-generation (e.g., capillary) sequencing technology (Eren et al., 2013, 2014; Callahan et al., 2017). Therefore, the Group “EV” clade is likely present in other lakes that were previously analyzed (e.g., lakes reported by Theroux et al., 2010). Future analyses of fresh and appropriately preserved samples from these lakes for molecular ecology work might reveal an even higher diversity of oligotypes (Willerslev & Cooper, 2005) than we present in this study.

Within the HLSU data, we found several dominant oligotypes, whereas there was only one dominant oligotype in the HSSU data: S-Oligotype 2 in the S-Oligotype Ia group. This is likely the result of inherently higher genetic variability in the LSU rRNA gene relative to the SSU rRNA gene due to higher mutation rates in LSU rRNA genes (Bittner et al., 2013) and therefore might provide additional insights into Group I diversity. We did observe a higher number of oligotypes within the HLSU dataset, despite the small number of samples. However, low branch support within the HLSU Group I suggested that better taxon sampling is needed before we are able to identify L-Oligotypes that correspond to distinct S-Oligotype clades within Group I. To test for congruence between the HSSU and HLSU oligotypes, we would need to sequence the region that spans both subunits, which was beyond the scope of this study but the subject of future efforts.

Our HSSU and HLSU results revealed another significant finding: Group I and III are more closely related than previous studies have demonstrated. Previous work reported that Groups I and II were more closely related (Theroux et al., 2010; Simon et al., 2013; Gran-Stadniczeňko et al., 2017). This provides insight into why there is such conservation in the alkenones of Groups I and III in contrast to Group II. Group I Isochrysidales are found in a range of environmental conditions: pH levels 5.9–9.4, salinities ranging from 0 to 4.43 g/L, alkalinity from 104 to 1,976 mEq/L, and water temperatures from 0–4°C to 12–16°C (D’Andrea & Huang, 2005; Theroux et al., 2010; Toney et al., 2010; D’Andrea et al., 2016; Longo et al., 2016, 2018; Plancq et al., 2018). Group II is found in a similarly broad range of environmental conditions: pH levels 7.3 to 10.5, salinities ranging from 0.05 to 270 g/L, and water temperatures from 8°C to 28°C (Chu et al., 2005; Sun et al., 2007; Theroux et al., 2010; Toney et al., 2010; Liu et al., 2011; Randlett et al., 2014; Longo et al., 2016, 2018; Plancq et al., 2018). We observed high

genetic diversity in both Groups I and II Isochyrsidales (Bendif et al., 2013; Egge et al., 2013; Edvardsen et al., 2016; Gran-Stadniczeňko et al., 2017), but we see differing alkenone conservation between Group I and Group II (Theroux et al., 2010; Toney et al., 2010; Randlett et al., 2014; D’Andrea et al., 2016; Longo et al., 2016, 2018; Zheng et al., 2016). In Group I, we see a conservation of tri-unsaturated alkenone isomers (C₃₇:3b Me, C₃₈:3b Et, C₃₈:3b Me, C₃₉:3b Et; Longo et al., 2016, 2018), whereas Group II culture studies point to the consistent absence of C₃₈ Me alkenones (Rontani et al., 2004; Sun et al., 2007; Ono et al., 2012; Theroux et al., 2013; Nakamura et al., 2014; Zheng et al., 2016). In contrast, we observe low genetic and alkenone diversity in Group III Isochyrsidales (Conte et al., 1995, 1998, 2006; Bendif et al., 2014). The low alkenone diversity in Groups I and III might point to similar mechanisms in streamlining their alkenone biosynthetic pathways, as they are more closely related to each other than to Group II Isochyrsidales (Figures 1 and 4).

4.2 Alaska case study: Group I oligotype succession in June

During the month of June, Lake E1 water column samples were collected during partial ice-cover, isothermal mixing, and summer stratification. Our findings demonstrate that Group I species composition varies throughout the season (Figure 2a). In Lake E1, at both 3- and 10-m depths we observed a higher number of oligotypes and relative read abundance for S-Oligotypes Ib in the early part of the season. We recovered the largest number of reads in the early part of the season, and we recovered fewer reads and observed an increase in S-Oligotype Ia and a decrease in S-Oligotype Ib relative read abundance as Lake E1 underwent isothermal mixing (June 6) and summer stratification (starting June 13 and completing stratification June 18). The temporal decreases in alkenone unsaturation in our dataset corresponded to changing lake water

temperatures at 3- and 10-m depths, which were both in the euphotic zone (Supporting Information, Figure S4).

In Lake E5, however, we did not see a similar change in HSSU oligotypes (Figure 2a), but we did see a decrease in the number of Group I oligotypes present in the lake, and the limited appearance of Group II oligotypes on June 25 at 2-m depth. In comparison with Lake E1, Lake E5 appears to have a longer isothermal mixing period and remained partially ice-covered until June 21 (Supporting Information, Figure S5).

We observed a comparatively high number of oligotypes present in the E1 datasets for HSSU and HLSU (Figure 2a, b). This was not apparent in the other Alaskan lakes, which had a lower number of oligotypes present in both the water column and surface sediment samples. In addition, we did not observe a major difference in the oligotypes found in the water column and surface sediment samples (Figure 2a). This suggests that the oligotypes we observed in the water column were getting preserved in the sediment. This also suggests that we were sampling the water column during the peak of the Group I Isochrysidales bloom. It should be noted that we observed a predominance of S-Oligotypes Ia in all of the sediment samples from Alaska (i.e., E1, E5, and Fog2; Figure 2a). Similarly, we saw a predominance of L-Oligotypes Ia in all of the surface sediment samples from Alaska (i.e., E1, E5, Fog2, S6, and Toolik; Figure 2b).

4.3 Conservation of alkenone signatures

Despite the large genetic diversity in Group I Isochrysidales, previous work suggested that variations in alkenone composition were due to temperature and that the high number of oligotypes did not play a major role in alkenone biomarker applications in these lakes (Longo et al., 2016, 2018). In Lake E1 from Alaska, for instance, we quantified alkenone concentrations

and sequenced DNA from the same water column samples. In our sequence data for HSSU, we observed extensive diversity in Group I throughout the month of June (Figure 2a), but we still observed a correlation between alkenones and temperature (Longo et al., 2016; Supporting Information, Figure S2). The largest variation that we observed in the alkenone profiles was in C_{37:4} Me, which corresponded to changes in lake water temperature.

Considering alkenone profiles from sediments for all of the sites analyzed in this study (Supporting Information, Figure S3), we saw that variability in the C₃₇ Me group was similar for sediment samples from the same temperature regime (note that Iceland samples from Baejarvotn and Skufnavotn were from a similar temperature regime, while samples from Vestra Gíslholtsvatn were from a warmer region). This corroborates a recent finding that C₃₇ Me alkenone production in response to temperature may be consistent among Group I Isochrysidales (Longo et al., 2018). In our study, we only present a small range of lakes that are typically cold biased. However, as mentioned earlier, previous studies showed that Group I alkenone signatures occur in a wide range of environmental conditions.

Comparison to Etang des Valleees, reported to have high concentrations of Group “EV” in the water column (Simon et al., 2013), showed that C_{37:3a} Me and C_{38a} Et were present in higher fractional abundance relative to the rest of the dataset (Supporting Information, Figure S3). However, this lake could experience warmer lake temperatures because it is shallow and experiences a mean annual air temperature of 10.4°C, which could also drive increases in the fractional abundance of longer chain alkenones. Therefore, it is difficult to draw any direct conclusions about the presence/absence of oligotypes and changes in alkenone abundance from our study, but this should be a focus of future efforts.

5. Conclusions

Samples from Alaska, Iceland, and Germany show that Group I haptophytes are more diverse and widespread than previously reported. By sequencing partial SSU rRNA genes, we identified a well-supported clade within Group I, which includes samples from previously described Group “EV” (Simon et al., 2013). This clade is distinctly different from the “Greenland phylotype” and was present in almost all of the samples analyzed in this study. We also report the first LSU rRNA gene sequence-based phylogeny for Group I Isochrysidales and the presence of distinct subclades; however, additional work is needed to better resolve these relationships and link them to SSU datasets. Using Lake E1 from Alaska as a case study, we noted a decrease in the relative read abundance of Group “EV” in the month of June 2016, and a corresponding increase in the “Greenland phylotype.” Despite the high genetic diversity in Group I Isochrysidales, we see an overall conservation of alkenones. In addition, we see a closer genetic relationship between Group I and III Isochrysidales.

Future work is needed to assess what controls the dominance of different oligotypes in lakes that are predominantly Group I. For example, when do Group “EV” Isochrysidales bloom relative to the “Greenland phylotype”? In addition, more work is needed to understand why Group I alkenones appear to be conserved across a chemically diverse set of lacustrine environments, and what role they play in the Isochrysidales life cycle.

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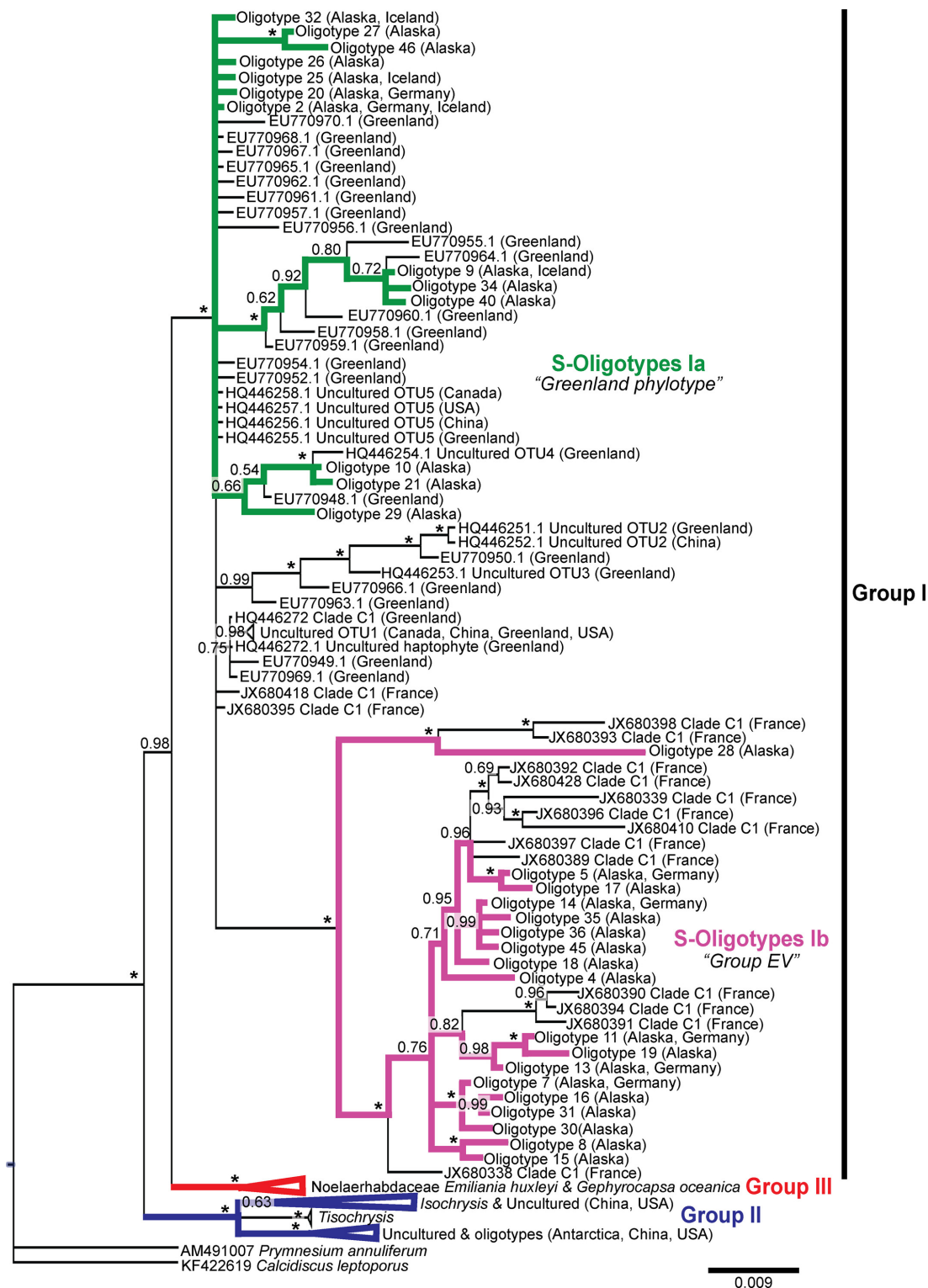


Figure 1. Haptophyte small-subunit phylogenetic tree with oligotypes analyzed in this study as indicated by the green and pink colored branches, corresponding to S-Oligotypes Ia ("Greenland phylotype") and S-Oligotypes Ib ("Group EV"), respectively. The posterior probability for each node is indicated, where "*" corresponds to a probability of 1.0. Note Groups I and III are closely related with 0.98 branch support, relative to Group II. An expanded version of the tree can be found in the Supporting Information Figure S6.

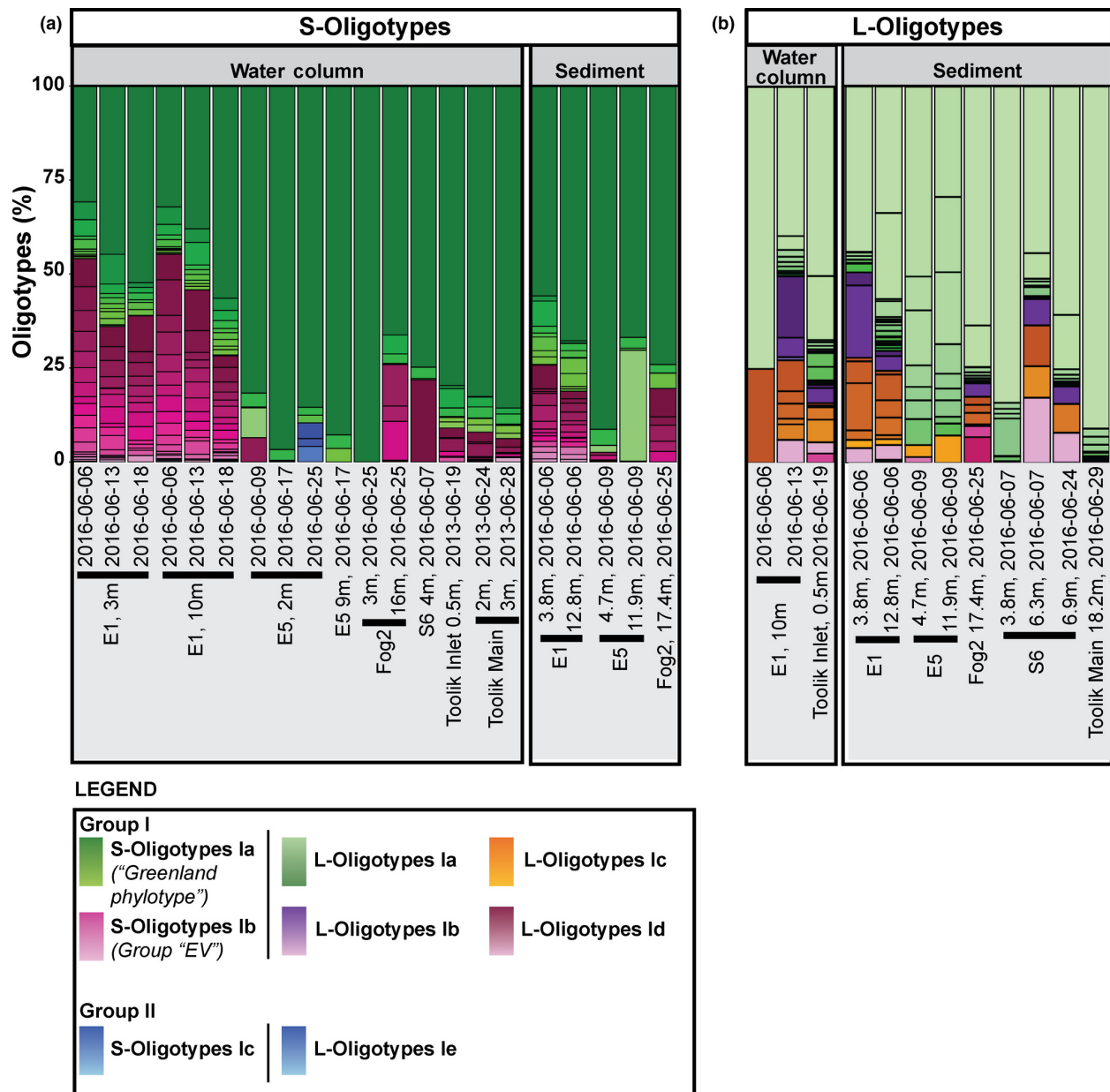
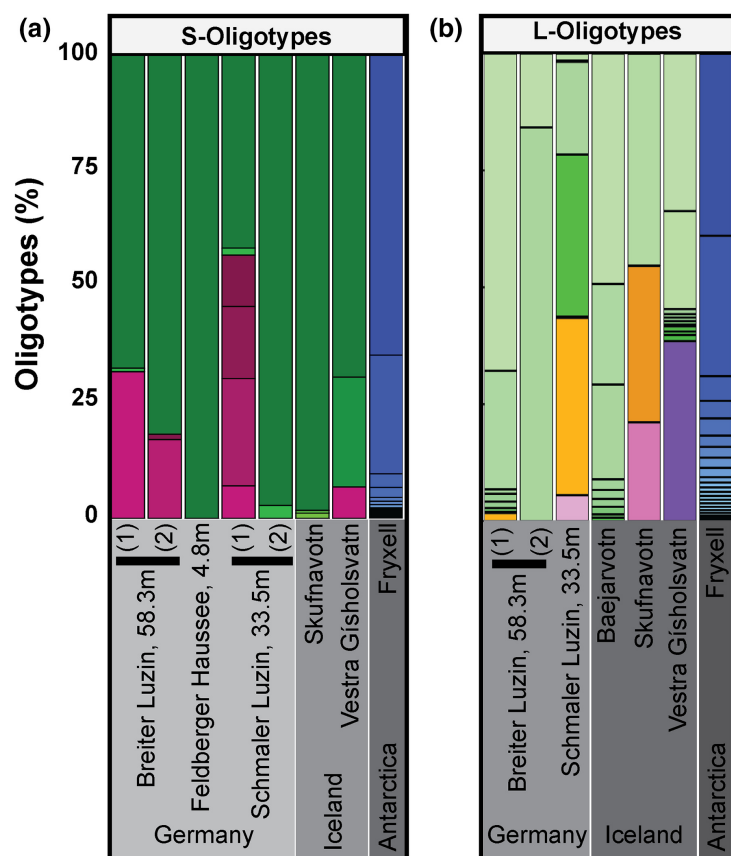


Figure 2. This figure shows the relative read abundance of (a) haptophyte small-subunit and (b) haptophyte large-subunit (HLSU) oligotypes for samples from Alaska analyzed in this study. (a) The green shades correspond to S-Oligotypes Ia, whereas the maroon shades correspond to S-Oligotypes Ib. (b) This figure reflects the relative read abundance of HLSU oligotypes for different samples analyzed in this study, where light green, purple, orange, and pink correspond to L-Oligotypes Ia, Ib, Ic, and Id, respectively. Group II oligotypes are shown in blue for both S-Oligotypes and L-Oligotypes. Group II L-Oligotypes were found in Toolik Lake; however, only two sequences were recovered, one for L-Oligotype 2 and another for L-Oligotype 3.



LEGEND

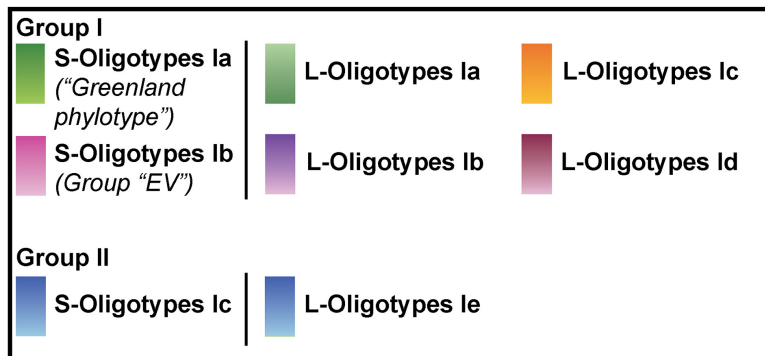


Figure 3. This figure reflects the relative read abundance of (a) haptophyte small-subunit and (b) haptophyte large-subunit (HLSU) oligotypes analyzed in this study from lakes in Germany, Iceland, and Antarctica. (a) The green shades correspond to S-Oligotypes Ia, whereas the maroon shades correspond to S-Oligotypes Ib. (b) This figure reflects the relative read abundance of HLSU oligotypes for different samples analyzed in this study, where light green, purple, orange, and pink correspond to L-Oligotypes Ia, Ib, Ic, and Id, respectively. Group II oligotypes are shown in blue for both S-Oligotypes and L-Oligotypes.

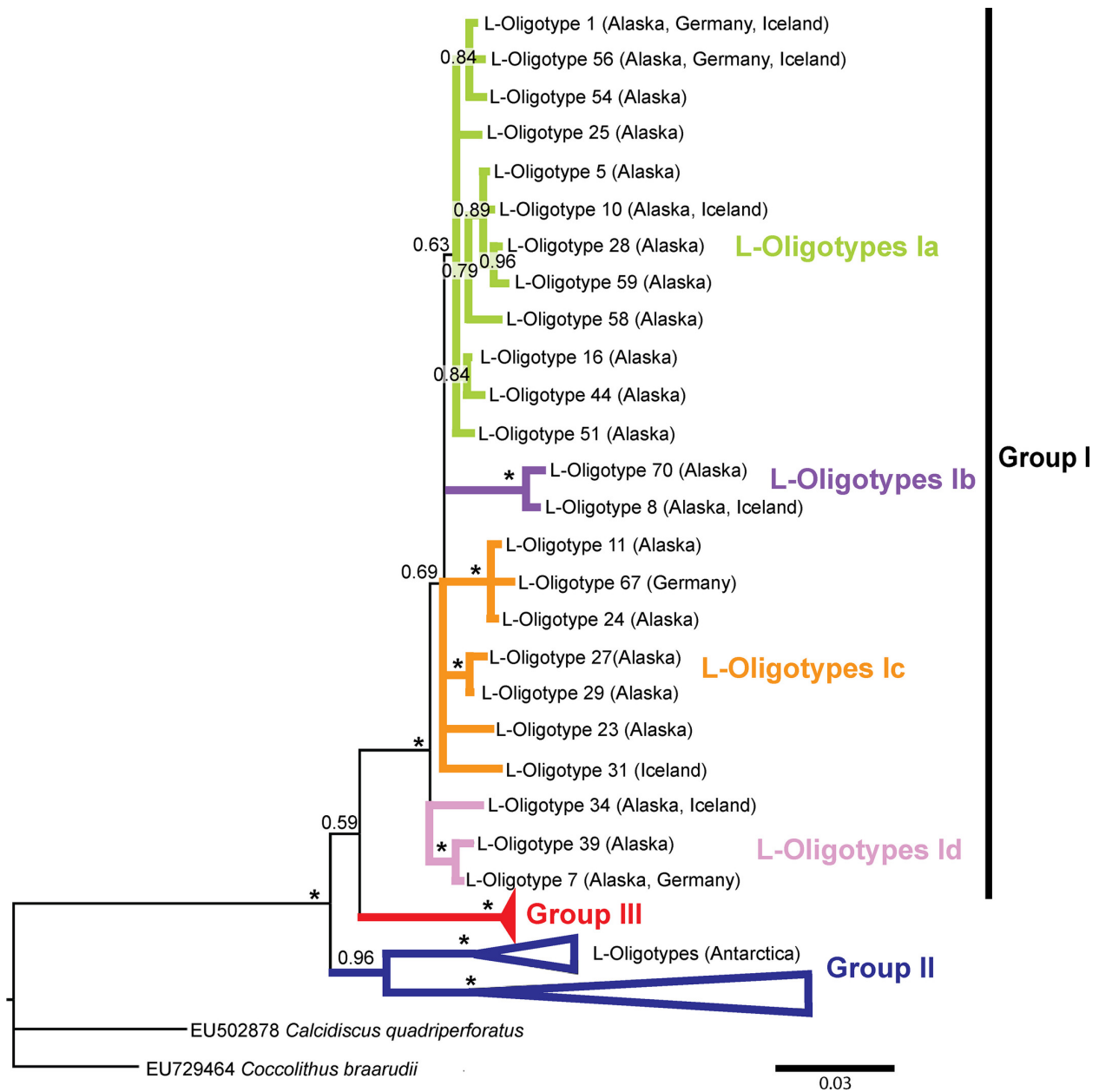


Figure 4. Haptophyte large-subunit (HLSU) phylogenetic tree with oligotypes analyzed in this study indicated by the light green, purple, orange, and pink branches (note that the different colors do not correspond to the haptophyte small-subunit oligotypes). The posterior probability for each node is indicated, where “*” corresponds to 1.0. An expanded version of the tree can be found in the supporting information, Figure S7.

Supplementary Tables.

Table S1a. Water column samples from Alaskan lakes.

Location			Sample Information			Environmental Parameters		
Lake	Latitude	Longitude	Date Sampled	Sample Depth (m)	Depth at Sampling Location (m)	pH	Water Temperature (°C)	Lake Physical Status
E1	68.63	-149.55	6-Jun-2016	3	12.82	7.08	3.63	Ice-covered
			6-Jun-2016	10	12.82	7.13	3.54	Ice-covered
			13-Jun-2016	3	12.43	7.51	5.39	Mixing
			13-Jun-2016	10	12.43	7.38	4.62	Mixing
			18-Jun-2016	3	11.93	7.48	8.06	Summer stratification
			18-Jun-2016	10	11.93	7.15	4.69	Summer stratification
E5	68.64	-149.46	9-Jun-2016	2	4.86	5.92	3.17	Mixing
			17-Jun-2016	2	10.12	6.05	4.55	Mixing
			17-Jun-2016	9	10.12	6.02	3.98	Mixing
			25-Jun-2016	2	9.5	6.73	8.37	Summer stratification
Fog2	68.679	-149.09	25-Jun-2016	3	16.59	7.22	5.72	Summer stratification
			25-Jun-2016	16	16.59	7.17	3.49	Summer stratification
S6	68.63	-149.64	7-Jun-2016	4	6.25	-	-	Mixing
Toolik Inlet	68.63	-149.6	19-Jun-2013	0.5	-	-	-	Summer stratification
Toolik Main	68.63	-149.6	24-Jun-2013	2	25	-	-	Summer stratification
			28-Jun-2013	3	25	-	-	Summer stratification

Table S1b. Sediment samples from Alaskan and global lakes.

Location				Sample Information			Environmental Parameters				Data Source
Lake	Country	Latitude	Longitude	Date Sampled	Sample Type	Lake Sampling Depth (m)	Maximum Lake Depth (m)	pH	Salinity (ppt)	Mean Spring Temperature (°C)	
E1	USA	68.63	-149.55	6-Jun-2016	Surface Sediment	3.8	13	7.76	0	-	Longo et al. 2016, 2018
				6-Jun-2016	Surface Sediment	12.8					
E5	USA	68.64	-149.46	9-Jun-2016	Surface Sediment	4.7	13	6.84	0	-0.25	Longo et al. 2016, 2018
				17-Jun-2016	Surface Sediment	11.9					
Fog2	USA	68.679	-149.09	25-Jun-2016	Surface Sediment	17.4	20	8.04	0	-	Longo et al. 2016, 2018
S6	USA	68.63	-149.64	7-Jun-2016	Surface Sediment	3.8	9	8.48	0	-0.025	Longo et al. 2016, 2018
				7-Jun-2016	Surface Sediment	6.3					
				24-Jun-2016	Surface Sediment	6.9					
Toolik Main	USA	68.63	-149.6	29-Jun-2016	Surface Sediment	18.2	26	7.61	0	-	Longo et al. 2016, 2018
Breiter Luzin	Germany	53.35	13.46	2016	Surface Sediment x1	58.3	58.3	8.70	0.2	2.05	Longo et al. 2018
				2016	Surface Sediment x2	58.3					
Schmaler Luzin	Germany	53.32	13.44	2016	Surface Sediment x1	33.5	33.5	8.50	-	2	Longo et al. 2018
				2016	Surface Sediment x2	33.5					
Felberger Haussee	Germany	53.35	13.45	2016	Surface Sediment	4.8	12.5	8.90	0.3	2.025	Longo et al. 2018
Baejarvotn	Iceland	65.73	-21.43	-	Core (8.5-10.5 cm)	23.2	23.2	7.40	0	-	Longo et al. 2018
Skufnavotn	Iceland	65.89	-22.12	9-Mar-2010	Core (19-21 cm)	4.5	5.8	-	-	-	Longo et al. 2018
Vestra Gisholtvatn	Iceland	63.95	-20.52	4-Aug-2008	Core (5.5-7.5 cm)	13.4	13.4	7.70	-	-	Longo et al. 2018
Fryxell	Antarctica	-77.61	85.54	-	Surface Sediment	-	18	8.04	-	-	Wharton et al. 1982; Yang et al. 2015

Table S2. Primers used in this study.

Primer Name	Target Group	Target Region	Direction 5' - 3'	Primer 5' - 3'	Length (bp)	References
V4F	Eukaryotes	18S-V4	Forward	CCAGCASCYGCGGTAATTCC	380	Stoeck et al. 2010
V4RB	Eukaryotes	18S-V4	Reverse	ACTTTCGTTCTTGATYRR	380	Balzano et al. 2015
528F_long	Haptophytes	18S	Forward	GCGGTAATTCCAGCTCCA	400	Egge et al. 2013
PRYM01+7	Haptophytes	18S	Reverse	GATCAGTGAAAACATCCCTGG	400	Egge et al. 2013
HAP_LSU_F	Haptophytes	28S	Forward	GGTRTCGGAGARGGTGAGAATCC	350-400	Modified from Bittner et al. 2013
LHapto20R_bis	Haptophytes	28S	Reverse	TCAGACTCCTTGGTCCGTGTTTCT	350-400	Bittner et al. 2013

Table S3. HSSU unique oligotypes showing the variable positions that were used to define each oligotype (note that this is not the entire sequence) with the number of sequences used to define each oligotype listed in the last column. The unique oligotype sequences are shown with nucleotide variations (relative to Oligotype 1) highlighted in blue to indicate what differences were used to define each oligotype.

HSSU Oligotypes	Unique Oligotypes	Sequences per Oligotype
Oligotype 1	GTGTTGCGTCGTCGCGCCAGCCTGGTGCGTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTTCG	73998
Oligotype 2	GTGTTGCGTCGTCGCGCCGTCCGCGCCGCGCCGCGGAAATGTTGGGCTTCAAAAGTGCGGCGCG	40771
Oligotype 3	GTGTTGCGTCGTCGCGTCAGCCTGGTGCGTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTTCG	29167
Oligotype 4	A-GTAGCGTCATCTGCGTCCGCGCCGCGCCGCGAA-ATGTTGGGCTTCAAAGAGTGTTGGCTG	5248
Oligotype 5	A-GTTGCGTCGTCGCGCCGTCCGCGCCGCGCCGCGGAAAAGTTGGCTTAAAGAGTGTTGGGCG	4286
Oligotype 6	GTGTTGCGTCGTCGCGCCAGCCTGGTGCGTGTCCGTGAAATGTTGGGCTTCAGAAGGCGCGGTTCG	3394
Oligotype 7	GTGTTGCGTCGTCGCGTCCGCGCCGCGCCGCGGAAATGTTGGGCTTCAAGAGTATTTGGGCG	3366
Oligotype 8	GTGTTGCGTGTGT-TGCGTCCGCGCCGACCGCGGAAATGTTGGGCTTCAAGAGTGTTGGGCG	3084
Oligotype 9	G-GTTGCGTCGTCGCGCGTCCGCGCCGCGCCGCGGAAATGTTGGGCTTCAAAAATGCGGCGCG	3021
Oligotype 10	GTGTTGCGTCGTCGCGCGTCCGCGCCGCGCCGCGGAAATGTTGGGCTTCAAAATATGCGACCG	3006
Oligotype 11	GTGTCGCTCGTCGCGTCCGCGCCGCGCCGCGGAAATGTTTGGCTTCAAGAGTGTTGGCTG	2513

Oligotype 12	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAGGTGCGGTTCG	2413
Oligotype 13	GTGTTCGCGTCGTCGCGCCGCGCCGCCGCGGAAATGTTTGGCTTCAGAGTGTGGGTG	2280
Oligotype 14	A-GTTGCGTCGTCGCGCCGCGCCGCCGCGGAAATGTTGGGCTTCAGAGTGTGGCCG	2121
Oligotype 15	GTGTTGCGTCGT-TGCCGTCTGCGCCGACGCGGAAATGTTGGGCTTCAGAGTGTGGCCG	1930
Oligotype 16	GTGTTGCGTCGTCGCGCCGCGCCGCCGCGGTAATATTGGGCTCCAGAGTATGGCCG	1899
Oligotype 17	A-GCTGCGTGTCTCCGCGCCGCCGCGGAAAAGTTGCGTTTAAGAGTGTGGCCG	1801
Oligotype 18	A-GTTGCGTCGTCGCGCCGCGCCGACGCGGAAATGTTGGGCTTCAGAGTGTGGCCG	1765
Oligotype 19	GTGTGCGTCGTCGCGCCGCGCCGCCGCGTAAATGTTTGGCTTCAGAGTGTGGGTG	1393
Oligotype 20	GTGTTGCGTCGTCGCGCCGCGCCGCCGCGGAAATGTTGGGCTTCAAAGGTGCGGCCG	1331
Oligotype 21	GTGTTGCGTCGTCGCGCCGCGCA GCCGCGGAAATGTTGGGC-TCAAATATGCGACCG	1288
Oligotype 22	GTGTTGCGTCGTCGCTCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAGGTGCGGTTCG	946
Oligotype 23	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTCA	859
Oligotype 24	GTGTTGCGTCGTCGCTCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTTCG	832
Oligotype 25	GTGTTGCGTCGTCGCGCCGCGCCGCCGCGGAAATGTTAGGCTTCAAAAGTGCGGCCG	609
Oligotype 26	GTGTTGCGTCGTCGCGCCGCGCCGCCGCTCGAAATGTTGGGCTTCAAAAGTGCGGCCG	555
Oligotype 27	GTGTTGCGTCGTCGCGTTCGCGCCGCCGTGCGAAATGTTGGGCTTCAAAAATGTGCGCG	430
Oligotype 28	GTGTTGCGTCGTCGCGCTTCGCGCCGTTCGCGTGA-ATGTTAGGCTTCAGAGTGTGGCCG	410
Oligotype 29	GTGTTGCGTCGCTCGCCGTCGCGCCGCCGCGTAAATGTTGGGCTTCAAAAGTGCGATCG	370
Oligotype 30	GTGTTGCGTCGTCGCGCCGCGCCGCCGCGGAATTTGTTGGGATCAAGAGTATGGCCG	367
Oligotype 31	GTGTTGCGTCGTCGCGCCGCGCCGCCGCGGAAATATTGGGCTCAAGAGTATGGCCG	362
Oligotype 32	GTGTTGCGTCGTCGCTGTCGCGCCGCCGCGGAAATGTTGGGCTTCAAAAGTGCGGCCG	331
Oligotype 33	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGCTGGGCTTCAGAAGTGCGGTTCG	325
Oligotype 34	GCGTTGCGTCGTCGCGCCGCGCCGCCGCGGAAATGTTGGGCTTCAAAAATGCGGCCA	320
Oligotype 35	A-GTTGCGTCGTCGCGCCGCGCCGCCGCGTAAATGTTGGGCTTCAGAGTGTGGCCG	276
Oligotype 36	A-GTTGCGTCGTCGCGCTTCGCGCCGCCGCGGAAATGTTGGGCTTCAGAGTGTGGCCG	269
Oligotype 37	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCGAAGTGCGGTTCG	265
Oligotype 38	GTGTTGCGTCGTCGCCCAGCCGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTTCG	247
Oligotype 39	GTGTTCGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAGTGCGGTTCG	244
Oligotype 40	GCGTTGCAATCGTCGCGCCGCGCCGCCGCGGA-ATGTTGGGCTTCAAAAATGCGGCCG	244

Oligotype 41	GTGTTGCGTCGTCGCCCAGCCTGGGCGCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	235
Oligotype 42	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	234
Oligotype 43	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	232
Oligotype 44	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	229
Oligotype 45	A-GTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	224
Oligotype 46	GTGTTGAGTCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	223
Oligotype 47	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	204
Oligotype 48	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	200
Oligotype 49	GTGTTGCGTCGTCGCCCAGCCTGGTGCCTGTCCGTGAAATGTTGGGCTTCAGAAAGTGCGGTTCG	182

Table S4. HLSU unique oligotypes showing the variable positions that were used to define each oligotype (note that this is not the entire sequence) with the number of sequences used to define each oligotype listed in the last column. The unique oligotype sequences are shown with nucleotide variations (relative to Oligotype 1) highlighted in blue to indicate what differences were used to define each oligotype.

HLSU Oligotype	Unique Oligotype	Sequences per oligotype
Oligotype 1	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCCGCGG	28165
Oligotype 2	CGCACCAGCTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCTAG	18775
Oligotype 3	CGCATTGCCAGTTATGTCCACCATGAAAGCAGGTCCTCCCTAACTCCGGTTAGCCTTGTCAGCGTG--TCGGCCCGCCGCTGG	14468
Oligotype 4	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCTCCCTTACCGCCGGTGTCCGCGG	7822
Oligotype 5	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCTCCCTTACCGCCGGTGTCTGCGG	3289
Oligotype 6	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCTCCCTTACCGCCAGTGTTCGCGA	3215
Oligotype 7	CGCCTTGTTTGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCTCCCTTCCGCCGGTGTCCGCGG	2788
Oligotype 8	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCTCCCTTACCGCCAGTGTTCGCGG	2605
Oligotype 9	CGCACCAGCTTATGTCCACCATGAAAGCAGGCACTCCCTAACTCCGGTCAGCCCCGTCAGCGTG--TTGGCCCGCCGCTCTGG	2545
Oligotype 10	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCTCCCGCCCTTACCGCCGGTGTCTGCGG	2426
Oligotype 11	CGCCTCGTTATCTATGTTACCATGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCTCCCTTCCCGCCGGTGTCCGCGG	2111

Oligotype 12	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	2093
Oligotype 13	CGCGTCGTTAGCTATGTCCAATCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	1902
Oligotype 14	CGCATGTGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	1800
Oligotype 15	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCCCTAACTCCGGTTAGCCTTGTCAGCGTG--TCGGCCCGCCGCCCTGG	1785
Oligotype 16	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCCCTCGC	1671
Oligotype 17	CGTCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCCGCGG	1271
Oligotype 18	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTGG	1149
Oligotype 19	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTGGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	1114
Oligotype 20	CGCATGTGCCAGTTATGTCCACCATGAAAGCAGGTCCTCCCTAACTCCGGTTAGCCTTGTCAGCGTG--TCGGCCCGCCGCCCTAG	1051
Oligotype 21	CGCATGTGCCAGTTATGTCCACCATGAAAGCAGGTCCTCCCTAACTCCGGTTGGCCTTGTCAGCGTG--TCGGCCCGCCGCCCTGG	967
Oligotype 22	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCCCTCGCTCCCTTAC	945
Oligotype 23	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACACCTCGC	943
Oligotype 24	CGCCTCGTTAGCTATGTTACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	938
Oligotype 25	CGCCTCGTTAACTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCCCTCGC	832
Oligotype 26	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCGACACCCCTCGCTCCCTTACCGCCGGTGTCCGCGG	775
Oligotype 27	CGCGTCGTTAGCTATGTCCAATCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	715
Oligotype 28	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCTCCCGC	678
Oligotype 29	CGCGTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	660
Oligotype 30	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCGAGGTCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCCGCGG	639
Oligotype 31	CGCCTCGTTAGCTAGGTCACCATGAAAAACGTCCTGCCTAGGTTACCTCCACCCCTCGC	623
Oligotype 32	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	576
Oligotype 33	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCCCGTCAGTGTG--TCGGCCCGCCGCCCTAG	555
Oligotype 34	CGCCTTGTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	512
Oligotype 35	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCTTGTCAGCGTG--TCGGCCCGCCGCCCTGG	490
Oligotype 36	CGCCTTGTGTTGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	475
Oligotype 37	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCCGCGG	471
Oligotype 38	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGTCCTCTCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	465
Oligotype 39	CGCCTTGTGTTGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTTCGCCTACACCCCTCGC	442
Oligotype 40	CGCATGTGCCAGTTATGTCCACCATGAAAGCAGGTCCTCCCTAACTCCGGTTAGCCTTGTCAGTGTG--TCGGCCCGCCGCCCTGG	414

Oligotype 41	CGCATTGCCAGTTATGTCCACCATGAAAGCAGGCGCTCCCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	395
Oligotype 42	CGCACCAACAGTTATGTCCACCATGAAAGCAGGCGCTCTCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	372
Oligotype 43	CGCATTGCCAGTTATGTCCACCATGAAAGCAGGCGCTCCCTAACTCCGGTTAGCCCTTGTCAGCGTG--TCGGCCCGCCGCCCTGG	366
Oligotype 44	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGTCTACACCCCTCGCCCCCTTACCGCCGGCGTCCGCGG	356
Oligotype 45	CGCCTCGTTAGCTAGGTCACCATG-AAAAGGTCCTGCCTAGGTTCACCTCCACCCCTCGCCCCCTTACCGCCGATGTCCGCGG	350
Oligotype 46	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGGACTCCCTAACTCCGGTCAGCCCCGTCAGCGTG--TTGGCCCGCCGTCCTGG	336
Oligotype 47	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCCCCTTACCGCTGGCGTCCGCGG	335
Oligotype 48	TGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCCCCTTACCGCCGGTGCTTGC GG	298
Oligotype 49	CGCACCAACAGTTATGTCCACCATGAAAGCAGGCGCTCCCTAACTCCGGTTAGCCCTTGTCAGCGTG--TCGGCCCGCCGCCCTGG	297
Oligotype 50	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGCTTGC GG	296
Oligotype 51	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGTTCCGCCCACACCCCTCGCCCCCTTACCGCCGGTGTCGCGG	293
Oligotype 52	CGCCTCGTTAGCTATGTCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCGCGG	292
Oligotype 53	CACCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCCCCTTACCGCCGGCGTCCGCGG	292
Oligotype 54	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTACCTAGGTCCGCCTACATCCCTCGCTCCCTTACCGCCGGTGTCGCGG	290
Oligotype 55	CGCATTGCCAGTTATGTCCACCATGAAAGCAGGGACTCCCTAACTCCGGTCAGCCCCGTCAGCGTG--TTGGCCCGCCGTCCTGG	282
Oligotype 56	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTGGTCCGCCTACACCCCTCGCTCCC--TCCGCCGGTGTCGCGG	280
Oligotype 57	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCGCTTACACCCCTCGCCCCCTTACCGCCGGTGTCGCGG	269
Oligotype 58	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCCCCCTTACCGCCGGTGCTTGC GG	269
Oligotype 59	CGCCTCGTTAGCTATGTCCACCATAAAAGCAGGTCCTGCCTAGGTCCGCCTACACCTCCGCCCCCTTACCGCCGGTGCTTGC GG	217
Oligotype 60	CGCACCAACAGTTATGTCCACCATGAAAGCAGGGACTCCCTAACTCCGGTCGGCCCCGTCAGCGTG--TTGGCCCGCCGTCCTGG	214
Oligotype 61	CGCCTCGTTAGCTATGCCACCATGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCCCCT-ACCGCCAGTGTTTCGCGG	208
Oligotype 62	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCACACCCCTCGCCCCCTTACCGCCGGTGTCGCGG	202
Oligotype 63	CGCACCGCCAGTTATGTCCACCATGAAAGCAGGCGCTCGCTAACTCCGGTTAGCCCCGTCAGCGTG--TCGGCCCGCCGCCCTAG	192
Oligotype 64	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTTTCGCGG	191
Oligotype 65	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCGACACCCCTCGCCCCCTTACCGCCGGTGTCGCGG	187
Oligotype 66	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACACCCCTCGCTCCCTTACCGCCGGTGTCGCGG	182
Oligotype 67	CGCCTCGTTAGCTATGTTCCCGGAAAGCAGGTCCTGCCTAGGTTCGCCTACACCCCTCGCCCCCTTCCCGCCGGTGTCGCGG	169
Oligotype 68	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCGAGGTCCGCCTACACCCCTCGCCCCCTTACCGCCGGTGTCGCGG	163
Oligotype 69	CGGCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCCGCCTACATCCCTCGCTCCCTTACCGCCGGTGTCGCGG	162

Oligotype 70	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTTGCGCCACACCCCTCGCCCCCT-ACCGCCAGTGTTCGCGA	153
Oligotype 71	CGCCTCGTTAGCTATGTCCACCATGAAAGCAGGTCCTGCCTAGGTCGCGCTACACCCCTCGCCCCCTTACCGCTGGTGTCCGCGG	152
Oligotype 72	CGCACCACAGTTATGTCCACCATGAAAGCAGGGAATCCCTAACTCCGGTCAGCCCCGTCAGCGTG--TCGGCCGCGGCCCTAG	148

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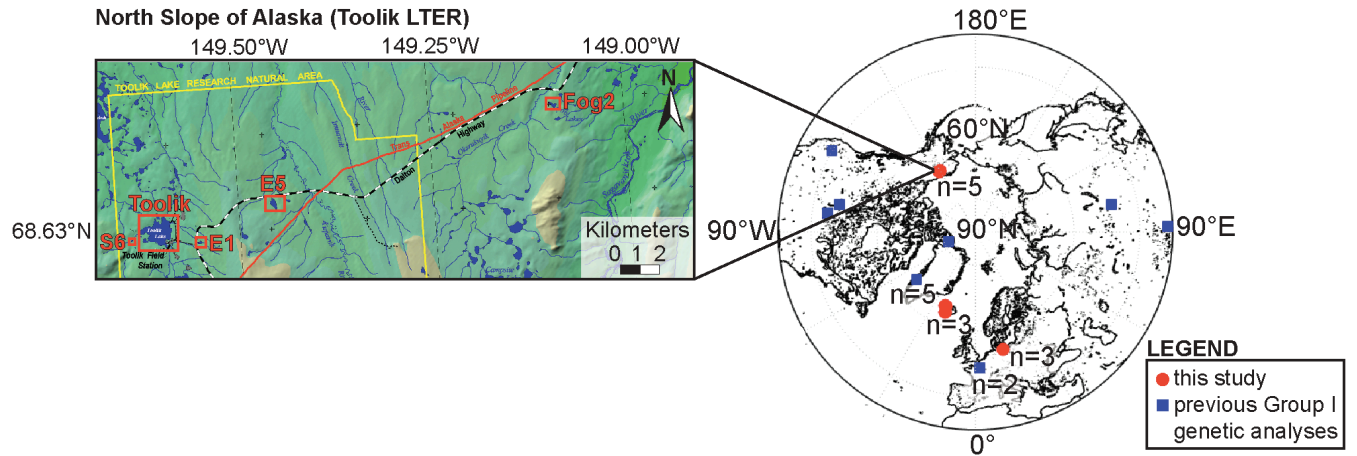


Figure S1. The map on the left, is a map of the Northern Hemisphere with samples used in this study indicated by red circles, and blue squares marking locations where previous genetic analyses confirmed the presence of Group I Isochrysidales. Markers with an “n” indicate the number of lakes represented at each location. On the right, there is a zoomed in map of the North Slope of Alaska of the area in and around the Toolik Long Term Ecological Research (LTER) station with the five lakes used in our case study indicated in red: E1, E5, Fog2, S6, and Toolik (this image was modified from a figure by the Institute of Arctic Biology, 2012).

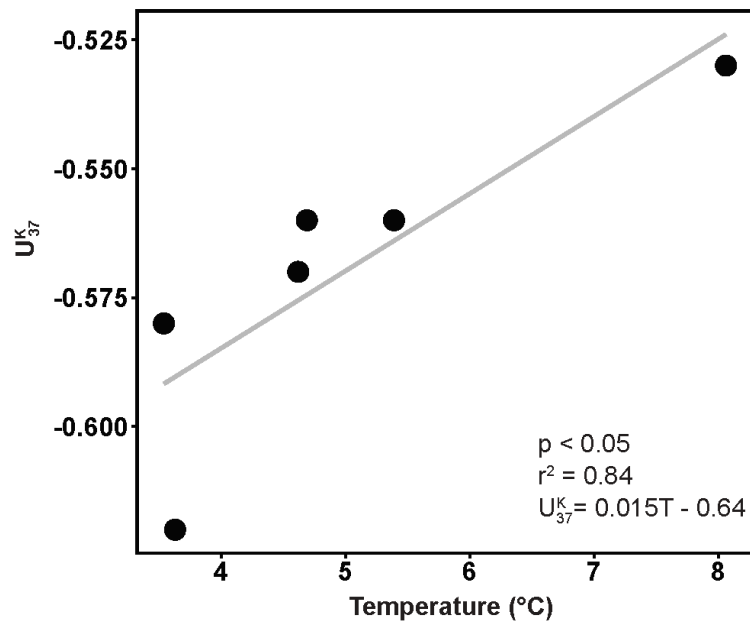
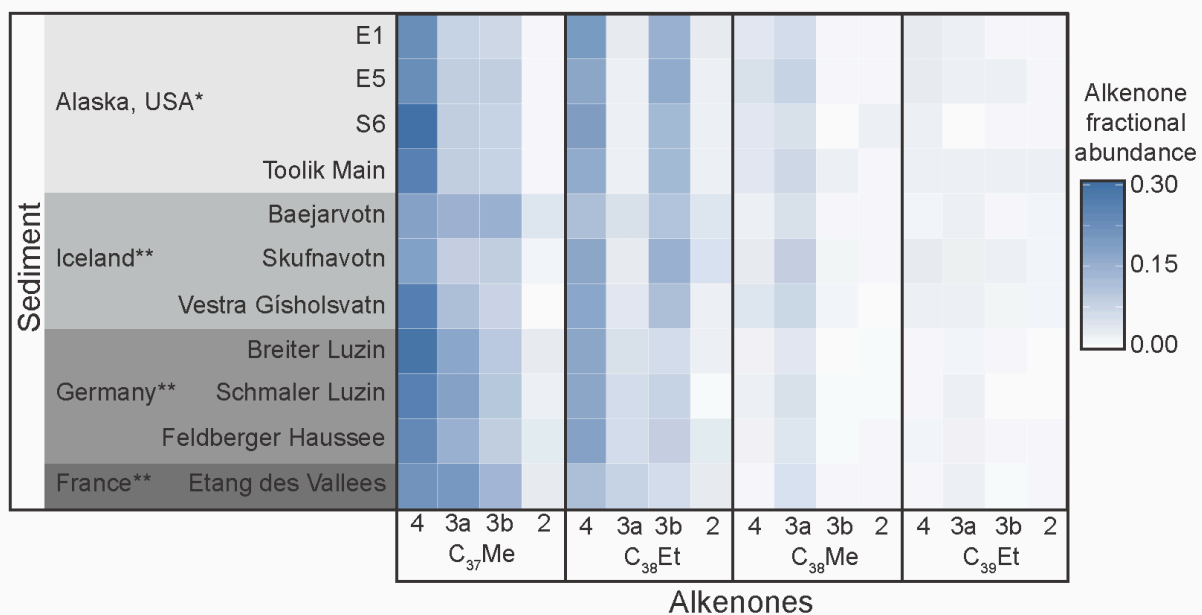


Figure S2. A comparison of U_{37}^K index with water column temperatures from Lake E1 at both 3- and 10-m depth from samples taken in June 2016.



*Alkenone values from Longo et al. (2016)

**Alkenone values from Longo et al. (2018)

Figure S3. Comparison of alkenone fractional abundance compiled from previous studies (Longo et al. 2016, 2018) for samples analyzed and discussed in this study.

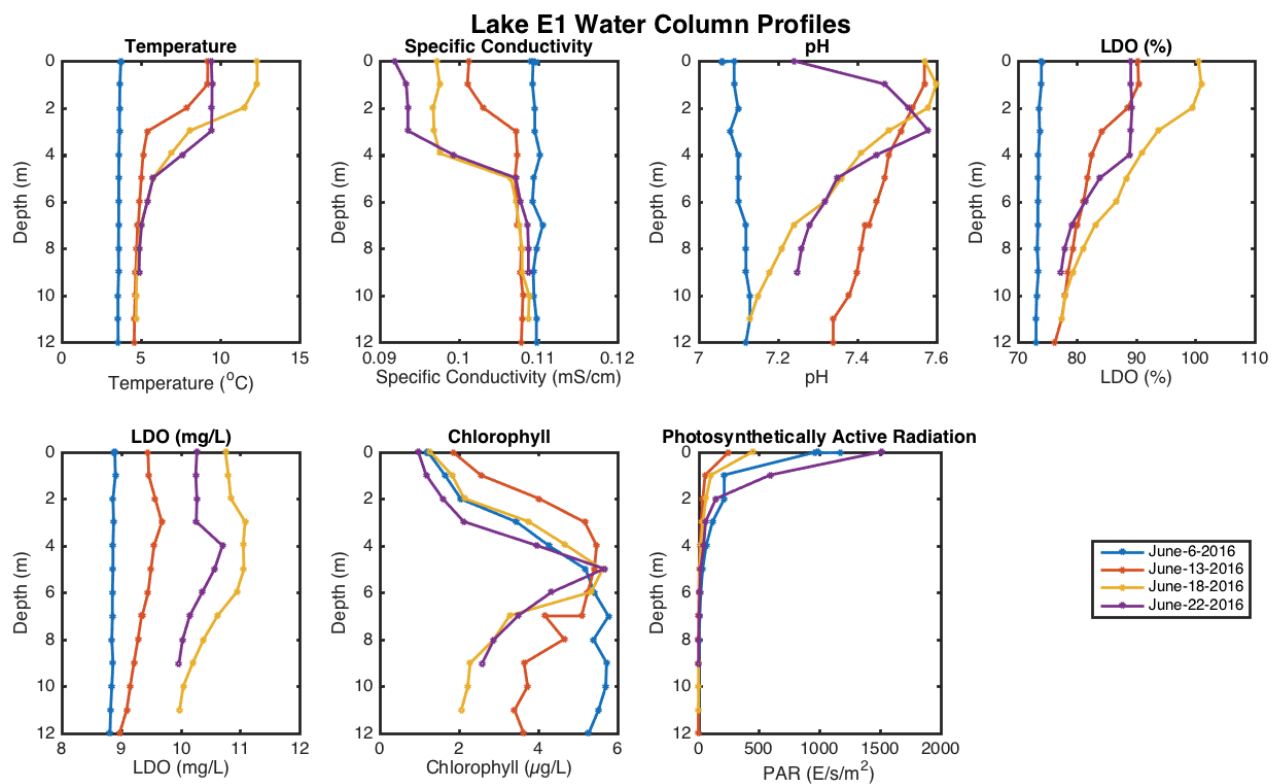


Figure S4. Water column profiles for Lake E1, Alaska from June 2016.

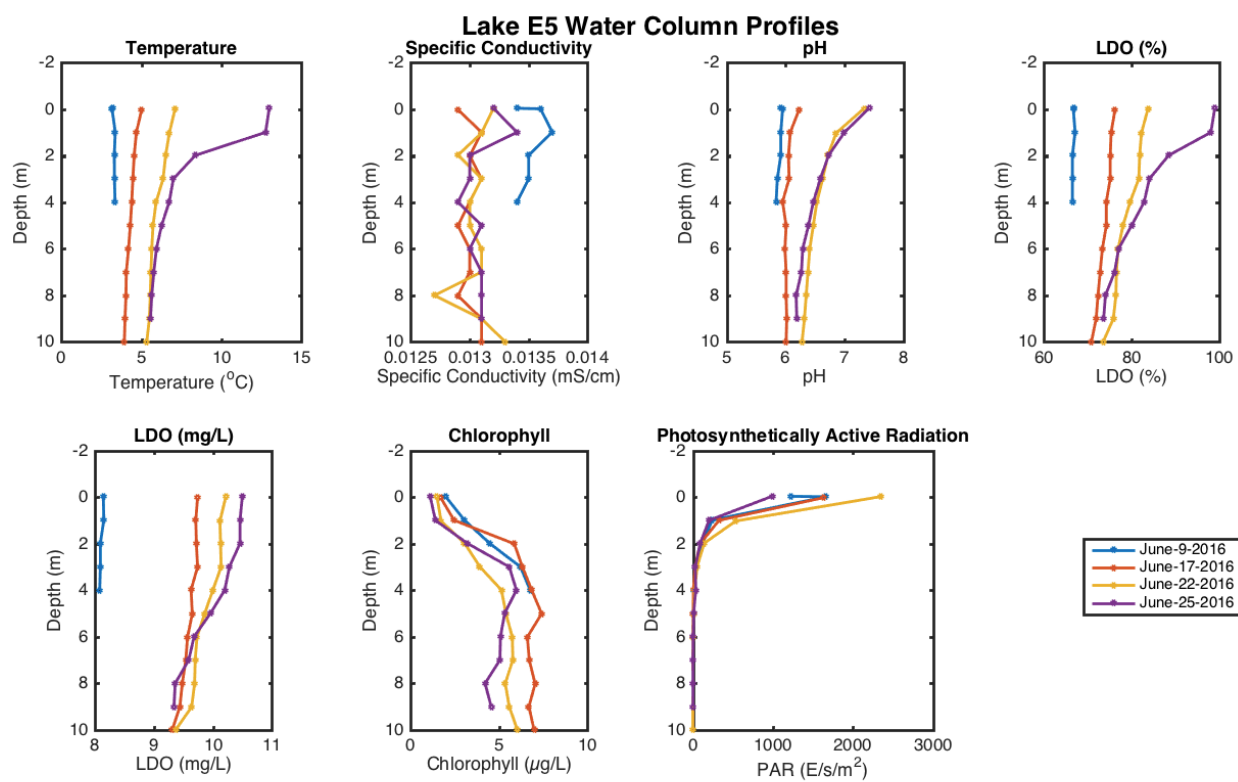
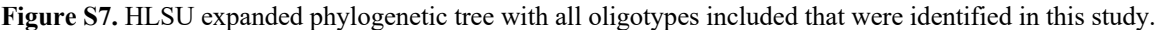


Figure S5. Water column profiles for Lake E5, Alaska from June 2016.



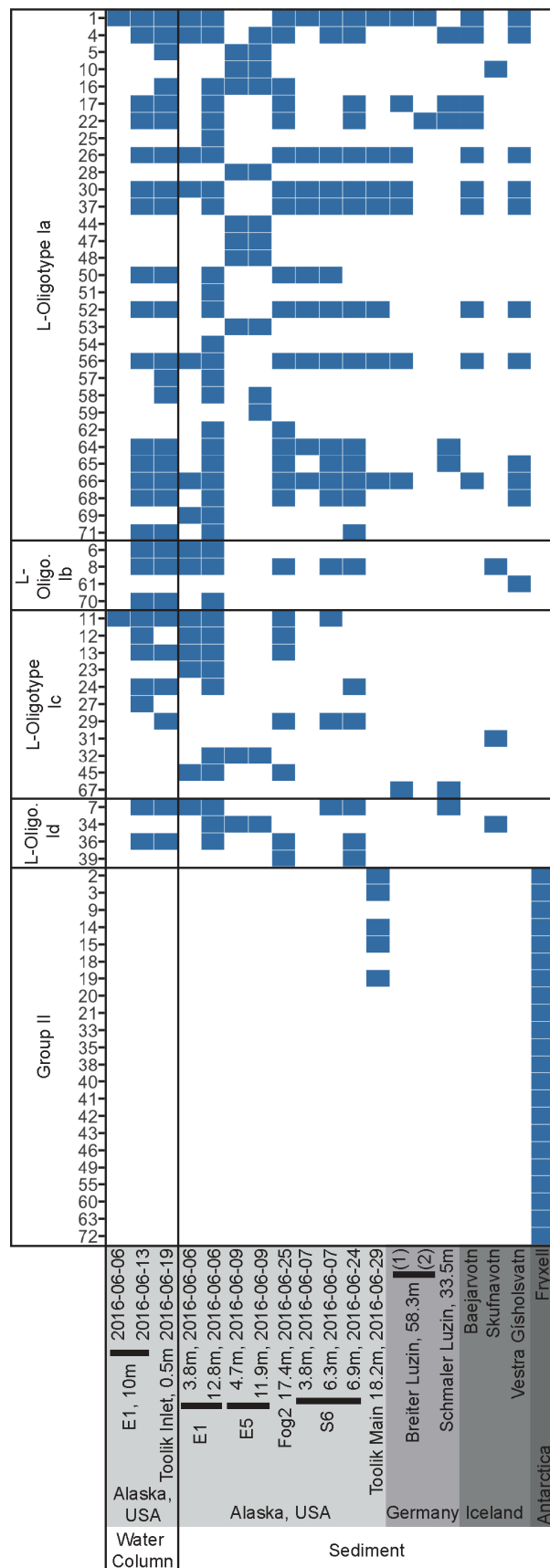


Figure S9. A binary heatmap, indicating the presence/absence of HLSU oligotypes from our samples where a blue square corresponds to the presence of a certain oligotype and a white space indicates the absence of a specific oligotype. The y-axis corresponds to the oligotypes identified (e.g., Oligotype 1) and the x-axis indicates the sampling location.

– CHAPTER 3 –

**Impacts of Norse settlement on terrestrial and aquatic ecosystems in Southwest
Iceland**

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Abstract

Norse colonization of North Atlantic islands in the 1st millennium of the Common Era led to drastic prehistoric environmental changes in these previously “pristine” landscapes. In Iceland, Norse settlement is associated with a rapid decline in birch trees and heightened soil erosion, yet the timing of Norse exploration in the North Atlantic coincided with large climate changes that also influenced Icelandic environments. To date, there are few records that disentangle climatic and human impacts on terrestrial ecosystems, and there has been very little work on the impacts of Norse arrival on Iceland’s aquatic ecosystems. Here we use a high-resolution lake sediment record from Vestra Gíslholtvatn (VGHV), southwest Iceland to investigate these processes during the last 2,000 years. Norse arrival (c. 870 CE) in Iceland was followed by a rapid increase in sedimentation rate and a transition in leaf wax *n*-alkanes indicating a decrease in trees and expansion of grasses. This transition coincides with limnological changes, including increased primary productivity (i.e. C₁₇ *n*-alkane and biogenic opal fluxes) and shifts in the Isochrysidales haptophyte algal community. Many of these changes are still apparent today. Comparisons with a new winter-spring alkenone temperature reconstruction from VGHV and marine sea-surface temperature records show little to no correlation between terrestrial and aquatic ecological changes and climate at this time. Similarly, volcanic eruptions recorded in VGHV are not associated with any long-term environmental changes. Rather, the VGHV record suggests that human settlement had a lasting impact on the catchment area of VGHV and changes within the lake ecosystem.

1. Introduction

Norse colonization, or *landnám*, of North Atlantic islands occurred during a warmer, climate that allowed the Norse to reach and settle the Faroe Islands (between c. 800-850 CE), Iceland (c. 874 CE), and eventually Greenland (c. 985 CE) (Richards, 2005). *Landnám* had major environmental consequences in Iceland and Greenland as the settlers imposed Norse agricultural practices on these new landscapes (Dugmore et al., 2005). Settlements in Iceland withstood variations in climate and other environmental changes, including volcanic eruptions and landscape degradation, whereas Greenlandic settlements were abandoned within a few centuries (Dugmore et al., 2005). Today, Iceland is still faced with environmental issues that were triggered by the Norse, but it is unclear how climate change and on-going human activities will continue to alter terrestrial and aquatic ecosystems. Thus, there is a need to understand the role of humans relative to past climate variability in shaping the modern-day landscape of Iceland.

Prior to human settlement, birch forests were dominant in the southern Icelandic lowlands, yet it is unclear how widespread or stable these forests were in the face of climate change before c. 870 CE (Ólafsdóttir et al., 2001; Dugmore et al., 2005; Streeter et al., 2015). Upon arrival in Iceland and Greenland, Norse settlers cut birch trees to clear space for agriculture and for use as building materials and fuel sources (Hallsdóttir, 1987; Dugmore et al., 2005; Massa et al., 2012a). Persistent grazing by cattle and sheep prevented reforestation, and contributed to the expansion of grasses, sedges, and weeds that are still apparent in the present-day vegetation (Dugmore et al., 2005; Hallsdóttir & Caseldine, 2005). Although there is evidence that soil erosion rates were already high prior to *landnám* (Geirsdóttir et al., 2009, 2013), breaks in vegetation and soil tilling for agriculture contributed to accelerated soil erosion in Iceland (Dugmore et al., 2009). However, other studies suggest that heightened soil erosion

only became a problem during the Little Ice Age as the climate cooled (Geirsdóttir et al., 2009, 2013). These interpretations are complicated by not accounting for changes in sedimentation rates and the use of proxies that are subject to multiple environmental and climatic factors (e.g. biogenic silica and total organic carbon).

Numerous studies have focused on changes in the terrestrial landscape during *landnám*, yet the resulting human and climate impacts on lacustrine ecosystems are poorly understood despite being an important resource for the Icelandic community. For instance, in northern Iceland and in the Faroe Islands lacustrine primary productivity increased shortly after Norse settlement, potentially due to nutrient loading (Hanon et al., 2005; Lawson et al., 2007). However, proxies for primary productivity (e.g. biogenic silica), which are potentially sensitive to human-driven changes over the past millennia, are often used as records of climate change (Geirsdóttir et al., 2009, 2013; Blair et al., 2015). This can result in false interpretations of warming or cooling that is instead a response by primary producers to changes in nutrients.

In this study, we used a high-resolution lake sediment record with a well-constrained age model from southwest Iceland to assess the impacts of climate change relative to human disturbances on the terrestrial landscape and aquatic ecosystems. Our paleo-ecological record from Vestra Gíslholtvatn (VGHV) utilizes proxies that directly reflect the magnitude of vegetation changes, the onset of heightened soil erosion, and variability in primary productivity. In addition, we use an alkenone paleothermometer, long-chain ketones produced by haptophyte algae that are sensitive to changes in temperature, to develop a high-resolution temperature record from the same core that allows us to differentiate climate changes from human impacts on the environment.

2. Methods

2.1 Study site

VGHV is a small lake (1.57 km²) in southwest Iceland (61 m a.s.l., 63°56'N, 20°31'W) that is situated near some of the initial Norse settlements (see Figure 1). The lake has no large inlets or outlets and sits on bedrock consisting of basalt (Hjartarson & Sæmundsson, 2014; Blair et al., 2015). Pollen records from VGHV suggest that the landscape around the lake was dominated by birch trees prior to 874 ± 2 CE, whereas today the catchment is used for planting hayfields and sheep grazing (Hallsdóttir & Caseldine, 2005; Blair et al., 2015). Lake sediment cores from VGHV were taken in 2008 using a Bolivia piston coring system (Blair et al., 2015). The cores were stored and sampled at the National Lacustrine Core Facility (LacCore) at the University of Minnesota. The cores were previously analyzed for physical and bulk organic and inorganic properties to infer changes in the catchment area, lake, and climate (Blair et al., 2015).

2.2 Age model

The sediment cores from VGHV were previously dated using tephras (Figure 2), which include seven historically recorded tephras and four tephras dated using various radiometric and stratigraphic methods, all deposited in the last 3,000 years (Christensen, 2013; Blair et al., 2015). The age model for this study was developed by applying a smooth spline to the tephra ages and depths in the classical age modelling (CLAM) program (Blaauw, 2010; Blair et al., 2015). Our resulting age model has an uncertainty of 5-15 yrs from -50 to 1200 yrs BP and an uncertainty of 18-83 yrs from 1201 to 2000 yrs BP (Figure 2). The resulting age model was used to estimate changes in sediment accumulation rates over the past 2,000 years.

2.3 Lipid analyses

We freeze-dried and extracted sediments using a DionexTM accelerated solvent extraction (ASE 350) system at 120°C and 1200 psi. We separated all of the extracts with silica gel (40-63 μm , 60 Å) flash chromatography to obtain alkane (hexane; Hex), ketone (dichloromethane; DCM, which contains alkenones), and polar (methanol; MeOH) fractions. Wax esters were removed from the ketone fraction via saponification, in which the fraction was dissolved in a 1 M potassium hydroxide solution in MeOH:H₂O (95:5, v/v) and heated for 3 hours at 65°C. A solution of 5% NaCl in H₂O was added, followed by acidification using 50% HCl in H₂O. Hex (100%) was then used to extract the desired lipid fraction. Ketone fractions were further purified using silver nitrate columns with a procedure modified from D'Andrea et al. (2007) using DCM (100%) and ethyl acetate (100%). If further cleaning of the alkenone fraction was necessary (as determined through gas-chromatography analyses), a modified urea adduction procedure from Salacup et al. (2019) was used. The ketone fraction was dried under N₂ gas, and dissolved in 1.5 mL of DCM:Hex (2:1, v/v). A 1.5 mL solution of 100 mg/mL urea in MeOH was added, the samples were dried under N₂ gas, and the resulting urea crystals were rinsed with Hex (100%) to remove the non-adduct. The urea crystals were dissolved with Milli-Q water, and the resulting adduct was extracted three times using Hex (100%). This process was repeated two additional times, and the cleaned adducts were analyzed. There were several samples with co-eluting compounds that we were unable to remove with this procedure, and in other samples the alkenone concentrations were too low to measure reliably. These samples were excluded from the final alkenone record.

All alkenones and *n*-alkanes were analyzed with an Agilent 6890N gas chromatography (GC) system and a flame ionization detector (FID). Alkenones were run on an Agilent VF-200ms capillary column (60 m x 250 μm x 0.10 μm) and were injected into a CIS-PTV inlet

operated in solvent vent mode (6.9 psi at 112°C). The oven program was started at 50°C, and was then ramped up to 235°C at 20°C/min, and ramped again at 1.39°C/min to 320°C where it was held isothermally for 5 min. All *n*-alkane samples were run using a Rtx-200 column (105 m x 205 µm x 0.25 µm) and were injected using a pulsed splitless mode (20.3 psi, 310°C). The oven program was started at 50°C and was ramped up to 315°C at 10°C/min where it was held isothermally for 30 min.

If additional compound identification or verification was required, samples were checked using an Agilent 6890N GC system coupled with an Agilent 5793 N quadrupole mass spectrometer (MS). Samples were injected using pulsed splitless injection mode (20 psi, 315°C) and were run on an Agilent VF-200ms capillary column (60 m x 250 µm x 0.10 µm). The initial oven program was set to 40°C for 1 min, and was increased to 255°C at 20°C/min and again to 315°C at 2°C/min where it was held isothermally for 10 min. The MS was set to an ionization energy of 70 eV and a scan range of 50-600 m/z.

2.4 Leaf-wax analysis

We used *n*-alkanes to assess changes in algal productivity and regional shifts in vegetation. Typically, shorter chain *n*-alkanes (C₁₇-C₂₁) are associated with phytoplankton (Cranwell et al., 1987). Thus, we can measure changes in C₁₇ *n*-alkane fluxes to test for changes in algal productivity. To track vegetation changes in the catchment area, we measured long-chain *n*-alkanes (C₂₅-C₃₃), which are produced by higher-level terrestrial plants (Cranwell, 1973; Maffei, 1996). Birch trees (e.g. *Betula pubescens* and *Betula nana*) produce a high abundance of *n*-alkanes with C₂₃, C₂₅, and C₂₇ chain lengths (Balascio et al., 2018), whereas herbs and graminoids produce C₂₇, C₂₉, and C₃₁ in higher proportions (Cranwell, 1973; Maffei, 1996; Balascio et al., 2018). To reconstruct shifts in terrestrial

vegetation we calculated the average chain length (ACL) for *n*-alkane homologs C₂₅ to C₃₁ as follows:

$$ACL_{25-31} = \sum \frac{C_i \times [C_i]}{[C_i]} \quad [1]$$

Where C_i represents the *n*-alkane chain length and $[C_i]$ represents *n*-alkane concentrations.

Lower ACL values (i.e. an increase in C₂₅-C₂₇) should indicate an increase in birch trees, whereas higher ACL values (i.e. an increase in C₂₉-C₃₁) indicate an increase in herbs and/or grasses.

2.5 Alkenones as a proxy for temperature and changes in species distributions

Alkenones are long-chain ketones with varying degrees of unsaturation that are produced by haptophyte algae (i.e. Isochrysidales) in both marine and lacustrine environments. Alkenone-producers can be divided into three main groups, with Group III occurring in marine environments, Group II in both marine and lacustrine environments, and Group I in freshwater lakes (Volkman et al., 1980, 1995; Conte et al., 1995; D'Andrea et al., 2006; Theroux et al., 2010). In all three groups, the distribution of alkenones is correlated with changes in temperature, such that alkenones with a higher degree of unsaturation (e.g. C_{37:4} Me) are produced in colder environments (Brassell et al., 1986). The U_{37}^K and $U_{37}^{K'}$ indices quantify the degree of unsaturation and are correlated to and widely used as proxies for temperature in marine environments (Brassell et al., 1986; Prahll & Wakeham, 1987; Prahll et al., 1988; Conte et al., 2006). The U_{37}^K index is also applied to lake sediments to reconstruct temperature using different calibrations (Zink et al., 2001; D'Andrea et al., 2011,

2012; Theroux et al., 2013; Randlett et al., 2014; Longo et al., 2016, 2018). The U_{37}^K index is calculated as follows:

$$U_{37}^K = \frac{[C_{37:2}Me] - [C_{37:4}Me]}{[C_{37:2}Me] + [C_{37:3}Me] + [C_{37:3*}Me] + [C_{37:4}Me]} \quad [2]$$

Although the U_{37}^K index has been used to reconstruct lake temperatures (Zink et al., 2001; D'Andrea et al., 2011, 2012; Randlett et al., 2014), different alkenone producers, in particular Group I vs. Group II, can have different U_{37}^K -temperature relationships (i.e. species-mixing effects) (Sun et al., 2007; Toney et al., 2012; Theroux et al., 2013; D'Andrea et al., 2016). We can test for impacts of species-mixing on reconstructions using the distinct alkenone signatures produced by Group I Isochrysidales. Group I produces tri-unsaturated isomeric compounds (e.g. $C_{37:3*}Me$) that can be used to test whether samples contain alkenones produced predominantly by Group I or Group II Isochrysidales. This is calculated using the RIK_{37} index (Longo et al., 2016):

$$RIK_{37} = \frac{[C_{37:3}Me]}{[C_{37:3}Me] + [C_{37:3*}Me]} \quad [3]$$

A RIK_{37} value of 1.0 indicates that $C_{37:3*}Me$ is not present in the samples and that Group II Isochrysidales is the main alkenone producer, whereas RIK_{37} values from 0.48-0.63 correspond to Group I Isochrysidales (Longo et al., 2016, 2018) presence. Previous surface sediment analyses using alkenones and DNA confirm that Group I Isochrysidales is the main alkenone producer in VGHV ($RIK_{37} = 0.60$) in the present day (Longo et al., 2018; Richter et al., 2019).

Group I Isochrysidales has so far only been identified in the Northern Hemisphere and blooms in the spring season during lake ice-off, such that Group I alkenones reflect changes in late winter and spring lake water temperatures (D'Andrea et al., 2011; Longo et al., 2016, 2018). Existing U_{37}^K -temperature calibrations provide unreasonable temperature estimates for our alkenone reconstruction. Thus, we present the U_{37}^K index as a qualitative estimate of late winter-spring lake water temperatures.

2.6 Sedimentological and bulk geochemical data

Physical and geochemical data from VGHV (Blair et al., 2015) was reanalyzed for comparison to our new organic geochemical data. Previous data included physical (density and magnetic susceptibility (MS)) and bulk organic and inorganic (total organic carbon (TOC), biogenic opal, and total nitrogen (TN)) measurements. To account for changes in the sedimentation rate, we calculated flux rates ($\text{mg cm}^{-2} \text{ yr}^{-1}$) for TOC, TN, and biogenic opal using downcore changes in sediment density measured with a GEOTEK multisensor core logger (MSCL) and sediment accumulation rates from our CLAM age model. Changes in water content and porosity were corrected for by measuring the mass loss before and after samples were freeze-dried. We note that biogenic opal in VGHV was measured using a single extraction method (Mortlock & Froelich, 1989) that can be problematic in distinguishing biogenic silica from volcanic glass. Although we assume that the measured silica should predominantly reflect changes in diatom productivity, caution should be used when interpreting these results.

2.7 Detection of change-points and transitions

For data analysis, all tephra layers were removed from the sediment depth, and the datasets

were re-sampled to the same time intervals with an average resolution of 20 years per sample. We used the “segmented” package in R (R Development Team) to conduct statistical break point analysis on our data to determine the timing of significant changes (Muggeo, 2008). A pooled standard error was calculated for each break point to account for age model uncertainties and are reported as 95% confidence intervals. Pearson correlations were also conducted in R.

3. Results

3.1 Short- and long-chain *n*-alkanes as proxies for primary productivity and vegetation

ACL₂₅₋₃₁ rapidly increases between 853 ± 10 and 966 ± 9 CE (Figure 3a, Table 1) reflecting an increase in grasses, sedges, and weeds and a decrease in woody plants, in particular birch trees (*Betula pubescens* ACL₂₁₋₃₃ = 25.4, and *Betula nana* ACL₂₁₋₃₃ = 25.6) (Cranwell, 1973; Maffei et al., 1996; Balascio et al., 2018). After this shift, ACL₂₅₋₃₁ remains stable from 966 ± 9 CE to the present. The C₁₇ *n*-alkane flux is slightly higher, but also more variable after c. 1120 ± 74 CE (Figure 4a). The largest increase in C₁₇ occurs after c. 1916 CE, indicating enhanced primary productivity during the 20th century.

3.2 Alkenones as indicators of species-mixing and temperature

The RIK₃₇ index suggests that Group I and II are both present in parts of our record. RIK₃₇ slightly increases after 514 ± 24 CE, with higher variability after 951 ± 22 CE (Figure 4b, Table 1), indicating shifts in the Isochrysidales community from predominantly Group I to both Group I and II over the past c. 1,000 years. Samples with high RIK₃₇ values, that is a high abundance of Group II, were removed from the temperature record. After removing these samples, the corrected U₃₇^K and RIK₃₇ indices are not correlated ($r = 0.11$, $p = 0.35$). The

remaining samples were used to calculate the U_{37}^K index to detect qualitative changes in late winter-spring temperature over time. The U_{37}^K record suggests that late winter-spring temperatures in southwest Iceland were gradually increasing over the past 2,000 years.

3.3 Re-analysis of physical and bulk organic and inorganic data

Although tephras were removed from our sediment stratigraphy, there are remnants of large volcanic eruptions in the MS signal (Figure 3d). This includes eruptions that occurred at c. 39 BCE, 58 CE, 435 CE, 452 CE, 617 CE, and 1500 CE. The largest shift in MS occurs after c. 833 ± 15 CE and is coincident with increases in TOC flux (884 ± 14 CE), TN flux (894 ± 19 CE), and sedimentation rate (898 ± 5 CE) (Table 1, Figure 3 and 4). An increase in biogenic opal commences about 1103 ± 12 CE and peaks c. 1281 ± 10 CE. This generally coincides with peaks in TOC (c. 1180 ± 9 CE) and TN fluxes (c. 1216 ± 13 CE). The decrease in biogenic opal, TOC, and TN fluxes coincide with decreasing sedimentation rates after c. 1222 ± 8 CE. Similarly, increasing sedimentation rates c. 1581 ± 5 CE correspond to increases in biogenic opal (c. 1676 ± 14 CE), TOC (c. 1513 ± 11 CE), and TN (c. 1641 ± 12 CE).

4. Discussion

4.1 Climatic and ecological changes prior to Norse settlement

Decreasing summer solar insolation led to long-term cooling in Northern Europe over the past c. 6,000-5,000 yrs BP. This is typically associated with a decrease in Fennoscandian forest extent (Seppä et al., 2009). Similarly, in Greenland and the Faroe Islands woody vegetation decreased after c. 3,000 yrs BP (Hannon et al., 2005; Massa et al., 2012b). Our U_{37}^K temperature record from VGHV, however, suggests late winter and spring warming during this

time, likely due to rising winter-spring insolation. There is also evidence for centennial climate variability in the VGHV U_{37}^K record and marine temperature reconstructions, with warming during the Roman Warm Period (RWP, c. 250 BCE-400 CE) and cooling during the Dark Ages Cold Period (DACP, c. 450-550 CE) (Figure 5) (Sicre et al., 2008, 2011; Seppä et al., 2009). Yet the relative abundance of birch trees and herb/grasses in southwest Iceland remain stable between c. 0-870 CE. In addition, there are no significant changes in VGHV primary productivity (i.e. biogenic opal and C_{17} *n*-alkanes) prior to *landnám* (Figure 4).

There is, however, a slight increase in the RIK_{37} values c. 514-560 CE, which could indicate that Group II Isochrysidales was present in the lake in low abundance (Figure 5d) (Longo et al., 2018). Previous studies suggest that shifts in Group I and II Isochrysidales communities are primarily driven by changes in salinity, with only Group I present in environments with a salinity of 0-0.5 psu and Group I and Group II both present at salinities between 0.5-5 psu (Longo et al., 2016). VGHV is a freshwater lake (0.1 psu) and given the low chemical weathering rates in the cool Icelandic climate, salt fluxes to the lake are very low. It is unlikely that short-term climate fluctuations would promote high enough evaporation rates to result in large salinity changes. Alternatively, the RIK_{37} values during this time period could correspond to Group I Isochrysidales but are outside of the recorded empirical range.

Prior to *landnám*, several major volcanic eruptions occurred in Iceland at 39 BCE, and subsequent eruptions in 58 CE, 435 CE, 452 CE, and 617 CE resulted in distinct tephra layers in VGHV (Figure 5f) and increases in MS (Blair et al., 2015). VGHV sedimentation rate increased after c. 600 CE, which could be associated with a volcanic eruption that occurred c. 617 CE and triggered long-term re-working of the volcanic tephra layer or localized soil erosion from breaks in vegetation (Dugmore et al., 2005, 2009). Alternatively, gradual increases in erosion could be

caused by long-term Neoglacial cooling trends that are associated with increased glacial activity and heightened wind erosion (Jennings et al., 2001; Dugmore et al., 2005, 2009; Geirsdóttir et al., 2009, 2013; Blair et al., 2015).

In existing paleo-reconstructions from Iceland there are discrepancies as to whether climate change led to a decrease in forest extent and increased soil erosion prior to *landnám* (Ólafsdóttir et al., 2001; Dugmore et al., 2009; Geirsdóttir et al., 2009, 2013; Streeter et al., 2015). The VGHV record from southwest Iceland suggests that overall the landscape was relatively stable and that there were no major aquatic shifts in response to climate perturbations or volcanic eruptions prior to Norse settlement.

4.2 Environmental changes during the Medieval Climate Anomaly and Norse settlement

Viking expansion and settlement of North Atlantic islands is thought to occur during a mild and relatively warm climate, typically referred to as the Medieval Climate Anomaly (MCA; c. 950-1250 CE) (Ogilvie et al., 2000; Sjöppa et al., 2009). Our U_{37}^K winter-spring temperature record indicates slight warming interspersed with brief cold anomalies between c. 900-1100 CE, whereas summer reconstructions suggest that peak warming occurred c. 1000-1300 CE (Figure 5) (Ogilvie et al., 2000; Sicre et al., 2008, 2011; Gathorne-Hardy et al., 2009; Holmes et al., 2016). This gradual warming coincides with an abrupt shift in the VGHV ACL_{25-31} record, indicating a rapid decline in birch trees between c. 853-966 CE. Similarly, pollen records from Iceland, including data from a low-resolution record from VGHV, suggest that birch woodlands in southern Iceland disappeared rapidly c. 870-920 CE and were replaced by grasses, sedges, and dwarf-shrub heath (Hallsdóttir, 1987; Dugmore et al., 2005; Hallsdóttir & Caseldine, 2005). This corresponds to the timing of Norse settlement in Iceland and introduction of agricultural practices that included extensive land clearance (Dugmore et al.,

2005). A very similar situation was observed in Greenland, with a rapid decline in woody vegetation shortly after settlement (Figure 6) (Massa et al., 2012a).

The decline of birch trees coincides with increases in MS and sedimentation rates recording heightened erosion and minerogenic inputs shortly after human settlement in 874 ± 2 CE (Figure 3). Some of the initial changes in sedimentation rate and MS could be associated with the *landnám* tephra (871 ± 2 CE), but both sedimentation rate and MS remain high after the volcanic eruption, indicating that human and/or climatic impacts led to sustained changes in the catchment area. In the Eastern Norse settlement of southern Greenland soil erosion rates also increased shortly after *landnám*, which is associated with land clearance and the introduction of sheep to the landscape (Edwards et al., 2008; Massa et al., 2012a).

Major changes in VGHV productivity (i.e. C_{17} *n*-alkane and biogenic opal fluxes), as well as changes in the RIK_{37} values, occur between c. 950-1150 CE (Figure 4). Increased primary productivity is potentially due to higher nutrient inputs associated with soil erosion and deforestation, and/or grazing and other agricultural processes (manuring). This could also explain the shifts observed in the haptophyte community, i.e. an increase in RIK_{37} values and therefore an increase in Group II abundance. An increase in Group II alkenone production was previously observed in anthropogenically influenced lakes from northeast China, which suggests that haptophyte communities are sensitive to both changes in salinity and nutrient inputs (Yao et al., 2019). In northern Iceland, productivity also increased shortly after *landnám* in Lake Helluvaðstjörn; however, there are no corresponding increases in soil erosion (Lawson et al., 2007). Heightened nutrient inputs into Lake Helluvaðstjörn are attributed to either forest clearance, resulting in more nutrient leaching from soils, or the introduction of livestock (Lawson et al., 2007). This is also observed in the Faroe Islands where there was

minimal forest cover prior to human settlement, yet productivity in many lakes increased from nutrient loading post-settlement (Hanon et al., 2005; Lawson et al., 2005). This contrasts to a settlement in southern Greenland, where there were no noticeable impacts on the trophic state of the lake as evidenced by stability in diatom species and no obvious changes in algal production (Figure 6) (Massa et al., 2012a). With some exceptions, increased lacustrine primary production appears to be a widespread response to Norse settlement in the North Atlantic, with or without large changes in terrestrial vegetation.

Certain proxies, such as TOC and biogenic opal, are often used to reconstruct climate assuming that a warmer climate heightens lacustrine primary productivity (Geirsdóttir et al., 2009, 2013; Blair et al., 2015). Although there is a weak correlation between C_{17} *n*-alkane fluxes and our U_{37}^K temperature record ($r = 0.20$, $p < 0.05$), there is no correlation between biogenic opal and the U_{37}^K index. In addition, multiple shifts in the VGHV environmental record coincide with the historical settlement of the Norse, suggesting that human settlement dominated terrestrial and aquatic ecosystem changes in the last ~1,100 years in southwest Iceland.

4.3 Post-settlement era and the Little Ice Age

The post-settlement period in Iceland and Greenland, along with settlements in Northern Norway, took different trajectories as the settlers, terrestrial, and aquatic ecosystems adjusted to changes in local climate and human activities. In the VGHV ACL_{25-31} record, vegetation stabilized after c. 966 CE but with an entirely different composition than observed prior to human settlement. In contrast, vegetation in northern Norway fluctuated between forests and agricultural types depending on changes in climate and human land-use (Balascio & Wickler, 2018). In Greenland, either environmental degradation, socioeconomic issues, or climatic changes resulted in the rapid demise of

Norse settlements in the 14th century (Edwards et al., 2008; Massa et al., 2012a). The abandonment of these settlements allowed for the recovery of native vegetation (i.e. the birch woodlands) and a decrease in soil erosion rates (Figure 5) (Massa et al., 2012a). In contrast, the Icelandic settlers withstood both environmental degradation of the landscape and fluctuations in climate.

Despite the continued presence of Norse settlers in Iceland, sedimentation rates decreased c. 1222 CE before increasing again c. 1581 CE. The temporary decline in erosion could be associated with stabilization of soils after initial changes in vegetation or less intensive land-use practices (Hallsdóttir & Caseldine, 2005; Dugmore et al., 2006). The VGHV MS record, however, remains relatively stable during the post-settlement era (aside from brief increases in MS shortly after tephra). These divergent lines of evidence could be reconciled if MS responds not only to total clastic sediment inputs but also to the composition of those inputs, such as magnetite (Bradshaw & Thompson, 1985; Hawley et al., 2017). The depletion of soils via erosion during Norse colonization could have mobilized large quantities of surface material to the lake that were relatively enriched in magnetite (Óskarsson et al., 2012; Hawley et al., 2017). Increases in sedimentation rates in the latter half of the last millennium are observed in numerous terrestrial and marine records in Iceland and are attributed to a cooling climate and heightened erosion rates from increased periglacial activity and windiness during the Little Ice Age (LIA) (Jennings et al., 2001; Sicre et al., 2008; Geirsdóttir et al., 2009, 2013; Streeter et al., 2015).

Heightened VGHV diatom productivity only lasts for a few centuries before gradually decreasing between c. 1300-1700 CE, whereas RIK₃₇ and C₁₇ values become more variable (Figure 4). Similarly, in northeast Iceland chironomid concentrations start to decrease c. 1200 CE, and algal productivity also declines (Lawson et al., 2007). This roughly corresponds

to the end of the MCA as observed in several marine and terrestrial records (although this is not reflected in the winter-spring VGHV temperature reconstruction) (Figure 5) (Sicre et al., 2008, 2011; Gathorne-Hardy et al., 2009; Holmes et al., 2016). Rather, winter-spring temperatures suggest there was a slight cooling c. 1200 CE, followed by increased climate variability. This change in primary productivity could indicate seasonal shifts in temperature that directly influenced changes in lacustrine ecosystems, or a reduction in human activities in the landscape (Ogilvie et al., 2000). Peaks in algal productivity in a northeastern Icelandic lake after c. 1700 CE and the rapid increase in C_{17} *n*-alkane flux after c. 1900 CE, could be related to either heightened nutrient inputs from increased agricultural activity in the catchment area and/or climate changes (Lawson et al., 2007). Thus, climate could have played a role in driving some of the centennial-scale environmental changes observed during the LIA, including changes in primary productivity within the lake and soil erosion.

5. Conclusions

Concurrent analyses of proxies that reflect climate and environmental changes provide new insights into how and why the Icelandic landscape changed over the past 2,000 years. Prior to Norse settlement (c. 0-870 CE), the lack of major changes in the VGHV catchment area or in primary productivity indicate that climate and volcanic eruptions did not have a significant influence on the terrestrial and aquatic ecosystems. There is some evidence of increased soil erosion, which could be an artifact of either gradual cooling or volcanic eruptions. However, the most severe and rapid environmental shifts in southwest Iceland took place shortly after Norse settlement. The ACL_{25-31} record provides an integrated perspective on the rate and magnitude of bulk vegetation changes in Iceland. In particular, there is a permanent shift in vegetation

composition that is accompanied by a rapid increase in soil erosion. Changes in the aquatic ecosystem were less stable and fluctuated more frequently after human settlement. This indicates that human activities were the primary drivers of environmental change in southwest Iceland after c. 870 CE, and these effects were exacerbated by climate and volcanic eruptions. Thus, caution should be used when interpreting and calibrating proxies that are sensitive to human induced changes, climate variability, and/or volcanic eruptions. Finally, the continued increase in primary productivity over the past century, suggests that human activities and/or a warming climate might lead to future environmental issues (e.g. eutrophication) in Icelandic lakes.

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Table 1. Breakpoint analysis for the various proxy records assessed in this study.

Variable	Segmented Breakpoint Analysis	
	Year (CE)	95% CI ($\pm$)
ACL ₂₅₋₃₁	853	10
	966	9
C ₁₇ Flux (mg cm ⁻¹ yr ⁻²)	1120	74
	1916	9
RIK ₃₇	514	24
	560	22
	951	22
	1007	20
Magnetic Susceptibility (SI)	140	37
	833	15
	922	13
TOC Flux (mg cm ⁻¹ yr ⁻²)	884	14
	1180	9
	1513	11
TN Flux (mg cm ⁻¹ yr ⁻²)	894	19
	1216	13
	1641	12
Sedimentation Rate (cm/yr)	618	22
	898	5
	1222	8
	1581	5
Biogenic Opal Flux (mg cm ⁻¹ yr ⁻²)	1103	12
	1281	10
	1676	14

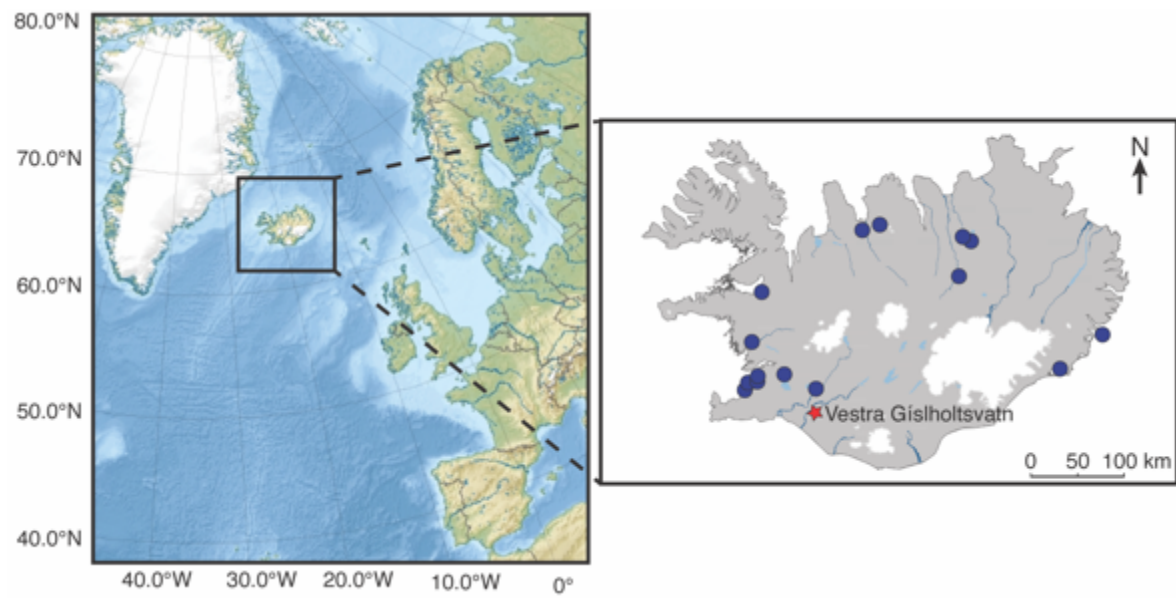


Figure 1. Map of Iceland with the study site, Vestra Gíslholtsvatn (VGHV), indicated by a red star. The blue circles correspond to initial Norse settlements (Zori, 2016). Raster data was obtained from the National Land Survey of Iceland and Natural Earth and maps were rendered in the Matlab.

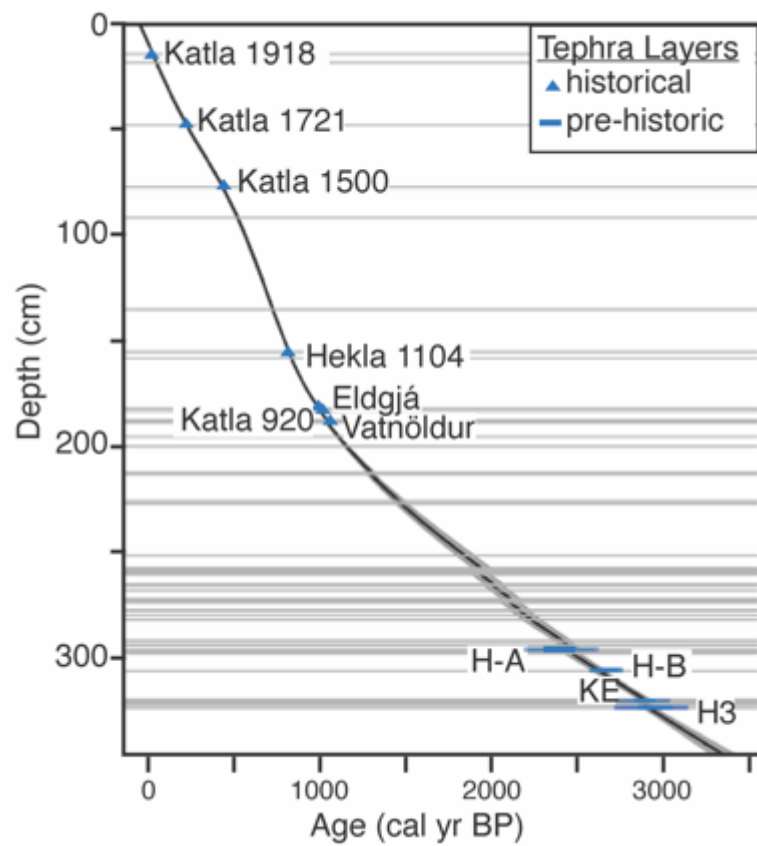


Figure 2. VGHV age model with historic and pre-historic tephras. Tephra slumps that were removed from analysis are indicated by gray lines.

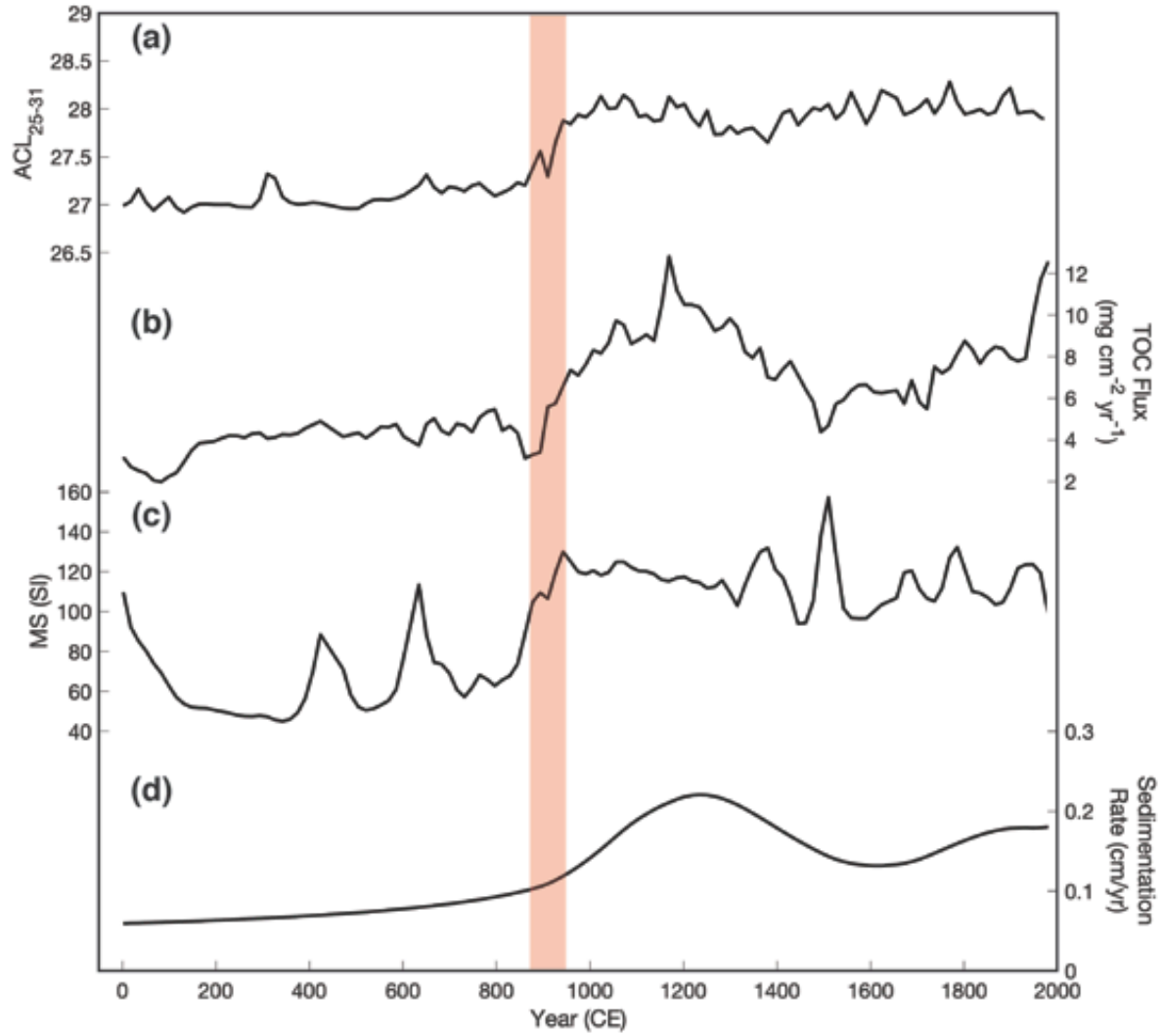


Figure 3. Reconstructions of changes in the catchment area of VGHV and shifts in sediment composition including **(a)** ACL_{25-31} indicating shifts in terrestrial vegetation, **(b)** total organic carbon flux (TOC), **(c)** magnetic susceptibility (MS), and **(d)** sedimentation rate. The red line indicates the historic date of *landnám* in Iceland (874 ± 2 CE).

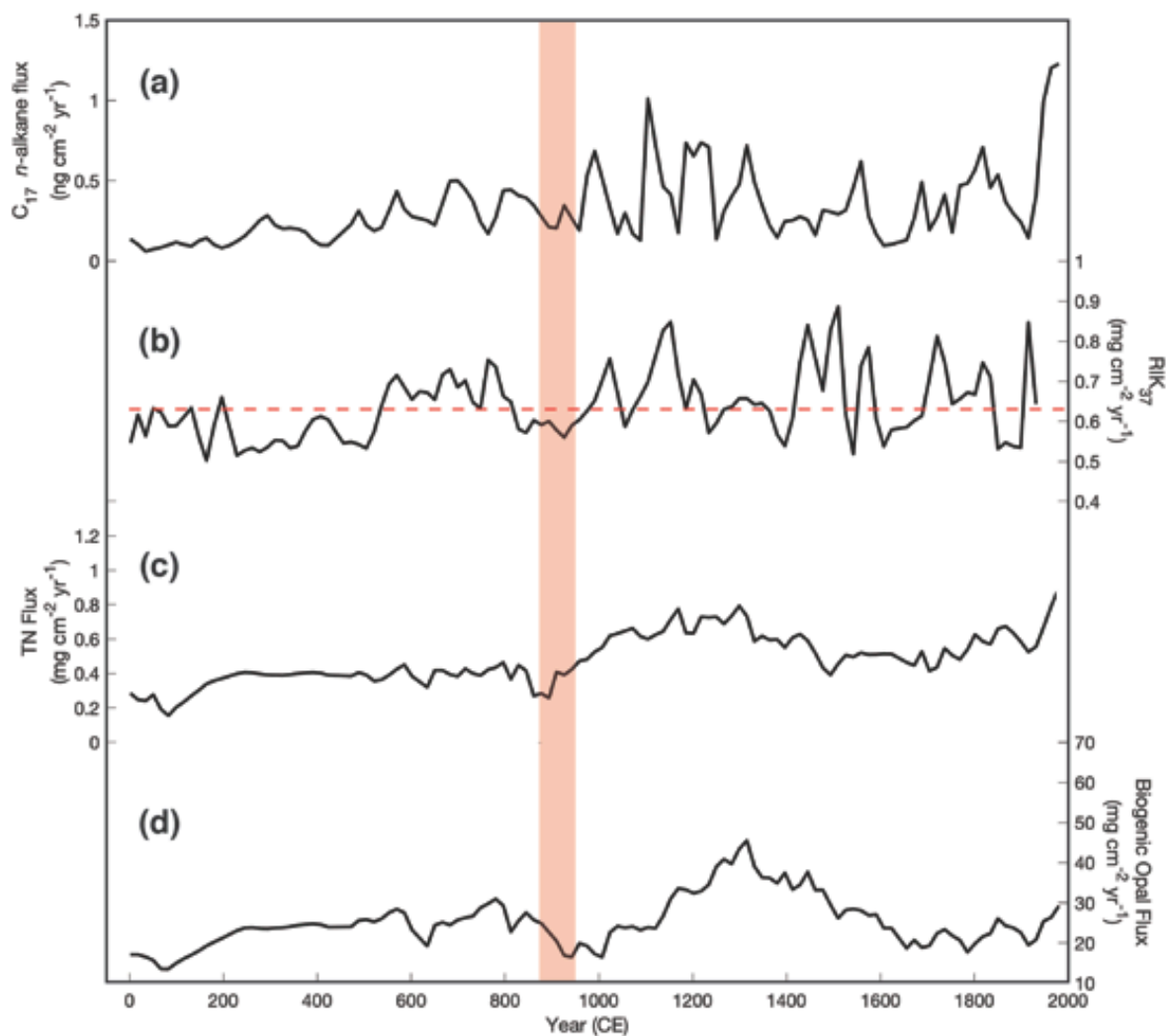


Figure 4. Shifts in VGHV (a) primary productivity as indicated by the C_{17} *n*-alkane flux and changes within the (b) Isochrysidales community where $RIK_{37} = 1.0$ corresponds to Group II and $RIK_{37} = 0.48-0.63$ indicates Group I is present in the water column (the red dashed line is the empirically defined cut-off for Group I, $RIK_{37} = 0.63$). These changes are compared with shifts in (c) total nitrogen (TN) deposition and (d) biogenic opal flux as a proxy for diatom productivity. The transparent red line represents the historic date of *landnám* in Iceland (874 ± 2 CE).

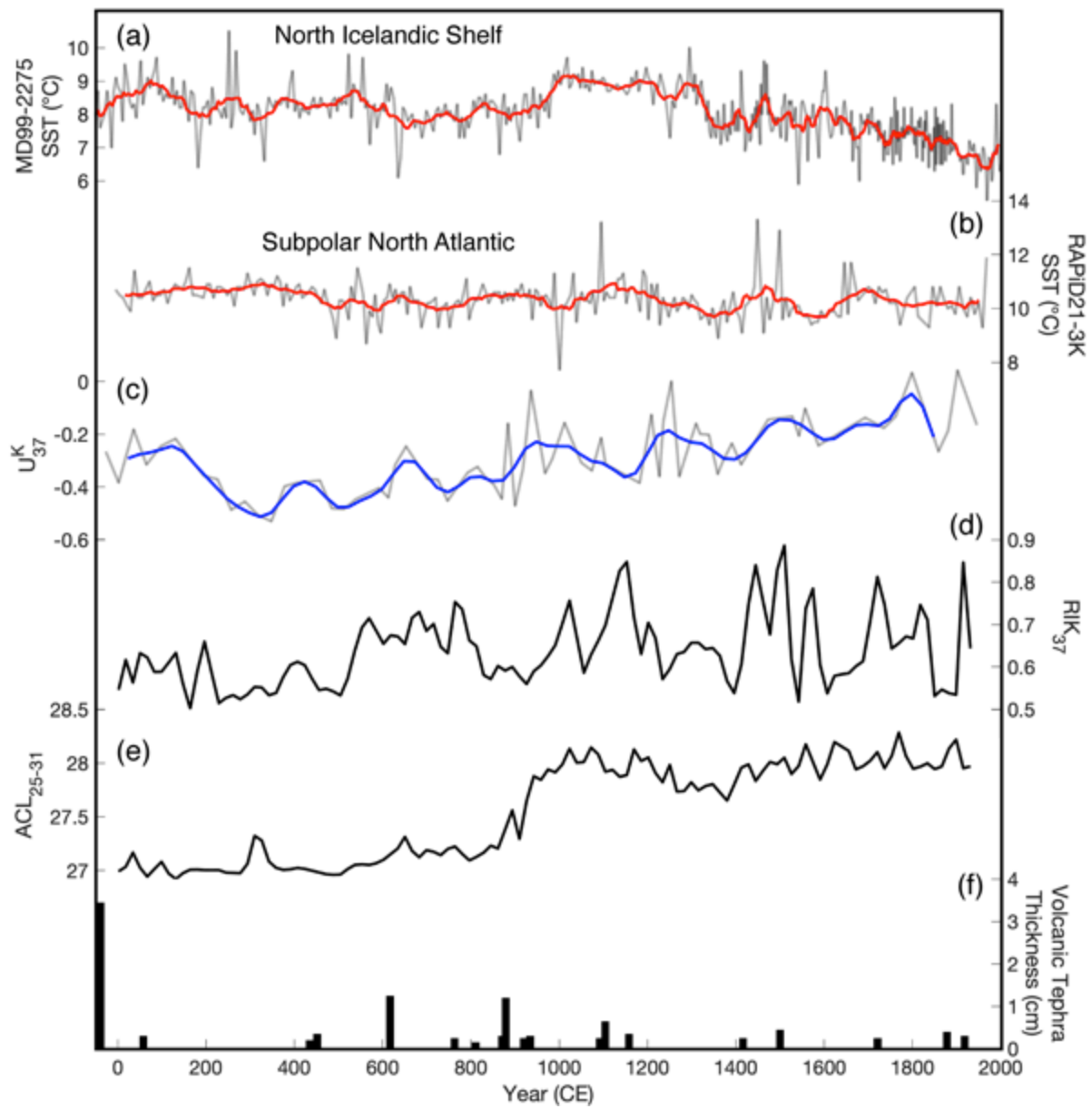


Figure 5. Alkenone temperature reconstructions in marine sediment records from **(a)** the North Icelandic shelf (Sicre et al. 2008, 2011) and **(b)** the subpolar North Atlantic (Sicre et al. 2011). For comparison, the **(c)** U_{37}^K winter-spring temperature reconstruction from VGHV is shown. Environmental changes in the lake are reflected by **(d)** RIK_{37} values and shifts in terrestrial vegetation are indicated by **(e)** ACL_{25-31} . Volcanic tephra deposition in VGHV is displayed as **(f)** tephra thickness.

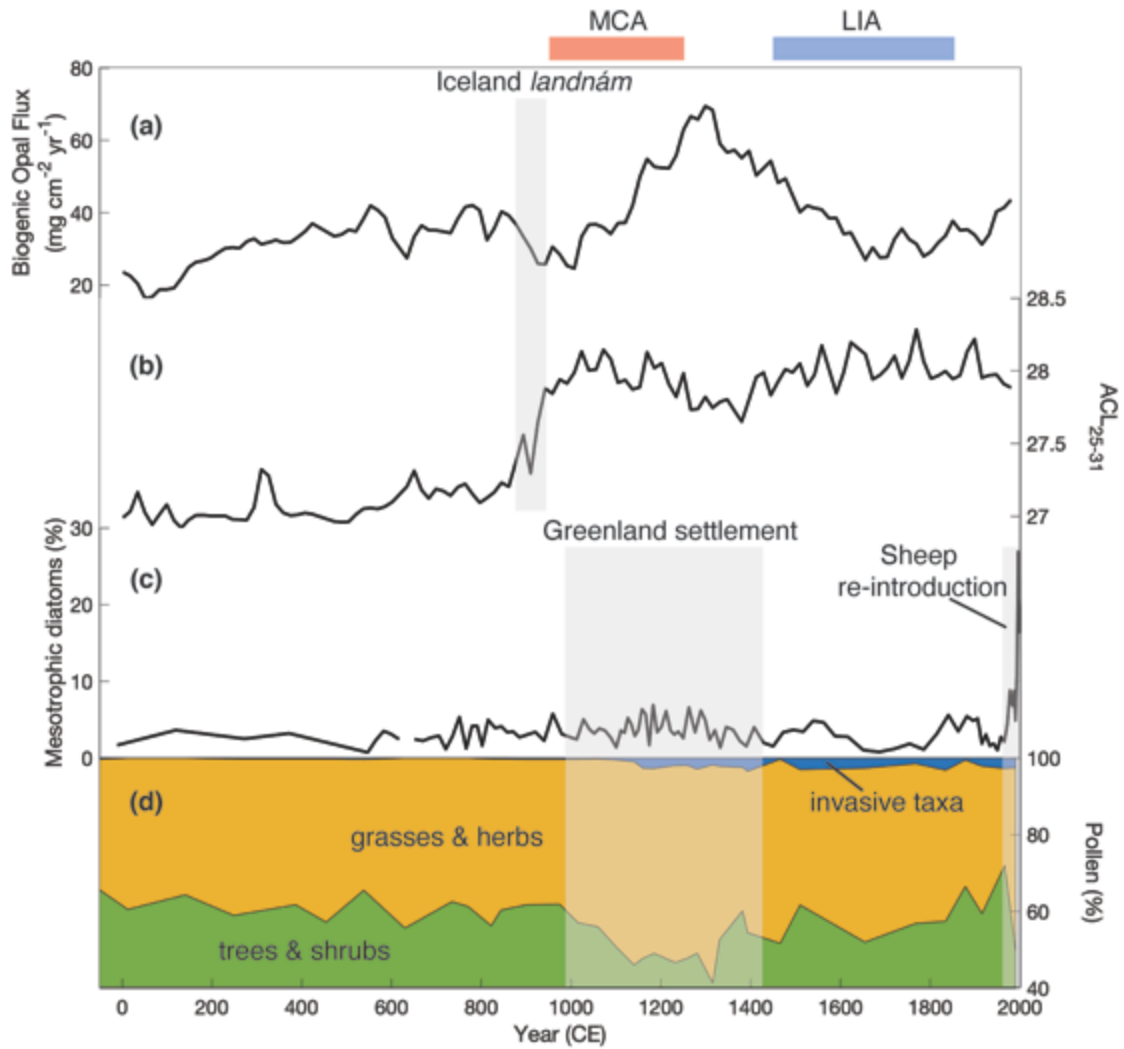


Figure 6. Changes in VGHV (a) biogenic opal (i.e. diatoms) and (b) ACL₂₅₋₃₁ (i.e. terrestrial vegetation), compared with ecological shifts in Greenland after the Norse settlement near lake Igaliku are reflected by (c) mesotrophic diatom species and (d) plant types (Massa et al. 2012a). The timing of the Medieval Climate Anomaly (MCA) and Little Ice Age (LIA) are indicated.

– CHAPTER 4 –

**Cold season warming in the North Atlantic during the last 2,000 years: Evidence
from Southwest Iceland**

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Abstract

Temperature reconstructions from the Northern Hemisphere (NH) generally indicate cooling over the Holocene, which is attributed to decreasing summer insolation. However, climate model simulations predict that rising atmospheric CO₂ concentrations and the collapse of the Laurentian ice sheet caused mean annual warming during this epoch. This contrast could reflect a bias in temperature proxies, and particularly a lack of proxies that record cold (late fall-early spring) season temperatures, or inaccuracies in climate model predictions of NH temperature. We reconstructed winter-spring temperatures during the Common Era (i.e. the last 2,000 years) using alkenones, lipids produced by *Isochrysidales* haptophyte algae that bloom during spring ice-off, preserved in sediments from Vestra Gíslholtsvatn (VGHV), southwest Iceland. Our record indicates local cold-season temperatures warmed during the last 2,000 years, in contrast to NH averages. Sensitivity tests with a lake energy balance model show that this warming is likely driven by increasing winter-spring insolation. We also found distinct seasonal differences in the decadal- to centennial-scale, cold-season temperature variations in VGHV compared to existing records of summer and annual temperatures from Iceland. Sustained or abrupt cooling in VGHV temperatures are associated with the cumulative effects of solar minima and volcanic eruptions, and potentially ocean and sea-ice feedbacks associated with cooling in the broader Arctic. However, multi-decadal to centennial-scale changes in cold season temperatures were strongly modulated by internal climate variability, i.e. the North Atlantic Oscillation, which can result in winter warming in Iceland even after a major negative radiative perturbation.

1. Introduction

Temperatures in the Northern Hemisphere (NH) are generally thought to have cooled over the past 2,000 years, culminating in the Little Ice Age (LIA, c. 1450-1850 CE) (Kaufman et al., 2009; Seppä et al., 2009; PAGES 2K Consortium, 2013, 2019; McKay & Kaufman, 2014). However, the majority of NH temperature reconstructions are based on proxies that respond to climate change during the warm season and may not capture trends in annual or winter and spring temperatures (Liu et al., 2014; PAGES 2K Consortium, 2019). This limits our understanding of major atmospheric phenomena in the NH, such as the North Atlantic Oscillation (NAO) which dominates wintertime variability, as well as changes in ocean circulation and other phenomena driving variability in the extent of Arctic sea ice.

Many oceanic and atmospheric processes that influence surface climate in the Atlantic and the broader NH are centered in the high North Atlantic region, making it an important location to study changes during the cold season (Hurrell, 1995; Yeager & Robson, 2017). Terrestrial paleoclimate records from Iceland, for instance, have the potential to resolve temperature changes during the cold season as this region is sensitive to the NAO and sits near the extent of Arctic sea ice (Hurrell, 1995; Hanna et al., 2004, 2006). The high sedimentation rates in Icelandic lakes, along with well-known volcanic eruptions that can be used as age constraints, make this an ideal location and archive to test how winter and spring temperatures evolved over the past 2,000 years. However, existing terrestrial records of temperature from Iceland are limited due to their sensitivity to the warm season, low temporal resolution and length, or compounding effects on proxies from human land-use or precipitation over the past 2,000 years (Axford et al., 2007, 2009; Geirsdóttir et al., 2009, 2019; Gathorne-Hardy et al., 2009; Larsen et al., 2011; Langdon et al., 2011; Holmes et al., 2016).

Here we present a reconstruction of winter-spring temperatures developed using well-dated lake sediments from southwest Iceland to assess seasonal temperature changes in the North Atlantic climate over the past 2,000 years. We take advantage of alkenone-production by Group I Isochrysidales (i.e. haptophyte algae) during the spring season to develop a record of winter-spring temperatures and investigate the forcings responsible for cold-season temperature changes using a lake energy balance model.

2. Methods

2.1 Study site

Vestra Gíslholtvatn (VGHV) is a small lake (1.57 km²) located in southwest Iceland (61 m a.s.l., 63°56'N, 20°31'W; Figure 1), about 25 km from the coast (Blair et al., 2015). Mean monthly temperatures range from -1.4°C during the winter months (DJF) to 10.4°C during the summer months (JJA) (station at Hella, 1958-2005; Icelandic Meteorological Office). Cores were collected in 2008 using a Bolivia piston coring system (Blair et al., 2015), and were sampled at the National Lacustrine Core Facility (LacCore) at the University of Minnesota for geochemical analyses.

2.2 Age model

The VGHV cores were dated using previously identified tephra, including seven historical and four pre-historical tephra beds (Blair et al., 2015 and references therein). The age model was developed using 'classical' age modeling (CLAM) with a smoothed spline fit. The resulting age model has an uncertainty of 5-15 yrs from -50 to 1200 yrs BP and 18-83 yrs from 1201-2000 yrs BP (Figure 2; Blaauw, 2010).

2.3 Lipid analyses

Sediments were freeze-dried and extracted using a DionexTM accelerated solvent extraction (ASE 350) system at 120°C and 1200 psi. All of the extracts were separated by silica gel (40-63 μm , 60 Å) flash chromatography to obtain alkane (hexane; Hex), ketone (dichloromethane; DCM), and polar (methanol; MeOH) fractions. Saponification was used to remove wax esters by dissolving the dried ketone fraction in a 1 molar potassium hydroxide solution with MeOH:H₂O (95:5, v/v) and heating the samples for 3 hours at 65°C. 5% NaCl in H₂O and 50% HCl in H₂O were added to the samples and the lipid fraction was extracted using Hex (100%). Ketone fractions were further purified using silver nitrate columns (D'Andrea et al., 2007) with DCM (100%) followed by ethyl acetate (100%) to elute the alkenones. If additional cleaning was needed, a modified procedure from Salacup et al. (2019) was used. The alkenone fraction was dried under N₂ gas and re-dissolved in 1.5 mL of DCM:Hex (2:1, v/v). To this, a 1.5 mL solution of 100 mg/mL urea in MeOH was added. The resulting crystals were dried under N₂ gas, and the urea addition was repeated two more times. The dried urea crystals were cleaned with Hex (100%) and extracted as the non-adduct. Milli-Q water was added to the vial to fully dissolve the urea crystals, and the adduct was extracted using Hex (100%). The samples were then analyzed for alkenones. For several samples, co-eluting compounds were still visible, or concentrations were too low for reliable quantification. These samples were not included in our final reconstruction.

The resulting alkenone fraction was analyzed using an Agilent 6890N gas chromatography (GC) system and flame ionization detector (FID) with an Agilent VF-200ms capillary column (60 m x 250 μm x 0.10 μm). Samples were injected into a CIS-PTV inlet in

solvent vent mode (6.9 psi at 112°C). The oven program was set to 50°C and increased to 235°C at 20°C/min and ramped to 320°C at 1.39°C/min where it was held isothermally for 5 min. For additional verification or identification of co-eluting compounds, samples were run on an Agilent 6890N GC system coupled with an Agilent 5793 N quadrupole mass spectrometer (MS). All samples were injected with pulsed splitless injection mode (20 psi at 315°C) and run on an Agilent VF-200ms capillary column (60 m x 250 µm x 0.10 µm). The oven program was started at 40°C for 1 min, ramped up to 255°C at 20°C/min, increased again to 315°C at 2°C/min, and then held isothermally for 10 min. The MS ionization energy was set to 70 eV with a scan range of 50-600 *m/z*.

2.4 Alkenones as a proxy for lake water temperatures

Alkenones are long-chain ketones produced by Isochrysidales haptophyte algae in both marine and lacustrine environments. Numerous marine-based culture and core-top studies show that variations in alkenone saturation (i.e., changes in C_{37:3}Me and C_{37:2}Me production) are inversely correlated with temperature, and can be linearly calibrated to temperature using either the U₃₇^K or U₃₇^{K'} index (Brassell et al., 1986; Prahl and Wakeham, 1987; Prahl et al., 1988; Müller et al., 1998; Conte et al., 2006). Similarly, culture studies, core tops, and *in situ* measurements in lakes show that changes in alkenone saturation are also correlated with temperature (Zink et al., 2001; Sun et al., 2007; Toney et al., 2010; D'Andrea et al., 2011, 2012, 2016; Wang & Liu, 2013; Nakamura et al., 2014; Longo et al., 2016, 2018; Zheng et al., 2016). The U₃₇^K index can be applied to lacustrine environments and is calculated as follows:

$$U_{37}^K = \frac{[C_{37:2}Me] - [C_{37:4}Me]}{[C_{37:2}Me] + [C_{37:3}Me] + [C_{37:3*}Me] + [C_{37:4}Me]} \quad [1]$$

Despite successful application of the U_{37}^K index in lakes (D'Andrea et al., 2011, 2012; van der Bilt et al., 2018), regional variability in the relationship between the U_{37}^K index and temperature often requires the development of local temperature calibrations (Wang & Liu, 2013; D'Andrea et al., 2016; Longo et al., 2016). Unfortunately, there is currently no local calibration for Icelandic lakes.

Sedimentary alkenones may derive from multiple alkenone-producing species, mainly Group I and II Isochrysidales, with distinct alkenone signatures and varying responses to temperature (Coolen et al., 2004; Sun et al., 2007; Theroux et al., 2010, 2013; Ono et al., 2012; Toney et al., 2012; Nakamura et al., 2014; D'Andrea et al., 2016). Group I Isochrysidales produces distinct tri-unsaturated alkenones (e.g., $C_{37:3}^*$ Me), which can be used to test for species-mixing effects with the RIK_{37} index (Longo et al., 2016):

$$RIK_{37} = \frac{[C_{37:3}]}{[C_{37:3} + C_{37:3}^*]} \quad (2)$$

A RIK_{37} value of 1.0 suggests a predominance of the $C_{37:3}^*$ Me and the presence of Group II Isochrysidales, while values from 0.48-0.63 are empirically shown to correspond to Group I Isochrysidales (Longo et al., 2016, 2018).

2.5 Seasonal temperature sensitivity of Group I alkenones in lakes

In Greenland and Alaska, Group I Isochrysidales bloom during the early spring in the photic zone as lake ice starts to melt (D'Andrea & Huang, 2005; D'Andrea et al., 2011; Longo et al., 2016, 2018). Alkenone production remains high as the lake undergoes

isothermal mixing and decreases when thermal stratification begins to develop (Longo et al., 2018). This holds true for other Group I-containing lakes in the NH, including lakes in Iceland, as evidenced by the positive correlation between the U_{37}^K index and mean spring air temperatures (Longo et al., 2018).

We investigated the controls on spring lake water temperatures and the timing of ice-melt in VGHV using a lake energy balance model (Dee et al., 2018). The model was initialized using ERA-Interim daily data (1979-2019) averaged over grid cells covering southwest Iceland (18.25°W-22.75°W by 63.00°N-64.5°N for a 0.75° x 0.75° grid). An initial control simulation was run for 30 years, followed by sensitivity tests where various perturbations were introduced.

The perturbation experiments focused on the effects of changes in seasonal air temperatures and shortwave and longwave radiation on lake surface temperatures and ice-off dates. We used instrumental data from Hella, Iceland (1958-2005) to determine the magnitude of seasonal air temperature changes (Icelandic Meteorological Office). In southwest Iceland, the average temperature range for each season is about $\pm 7^\circ\text{C}$, whereas the interannual variability is about $\pm 3^\circ\text{C}$. Based on this we perturbed the ERA interim seasonal air temperature values by -7°C , -3°C , 0°C , $+3^\circ\text{C}$, and $+7^\circ\text{C}$ and re-ran the lake model with the adjusted parameters. We repeated these experiments, but instead perturbed surface incident shortwave radiation to test how external forcings can drive changes in temperature. Incoming (top of the atmosphere) insolation at 63°N has increased in winter-spring (DJFMAM) by 2.5 W/m^2 and in spring (MAM) by 3.7 W/m^2 over the past 2,000 years. We therefore tested insolation forcing by perturbing seasonal changes in surface incident shortwave radiation by -4 W/m^2 , -2 W/m^2 , 0 W/m^2 , $+2 \text{ W/m}^2$, and $+4 \text{ W/m}^2$. The effects of volcanic eruptions on temperature and ice-off dates were

also tested by changing shortwave radiation by -30 W/m², -10 W/m², 0 W/m², +10 W/m², and +30 W/m². These values were based on regional radiative feedback studies from the 1783 Laki (Oman et al., 2006) and 2010 Eyjafjallajökull (Hirtl et al., 2019) eruptions in Iceland. It should be noted that Iceland receives minimal light during the winter months, and values were set to 0 W/m² if a negative perturbation decreased shortwave radiation below 0 W/m². To assess the effects of longwave radiation on lake water temperatures and ice-off dates, we decreased and increased incoming longwave radiation by -0.2 W/m², 0 W/m², +0.2 W/m². These values reflect the forcing from well-mixed greenhouse gas (GHG) radiation during the pre-industrial period (Schmidt et al., 2011).

3. Results

3.1 U_{37}^K index: corrections for species-mixing

Our U_{37}^K index from VGHV suggests that there was substantial variability in temperature during the last 2,000 years, but there was also variability in the community of alkenone-producers (Figure 3a). Alkenones in VGHV surface sediments have a RIK_{37} value of 0.60 and genetic analyses confirm that Group I Isochrysidales is the main alkenone-producer (Longo et al., 2018; Richter et al., 2019). However, the RIK_{37} values increase slightly above the Group I cut-off of 0.63 about c. 500 CE, and then show a more sustained increase after human settlement in Iceland (c. 870 CE), suggesting that Group II alkenone-producers were also present in the lake (Figure 3b).

To evaluate the impacts of species mixing on the U_{37}^K record, samples with a high abundance of Group II alkenones were removed. We tested several different cut-offs for the RIK_{37} index and compared changes in the mean U_{37}^K values (Figure 4). If no correction is

applied ($RIK_{37} = 1.0$), then $U_{37}^K = -0.34 \pm 0.12$ from 0-1000 CE and $U_{37}^K = -0.14 \pm 0.13$ from 1000-2000 CE. The empirically defined cut-off of 0.63 yields a mean U_{37}^K index of -0.37 ± 0.12 from 0-1000 CE and -0.20 ± 0.10 from 1000-2000 CE. A less stringent RIK_{37} cut-off at 0.71, results in no significant difference in the mean or the variability of the data (0-1000 CE $U_{37}^K = -0.36 \pm 0.10$ and 1000-2000 CE $U_{37}^K = -0.21 \pm 0.11$). Regardless of the correction applied to the data, there is an increase in the mean U_{37}^K values (which we interpret as warming) from 0-1000 CE to 1000-2000 CE.

For a RIK_{37} cut-off of 0.71, the corrected U_{37}^K values and RIK_{37} index are not correlated ($r = 0.11$, $p = 0.35$), indicating that species-mixing effects were removed from the final temperature calibration. The resulting U_{37}^K values can be interpreted as a record of temperature changes from Group I alkenones.

3.2 Controls on spring lake water temperature

Results from the lake energy balance model show that seasonal perturbations can have a strong influence on spring lake water temperatures and ice-off dates in VGHV (Fig 5; Tables S1 & S3). The control run yields an average ice-off date of April 15th with water temperatures on May 1st about 6.6°C. Air temperature perturbations during the winter (DJF) and spring (MAM) alter the timing of ice-off and how rapidly surface water temperatures warm, with warmer air temperatures leading to warmer water temperatures and earlier ice-off dates (Figure 5a-b). In addition, an increase in shortwave solar radiation during the spring season (MAM) leads to earlier ice-off dates and warmer lake water temperatures (Figure 5c-d, Table S2). Shorter days during the winter months (DJF) limits the amount of shortwave radiation reaching Iceland, and therefore has a minimal influence on Icelandic temperatures (Figure 5d & f). Shortwave radiative

perturbations from volcanic eruptions during the winter season result in small changes in spring lake water temperatures and ice-off dates, while eruptions during the spring lead to much colder spring water temperatures and later ice-off dates (Figure 5e-f, Table S2). The increase in longwave radiation from GHGs during the pre-industrial period is relatively small and has no significant influence on either lake water temperatures (change from control = 0.01 ± 1.02 °C) or ice-off dates (change from control = -0.03 ± 8.77 days) (Figure S1, Table S3). Thus, the timing of the alkenone bloom and the water temperatures recorded by the alkenones are most likely responding to changes in air temperature and temperature changes driven by shortwave solar radiation during the late winter and spring season (DJFMAM).

3.3 Long-term trends and short-term variability in the U_{37}^K record and temperature

The U_{37}^K index can provide temperature estimates using linear relationships that are calibrated in lakes with Group I alkenone-producers (D’Andrea et al. 2011, 2016; Longo et al., 2016, 2018). However, existing lacustrine temperature calibrations provide unreasonable temperature estimates for our site, with large changes in temperature over the past 2,000 years (e.g. calibrations give estimates of 10.2 to 33.5°C (D’Andrea et al., 2011), 7.1 to 34.4°C (Longo et al., 2016), 4.4 to 24.5°C (D’Andrea et al., 2016), -1.4 to 18.3°C (Longo et al., 2018)). Given the sensitivity of VGHV lake water temperatures to cold season perturbations and the large variability in spring temperatures observed in instrumental data, it might be plausible to observe temperature swings close to 10°C during the spring transitional season (Figure 5b). Nevertheless, the U_{37}^K index is known to be highly sensitive to temperatures in NH lakes, suggesting that the extreme amplitude of reconstructed temperatures stems from the lack of a local calibration.

Therefore, we use the U_{37}^K index to infer and evaluate qualitative changes in temperature trends and variability during the past 2,000 years.

The corrected U_{37}^K record for VGHV is characterized by a long-term trend towards warmer spring lake water temperatures over the past 2,000 years as well as strong decadal- to centennial variability (Figure 6). In particular, a warmer period from the start of our record to c. 200 CE roughly coincides with the Roman Warm Period (RWP) in Northern Europe (Seppä et al., 2009). Cooler temperatures from c. 250 to 600 CE correspond to the Dark Ages Cold Period (DACP). More variable temperatures, but on average warmer temperatures between c. 850-1100 CE could be associated with the Medieval Climate Anomaly (MCA). The time period usually associated with the LIA is characterized by a warming trend with an abrupt decrease in the U_{37}^K index from c. 1800-1900 CE. However, caution should be used when interpreting results after c. 1400 CE because of low sampling resolution (c. 50 yrs between each sample).

4. Discussion

4.1 Long-term seasonal climate trends in North Atlantic paleoclimate records

In global climate model simulations, rising GHG concentrations and retreating ice sheets during the Holocene lead to warming of global mean annual temperatures, including mean annual temperatures in the NH (Liu et al., 2014). However, temperature reconstructions from the NH typically exhibit a long-term cooling trend during the Holocene (Figure 6b) (Kaufman et al., 2009; Seppä et al., 2009; PAGES 2K Consortium, 2013, 2019; McKay & Kaufman, 2014). This cooling is often interpreted as a response to decreasing summer insolation. This discrepancy could suggest that existing NH temperature reconstructions are biased towards the warm season, highlight important deficiencies in climate models, and/or feedbacks to NH summer

temperatures that influence the temperatures in other seasons (Liu et al. 2014). Alternatively, this could indicate that insolation has a large influence on seasonal temperatures, with summer cooling and winter warming controlled by increasing and decreasing seasonal insolation, respectively (Figure 6a). In this case, mean annual temperatures could still be primarily controlled by rising greenhouse gases, particularly at the global scale (Liu et al., 2014).

Winter-spring temperatures from VGHV warm over the past 2,000 years (Figure 6), and our lake energy balance modeling results show that increasing shortwave radiation and air temperatures during the winter and spring season result in warmer water temperatures and earlier ice-off dates. In contrast, mean annual long-wave radiative forcing, i.e. GHGs, over the pre-industrial period have a minimal influence on water temperatures and ice-off dates. This suggests that the long-term warming trend in VGHV is driven by air temperature changes and solar insolation during the winter and spring season.

Long-term warming trends over the Holocene were also observed in other records of cold-season temperatures in the NH. For instance, pollen records of cold-season temperatures from North America and Europe (Mauri et al., 2015; Marsicek et al., 2018) and an alkenone reconstruction of winter-spring temperatures from Alaska (Longo, 2017) suggest that increasing winter orbital insolation over the Holocene drove warming during the winter season. Winter warming over the Holocene, including the Common Era, was also inferred from chrysophyte cysts in Spain (Pla & Catalan, 2005). An increase in winter sea surface temperatures (SSTs) and a decrease in seasonality over the Holocene is observed in a marine record directly south of Iceland from the North Atlantic subpolar gyre (Van Nieuwenhove et al., 2018). In each of these studies, insolation is proposed as the primary mechanism that is driving changes in seasonal temperatures over the Holocene, supporting the results of our energy balance model.

Although there are existing reconstructions of NH cold season temperatures during the Common Era, the high-resolution reconstruction of winter-spring temperatures from VGHV is one of the few sites in the Northern high latitudes where the effects of seasonal insolation on winter temperature can be tested without having to account for the influence of compounding factors on proxy records. For instance, spring warming inferred from a varve thickness record in Finland might be influenced by human activities in the catchment area (Ojala & Alenius, 2005), while the cooling trend inferred from another cold season varve thickness record could be affected by variations in snow accumulation, the timing of spring melt, and changes in precipitation (Haltia-Hovi et al., 2007). Varve thickness records from the Arctic that record changes in snow or glacial melt are also used to infer long-term cooling during the melt season; however, the melt season in the high Arctic often extends well into the summer months (Cook et al., 2009; Larsen et al., 2011). Water isotope records from ice cores from Svalbard and a stalagmite from the Central Alps are sensitive to winter air temperatures during the instrumental period, but changes in the moisture source and seasonality of precipitation over time can alter long-term temperature interpretations (Isaksson et al., 2005; Mangini et al., 2005; Divine et al., 2011a, b). In Iceland, records of increasing drift ice along the North Icelandic shelf are sensitive to cold season temperature but are also strongly influenced by different ocean currents and are in direct contrast to records from Western Iceland (Ogilvie et al., 1984; Hopkins 1991; Moros et al., 2006; Andrews et al., 2019). Although our record lacks a local calibration, we argue that the U_{37}^K record from VGHV provides a robust, albeit qualitative, record of cold season temperature given the unique seasonal growth ecology of Group I haptophytes in NH lakes.

The warming trend observed in our new record from Iceland and other records of cold-season temperatures from the NH suggest that long-term temperature changes are driven by a

common forcing, mostly orbitally-driven changes in winter and spring insolation (Pla & Catalan, 2005; Mauri et al., 2015; Longo, 2017; Marsicek et al., 2018; Van Nieuwenhove et al., 2018).

Our lake model results confirm that water temperatures are sensitive to perturbations in shortwave radiation, indicating that the long-term warming trend observed in the VGHV record is most likely being driven by increasing winter-spring insolation.

4.2 Seasonal multi-decadal to centennial climate variability in the North Atlantic

The VGHV reconstruction of winter-spring temperatures provides an opportunity to test how changes in internal climate variability and external forcings (volcanic eruptions and total solar irradiance (TSI)) influence temperature changes during the cold season in the North Atlantic region. This is important for understanding multi-decadal to centennial climate changes during the cold season and the important role of the Atlantic Multi-decadal Variability/Oscillation (AMV/AMO), a low-frequency basin-wide North Atlantic SST anomaly that varies in response to external forcings and internal variability, in driving and/or responding to changes during the cold season (Kerr, 2000; Wang et al., 2017; Yeager & Robson, 2017).

Whether forced or unforced, variability in winter atmospheric circulation, including the NAO, and sea ice extent are often linked to multi-decadal and centennial climate change in the North Atlantic region, particularly over Iceland (e.g. Hanna et al., 2006; Massé et al., 2008; Wang et al., 2017; Yeager & Robson, 2017). In the VGHV record of winter-spring temperatures there is a sharp decrease in the U_{37}^K index c. 250 CE and a corresponding increase in drift ice along the North Icelandic shelf (c. 400-900 CE), which coincides with the RWP to DACP transition (Figure 7) (Cabedo-Sanz et al., 2016). Cooling during the DACP is typically attributed to volcanic eruptions and a minimum in solar activity (c. 400-700 CE) (Figure 7a-b); however,

the DACP was not uniform across the NH and records differ as to when peak cooling occurred (Steinhilber et al., 2009; Sigl et al., 2015; Helama et al., 2017). For instance, there is no distinct or prolonged cooling in Iceland SST records during the DACP (Figure 7c & d), whereas terrestrial records indicate that temperatures were on average cooler between c. 450-700 CE in the Arctic and Northern Europe or c. 500-650 CE in Europe (Sicre et al., 2008, 2011; Kaufman et al., 2009; Miettinen et al., 2012; Jiang et al., 2015; Sigl et al., 2015; Helama et al., 2017). The heterogeneous temperature response is commonly attributed to a prolonged negative phase in the NAO, which would result in more pronounced cooling in Icelandic and Northern European winter temperatures (Helama et al., 2017). Our record would support this interpretation. A negative winter NAO phase also does not cause colder NH summer temperatures, which might explain why cooling is not observed in summer temperature reconstructions from tree rings or SSTs before 500 CE (Sicre et al., 2008, 2011; Miettinen et al., 2012; Jiang et al., 2015; Sigl et al., 2015; Helama et al., 2017).

Winter and spring temperatures in VGHV were on average higher between c. 880-1100 CE than temperatures during the DACP but were not stable or particularly warm, unlike the climate usually associated with the Norse settlement of Iceland between c. 870-1100 CE (Figure 7) (Ogilvie et al., 2000). This time period corresponds to a peak in TSI and weak volcanic activity, which could have triggered a positive phase in the AMV/AMO and warmer summer and annual SSTs near Iceland (Sicre et al., 2008, 2011; Justwan et al., 2008; Ran et al., 2011; PAGES 2K Consortium, 2013; Otto-Bliesner et al., 2016; Wang et al., 2017). Observational data and model results suggest that a positive AMV/AMO can result in more frequent negative NAO phases during the winter, which could explain the cold excursions observed in the VGHV

reconstruction and reports of several severe winters in historical records (Ogilvie et al., 1984; Omrani et al., 2014, 2016; Peings & Magnúsdóttir, 2014).

The 14th-15th centuries mark the start of the LIA and are often associated with a colder-than-average climate in Iceland (Ogilvie et al., 1984; Ogilvie & Jónsson, 2001). In contrast, the VGHV record indicates there was no prolonged cold period during the winter and spring season between c. 1300-1800 CE. Rather, temperatures inferred from the VGHV record varied on multi-decadal to centennial timescales. Cooling during this time period is associated with major volcanic eruptions and decreases in TSI that most likely resulted in a negative AMV/AMO phase and temporary increases in sea ice (Figure 7) (Moros et al., 2006; Massé et al., 2008; Steinhilber et al., 2009; PAGES 2K Consortium, 2013; Cabedo-Sanz et al., 2016; Otto-Bliesener et al., 2016; Wang et al., 2017). In some marine records from Iceland this led to cooling of summer/annual SSTs between c. 1400-1900 CE (Figure 7c & e) (Jiang et al., 2005, 2015; Sicre et al., 2008; Ran et al., 2011).

In the VGHV record, multi-decadal variability and an inconsistent temperature response to major radiative forcings during the LIA suggest that temperature anomalies during the cold season are driven by both forced and unforced variability. For instance, the strong negative radiative forcing after the Samalas eruption (1258 CE) and the Wolf solar minimum (c. 1280-1350 CE) correspond to an increase in drift ice along the North Icelandic shelf and cooling in the VGHV temperature record (Massé et al., 2008; Steinhilber et al., 2009; Sigl et al., 2015; Cabedo-Sanz et al., 2016). Similarly, the cumulative effects of the Dalton solar minimum c. 1790-1830 CE and multiple major volcanic eruptions (i.e., Laki 1783, unidentified 1809, and Tambora 1815) in the late 18th and early 19th century could have resulted in enhanced sea ice feedbacks and cooling in VGHV temperatures between c. 1800-1900 CE (Massé et al., 2008; Steinhilber et

al., 2009; Zanchettin et al., 2012; Sigl et al., 2015; Cabedo-Sanz et al., 2016; Toohey & Sigl, 2017). Enhanced cooling in the Arctic or colder initial conditions associated with a solar minimum could have dampened the positive NAO response, i.e. winter warming in Iceland and Northern Europe, that is usually observed for 2-5 winters after volcanic eruptions (Yoshimori et al., 2005; Zanchettin et al., 2012; Ortega et al., 2015; Smith et al., 2016; Sjolte et al., 2018). The inconsistent response in VGHV temperatures to volcanic eruptions and solar minima between c. 1450-1750 CE could be associated with a return to a positive NAO phase that counteracted the effects of negative radiative forcings and led to winter warming over Iceland.

On multi-decadal to centennial timescales, changes in the VGHV record do not consistently correspond to major temperature anomalies observed during the summer months. The differences in seasonal climate responses to external forcings imply that the regional manifestation of these events depends on the initial state of the atmosphere and ocean but is also modulated by internal climate variability (Yoshimori et al., 2005; Zanchettin et al., 2012; Otto-Bliesner et al., 2016; Anchukaitis et al., 2019). Discrepancies in regional temperature responses to radiative forcings suggest that unforced variability, namely the NAO, significantly modulates the climate system response on multi-decadal timescales (Yoshimori et al., 2005; Zanchettin et al., 2012; Anchukaitis et al., 2019).

5. Conclusions

The most striking feature of the VGHV record of winter-spring temperatures is a long-term warming trend from c. 250 CE to the present. Gradual warming in cold-season temperatures is most likely driven by increasing winter and spring solar insolation over the last 2,000 years, and contrasts with inferences of mean annual and summer time warming elsewhere in the NH.

On multi-decadal timescales winter-spring temperatures are sensitive to strong radiative perturbations, but regional temperature responses can be masked by internal climate variability, namely the NAO. These processes can cause strong contrasts between cold season, warm season, and mean annual temperatures. In general, this highlights a need for more winter and spring temperature reconstructions to improve our understanding of the magnitude and direction of cold-season temperature changes over the Common Era.

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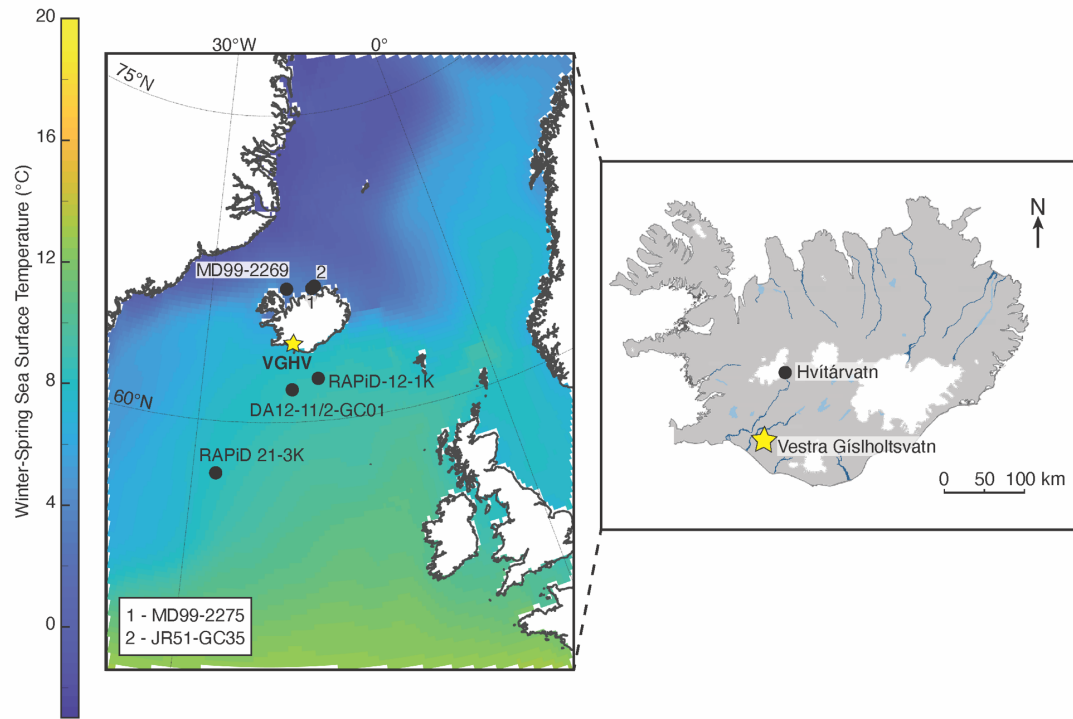


Figure 1. Map of mean winter-spring (DJFMAM) sea surface temperatures from 1955-2017 in the high North Atlantic region. The marine sediment cores MD99-2269 (Moros et al., 2006; Justwan et al., 2008; Cabedo-Sanz et al., 2016), MD99-2275 (Massé et al., 2008; Sicre et al., 2008; Jiang et al., 2015), JR51-GC35 (Cabedo-Sanz et al., 2016), DA12-11/2-GC01 (Van Nieuwenhove et al., 2019), and RAPiD 21-3K (Sicre et al., 2011; Miettinen et al., 2012) that are discussed in the text are indicated. The terrestrial record, Hvítárvatn, (Larsen et al., 2011) that is discussed in the text is shown on the left. The study site, Vestra Gíslholtvatn (VGHV), is marked by a yellow star. The maps were made using data from Natural Earth, the National Land Survey of Iceland, and the National Oceanic and Atmospheric Administration (NOAA) World Ocean Database.

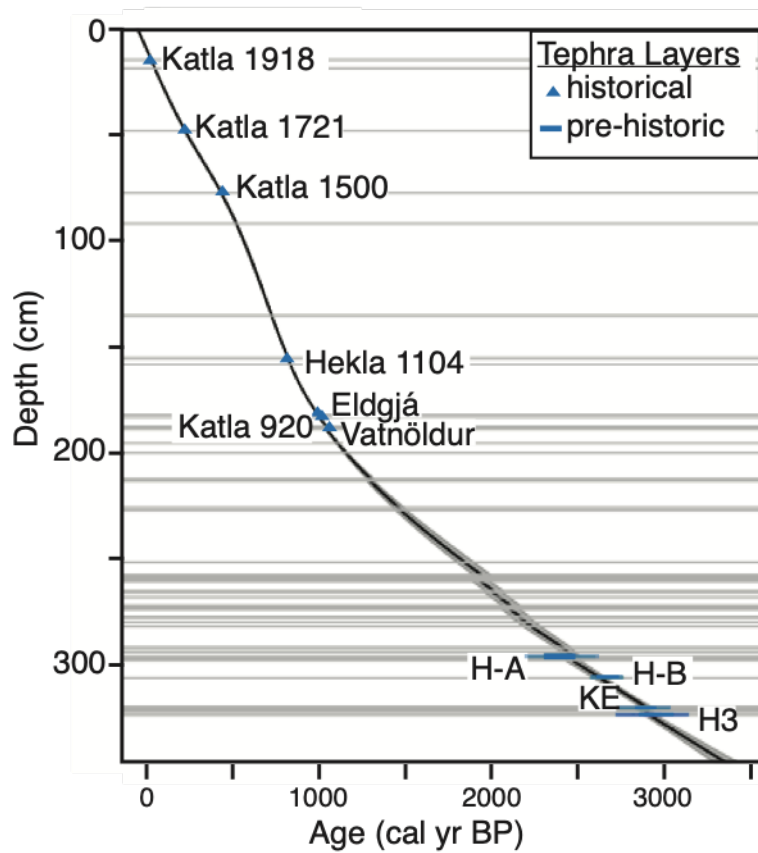


Figure 2. Age model for Vestra Gíslholtsvatn with historic and pre-historic tephra layers used for dating indicated. The gray lines represent tephra layers that were removed from the age model.

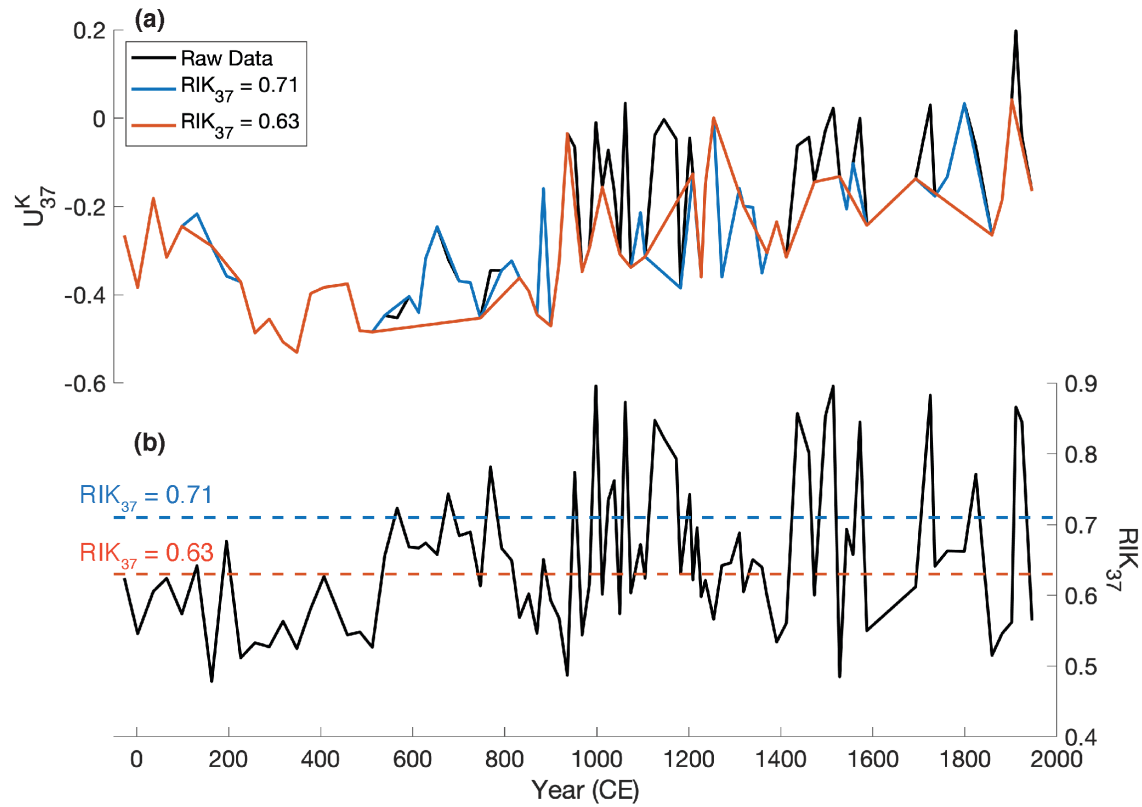


Figure 3. (a) The raw data for the U_{37}^K index is indicated in black, and the corrected U_{37}^K index with a RIK_{37} cut-off of 0.63 and 0.71 are shown in orange and blue, respectively. **(b)** The original RIK_{37} index is shown below for comparison with the empirical cut-off ($RIK_{37} = 0.63$) and cut-off for $RIK_{37} = 0.71$ indicated.

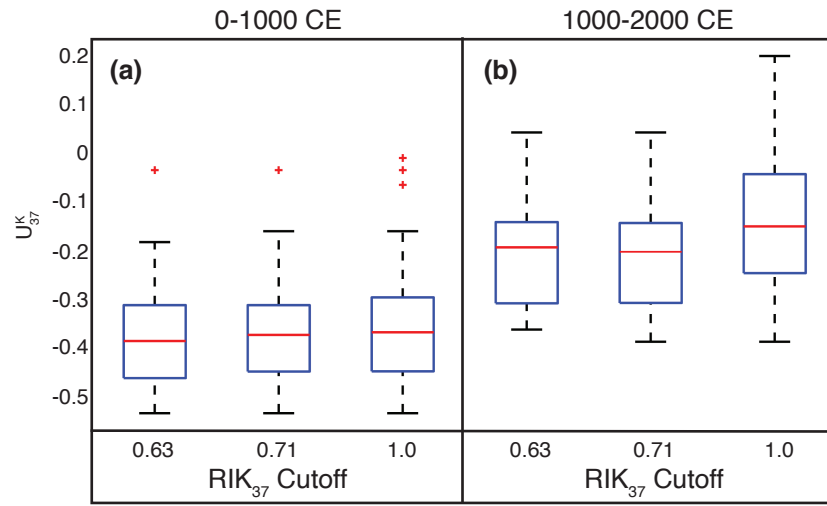


Figure 4. Different RIK_{37} cutoffs applied to the U_{37}^K index for **(a)** 0-1000 CE and **(b)** 1000-2000 CE. A RIK_{37} value of 1.0 indicates that the data was not corrected for species-mixing effects, while $RIK_{37} = 0.63$ corresponds to the empirically defined cut-off for Group I and II (Longo et al., 2018).

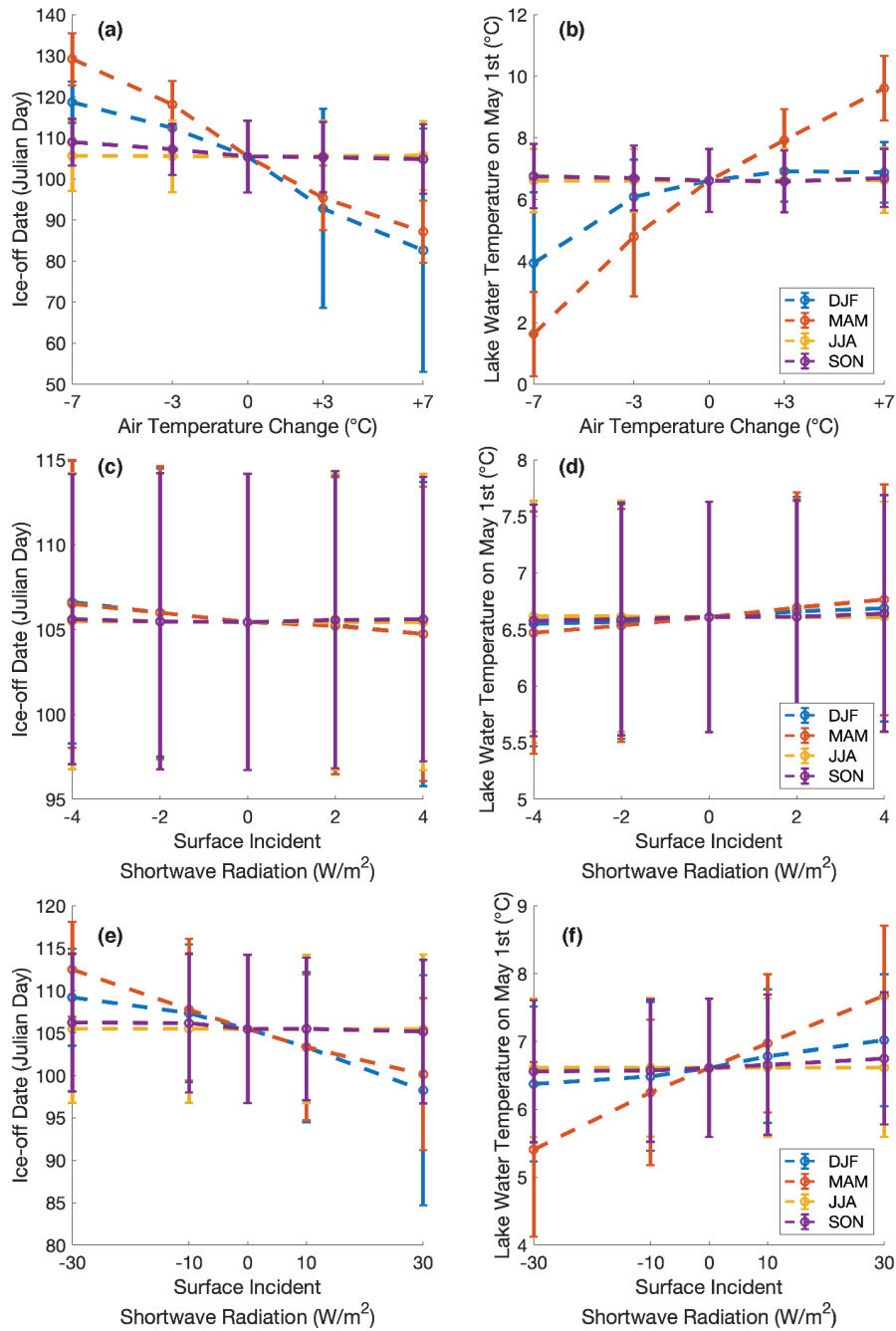


Figure 5. Lake model sensitivity tests showing the impacts of air temperature perturbations on (a) ice-off dates and (b) lake water temperatures on May 1st. Similarly, the results for shortwave radiation perturbations that reflect changes in orbital insolation for (c) ice-off dates and (d) lake water temperatures and changes from volcanic eruptions for (e) ice-off dates and (f) lake water temperatures are shown.

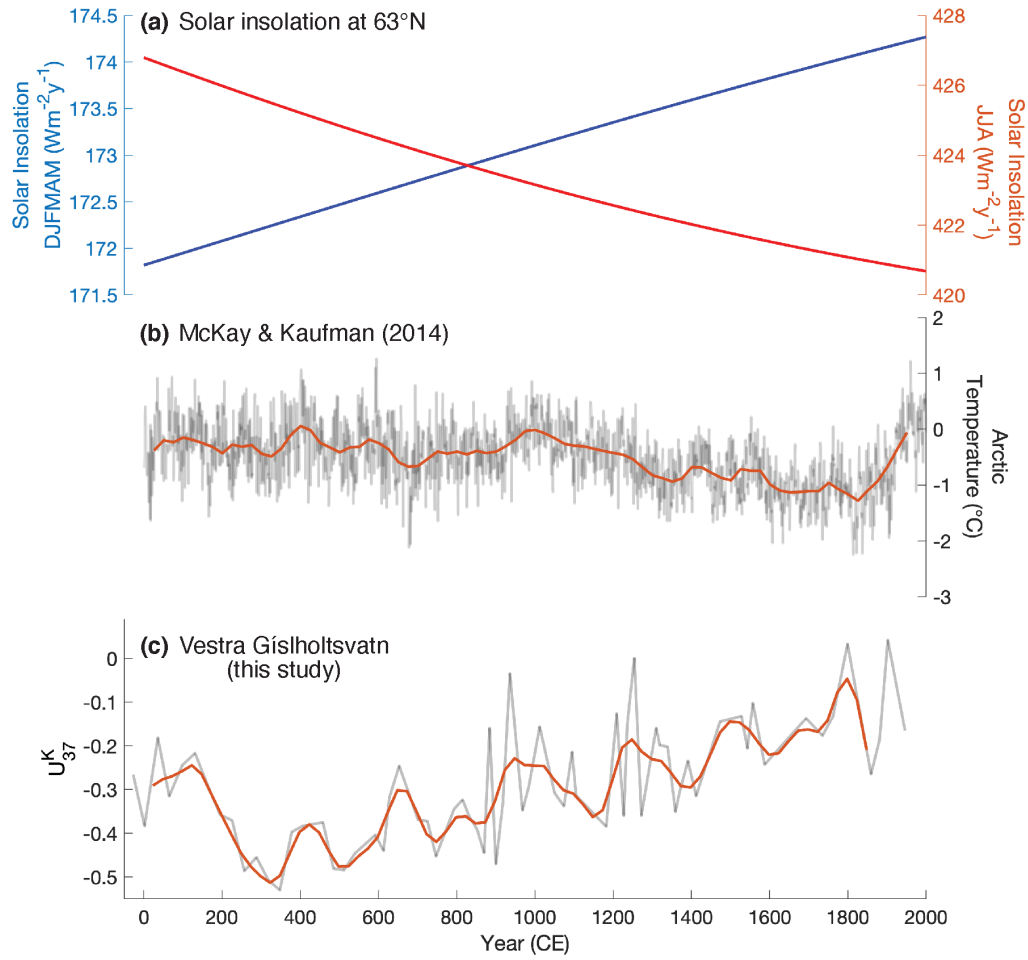


Figure 6. (a) Changes in solar insolation at 63°N for winter-spring (DJFMAM) and summer (JJA) are shown for the past 2,000 years. In addition, (b) a compilation of Arctic temperature reconstructions (McKay & Kaufman, 2014) is shown for comparison with the (c) winter-spring temperature reconstruction from VGHV (a lowpass filter with data resampled to every 25 years is shown in orange to capture the low-frequency variability in the datasets).

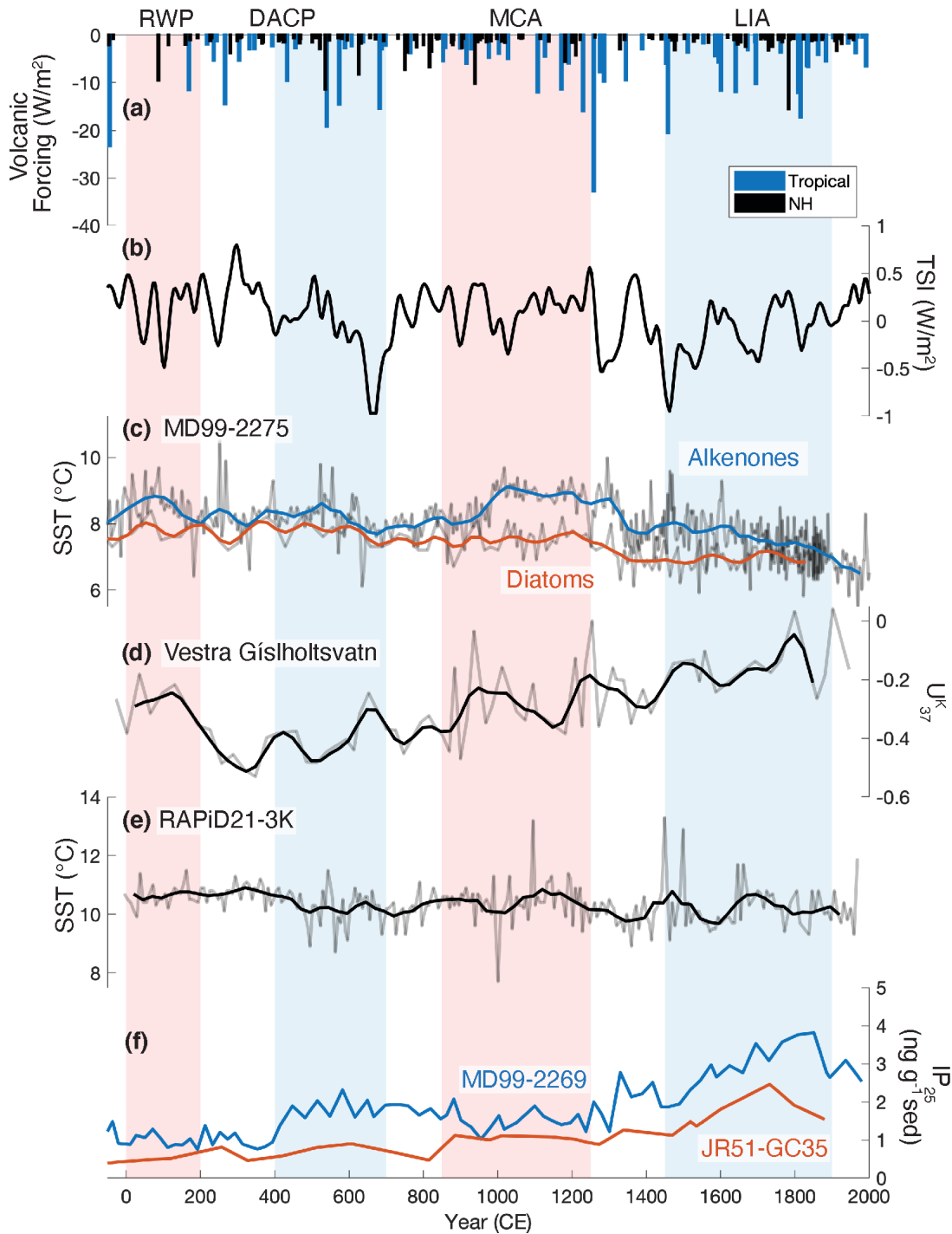


Figure 7. Major changes in radiative forcings over the past 2,000 years, including (a) volcanic forcing from tropical and NH eruptions (Sigl et al., 2015) and (b) changes in total solar irradiance (TSI) (Steinhilber et al., 2009) are shown. Marine and terrestrial reconstructions from Iceland are shown, including (c) alkenone and diatom sea surface temperature (SST) reconstructions from core MD99-2275 on the North Icelandic shelf (Sicre et al., 2008; Jiang et al., 2015), (d) an alkenone winter-spring temperature reconstruction from VGHV in southwest Iceland (this study), (e) an alkenone SST record from core RAPiD21-3K in the sub-polar North Atlantic (Sicre et al., 2011), and (f) sea-ice reconstructions developed using IP₂₅ from cores MD99-2269 (blue) and JR51-GC35 (orange) from the North Icelandic shelf (Cabedo-Sanz et al., 2016). The timing of these periods is inferred from Iceland climate records and include: the Roman Warm Period (RWP, c. 0-200 CE), Dark Ages Cold Period (c. 400-700 CE), Medieval Climate Anomaly (MCA, c. 850-1250 CE), and Little Ice Age (LIA, c. 1450-1900 CE).

Supplementary Information

Table S1. Results from the lake energy-balance model for air temperature perturbations.

Temperature Perturbation	Ice-off Date (Julian Day)	Water Temperature on May 1 st (°C)
Control 0°C	106 ± 9	6.6 ± 1.0
DJF -7°C	119 ± 5	3.9 ± 2.3
DJF -3°C	112 ± 5	6.1 ± 1.2
DJF +3°C	93 ± 24	6.9 ± 1.0
DJF +7°C	83 ± 30	6.9 ± 1.0
MAM -7°C	129 ± 6	1.6 ± 1.4
MAM -3°C	118 ± 6	4.8 ± 1.9
MAM +3°C	95 ± 8	7.9 ± 1.0
MAM +7°C	87 ± 8	9.6 ± 1.0
JJA -7°C	106 ± 9	6.6 ± 1.0
JJA -3°C	106 ± 9	6.6 ± 1.0
JJA +3°C	106 ± 9	6.6 ± 1.0
JJA +7°C	106 ± 8	6.6 ± 1.0
SON -7°C	109 ± 6	6.8 ± 1.0
SON -3°C	107 ± 6	6.7 ± 1.0
SON +3°C	105 ± 9	6.6 ± 1.0
SON +7°C	105 ± 9	6.7 ± 1.0

Table S2. Results from the lake energy-balance model for shortwave solar radiation perturbations.

Shortwave Solar Radiation Perturbation	Ice-off Date (Julian Day)	Water Temperature on May 1st (°C)
Control	105 ± 9	6.6 ± 1.0
DJF x 0.50	108 ± 8	6.5 ± 1.0
DJF x 0.25	107 ± 8	6.5 ± 1.0
DJF x 1.25	105 ± 9	6.7 ± 1.0
DJF x 1.50	104 ± 9	6.8 ± 1.0
MAM x 0.50	124 ± 8	1.8 ± 1.6
MAM x 0.25	112 ± 9	4.5 ± 1.6
MAM x 1.25	100 ± 8	8.2 ± 1.2
MAM x 1.50	95 ± 7	9.8 ± 1.3
JJA x 0.50	106 ± 9	6.6 ± 1.0
JJA x 0.25	106 ± 9	6.6 ± 1.0
JJA x 1.25	106 ± 9	6.6 ± 1.0
JJA x 1.50	106 ± 8	6.6 ± 1.0
SON x 0.50	106 ± 8	6.5 ± 1.0
SON x 0.25	106 ± 9	6.6 ± 1.0
SON x 1.25	106 ± 8	6.6 ± 1.0
SON x 1.50	105 ± 8	6.7 ± 1.0

Table S3. Results from the lake energy-balance model for longwave radiation perturbations.

Longwave Radiation Perturbation	Ice-off Date (Julian Day)	Water Temperature on May 1st (°C)
Control	105 ± 9	6.6 ± 1.0
-0.2 W/m²	105 ± 9	6.6 ± 1.0
+0.2 W/m²	105 ± 9	6.6 ± 1.0

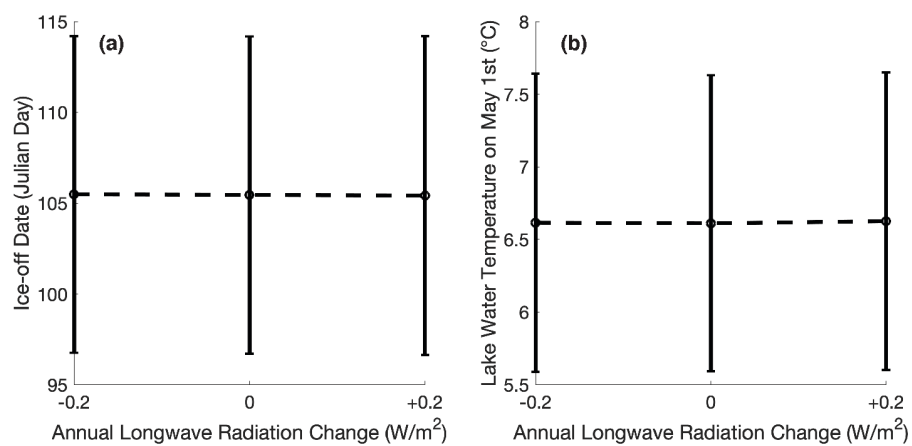


Figure S3. Lake model sensitivity tests showing the effect of annual longwave radiation perturbations on **(a)** ice-off dates and **(b)** lake water temperatures on May 1st.

– CHAPTER 5 –

**Tracking hydroclimate changes and human impacts over the last millennium in
lake sediment records from Flores Island, the Azores**

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Abstract

Human land use and climate change threaten ecosystems and natural resources on islands, such as the Portuguese Azores Archipelago in the Atlantic. The Azorean landscape has been extensively modified by human activities since Portuguese settlement in the 15th century, with invasive plants dominating the present-day vegetation and evidence of eutrophication in numerous lakes. However, it is unclear whether changes in precipitation played a role in shaping terrestrial and aquatic ecosystems and influenced human settlement in the Azores. Further, the timing of when humans first arrived and began to modify the landscape of the islands is still debated. To evaluate changes in terrestrial and aquatic ecosystems in the Azores, we developed complementary paleoecological and paleoclimate records from Lake Funda on Flores island that span the last millennium. Hydroclimate variability in the Azores is strongly modulated by the North Atlantic Oscillation (NAO), and prior to c. 1400 CE precipitation changes fluctuated between dry and wet periods. Although there is no evidence of major land use changes prior to c. 1400 CE, coprophilous fungal spores and increased primary productivity in Lake Funda could be evidence of livestock on Flores island about 100 years before the official arrival of settlers. Early exploration and settlement of the Azores by the Portuguese in the 15th century coincides with a major climate shift from drier to wetter conditions, which might have contributed to the intermittent settlement of the islands. Nevertheless, our records indicate a major change in vegetation shortly after the Portuguese arrived in the Azores. Land use changes also led to a permanent shift in the lake trophic state with a further increase in primary production and evidence of hypoxic conditions in the lake bottom water beginning about 100 years after the Portuguese arrived. The introduction of trees to Flores and a drier climate in the 20th century most likely reduced nutrient leaching and soil erosion in the catchment area of Lake Funda, yet

eutrophication continues to be a problem. Despite harsh climate conditions in the Azores, humans had a major influence on the Azorean landscape and the cumulative effects of multiple disturbances have resulted in a permanent shift in the trophic state of Lake Funda. Many of these environmental issues could be exacerbated in the Azores in the near future with warming temperatures and changing precipitation patterns.

1. Introduction

Rising sea levels, changing precipitation patterns, and warmer temperatures threaten islands that are already under pressure from major human land use changes (Frias, 2000; Santos et al., 2004; Hoegh-Guldberg et al., 2018). The landscape of the Azorean Archipelago, for instance, was shaped by the first Portuguese settlers in the 15th century who replaced the dense native laurel forests with exotic plants and agricultural fields, which led to a destabilization of the landscape and left only a few places with native flora and fauna (Dias et al., 2005; Connor et al., 2012; Rull et al., 2017). Today the islands are at risk from landslides, floods, and coastal erosion, and this risk is expected to increase under projected climate change scenarios (Frias, 2000; Santos et al., 2004; Hoegh-Guldberg et al., 2018). A more in-depth assessment of past land use changes and climate variability is needed to understand the current state and vulnerability of ecosystems in the Azores.

Portuguese settlement of the Azores occurred about the onset of the Little Ice Age (LIA, c. 1450-1850 CE), however, existing paleorecords disagree whether this occurred during a persistent negative phase in the North Atlantic Oscillation (NAO) and wetter climate in the Azores or a positive NAO phase and drier conditions (Björck et al., 2006; Esper et al., 2007; Baker et al., 2015; Hernández et al., 2017). Historical records from this time period describe a harsh climate that could be related to a negative NAO phase that dissuaded many early colonists from settling the Azores Archipelago (Smith, 2010). A high-resolution reconstruction of precipitation changes in the Azores and a complementary record of human land use changes could provide insights on how climate change over the last millennium influenced both ecosystems and human settlements in the Azores Archipelago.

Ongoing eutrophication in lakes on the Azores is attributed to human activities, including indirect effects such as nutrient loading from the catchment area, and direct effects such as fish introduction (Skov et al., 2010; Antunes & Rodrigues, 2011; Cruz et al., 2015; Raposeiro et al., 2017). However, this does not explain the high rates of eutrophication observed in lakes isolated from direct human impacts (Antunes & Rodrigues, 2011). For instance, Lake Funda on Flores island is considered to be less impacted by human activities relative to the rest of the Azores Archipelago but the catchment area is still dominated by exotic plants and the lake itself is eutrophic (Connor et al., 2012; P.M. Raposeiro and V. Gonçalves, *pers. commun.*). It is unclear whether eutrophication in Lake Funda is natural or human-induced. Rising temperatures, changing precipitation patterns, and continued human activities all pose potential risks to these freshwater ecosystems, and threaten their use as a water supply for the archipelago (Antunes & Rodrigues, 2011).

To understand the role of humans and natural climate variability in shaping the present-day landscape, we developed complementary paleoecological and paleoclimate records for Lake Funda on Flores island. Fecal biomarkers and changes in vegetation composition were used to determine when human activities began in the catchment area. In addition, bulk organic and inorganic geochemical proxies, elemental analyses and sterol hydrogenation, and archaeal lipids are used to trace changes in organic matter inputs to the lake, redox conditions, and biogeochemical cycles, respectively. To track changes in hydroclimate, a complementary high-resolution record was developed using leaf wax hydrogen isotopes.

2. Methods

2.1 Study site

Funda is a maar lake (371 m a.s.l., 39°24'N 31°13'W; Figure 1) on the island of Flores in the Azores Archipelago. The lake is monomictic and thermally stratifies during the summer and into the early fall (usually June/July to October/November) and the water column mixes between November/December to May/June (Figure S1). The lake is considered to be eutrophic and is usually phosphorous limited. However, heightened primary productivity during the spring/summer can lead to rapid oxygen depletion in the water column and hypoxic conditions in the bottom water that results in internal phosphorous loading (P.M. Raposeiro and S. Giralt *pers. commun.*). The lake is surrounded by a steep catchment, mostly consisting of forested area and some agriculture.

2.2 Age model

The age model was developed using ^{210}Pb concentration profiles and ^{14}C dates measured on plant macrofossils in the sediment core (Figure 2 & Table 1). ^{210}Pb was measured in the top 80 cm of the core at the Environmental Radioactivity Laboratory in the Unviersitate Autònoma de Barcelona using a Passivated Implanted Plana Silicon (PIPS) detector (CANBERRA, Mod. PD-450, 18 A M). These measurements were verified with ^{226}Ra measurements along the core using gamma-spectroscopy with a high-purity germanium detector (CANBERRA, mod. GCW3523). AMS ^{14}C dates were measured by Beta Analytic Laboratory (Florida, USA). All radiocarbon dates were calibrated to calendar years (cal CE) using the CALIB 7.1 software and the INTCAL13 curve (Reimer et al., 2013). All slumps were removed from the record, and the age-depth model was determined using CLAAM (Blaauw, 2010).

2.3 Sample preparation and analysis

Sediment cores were retrieved from Lake Funda in June 2017 using a UWITEC piston corer installed on a UWITEC platform. The cores were sealed and shipped to the Institute of Earth Sciences Jaume Almera (CSIC), where they were kept in a dark room at +4 °C until they were prepared for analysis. Cores were split longitudinally and imaged using the high-resolution line scan camera installed on the XRF AVAATECH Core Scanner at the Universitat de Barcelona (UB). Samples were measured for total carbon (TC) and total nitrogen (TN) and the respective isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) using a Finnigan Delta Plus Elemental Analyzer-Continuous Flow-Isotope Ratio Mass Spectrometer (EA-CF-IRMS) at the Centres Científics i Tecnològics at the Universitat de Barcelona (CCiTUB). Mineralogical analyses were also conducted with a SIEMENS-D500 automatic X-ray diffractometer (Cu-K α , 40 kV, and 30 mA) and a graphite monochromator at the Institute of Earth Sciences Jaume Almera (CSIC). The carbonate content of these samples was below the detection limit of the X-ray diffractometer, and therefore TC is considered to be equivalent to total organic carbon (TOC). TOC/TN (or C/N) molar ratios were calculated for all of the measured samples. X-Ray Fluorescence (XRF) was measured on the archived half of the core with the AVAATECH XRF II core scanner at 2 mm intervals. Biogenic silica content was determined every 4 cm following Bernárdez et al. (2005) and Mortlock and Froelich (1989), using an Auto Analyzer Technicon AAII at the Institute of Marine Research (CSIC) in Vigo.

The cores were also sampled for lipid analyses and samples were shipped to Brown University for processing. Sediment samples were freeze-dried and extracted using a DionexTM accelerated solvent extraction (ASE 350) system at 120°C and 1200 psi. The resulting extracts

were split, and one fraction was processed for glycerol dialkyl glycerol tetraethers (GDGTs) and *n*-alkanes and the other fraction was prepared for fatty acid and sterol and stanol analyses.

The fraction for GDGT samples was separated using aluminum oxide (Al_2O_3) columns to obtain a non-polar (hexane:dichloromethane (Hex:DCM), 9:1 v/v) fraction containing *n*-alkanes and a polar (DCM:Methanol (MeOH), 1:1 v/v) fraction for GDGTs. The resulting polar fraction was dried using N_2 gas, and re-dissolved in Hex:Isopropanol (99:1, v/v) and filtered through a 0.4 μm PTFE prior to analysis.

The acid/polar fraction was separated into neutral (DCM:Isopropanol, 2:1, v/v) and acid (ethyl ether:acetic acid, 24:1, v/v) fractions using an aminopropylsilyl (NH_2) column. The acid fraction was methylated at 60°C for 2 hrs with acidified anhydrous methanol of a known isotopic composition, and the resulting fatty acid methyl ethers (FAMES) were purified via silica gel (40-63 μm , 60 Å) flash chromatography. The neutral fraction was further separated by silica gel flash chromatography into alkane (Hex), ketone (DCM), and polar (MeOH) fractions. The polar fraction was then saponified by dissolving the sample in a 1 M potassium hydroxide solution with MeOH:H₂O (95:5, v/v) and heating it for 3 hrs at 65°C. To this 5% NaCl in H₂O and 50% HCl in H₂O was added, and the lipid fraction was extracted using Hex (100%). The saponified samples were cleaned on a short silica gel column and dried using N_2 gas for derivatization. Pyridine (50 μL) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 50 μL) were added to the dried sample, and the sample was capped under N_2 gas and heated for 2 hrs at 60°C. The derivatized samples were stored in pyridine until analysis, when they were dried and re-dissolved in toluene.

Samples for GDGTs were analyzed at the Royal Netherlands Institute for Sea Research (NIOZ) using an Atmospheric Pressure Chemical Ionization/High Performance Liquid Chromatography-Mass Spectrometer (APCI/HPLC-MS) following the method described in

Hopmans et al. (2016). The analyses were run using selective ion monitoring to track m/z 1302, 1300, 1298, 1296, 1292, 1050, 1048, 1046, 1036, 1034, 1032, 1022, 1020, 1018, and 744.

All *n*-alkanes and fatty acids were quantified with an Agilent 6890N gas chromatography (GC) system and a flame ionization detector (FID). Samples were injected using pulsed splitless mode (20.3 psi, 310°C) onto a Rtx-200 column (105 m x 205 μm x 0.25 μm). The oven program was started at 50°C, ramped up to 315°C at 10°C/min, and then held isothermally for 30 min. All *n*-alkanes were quantified using hexamethylbenzene as an internal standard.

Compound specific isotope ratios (δD_{wax}) of C_{30} FAMES were measured on an Agilent 6890 GC equipped with a ZB-1MS (30 m x 320 μm x 0.25 μm) coupled to a Thermo Delta V Plus Isotope Ratio Mass Spectrometer (IRMS). The GC method was run using a pulsed splitless injection mode (30 psi, 320°C), and the oven program was started at 40°C for 1 min, and then ramped up to 230°C at 30°C/min where it was held for 1 min. The temperature was increased again to 310°C at 10°C/min and held isothermally for 10 min. The pyrolysis reactor temperature for the IRMS was set at 1450°C. The reference gas $^2\text{H}/^1\text{H}$ was measured using certified C_{29} and C_{31} *n*-alkane standards. The H^{3+} factor was determined every day (2.41 ± 0.07). An internal standard mixture containing C_{16} , C_{18} , C_{22} , C_{24} , C_{28} , and C_{30} *n*-acids (see Table S1 for analytical uncertainty) was analyzed between every 3 to 4 injections to monitor instrument performance and drift, and a certified C_{30} methyl ester standard of known composition was run to monitor instrument accuracy (lab measured: $-186.4 \pm 3.9 \text{ ‰}$ for $n = 71$, actual: $-189.4 \pm 2 \text{ ‰}$ for $n = 5$ (Schimmelmann, 2018)). Each sample was measured three times, and isotopic values were accepted for a voltage response between 2.5 and 7 volts. Corrections were made on a daily basis for offsets between measured and reported standard values.

Sterol and stanol samples were analyzed on an Agilent 7890B gas chromatography (GC) system equipped with an Agilent 5977B quadrupole mass spectrometer (MS). Samples were injected using pulsed splitless mode (320°C, 1.3 psi) and run on a ZB-1MS (30 m x 320 μ m x 0.25 μ m) column. The oven program was started at 40°C for 1 min, and then ramped up to 255°C at 20°C/min and ramped to 315°C at 4°C/min and held isothermally for 10 min. The MS ionization energy was set to 70 eV with a scan range of 50-650 m/z . Samples were quantified using select ion monitoring mode and concentrations were determined using 5 α -cholestan-3-one as an internal standard (see Table S2).

2.4 Data processing and breakpoint analysis

Lipid and sediment fluxes were calculated by correcting for changes in dry bulk density and sedimentation rates in the sediment core. To determine when major changes occurred in the sediment record, all datasets were re-sampled first to a 20-yr resolution (except for the sterol and stanol samples) and to a 60-yr resolution to include the sterol and stanol samples. Breakpoint analysis was conducted on both re-sampled datasets using the “segmented” package in R (R Development Team; Muggeo, 2008). The breakpoints that were common to both re-sampled datasets are reported. The 95% confidence intervals were determined by calculating the pooled uncertainty from the breakpoint analysis and age model.

2.5 Controls on leaf wax hydrogen isotopes in the Azores

The hydrogen isotope composition of leaf waxes (δD_{wax}) in higher terrestrial plants (i.e. C₂₈-C₃₂ n -acids) is correlated with changes in δD of precipitation (δD_{precip}) (Sachse et al., 2012). The variability in δD_{precip} reflects a combination of processes, including water source, transport

history, and Rayleigh-type processes related to evaporation and condensation (Craig, 1961; Dansgaard, 1964; Sachse et al., 2012). However, in the Azores changes in precipitation amount are dominated by shifts in the high-pressure Azores anticyclone, resulting both intra- and inter-annual variability associated with the North Atlantic Oscillation (NAO) (Hurrell, 1995; Santos et al., 2004; Cropper & Hanna, 2014; Hernández et al., 2016). In general, changes in precipitation amount are negatively correlated with the NAO, and the hydrogen isotope composition of precipitation during the winter (DJF; $r = 0.52$, $p < 0.01$) and spring (MAM; $r = 0.63$, $p < 0.01$) are positively correlated with the NAO (Cropper & Hanna 2014; Hernández et al., 2016; Global Network of Isotopes in Precipitation (GNIP)). This suggests that δD_{wax} of C_{30} FAMES should predominantly respond to variations in precipitation amount and this is strongly influenced by fluctuations in the NAO.

2.6 Leaf waxes as a proxy for vegetation change

Shifts in vegetation were assessed by measuring changes in the *n*-alkane distribution. Higher-level plants produce longer-chain *n*-alkanes (e.g., C_{25} - C_{33}) which can differentiate woody plants (C_{27} and C_{29}) from grasses and shrubs (C_{31}) (Cranwell, 1973; Maffei, 1996). Therefore, shifts in the average chain length (ACL) are often used to reconstruct changes in vegetation as follows:

$$ACL_{27-33} = \sum \frac{C_i \times [C_i]}{[C_i]} \quad [1]$$

where $[C_i]$ represents the concentration of *n*-alkanes and C_i corresponds to the hydrocarbon chain-length.

2.7 Sterols and stanols as fecal biomarkers and indicators of water column redox conditions

Sterols and stanols are structurally diverse and relatively stable compounds in the geologic record, making them useful tracers for different inputs to the sediment (e.g. Nishimura & Koyama, 1977; Volkman et al., 1986; Leeming et al., 1996). For instance, $5\alpha(H)$ -stanols are mainly sourced from primary producers (e.g. diatoms and dinoflagellates) in the water column, but under anoxic conditions hydrogenation of Δ^5 -double bond of sterols also results in the production of $5\alpha(H)$ -stanols in the sediment (Gaskell & Eglinton 1975; Nishimura, 1977; Nishimura & Koyama, 1977). The ratio of $5\alpha(H)$ -stanols/sterols can thus provide insight into changes in the redox conditions of the water column and sediment, and is calculated as follows:

$$\frac{5\alpha(H)\text{-stanols}}{\text{sterols}} = \frac{\text{cholestanol} + \text{campestanol} + \text{stigmastanol} + \text{sitostanol}}{\text{cholesterol} + \text{campesterol} + \text{stigmasterol} + \beta\text{-sitosterol}} \quad [2]$$

Where an increase in the $5\alpha(H)$ -stanols/sterols would indicate more reducing conditions in the water column/sediment and a decrease corresponds to oxidizing conditions in the water column/sediment.

Certain 5β -stanols are produced in high abundance in the gastrointestinal tract of higher mammals, making them ideal fecal biomarkers (Leeming et al., 1996). For instance, carnivores and omnivores, particularly humans, consume large quantities of cholesterol that gets microbially reduced to coprostanol (5β -cholestan- 3β -ol) by their gut microbiome (Leeming et al. 1996). Ruminants, on the other hand, reduce a high proportion of plant sterols, e.g. sitosterol and stigmasterol, to 24-ethylcoprostanol and 5β -stigmastanol, respectively (Leeming et al., 1996; Bull et al., 2002).

3. Results

3.1 Core stratigraphy

Prior to c. 1000 CE the core is composed of light brown that is interspersed with numerous events consisting of poorly sorted pebbles and sand with numerous decimetric erosive-based fining-upward siliciclastic sequences that are most likely associated with mass-wasting events. Between c. 1000-1200 CE there are only a few deposits related to mass-wasting events as indicated by thin layers of sand and one layer of gravel-sized clasts c. 1200 CE. The uppermost section of the core consists of laminated sediments between c. 1450-2000 CE. The laminations consist of darker lamina composed of fine terrigenous material and lighter layers composed of diatom oozes. At the very top of the core (c. 2000-2015 CE) there is a shift back to massive brown-black mud.

3.2 Hydroclimate variability in the Azores

In the δD_{wax} record of C₃₀ FAMES there is low variability ($\sigma^2 = 5$) between c. 800-1400 CE (Figure 3). A breakpoint at 1420 ± 23 CE and 1568 ± 17 CE correspond to a change from more enriched (drier) to depleted (wetter) values in δD_{wax} (Table 3). After c. 1650 CE δD_{wax} variability increases ($\sigma^2 = 14$) and there is also a gradual increase in δD_{wax} to more enriched (drier) values between c. 1950 CE to 2000 CE.

3.3 Changes in bulk geochemical and elemental properties

Before c. 1300 CE total carbon to total nitrogen (C/N) values are around 18 but decrease between 1297 ± 29 CE and 1604 ± 11 CE to about 10 (Figure 4 & Table 3). Biogenic silica

(BSi) increases from 1266 ± 27 CE to 1395 ± 33 CE and increases again after 1525 ± 24 CE. This change coincides with an abrupt decrease in $\delta^{15}\text{N}$ at 1341 ± 37 CE and a further decrease between 1500 ± 12 CE and 1676 ± 8 CE (Figure 4). There is also a gradual decrease in $\delta^{13}\text{C}$ values after 1624 ± 20 CE (Figure 5).

3.4 Changes in the lake catchment area and redox conditions in the sediment

To infer specific changes in the catchment area, ACL_{27-33} is used to track vegetation composition and sterols/stanols are used as proxies for livestock and humans and as an indicator of changing redox conditions in the lake sediment. Breakpoints for ACL_{27-33} occur at 1392 ± 35 CE and 1586 ± 12 CE, marking a decrease in ACL_{27-33} (Figure 3 & Table 3). Unfortunately, we do not have access to plants from the Azores to determine the *n*-alkane signature of native and endemic plants, so we interpret this change in *n*-alkane distribution in conjunction with pollen reconstructions. The decrease in ACL_{27-33} values corresponds to a decrease in *Juniperus brevifolia* and *Picconia azorica* and a gradual increase of native grasses (Poaceae) and shrubs (e.g. *Erica azorica* and *Myrsine*) in the pollen record (Connor et al., 2012; S. Giralt and E. de Boer, *pers. commun.*). 5β -stigmastanol first occurs c. 1500 CE and is followed by an increase in $5\alpha(H)$ -stanols/sterols after 1521 ± 13 CE (Figure 5 & Table 3). ACL_{27-33} values increase again after 1947 ± 6 CE, which corresponds to the first occurrence of *Cryptomeria* in the pollen record (S. Giralt and E. de Boer, *pers. commun.*).

3.3 isoGDGTs as tracers of biogeochemical cycles in the lake water column

Isoprenoidal glycerol dialkyl glycerol tetraethers (isoGDGTs) are found in a wide range of environments, including lakes, and are commonly used to infer changes in temperature and pH

(e.g. Sinninghe Damsté et al., 2009; Schouten et al., 2013). However, changes in lake trophic state and dissolved oxygen content can also drive shifts in these communities, making them useful tracers for paleo-ecological studies (e.g., Blaga et al., 2009; Naeher et al., 2014). In Lake Funda the composition of isoGDGTs is distinctly different from the GDGT composition of the soils in the catchment area, indicating that most of the GDGTs in the sediment are produced in the water column (Figure S2).

Throughout the sediment record GDGT-0 is present in higher abundance relative to other isoGDGTs. Crenarchaeol (Cren) and its regioisomer, Crenarchaeol' (Cren'), which are specific to *Thaumarchaeota* or ammonia-oxidizers, are both present in this section of the sediment core (Pouliot et al., 2009; Sinninghe Damsté et al., 2009; Auguet et al., 2011; Blaga et al., 2011; Buckles et al., 2013). GDGT-0/Cren values remain around 10 between c. 1000-1500 CE, and GDGT-1 and -2 are present in low abundance. Two distinct breakpoints occur in the isoGDGT composition: a decrease in Cren'/Cren between 1374 ± 46 CE and 1399 ± 28 CE and an increase in GDGT-0/Cren about 1530 ± 14 CE (Figure 5). The later increase in GDGT-0 relative to Cren also coincides with a decrease in GDGTs-1 and -2 (Figure S3).

4. Discussion

4.1 Evidence of early human activities in the Azores?

The Portuguese are typically credited with the discovery and settlement of the Azores in the 15th century (Crosby, 2004). However, the Canary Islands, and possibly the Azores and Madeira, were already known to the Romans (c. 500 BCE) and Isidor de Sevilla (c. 600 CE) (Schäfer, 2003; Crosby, 2004). The Azores Archipelago was first noted on maps by Dulcert

(1339) and Mediceu (1352), which predates the Portuguese arrival (Schäfer, 2003). However, it is unclear whether early explorers had any lasting impacts on these local ecosystems.

Between c. 1000-1500 CE there are very few changes in the ACL₂₇₋₃₃ record, and pollen data indicates that dense laurel forests dominated the catchment area of Lake Funda (Connor et al., 2012; E. de Boer and S. Giralt *pers. commun.*). However, there is a decrease in C/N values that corresponds to a transition from terrestrially dominated to aquatically sourced organic matter inputs after c. 1300 CE and an increase in BSi (%) (Figure 4). A decrease in Cren'/Cren could also be a result of heightened primary productivity as ammonium concentrations increase in the water column from the decomposition of particulate organic matter (Blaga et al., 2011; Kumar et al., 2019). This could promote increased ammonia oxidation by *Thaumarchaeota* group I.1a at the oxycline/thermocline and nitrocline, resulting in increased Cren production relative to Cren' (Sinnighe Damste et al., 2009; Auguet et al., 2011; Blaga et al., 2011; Kumar et al., 2019). The decrease in $\delta^{15}\text{N}$ at c. 1350 CE differs from the expected enrichment of $\delta^{15}\text{N}$ that is usually associated with increased primary productivity and a decrease of terrestrial material (Hodell & Schelske, 1998; Brenner et al., 1999; Meyers et al., 2003). However, many lakes in the Azores, including Lake Funda, are phosphorous limited and increased phosphorous inputs to the lake could lead to increased N₂-fixation by cyanobacteria and lower $\delta^{15}\text{N}$ values (Figure S1) (Brenner et al., 1999; Meyers, 2003; Cruz et al., 2015).

Coprophilous fungal spores (i.e., *Sporormiella*) are also found in the sediment core between c. 1300-1350 CE (S. Giralt and E. de Boer, *pers. commun.*), which marks the first introduction of mammals (aside from bats) to Flores island. Similarly, on the Azorean island of São Miguel evidence of earlier human settlement is identified by the appearance of cereal grains and *Sporormiella* in lake sediments c. 1287 CE (Rull et al., 2017). Although there are no

significant changes in vegetation composition or soil erosion, the introduction of livestock could enhance nutrient cycling in the landscape and result in more open patches of vegetation leading to increased nutrient leaching from soils into the lake from the catchment area (e.g. McNaughton et al., 1997). Even a slight increase in nutrient inputs to Lake Funda could result in increases in primary productivity. This is also observed in the Faroe Islands and Iceland, where the introduction of livestock resulted in increased nutrient loading and changes in lake trophic state before noticeable changes in soil erosion and vegetation composition occurred (Hannon et al., 2005; Lawson et al., 2007). These early perturbations to Lake Funda may have made the lake more susceptible to later disturbances in the catchment area.

4.2 Portuguese settlement of the Azores Archipelago and broader Macaronesia

The European Age of Discovery was marked by Portuguese exploration along the North Atlantic coast of Africa as they sought new routes to Asia, leading to the accidental “discovery” and settlement of Macaronesia (Diffie & Winius, 1977). The settlement of the Azores began in 1432 CE when the Portuguese took possession of Santa Maria and São Miguel, and eventually attempts were made to establish colonies on Flores in 1472 CE (Schäfer, 2003; Connor et al., 2012; Rull et al., 2017). Settlement in the Azores coincides with more depleted δD_{wax} values or a wetter climate combined with generally cooler conditions during the LIA, which could explain why settlement of the islands was gradual and intermittent (Smith, 2010). Despite the relatively recent settlement of the islands, the present-day landscape has been completely altered and it is uncertain how vulnerable this has made the current island ecosystems to climate change (e.g. Antunes & Rodrigues, 2011; Connor et al., 2012; Cruz et al., 2015; Rull et al., 2017).

Evidence of 5 β -stigmastanol c. 1500 CE corresponds to the widespread release of livestock on the islands to provide food for later settlers (Figure 3) (Schäfer, 2003; Smith, 2010). This was a common practice among the Portuguese as they settled Macaronesia, for instance, in Cabo Verde the timing of human settlement (c. 1450-1600 CE) coincides with an abrupt increase in non-obligate coprophilous fungi from livestock feces, followed by the gradual proliferation of newly introduced flora and increased erosion (c. 1600-1700 CE) (Castilla-Beltrán et al., 2019). On Flores island, the introduction of livestock was followed by a decrease in ACL₂₇₋₃₃ between c. 1400-1600 CE, as native vegetation (i.e., *Juniperus brevifolia* and *Picconia azorica*) was cleared to create a more open landscape for livestock (Connor et al., 2012; Rull et al., 2017; S. Giralt and E. de Boer, *pers. commun.*). Similarly, in the Canary Islands early European settlers cleared forests in the lowlands for agriculture and pastures for livestock (de Nascimento et al., 2009).

The effects of Portuguese activities on Lake Funda are apparent shortly after c. 1450 CE. Diatom oozes and a further increase in BSi occurs after c. 1450 CE, which would suggest that nutrient loading from the catchment area was driving increases in primary productivity (Figure 4). This also coincides with a further decrease in $\delta^{15}\text{N}$ values, suggesting another increase in N_2 fixation by cyanobacteria in the water column (Brenner et al., 1999; Meyers, 2003). A corresponding shift to more reducing conditions in the sediment, i.e. an increase in 5 α (H)-stanols/sterols, from oxygen depletion in the water column occurs after the initial increase in primary productivity c. 1500-1550 CE (Figure 5) (Gaskell & Eglinton 1975; Nishimura, 1977; Nishimura & Koyama, 1977; Kalff, 2002). In addition, increases in GDGT-0/Cren and a decrease in GDGT-1 and -2 suggest that there could be production of isoGDGTs by methanogenic archaea in the bottom water of the lake (Figure S3) (Pancost et al., 2000; Blaga et

al., 2009; Naeher et al., 2014). This is supported by the gradual decrease in bulk $\delta^{13}\text{C}$ values after c. 1630 CE as more depleted carbon is added to the epilimnion from increased methanogenesis (e.g., Hollander & Smith, 2000).

Increased primary production and evidence of hypoxic conditions in the lake bottom water suggest that Lake Funda underwent a relatively rapid period of eutrophication that occurred c. 100 years after major land use changes began in c. 1400 CE. In contrast, Lake Azul in São Miguel responded more gradually to human impacts in the catchment area, with the lake remaining relatively pristine and oligotrophic until the introduction of exotic fish in the 1800s (Raposeiro et al., 2017). A rapid response in lacustrine ecosystems to human activities is observed during the settlement of Iceland (Figure 7) and the Faroe Islands, but less so in Greenland (Hannon et al., 2005; Lawson et al., 2005, 2007; Massa et al., 2012; Richter et al., *submitted*). The varied ecosystem response of these lakes to human impacts highlights the different sensitivities of these lake ecosystems or differences in the severity of human land use changes.

Despite a shift to drier but also more variable conditions between c. 1550-1900 CE, the vegetation composition (ACL_{27-33}) stabilized between c. 1600-1950 CE in the catchment area of Funda (Figure 3). However, $5\alpha(H)$ -stanols/sterols and GDGT-0/Cren values continue to increase during this time period, which would suggest that heightened nutrient inputs continued to cause eutrophication and hypoxic conditions in the bottom water of the lake.

4.3 Ecological changes in the Azores in the 20th and 21st centuries

The transition from the 19th to early 20th century was marked by major environmental and climatic changes in broader Macaronesia. In Flores, the gradual enrichment of $\delta\text{D}_{\text{wax}}$ between c.

1900-1950 CE marked a shift to drier conditions, and a more frequent positive NAO phase (Figure 6) (Cropper & Hanna 2014; Baker et al., 2015; Hernández et al., 2016, 2017). A sharp increase in ACL_{27-33} values c. 1950 CE in Lake Funda (Figure 3) also marks the start of major reforestation efforts, as the local Forestry Service began to actively plant *Cryptomeria japonica* in the Azores (Rull et al., 2017; S. Giralt and E. de Boer, *pers. commun.*). Similarly, in Cabo Verde exotic trees, such as *Pinus* and *Acacia*, were introduced in the early 20th century to help reduce land degradation (Castilla-Beltrán et al., 2019).

The combination of a drier climate and more trees in the catchment area should act to stabilize the soil, which in theory should lead to reduced erosion and external nutrient loading and therefore lower primary productivity and improve oxygenation in the water column. In Lake Funda, but the lack of changes in $5\alpha(H)$ -stanols/sterols and GDGT-0/Cren suggest that there is little change in the response of microbial communities (Figure 5). Limnological surveys over the last two decades confirm that eutrophication continues to be a problem for Lake Funda and hypoxic, and sometimes fully anoxic, conditions develop in the lake bottom water during the summer and fall months (Figure S1) (P.M. Raposeiro and S. Giralt *pers. commun.*). Continued internal loading of phosphorous and other changes in the nitrogen and carbon cycle could promote microbially mediated hydrogenation of sterols to $5\alpha(H)$ -stanols and methanogenesis in the bottom water (Gaskell & Eglinton 1975; Nishimura, 1977; Nishimura & Koyama, 1977; Kalff, 2002).

Eutrophication is a problem for numerous lakes in the Azores Archipelago, and as exemplified by this case study in Lake Funda and several other paleoecological studies, these changes in trophic state can be attributed to past human land use changes and/or the introduction of fish (Skov et al., 2010; Antunes & Rodrigues, 2011; Cruz et al., 2015; Raposeiro et al., 2017).

In contrast to the lacustrine ecosystems in the high North Atlantic (e.g. Iceland, Faroe Islands, and Greenland), Lake Funda underwent what appears to be a permanent shift in trophic state in response to major human activities in the landscape (Figure 7) (Lawson et al., 2005, 2007; Massa et al., 2012; Richter et al., *submitted*). This suggests that Lake Funda was more sensitive to human-induced perturbations in the catchment area relative to lakes in the high North Atlantic either due to more intensify agricultural practices in the Azores or internal phosphorous loading in Lake Funda that led to a non-linear transition in the lake (Langdon et al., 2016). To prevent further eutrophication in Lake Funda might require more aggressive remediation strategies to reduce internal nutrient loading.

5. Conclusions

Fecal markers in lake sediment records from Funda are evidence for the introduction of mammals, most likely livestock, to the Azores Archipelago prior to the official arrival of Portuguese settlers in the 15th century. Although there were no major changes in vegetation or soil erosion at that time, there are increases in primary productivity within Lake Funda that could indicate a response to heightened nutrient loading from the catchment area. The Portuguese arrival in the Azores during the 15th century is characterized by harsh and wet climate conditions. Despite the slow establishment of settlements, rapid land use changes, including the introduction of more livestock and active clearing of vegetation occurred shortly after the Portuguese arrived in the Azores (1432 CE). Increased nutrient inputs to the lake resulted in a permanent shift in the lake trophic state as marked by a further increase in primary productivity and the onset of hypoxic conditions in the lake bottom water. Despite recent reforestation efforts to reduce soil erosion and drier climate conditions in the 20th century, Lake Funda has remained eutrophic

either from sustained nutrient inputs or internal loading of phosphorous. Many of the environmental issues in the Azores are likely to be exacerbated by a warming climate and changes in precipitation, and environmental management strategies need to account for the potential impacts this might have on already vulnerable lake ecosystems.

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Table 1. Radiocarbon dates and calibrated ages for the sediment cores from Lake Funda.

Lab ID	Depth (cm)	Radiocarbon Age (yrs BP)	Median probability (cal yrs CE)	2 σ (cal yrs CE)
Surface	0	N/A	2017	2015-2019
ULA7746	60.8	(previous core)	1956	1954-1958
ULA7657	221.2	510 $\pm$ 15	1422	1409-1435
ULA7745	295.2	550 $\pm$ 20	1379	1323-1418
ULA7744	435.0	785 $\pm$ 20	1245	1219-1271
ULA7739	712.0	1100 $\pm$ 20	944	895-990

Table 2. Segmented breakpoint analysis for the proxy records discussed in this study with the pooled uncertainty shown.

	Proxy	Segmented Breakpoint Analysis	
		Year (CE)	95% CI ($\pm$)
Climate	δD_{wax} C ₃₀ FAMES (‰)	1420	23
		1568	17
Catchment area	ACL ₂₇₋₃₃	1392	35
		1586	12
		1947	6
Organic matter sources & lake biogeochemical cycles	C/N	1297	29
		1604	11
	TOC (%)	1321	33
		1447	15
		1953	8
	BSi (%)	1266	27
		1395	33
		1525	24
	$\delta^{15}N$ (‰)	1341	37
		1500	12
		1676	8
	Cren'/Cren	1374	46
		1399	28
		1882	10
	GDGT-0/Cren	1530	14
	$\delta^{13}C$ (‰)	1624	20
Redox conditions	5 $\alpha(H)$ -stanols/sterols	1521	13

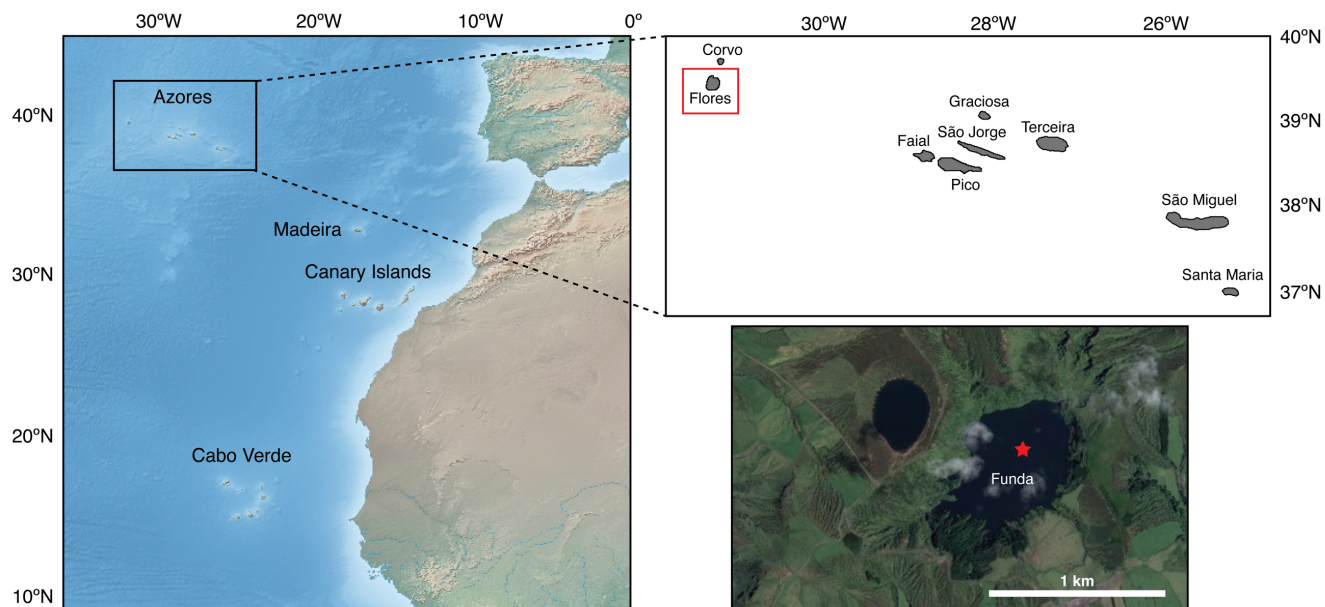


Figure 1. The islands that make up Macaronesia are shown with a close-up of the Azores Archipelago. Lake Funda is located on Flores island, and the location of the cores analyzed in this study are marked by a red star. Raster data was obtained are from Natural Earth and Google Earth and the maps were rendered in Matlab.

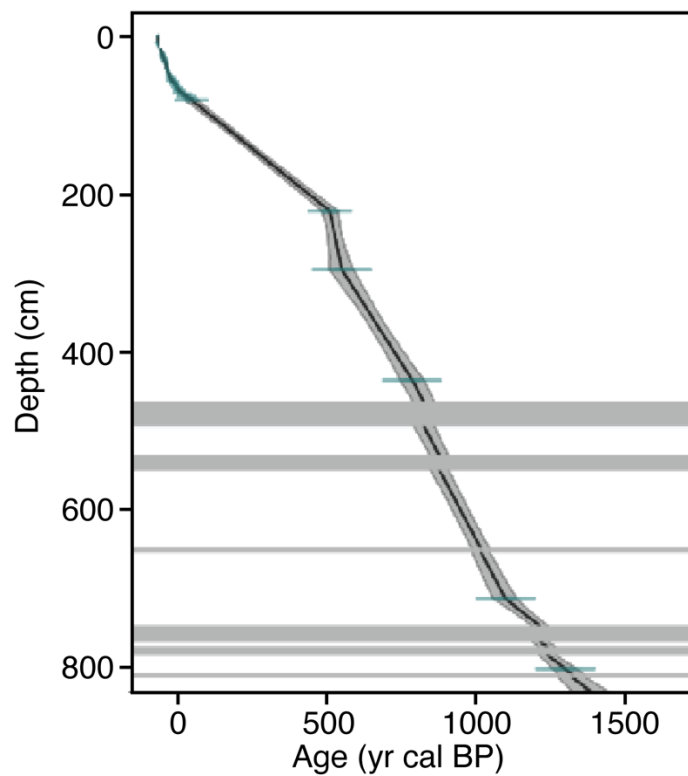


Figure 2. Age-depth model for Lake Funda developed in CLAM using ^{210}Pb and ^{14}C dates with the 2σ uncertainty indicated by gray shading in the age model. Slumps were removed from the analysis as their sedimentological features suggest that they were deposited instantaneously during mass-wasting events and are indicated by the horizontal gray bars.

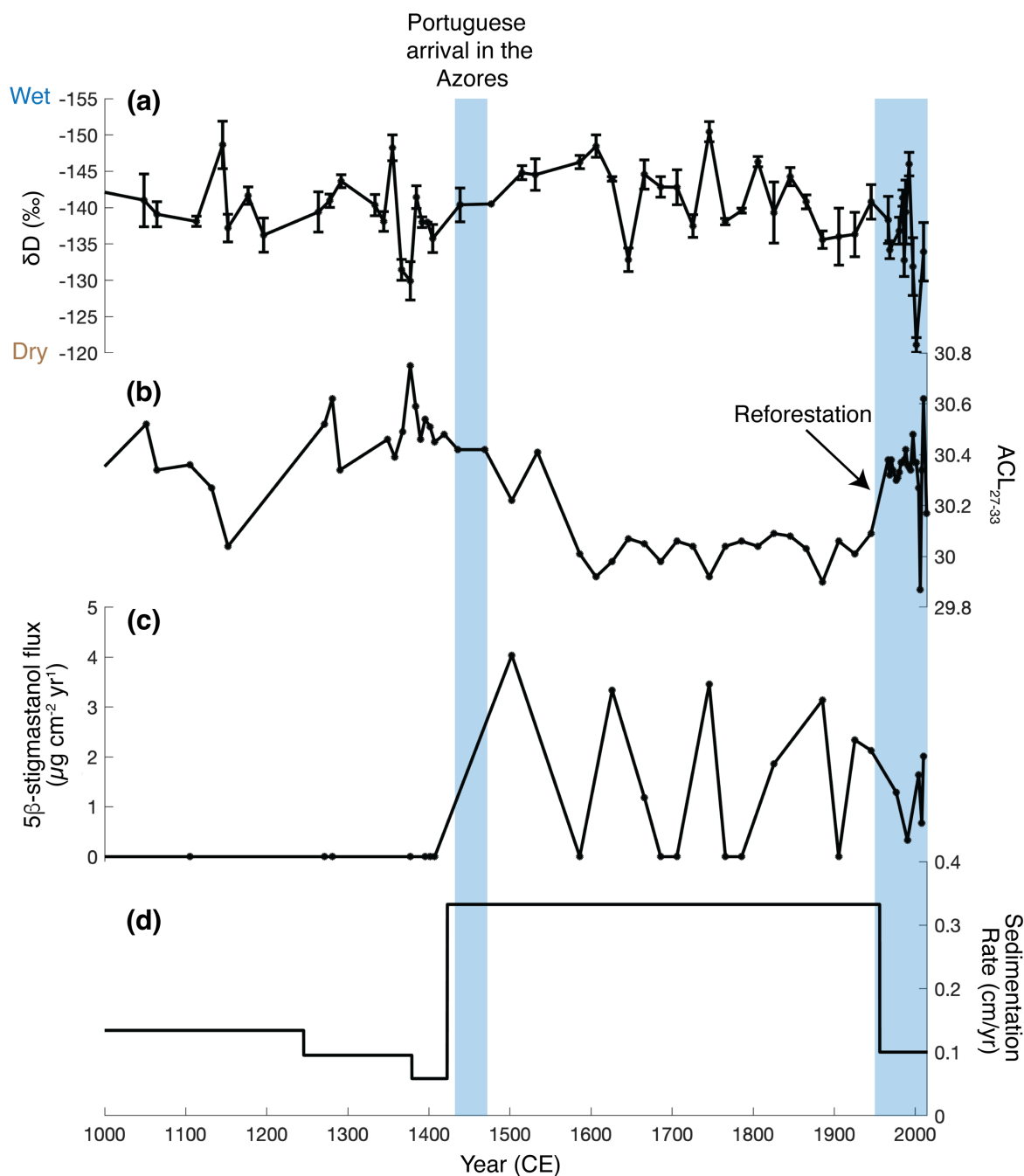


Figure 3. (a) Changes in hydroclimate are noted by shifts in FAMES C_{30} δD_{wax} , where more depleted isotopes correspond to wetter conditions and enriched isotopes correspond to drier conditions. Major changes in vegetation composition are noted by shifts in (b) ACL_{27-33} and are compared with (c) 5β -stigmastanol to determine when livestock were present in the catchment area. (d) Estimated changes in sedimentation rate are also shown for comparison. The first blue bar corresponds to when the Portuguese first arrived in the Azores and when the official settlement of Flores island began (1432-1472 CE). The second blue bar corresponds to when reforestation efforts began in the Azores c. 1950 CE.

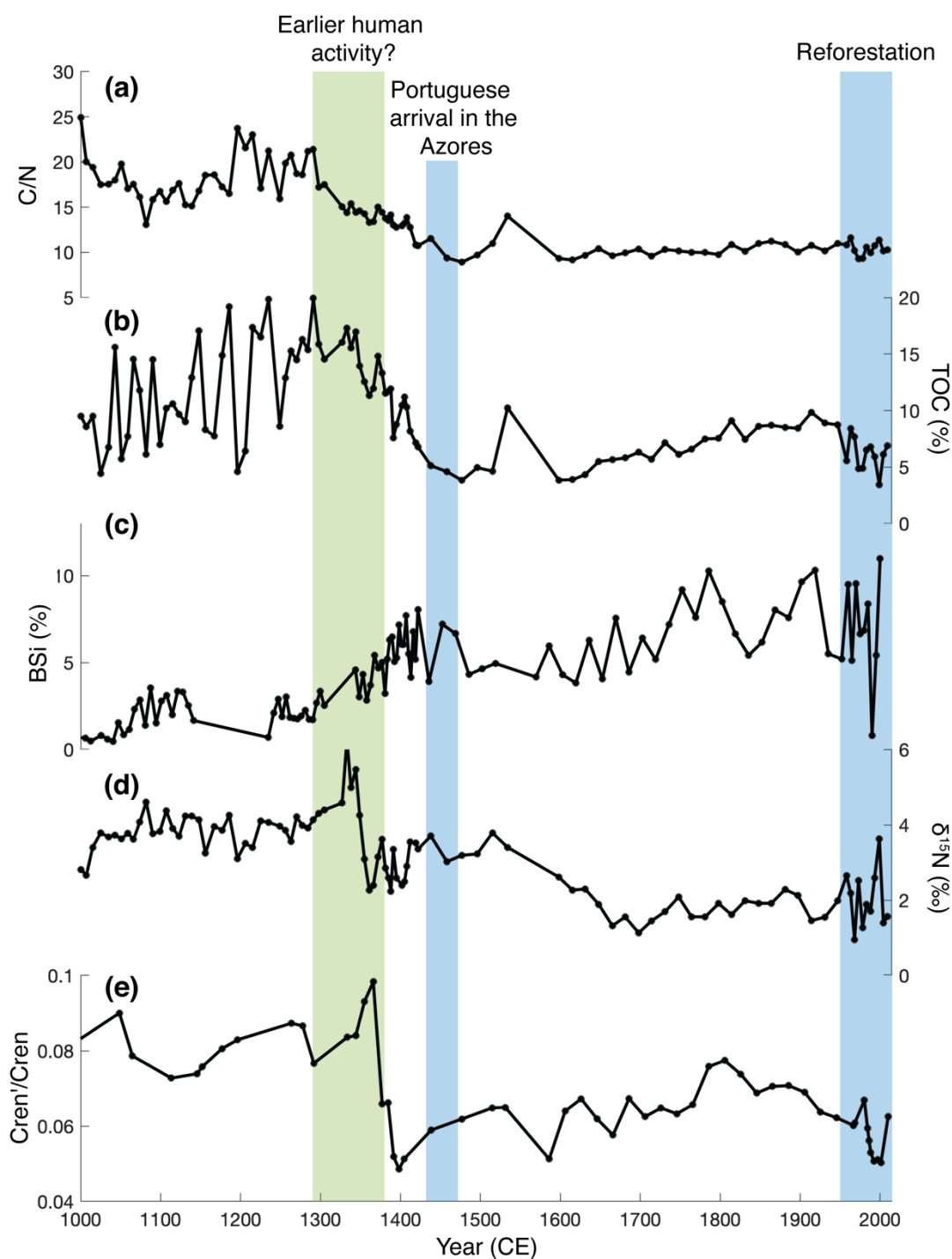


Figure 4. Changes in organic matter inputs to the lake are noted by shifts in (a) C/N and are compared with (b) changes in TOC (%) and (c) diatom productivity, BSi (%). Further changes in the lake are noted by (d) $\delta^{15}\text{N}$ and (e) Cren'/Cren correspond to changes in the *Thaumarchaeota* community. The green bar corresponds to ecological transitions in the lake that coincide with early evidence of coprophilous fungal spores. The first blue bar corresponds to the Portuguese arrival in the Azores and settlement in Flores (1432-1472 CE) and the second blue bar corresponds to the start of reforestation efforts c. 1950 CE.

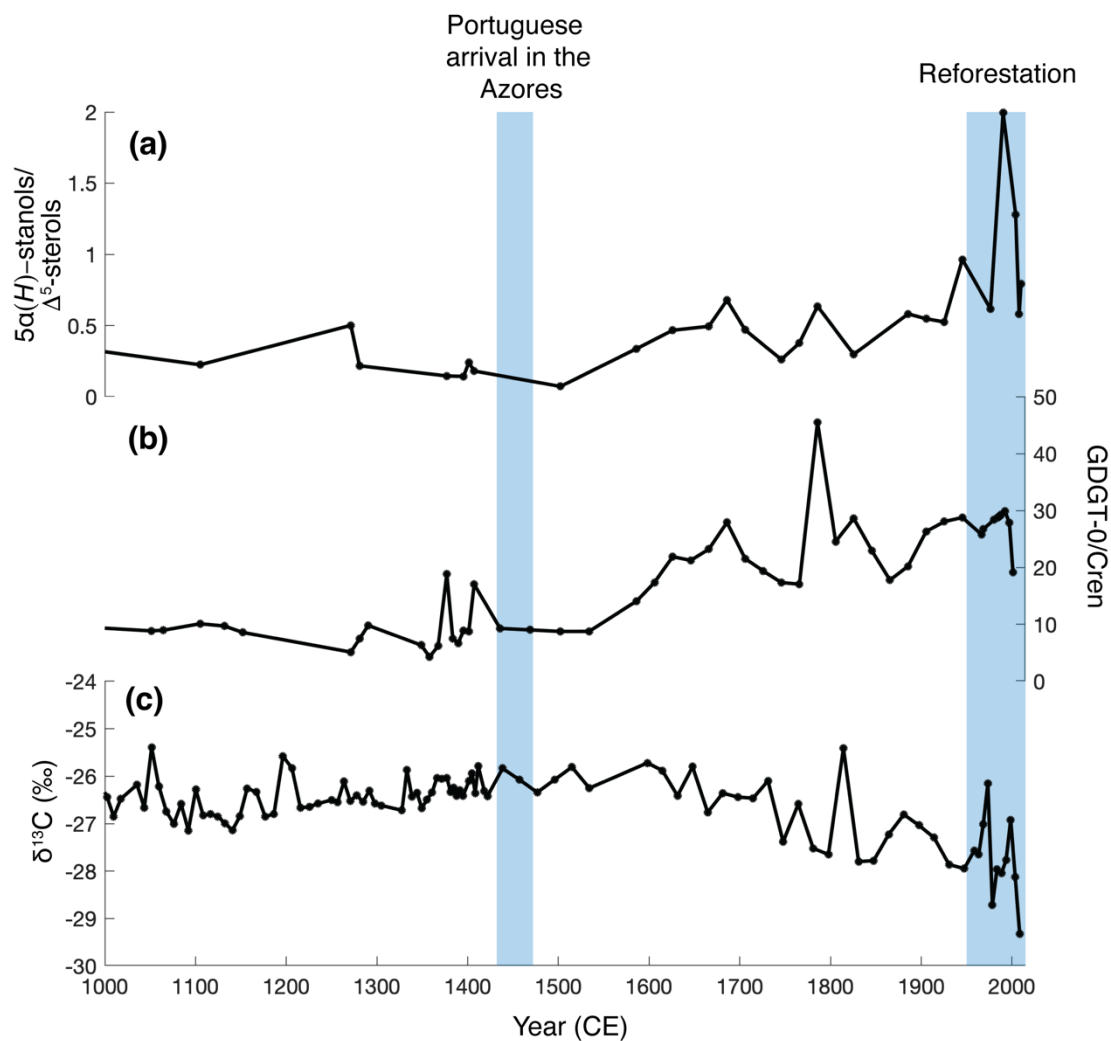


Figure 5. Redox conditions in the lake bottom water are noted by changes in **(a)** $5\alpha(H)\text{-stanols}/\Delta^5\text{-sterols}$. Similarly, **(b)** GDGT-0/Cren reflects changes in lake bottom water processes and changes in **(c)** $\delta^{13}\text{C}$ are shown for comparison. Portuguese arrival in the Azores and settlement in Flores are noted by the first blue bar (1432-1472 CE), and the second blue bar corresponds to reforestation efforts that began c. 1950 CE.

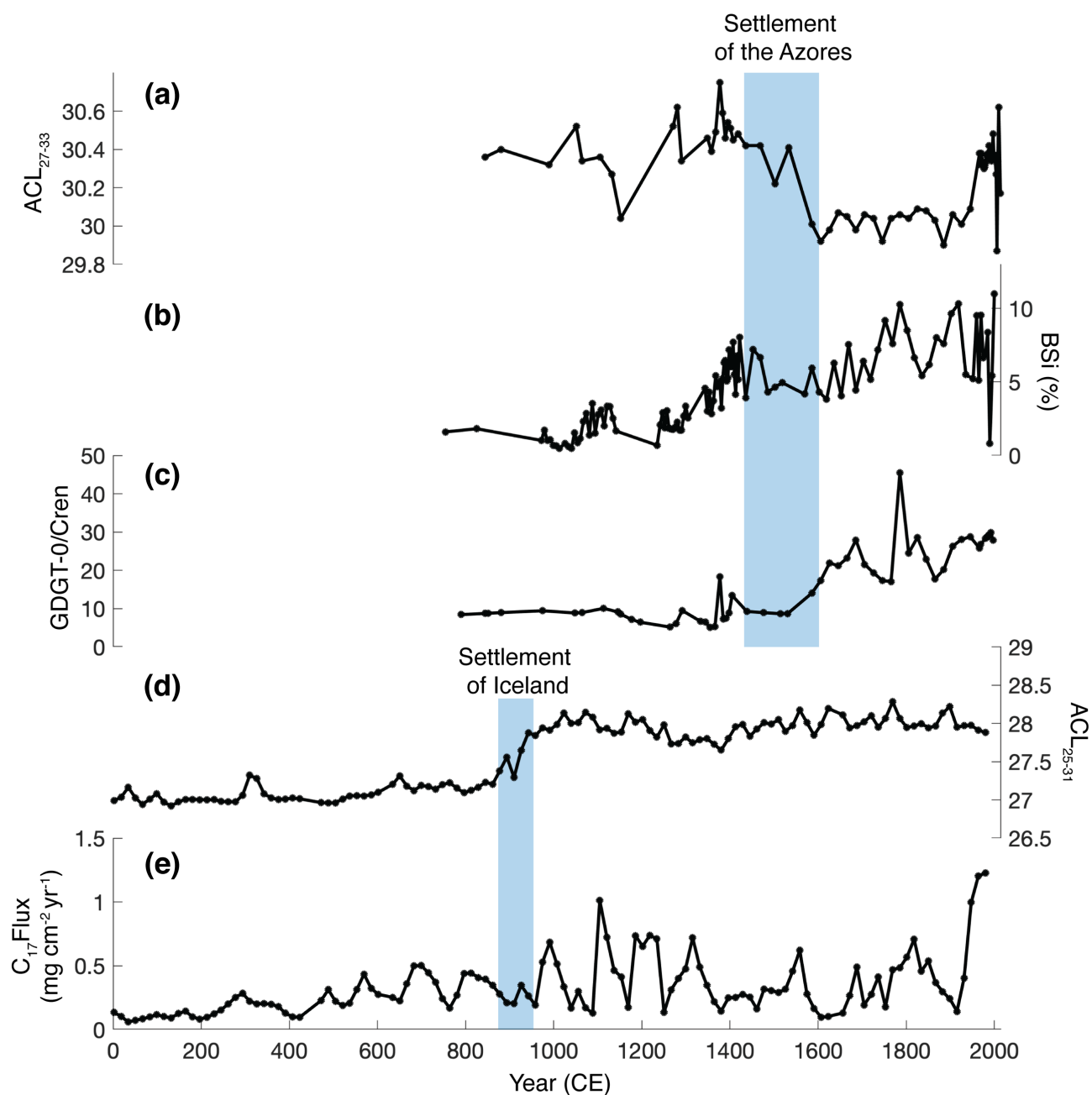


Figure 6. Environmental changes observed in Lake Funda over the last millennium are shown, including (a) changes in vegetation composition (ACL_{27-33}), (b) diatom productivity or BSi (%), and (c) GDGT-0/Cren reflecting changes in methanogenesis in the lake bottom water. For comparison, major changes in (d) vegetation composition (ACL_{25-31}) and (e) primary productivity, C_{17} flux, from Vestra Gíslholtvatn in Iceland are shown. The blue bars mark when European settlement began in the Azores (1432 CE) and Iceland (874 ± 2 CE).

Supplementary Information

Table S1. Summary of pooled analytical uncertainties for δD_{wax} measurements described in text, which include the pooled uncertainty for the sample measurements (56 samples that were measured in triplicate when possible).

FAME	Standard		Sample	
	1 σ	n	1 σ	n
C ₁₆	4.27	146	-	-
C ₁₈	3.28	146	-	-
C ₂₂	3.57	146	-	-
C ₂₄	3.98	146	-	-
C ₂₈	2.77	146	-	-
C ₃₀	2.35	103	2.09	56

Table S2. Sterol and stanol names and mass ions used for quantification in select ion monitoring mode on the GC-MS. Mass fragments are reported as trimethylsilyl (TMS) ethers.

Trivial Name	Systematic Name	Carbon Num.	Mass Fragment (<i>m/z</i>)	Quant. (SIM mode)
5 α -cholestan-3-one	5 α -cholestan-3-one	C ₂₇	386	231
Coprostanol	5 β -Cholestan-3 β -ol	C ₂₇	460	215
Epi-coprostanol	5 β -Cholestan-3 α -ol	C ₂₇	460	215
Cholesterol	Cholest-5-en-3 β -ol	C ₂₇	458	129
Cholestanol	5 α -Cholestan-3 β -ol	C ₂₇	460	215
Campesterol	24 α -Methyl-5-cholesten-3 β -ol	C ₂₈	472	129
Campestanol	24-Methyl-5 α -cholestan-3 β -ol	C ₂₈	474	215
Stigmasterol	24-Ethylcholesta-5,22E-dien-3 β -ol	C ₂₈	484	129
β -Sitosterol	24-Ethylcholest-5-en-3 β -ol	C ₂₉	486	129
Stigmastanol	24-Ethyl-5 α -cholest-22E-en-3 β -ol	C ₂₉	488	215
5 β -Stigmastanol	24-Ethyl-5 β -cholestan-22E-en-3 β -ol	C ₂₉	486	215

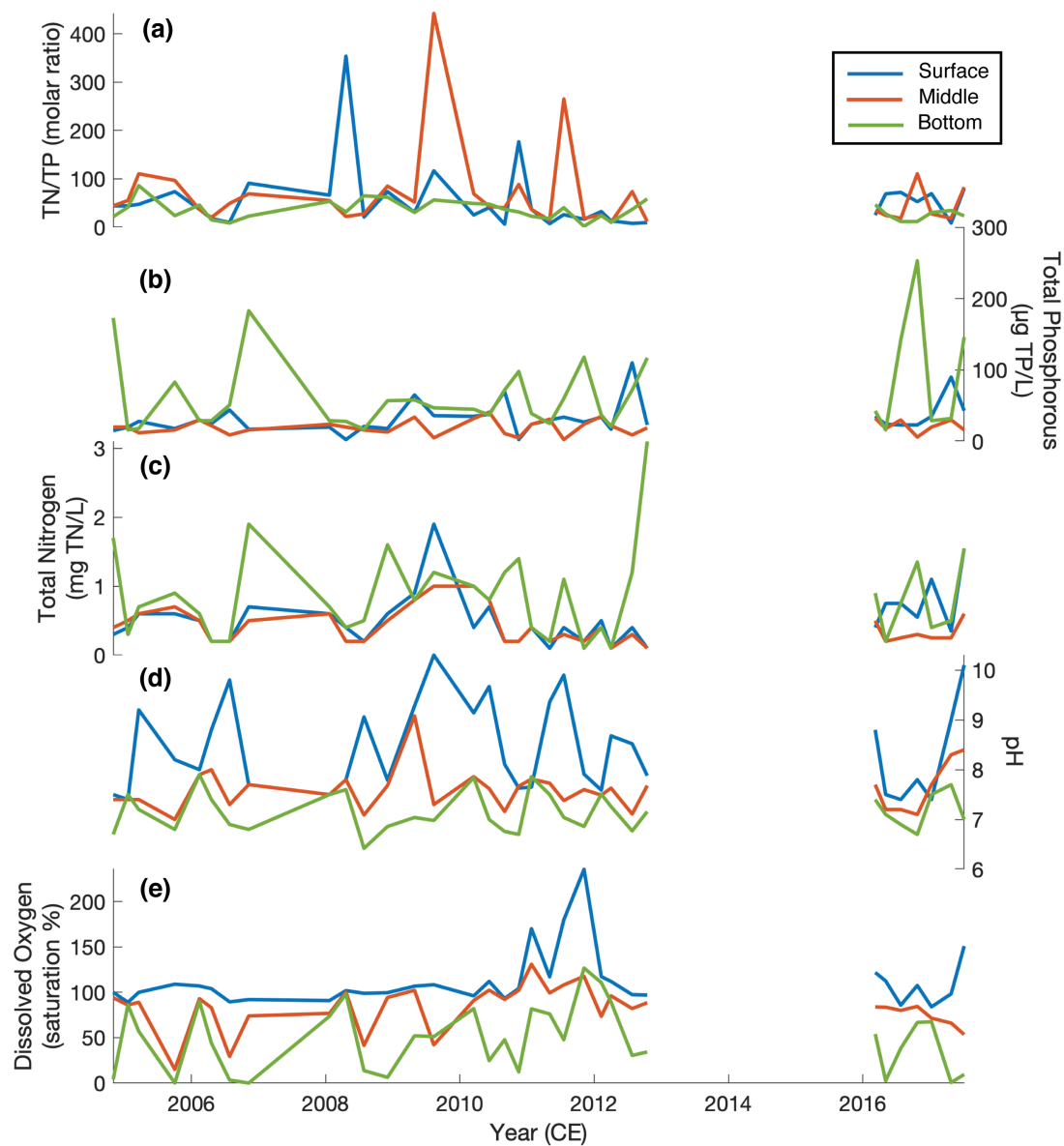


Figure S1. Water column properties for Lake Funda including the molar ratio for **(a)** total nitrogen/total phosphorous (TN/TP), **(b)** total phosphorous concentrations, **(c)** total nitrogen concentrations, **(d)** pH, and **(e)** dissolved oxygen content for the surface, middle, and bottom water from 2004-2017 (data was collected by the long-term monitoring program run through the Universidade dos Açores).

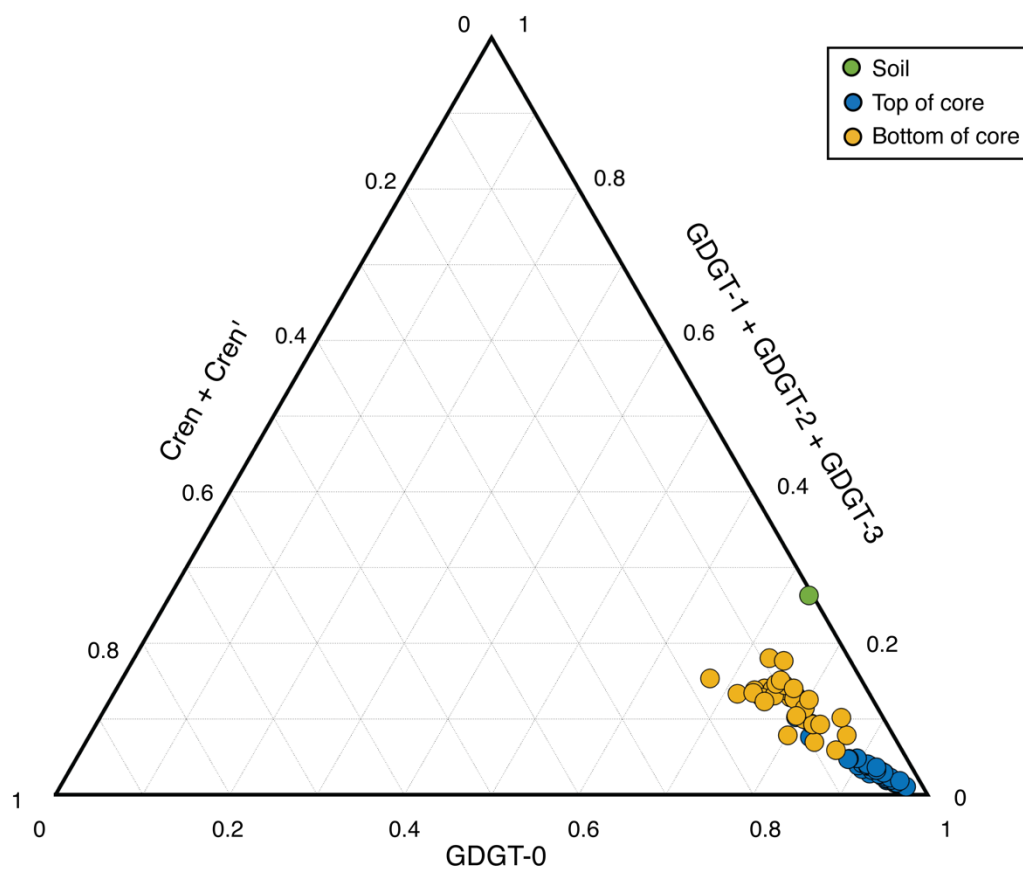


Figure S2. isoGDGT composition from soil samples taken in the catchment area and in different parts of the core. Fraction of isoGDGTs were calculated relative to GDGT-0, GDGT-1, GDGT-2, GDGT-3, Cren, and Cren'.

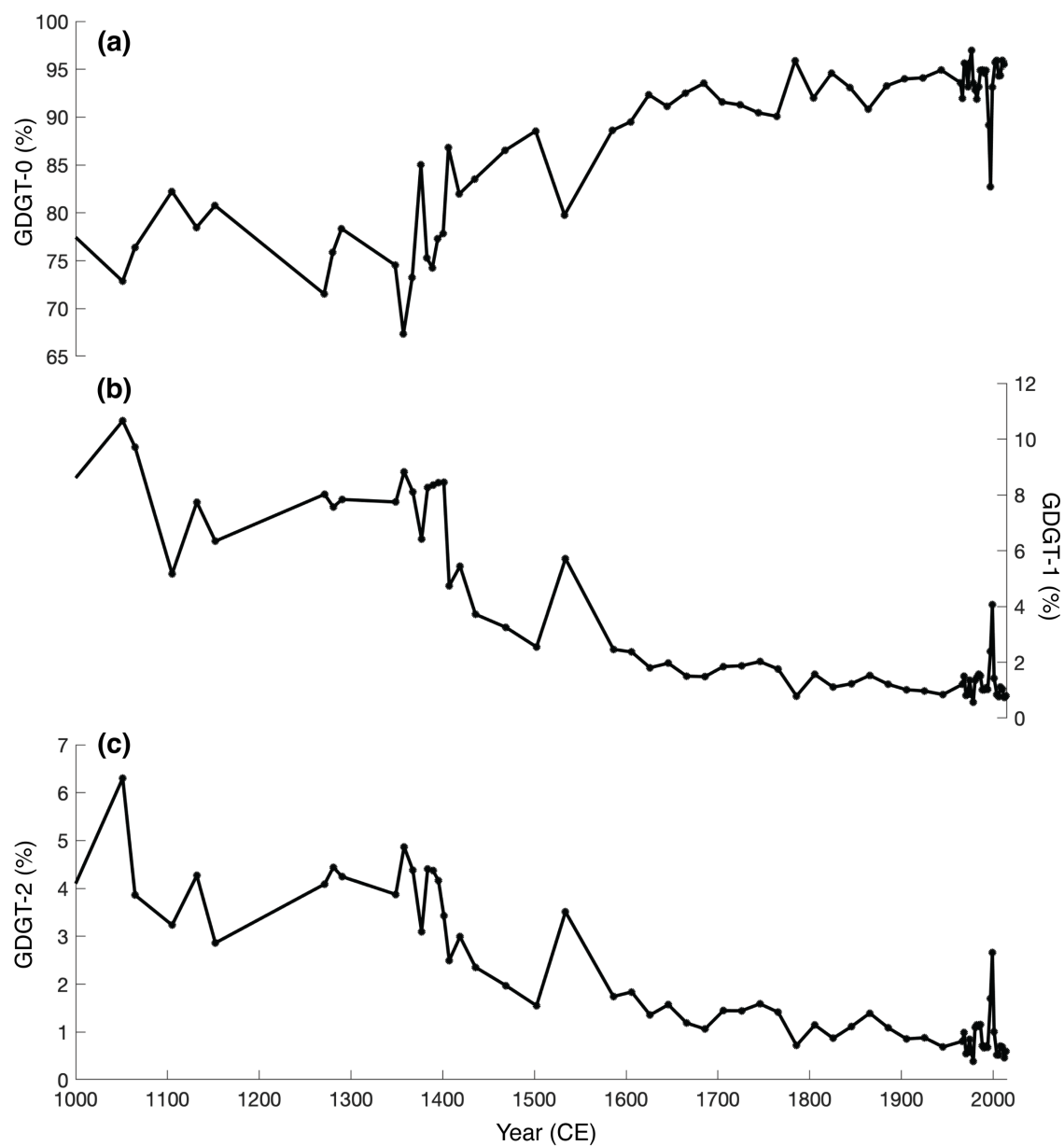


Figure S3. Changes in the relative composition of isoGDGTs for (a) GDGT-0, (b) GDGT-1, (c) GDGT-2. Percent of isoGDGTs are calculated relative to GDGT-0, GDGT-1, GDGT-2, GDGT-3, Cren, and Cren'.

– APPENDIX 1 –

**Hydroclimate changes in the sub-tropical North Atlantic over the last millennium with
implications for the North Atlantic Oscillation**

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Abstract

Precipitation patterns during the winter months in the North Atlantic region are strongly influenced by variability in the North Atlantic Oscillation (NAO), the difference in sea level pressure between the Icelandic Low in the high North Atlantic and the Azorean High. Changes in the strength and position of the Azorean High accounts for most of the variability in inter- and intra-annual hydroclimate changes in the Azores. However, multi-decadal to centennial changes in the NAO and sub-tropical hydroclimate are poorly constrained. We developed a hydroclimate record from the Azores over the last millennium using hydrogen isotopes (δD_{wax}) from long-chain leaf waxes (C_{30} fatty acid methyl esters) preserved in sediments from Lake Funda on Flores island. Our record indicates that precipitation patterns varied between drier and wetter conditions during the Medieval Climate Anomaly (MCA, c. 950-1250 CE). This would suggest that there was no prolonged positive NAO phase during the MCA, which is consistent with a reconstruction of cold season temperatures from Iceland. During the Little Ice Age (c. 1450-1850 CE), the δD_{wax} record from the Azores shows opposing but similar changes to a speleothem record from Northern Scotland, which resembles the spatial pattern of the NAO. In particular, more depleted δD_{wax} values (wetter conditions) between c. 1450-1630 CE and corresponding drier conditions in Scotland could be related to a negative NAO excursion in response to the Spörer solar minimum and major volcanic eruptions between c. 1450-1460 CE. Similarly, more depleted δD_{wax} values c. 1650-1760 and c. 1800-1850 CE correspond to periods associated with major volcanic eruptions and solar minima. Comparisons between the δD_{wax} record from the Azores and reconstructions from the high North Atlantic suggest that both unforced climate variability and external forcings are important drivers of precipitation changes in the Azores on multi-decadal to centennial timescales.

1. Introduction

Variability in atmospheric circulation, including changes in temperature, precipitation, and storm tracks, during the winter months are often associated with the North Atlantic Oscillation (NAO) in the North Atlantic region (Hurrell, 1995). The NAO is an intrinsic mode of climate variability; however, there is evidence that multi-decadal to centennial changes in Atlantic sea surface temperatures, i.e. the Atlantic Multi-decadal Variability (Omrani et al., 2014, 2016; Gastineau & Frankignoul, 2015), or changes in radiative forcings (i.e., volcanic eruptions and solar variability) (Yoshimori et al., 2005; Zanchettin et al., 2012; Ortega et al., 2015; Smith et al., 2016; Sjolte et al., 2018) can influence the NAO on seasonal to multi-decadal timescales. The spatial and temporal variability of the NAO over the last millennium is poorly constrained, and existing reconstructions differ as to the direction and phase of the NAO during key climate events, namely the Medieval Climate Anomaly (MCA, c. 950-1250 CE) and the Little Ice Age (LIA, c. 1450-1850 CE) (Esper et al., 2007; Wassenburg et al., 2013; Baker et al., 2015; Ortega et al., 2015; Ait Brahim et al., 2017; Cook et al., 2019). A hydroclimate reconstruction from the Azores can provide insights into the southern extent and variability of the NAO. In this study we present a hydroclimate reconstruction using hydrogen isotopes from long-chain leaf waxes preserved in a sediment record from Lake Funda on Flores island, the Azores. Our record spans the last millennium and captures the multi-decadal to centennial variability of precipitation changes in the North Atlantic region.

2. Methods

2.1 Study site

Lake Funda is a maar lake (371 m a.s.l., 39°24'N 31°13'W; Figure 1) located on the island of Flores in the Azores Archipelago. Precipitation in the Azores is strongly influenced by the high-pressure Azores anticyclone, which dominates the climate during the late spring to summer (Hurrell, 1995; Santos et al., 2004; Cropper & Hanna, 2014; Hernández et al., 2016). From March to September the Azores anticyclone is displaced latitudinally, such that the North Atlantic storm tracks cross the Azores more frequently (Santos et al., 2004; Cropper & Hanna, 2014; Hernández et al., 2016). This results in large intra- and inter-annual variability in rainfall that can largely be ascribed to the NAO (Hurrell, 1995; Cropper & Hanna, 2014; Hernández et al., 2016). In general, winter (DJF) and summer (JJA) precipitation are negatively correlated with changes in the NAO (Cropper & Hanna, 2014; Hernández et al., 2016).

2.2 Age model

The age model for the core is based on both ^{210}Pb concentration profiles and ^{14}C dates (see Chapter 5 for details). The concentration profile for ^{210}Pb was measured in the top 80 cm of the core at the Environmental Radioactivity Laboratory in the Unviersitate Autònoma de Barcelona using a Passivated Implanted Plana Silicon (PIPS) detector (CANBERRA, Mod. PD-450, 18 A M). Concentrations for ^{226}Ra were measured for select samples using gamma-spectroscopy with a high-purity germanium detector (CANBERRA, mod. GCW3523). AMS ^{14}C dates were measured at Beta Analytic Laboratory (Florida, USA). The radiocarbon ages were calibrated using the CALIB 7.1 software and the Intcal13 curve (Reimer et al., 2013). Erosive

events identified in the sediment record were removed, and the age-depth model was determined using CLAAM (Blaauw, 2010).

2.3 Sample preparation and analysis

The sediment cores from Lake Funda were taken in June 2017 using a UWITEC piston corer on a UWITEC platform. All of the cores were sealed and shipped to the Institute of Earth Sciences Jaume Almera (CSIC), where they were stored at +4 °C until they were sampled. Samples were taken for lipid analyses and shipped to Brown University for processing.

All sediment samples were freeze-dried and then extracted using a DionexTM accelerated solvent extraction (ASE 350) system at 120°C and 1200 psi. The resulting extract was separated on an aminopropylsilyl (NH₂) column into neutral (DCM:Isopropanol, 2:1, v/v) and acid (ethyl ether:acetic acid, 24:1, v/v) fractions. The acid fraction was methylated with acidified anhydrous methanol of a known isotopic composition at 60°C for 2 hrs. The fatty acid methyl ethers (FAMES) were then purified using silica gel (40-63 µm, 60 Å) flash chromatography.

FAMES were quantified on an Agilent 6890N gas chromatography (GC) system and a flame ionization detector (FID). The samples were injected onto a Rtx-200 column (105 m x 205 µm x 0.25 µm) using pulsed splitless mode (20.3 psi, 310°C). The oven program was set at 50°C and then ramped up to 315°C at 10°C/min where it was held isothermally for 30 min.

All compound specific isotope ratios (δD_{wax}) for C₃₀ FAMES were then measured on an Agilent 6890 GC coupled to a Thermo Delta V Plus Isotope Ratio Mass Spectrometer (IRMS). On the GC, samples were injected onto a ZB-1MS (30 m x 320 µm x 0.25 µm) column with pulsed splitless injection mode (30 psi, 320°C). The oven program was started at 40°C for 1 min, and then ramped up to 230°C at 30°C/min where it was held isothermally for 1 min. The oven

temperature was increased again to 310°C at 10°C/min where it was held for 10 min. The reactor temperature for the IRMS was set to 1450°C for pyrolysis. The reference gas $^2\text{H}/^1\text{H}$ was determined with certified C_{29} and C_{31} *n*-alkane standards, and the H^{3+} factor was measured every day (2.41 ± 0.07). To monitor instrument performance and drift, an internal standard mixture with C_{16} , C_{18} , C_{22} , C_{24} , C_{28} , and C_{30} *n*-acids was analyzed after every 4 injections (see Chapter 5 for analytical uncertainty). Instrument accuracy was monitored with a certified C_{30} methyl ester standard of known composition (lab measured: -186.4 ± 3.9 ‰ for $n = 71$, actual: -189.4 ± 2 ‰ for $n = 5$ (Schimmelmann, 2018)). All samples were measured in triplicate and values were accepted for a voltage response between 2.5 and 7 volts. Offsets between measured and reported standard values were corrected on a daily basis.

2.4 Controls on leaf wax hydrogen isotopes in the Azores

Long-chain leaf waxes, such as C_{30} fatty acids, are primarily derived from higher terrestrial plants, and the hydrogen isotope composition of these leaf waxes ($\delta\text{D}_{\text{wax}}$) is strongly influenced by changes in δD of precipitation ($\delta\text{D}_{\text{precip}}$) (Sachse et al., 2012). In the Azores, variability in the NAO has a strong influence on seasonal precipitation amount, but also $\delta\text{D}_{\text{precip}}$. During the winter and spring season $\delta\text{D}_{\text{precip}}$ is negatively correlated with the amount of precipitation in the Azores and is positively correlated with the station-based NAO (Table 1) (Baldini et al., 2008; Rubio de Inglés, 2016). Although the source of the vapor mass changes (i.e., North America, North Atlantic, Gulf of Mexico, Europe, and the Sahara), the vapor mass that reaches the Azores Archipelago re-charges over the North Atlantic Ocean and reduces the isotopic variability associated with source changes (Araguás-Araguás et al., 2000; Rubio de Inglés, 2016). In addition, a temperate oceanic climate leads to small variations in air

temperature in Flores, ranging from 15°C during the winter months (DJF) to 21°C during the summer (JJA), and has a minimal influence on δD_{precip} (Dansgaard, 1964; Baldini et al., 2008; Rubio de Inglés, 2016). Therefore, δD_{wax} of C₃₀ FAMES is primarily influenced by changes in precipitation amount and also variations in the NAO, particularly during the winter and spring season.

3. Results and discussion

In the Azores, the MCA is characterized by small fluctuations between more enriched and depleted δD_{wax} of C₃₀ FAMES values from c. 800-1400 CE (Figure 2). Overall drier conditions in the Azores correspond to lower lake levels on Pico island and reduced precipitation in Morocco prior to c. 1350 CE, whereas Scotland is characterized by wetter conditions (Esper et al., 2007; Björck et al., 2006; Wassenburg et al., 2013; Baker et al., 2015; Ait Brahim et al., 2017). This broadly resembles the spatial fingerprint of a positive or neutral NAO phase. However, variability in the δD_{wax} record suggests that there was no multi-centennial positive NAO phase during the MCA; rather this time period was most likely characterized by fluctuations in unforced climate variability (Ortega et al., 2015). Similarly, cold season temperatures in Iceland are on average warmer between c. 880-1100 CE than previous centuries; however, multiple cold temperature excursions during this time period could be related to negative NAO phases (Figure 2) (Richter et al., *in prep.*). The Samalas eruption (1258 CE) and Wolf solar minimum (c. 1280-1350 CE) correspond to a negative δD_{wax} excursion (Figure 2), or increased precipitation in the Azores, between c. 1270-1370 CE and a similar increase in precipitation in Morocco (Ait Brahim et al., 2017). Cooling in cold season temperatures in

Iceland between c. 1250-1400 CE could also be attributed to the cumulative effects of multiple negative radiative forcings during this time period (Richter et al., *in prep.*).

The δD_{wax} reconstruction from Funda shows considerable multi-decadal to centennial hydroclimate variability over the past millennium particularly after the onset of the LIA (c. 1400 CE). Multi-decadal to centennial hydroclimate variability in the Azores strongly resembles opposing precipitation changes in speleothem records from Northern Scotland (Figure 2), i.e. wetter conditions in the Azores correspond to drier conditions in Northern Scotland (Baker et al., 2015). This would suggest that a common forcing, namely the NAO, could account for the precipitation changes observed in both of these records after c. 1400 CE.

δD_{wax} values decrease between c. 1450-1630 CE, which also coincides with wetter conditions in Morocco and the western Mediterranean and a drier climate in Northern Europe (Esper et al., 2007; Baker et al., 2015; Anchukaitis et al., 2019). The timing of these hydroclimate changes occurs shortly after a major volcanic eruption in 1458 CE and during the Spörer minimum (Steinhilber et al., 2009; Sigl et al., 2015). However, a cooling response is not observed in cold season temperatures in Iceland during this time period, which either highlights the importance of background conditions, additional feedbacks from sea ice, and/or a change in the strength and position of the NAO during this time period (Hurrell & Deser, 2010; Zanchettin et al., 2012; Slawinska & Robock, 2018; Ortega et al., 2015; Richter et al., *in prep.*). A coherent response to negative radiative forcings is observed in Icelandic temperatures and Azorean precipitation between c. 1800-1900 CE, which could be related to a negative NAO phase. Changes in the relationship between Icelandic temperatures and precipitation in the Azores could be explained by the non-stationarity of the NAO, i.e. spatial changes in the strength and position of Icelandic Low and Azorean High (Hurrell & Deser, 2010; Ortega et al., 2015).

In the δD_{wax} record from the Azores, internal climate variability is a more prominent feature during the MCA and leads to changes between wetter and drier conditions in the Azores. The cumulative effects of multiple negative radiative forcings either sustains or promotes a negative NAO phase, i.e. wetter conditions in the Azores and drier and colder conditions in the Northern high latitudes (Baker et al., 2015; Slawinska & Robock, 2018; Richter et al., *in prep.*). However, the non-stationarity of the NAO has to be accounted for and a multi-proxy comparison with records from across the North Atlantic region is needed to understand long-term changes in the spatial extent and magnitude of the NAO.

4. Conclusions

In the Azores, fluctuations between drier and wetter conditions during the MCA suggest that there was no prolonged positive NAO phase during this time period. Over the past c. 600 years variability in the NAO could explain the opposing but coherent hydroclimate shifts observed in the Azores and Northern Scotland. The non-stationarity of the NAO or changes in regional feedbacks and background conditions could explain changes in the relationship between Icelandic temperatures and precipitation in the Azores over time. Depleted δD_{wax} values or wetter conditions in the Azores between c. 1270-1370 CE, c. 1450-1630 CE, c. 1650-1760, and c. 1800-1850 CE correspond to periods with strong negative radiative forcings that are associated with major volcanic eruptions and solar minima. This suggests that both external forcings and internal climate dynamics contribute to the multi-decadal and centennial variability observed in the δD_{wax} record from the Azores.

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Table 1. Spearman rank correlation coefficients used to assess the relationship between temperature, precipitation amount, large-scale circulation patterns, and δD_{precip} . The instrumental data is obtained from Ponta Delgada (1963-2014) (Hernández et al., 2016; Global Network of Isotopes in Precipitation (GNIP)) and is compared with a station-based NAO record (Azores - Iceland) (Cropper & Hanna, 2015).

	Air Temperature vs. δD_{precip}		Precipitation amount vs. δD_{precip}		Precipitation Amount vs. NAO		NAO vs. δD_{precip}	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Winter (DJF)	-0.09	0.63	-0.35	0.07	-0.70	<0.01	0.52	<0.01
Spring (MAM)	0.30	0.14	-0.72	<0.01	-0.60	<0.01	0.63	<0.01
Summer (JJA)	-0.07	0.74	-0.49	<0.05	-0.42	<0.01	0.15	0.45
Fall (SON)	0.13	0.51	-0.41	<0.05	-0.15	0.35	0.07	0.75

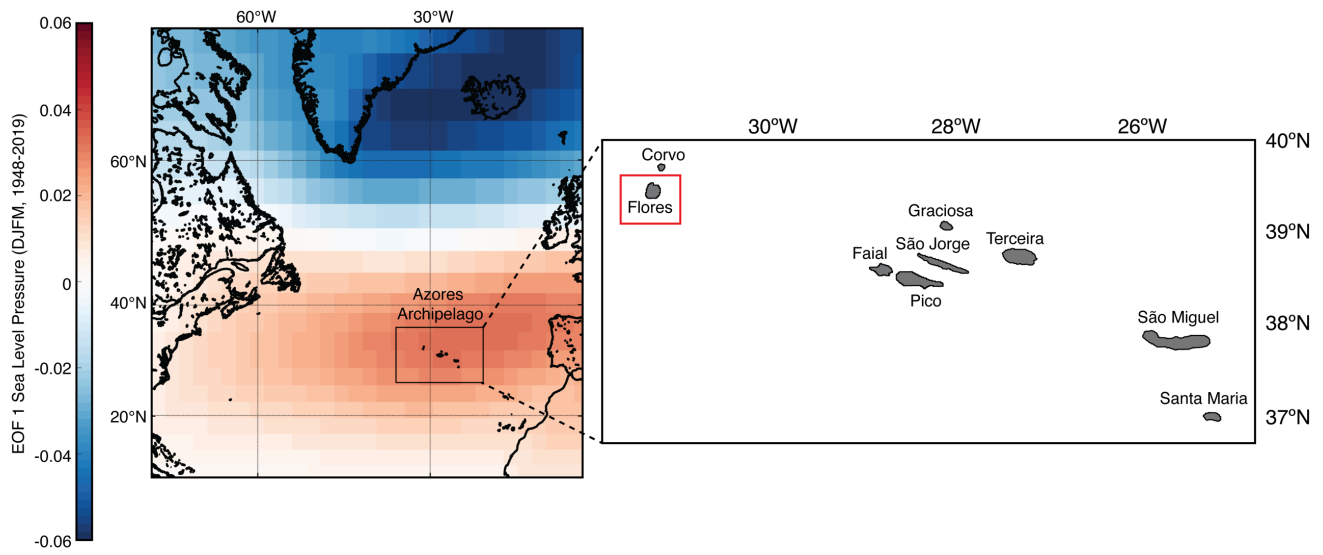


Figure 1. Map of the North Atlantic region with the leading empirical orthogonal function (EOF1) for sea level pressure over the Atlantic (20°N-80°N, 90°W-40°E) for the winter months (DJFM) from 1948-2019 (Kalnay et al., 1996). The Azores Archipelago with Flores island are shown on the right. These maps were rendered in Matlab using the Climate Data Toolbox (Greene et al., 2019).

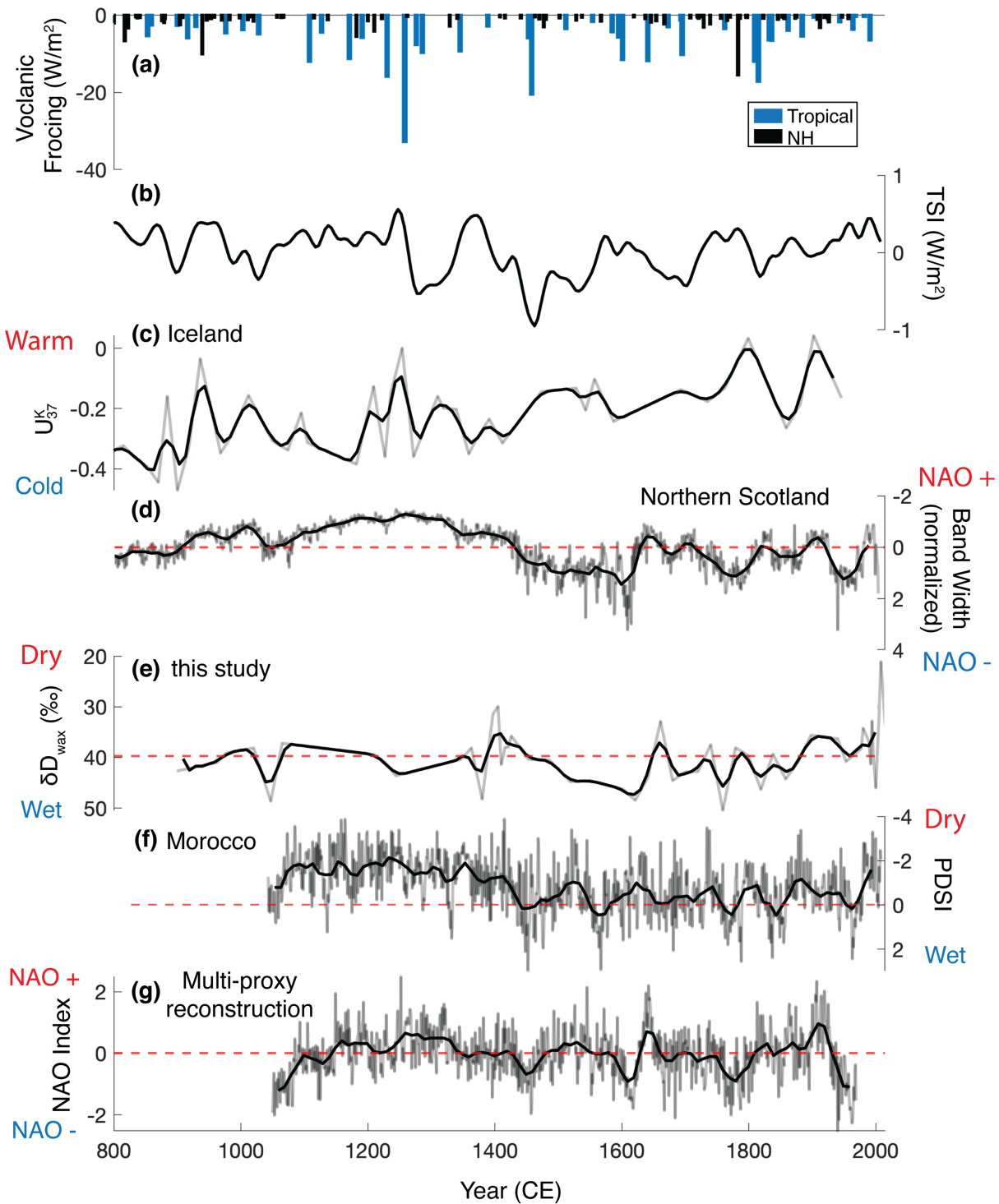


Figure 2. Changes in radiative forcings for (a) tropical and NH eruptions (Sigl et al., 2015) and (b) changes in total solar irradiance (TSI) (Steinhilber et al., 2009) are shown. This is compared with (c) a winter-spring temperature reconstruction from Vestra Gíslholtvatn, southwest Iceland and hydroclimate records from the North Atlantic region, including (d) a speleothem record from northwest Scotland (Baker et al., 2015), (e) hydroclimate changes in the Azores recorded by $\delta\text{D}_{\text{wax}}$ C₃₀FAMES, and (f) the Palmer Drought Severity Index (PDSI) reconstructed from tree rings in Morocco (Esper et al., 2007; Wassenburg et al., 2013). (g) These are compared with a model-constrained proxy-ensemble NAO reconstruction (Ortega et al., 2015).

– APPENDIX 2 –

**Optimizing the yield of transient mono-dimethyl disulfide adducts for elucidating double
bond positions of long chain alkenones**

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Abstract

Long chain alkenones (LCAs) are among the most successful biomarkers for paleotemperature reconstructions. However, fundamental questions regarding the biosynthesis and cellular functions of alkenones in haptophyte algae remain poorly understood. Recent discoveries of LCAs with double bond positions and chain lengths that differ from common structures further highlight the importance of continued research into structural variations of this important class of lipid biomarkers to improve LCA applications as temperature proxies. Double bond positions on alkyl chains can be effectively determined by preparing mono-double bond adducts with dimethyl disulfide (DMDS-1), and subsequent gas chromatography–mass spectrometry (GC–MS) analysis. However, previously published procedures for adduct preparation were originally designed for mono-unsaturated fatty acids, and generally produce low product yields when applied to alkenones. Here we demonstrate that the problem originates mainly from DMDS and alkenone overreaction at high temperatures for long time periods, and, secondarily, insufficient amount of iodine catalyst. The overreaction results in DMDS reacting with multiple double bonds, and the possible formation of intermolecular linkages, creating non-volatile products. These products are of little use for elucidating alkenone structures. We demonstrate that by reducing the reaction temperature and time, and by using an optimal amount of iodine, we can maximize the yield of transient DMDS-1 adducts for alkenone structure determination.

1. Introduction

Long chain alkenones (LCAs) are a class of unsaturated methyl and ethyl ketones produced by specific haptophyte algae in the ocean and terrestrial aquatic environments across the globe (de Leeuw et al., 1980; Volkman et al., 1980; Brassell et al., 1986; Zink et al., 2001; Longo et al., 2016). The number of LCA double bonds respond linearly to growth temperatures between 5 and 25 °C (Prahl & Wakeham, 1987; Prahl et al., 1988; Conte et al., 1998), yet they exhibit a nonlinear response at more extreme temperatures (Prahl et al., 1988; Conte et al., 1998). LCAs have been successfully applied to paleotemperature reconstructions in marine environments for the past four decades (Brassell et al., 1986; Prahl & Wakeham, 1987; Prahl et al., 1988; Müller et al., 1997; Herbert, 2006). Recently major efforts have been made to apply alkenone temperature proxies in lacustrine and estuarine systems; however, these systems are generally more complex. Lakes and estuaries generally harbor a more diverse group of alkenone-producing haptophytes, complicating temperature calibrations (Theroux et al., 2010). Studies are revealing that haptophytes in these environments produce a wider range of alkenones with variations in double bond positions (Rontani et al., 2006; Longo et al., 2013; Dillon et al., 2016), the degree of unsaturation (Jaraula et al., 2010), and chain lengths (Xu et al., 2001; Prahl et al., 2006; van Soelen et al., 2014; Zhao et al., 2014) in response to various environmental parameters (Prahl et al., 2006) or post-depositional changes (Rontani et al., 2006). Longo et al. (2016) proposed that temporal variations in salinity levels could favor certain species at a given time, indicating the need for a more in-depth understanding of alkenone production under different environmental conditions.

To further improve alkenone paleothermometry in both lacustrine and marine environments, convenient and reproducible methods are needed to identify double bond positions

for alkenones. Double bond positions in alkenones can be determined through osmium tetroxide (OsO₄) oxidation followed by the addition of trimethylsilyloxy groups (de Leeuw et al., 1980; Rontani et al., 2001; Rontani et al., 2006) or through imine derivatization of the ketone (López & Grimalt, 2006), followed by gas chromatography–mass spectrometry (GC–MS) analyses. Comparisons to analytical standards indicate that alkenone double bonds mainly occur in the *trans* configuration (Rechka & Maxwell, 1988), but have also been found as *cis/trans* or *trans/cis* isomers resulting presumably from the action of extracellular bacterial isomerases during the diagenesis stage (Rontani et al., 2005). However, the formation of the trimethylsiloxy derivatives requires a substantial addition of molecular mass to each double bond and increases the boiling point of the final product, which has limited this method to alkenones with at most two double bonds (Zheng et al., 2015). Imine derivatization of the alkenone carbonyl group produces useful fragments for double bond position identification (López & Grimalt, 2004, 2006; Dillon et al., 2016), but also yields additional mass fragments and sometimes non-unique assignments of double bond positions (López and Grimalt, 2004; López & Grimalt, 2006; Zheng et al., 2015).

We recently reported double bond positions of tri-unsaturated alkenone isomers (Dillon et al., 2016, Zheng et al., 2016) by imine derivatization of the alkenone carbonyl group and dimethyl disulfide (DMDS) double bond addition, using a new gas chromatographic separation method that provides higher selectivity of derivatized alkenones than conventional methods (Longo et al., 2013). In contrast to the OsO₄ oxidation method (de Leeuw et al., 1980; Rontani et al., 2001; Rontani et al., 2006), DMDS can separately and consecutively bind to individual double bonds in an alkenone molecule (termed DMDS-1), creating products with distinct mass fragments at any given double bond position without significantly decreasing the product volatility (Buser et al., 1983; Dillon et al., 2016). To account for fragments that are structurally

symmetric (i.e., ions which may be assigned to two different double bond positions on opposite sides of the alkyl chain based on the mass spectra for DMDS adducts), compounds can be further derivatized using cyclobutylimine to arrive at a structurally unique solution (Zheng et al., 2016).

Despite our initial success (Longo et al., 2013; Dillon et al., 2016; Zheng et al., 2016), there are major problems with published procedures for alkenone DMDS adduction. In our experience, applying published DMDS adduction procedures (Francis, 1981; Leonhardt & DeVilbiss, 1985; Yamamoto et al., 1991; Xu et al., 2001; van Soelen et al., 2014; Zhao et al., 2014) to alkenones, has frequently resulted in the lack of alkenone adducts in GC–MS analyses and even the complete absence of the original alkenones (i.e., total sample loss). Discussions with experienced alkenone researchers confirm similar experiences with DMDS derivatization (Maureen Conte, personal communication, April 8, 2015). Notably, the DMDS derivatization method was originally designed for mono-unsaturated fatty acids (reviewed by Christie, 1998). In the case of di-unsaturated and tri-unsaturated, methylene-interrupted fatty acids (with one, two, or three methylene interruptions), the products often contain cyclized sulfur products, making the interpretation of double bond positions difficult or impossible (Vincenti et al., 1987; Pepe et al., 1997; Méjanelle et al., 2002). Numerous studies using the DMDS derivatization method have tried to modify the procedure. In general, the experiments still involve heating at temperatures ranging from room temperature to 70 °C for 30 min to 24 h (Francis, 1981; Buser et al., 1983; Leonhardt & DeVilbiss, 1985; Shibahara et al., 1985; Shibahara et al., 2008; Vincenti et al., 1987; Yamamoto et al., 1991; Pepe et al., 1997; Xu et al., 2001; Méjanelle et al., 2002; van Soelen et al., 2014; Zhao et al., 2014; Dillon et al., 2016; Zheng et al., 2016). In all of the reactions iodine is used as a catalyst, however, the amount of I₂ added varies from 0.1 to 4.0 mg (Francis, 1981; Buser et al., 1983; Leonhardt and DeVilbiss, 1985; Vincenti et al., 1987;

Yamamoto et al., 1991; Xu et al., 2001; Shibahara et al., 2008). Further complications arise when applying these procedures to alkenones. For instance, alkenones can have up to five double bonds (Jaraula et al., 2010), such that if DMDS is bonded to all double bond positions, the molecular weight of the product is too high for conventional GC–MS analysis.

Obtaining derivatives with DMDS mono-adducts can aid in structure identification. Yamamoto et al. (1991) recognized the significance of DMDS mono-adducts when applying this method to fatty acids. The reaction was performed at a lower temperature (35 °C) and for a shorter time (30 min) to obtain mono-adducted dienoic esters (Yamamoto et al., 1996), yet it remains unclear how product yield changes under varying temperature and catalyst conditions. This methodology was recognized and applied to some fatty acid studies (Carballeira et al., 1994; Urbanová et al., 2004), however, it did not become a commonly accepted method for fatty acids. There were also no reports on an optimized method for obtaining DMDS mono-adducts. DMDS addition was also applied to alkenones for structure elucidation (Zhao et al., 2014; Dillon et al., 2016; Zheng et al., 2016); but most alkenone studies used long reaction times and high temperatures to generate DMDS adducts where all the alkenone double bonds were derivatized (Xu et al., 2001; Méjanelle et al., 2002; van Soelen et al., 2014).

In our recent studies, we were occasionally successful at obtaining visible, but low, DMDS-1 adducts after multiple attempts at derivatizing alkenones (Zhao et al., 2014; Dillon et al., 2016; Zheng et al., 2016). The difficulty in obtaining a consistent and reasonable DMDS-1 yield could be a result of mono-DMDS adducts being transient products, such that the yields are very sensitive to reaction conditions and duration. In this study, we sought to address this problem. Taking advantage of the exceptionally high alkenone abundance in Lake Braya Sø, Greenland, we are able to perform repeated experiments under systematically varied reaction

conditions. The overall objective was to improve DMDS addition to alkenone double bonds by identifying optimal reaction conditions that maximize product yield with reproducible results.

2. Experimental

2.1 Reagents

All solvents were HPLC grade from Fisher Scientific (USA). Diethyl ether (Et_2O , > 99%), dimethyl disulfide (> 98%), iodine (> 99.8%), sodium thiosulfate (> 99%) and sodium sulfate (anhydrous, > 99%) were purchased from Sigma Aldrich (USA).

2.2 Sample preparation

Alkenone samples used for the DMDS optimization reactions were extracted from sediments retrieved from Lake Braya Sø, western Greenland, which is known for its extremely high alkenone concentrations (D'Andrea & Huang, 2005). Sediment samples were freeze-dried and homogenized, prior to extraction using dichloromethane/methanol (DCM/MeOH; 9:1, v:v) with an Accelerated Solvent Extractor ASE200 (Dionex) at 120 °C and 1200 psi. Solvent extracts were separated into acid and neutral fractions by flash chromatography (LC- NH_2 , 40–63 μm) using 4% acetic acid in Et_2O to elute the acid fraction and DCM/isopropanol (2:1, v:v) to extract the neutral fraction. The neutral fraction was evaporated to dryness with nitrogen, and then re-dissolved in hexane. The resulting solution was separated via flash chromatography (SiO_2 , 40–63 μm) yielding hexane (alkane), DCM (ketone), and methanol (alcohol) fractions. The ketone fraction, which contains alkenones, was evaporated to dryness, and re-dissolved in hexane for quantification using gas chromatography–flame ionization detection (GC–FID).

2.3 Preparation of alkenone dimethyl disulfide derivatives

The experiments in this study were modified based on the method described by Xu et al. (2001). For each reaction, 32.8 ± 4.1 μg of LCAs from Lake Braya Sør was dissolved in 50 μL of hexane. To initiate the reaction, a 40 μL iodine solution with variable concentrations (15–225 mg I_2 in 1 mL of Et_2O) and 200 μL of DMDS were added to the dissolved LCAs. After briefly flushing with N_2 gas for 30 s, the reaction mixture was capped and held at a constant temperature while being stirred at 375 rpm for a specified time period. The reaction was stopped using 5% sodium thiosulfate in deionized water, and the resulting products were extracted using hexane. The organic phase was transferred to a clean vial and anhydrous sodium sulfate was added, and then allowed to sit for 30 s. The resulting reaction mixture was isolated and evaporated to dryness. Samples were quantified using a GC–FID with 18-pentatriacontanone as an internal standard. For each of the samples, the percent yield was calculated relative to the initial amount of LCAs used for the reaction.

To determine how we should modify the procedure proposed by Xu et al. (2001), we performed an initial series of reactions that varied time, temperature, stirring/non-stirring, iodine amounts and solvents. These results indicated that the duration of the reaction and the temperature at which it was held were the primary factors controlling the reaction yield. In Xu et al. (2001), the reaction mixture was heated for 20 h at 40 °C. In our experiments, we used a design of experiment (DoE) framework (Telford, 2007; Moseley & Murray, 2014) to map out a 2-factor, 3-level design for systematic time and temperature experiments with temperatures ranging from 0 to 75 °C and reaction times from 10 min to 24 h (Supplementary Table S1). We found that the amounts of iodine is a secondary factor influencing the product yield. After

optimizing the time and temperature conditions, we used an additional DoE framework with a 3-factor, 3-level design to maximize time, temperature and the amount of iodine.

To avoid degradation of DMDS adducts and to ensure that no other reactions occurred, all products were analyzed immediately after the reactions were performed or blown down with N₂ gas and kept frozen until the samples were analyzed. Additional tests were done to track the degradation of DMDS adducts after samples were blown down with N₂ gas and kept frozen overnight. These experiments showed that $58.4 \pm 3.0\%$ of the products were lost after the samples were measured, stored in a freezer overnight, and then re-measured on the GC–FID after two days.

2.4 Analysis of alkenone derivatives by GC–FID and GC–MS

Alkenone dimethyl disulfide adducts were analyzed using a GC–FID (Agilent 6890N) and GC–MS (Agilent 6890N GC, Agilent 5973N quadrupole mass spectrometer) with a VF-200 ms capillary column (30 m for GC–FID and 60 m for GC–MS, 250 μm i.d., 0.10 μm film thickness) with the method described by Longo et al. (2013). Samples were injected onto the column using a split/splitless injector (splitless mode) with the injector port and transfer line temperature set to 330 °C. The carrier gas (He) was held at a constant flow rate of 1.3 mL/min. The initial oven temperature was set to 75 °C and held for 1 min, then increased to 320 °C at 10 °C/min and held for 30 min. The mass spectrometer was operated in EI mode (70 eV) with a scan range of 50–750 Da. Gas chromatograms for the GC–FID were analyzed by first establishing a baseline for each cluster of identifiable peaks (i.e., C₃₇Me, C₃₈Et, C₃₈Me, and C₃₉Et) for the original alkenone and the visible DMDS adducts. GC–FID peaks were then quantified in clusters by summing the areas under the identified peaks to obtain totals for the

original alkenones and DMDS adducts. All GC–FID and GC–MS data were exported from Agilent ChemStation for further processing in MATLAB (R2016a) using custom scripts and data processing routines available at <https://www.github.com/chemplexity/chromatography>.

3. Results and discussion

3.1 Key variables affecting alkenone dimethyl disulfide reactions

An initial set of experimental trials identified three key factors affecting DMDS-1 yields: reaction time, temperature, and iodine amount. In our first assessment, we compared the yield of DMDS-1 at 20, 50 and 75 °C for a reaction time of 210 min. At 20 °C, the products included DMDS binding to multiple double bonds (for consistency with DMDS-1, we designate those with two double bonds adducted by DMDS as DMDS-2, and similarly three double bonds as DMDS-3, etc.; Figure 1). At 50 °C, DMDS-1 products were still visible on the GC–FID chromatograms, but with low yields (6.0% at 30 min, 1.0% at 120 min, and 0.3% at 210 min). A further increase in reaction temperature to 75 °C, led to a further decline in yield and even the complete absence of DMDS adducts from the gas chromatograms (1.6% at 30 min, and 0.0% at 120 and 210 min). These results suggest that the reaction yields for DMDS-1 were higher at lower temperatures. Most literature reports using DMDS adduction carried out reactions at 40–50 °C (Buser et al., 1983; Leonhardt & DeVilbiss, 1985; Vincenti et al., 1987; Pepe et al., 1997; Xu et al., 2001; Méjanelle et al., 2002; van Soelen et al., 2014; Dillon et al., 2016; Zheng et al., 2016), although others reported performing DMDS reactions at a temperature of 70 °C (Zhao et al., 2014). Our initial results thus suggest lower temperature could increase the DMDS-1 product yields. This is readily explained by the fact that DMDS-1 is a transient product in the reaction

between DMDS and alkenones with multiple double bonds: lower temperatures slow down the overall reaction and reduce adduction onto multiple double bonds.

We also found that prolonged reaction times (e.g., overnight) resulted in a significant loss or even the complete disappearance of DMDS-1 adducts and the original alkenones. For example, when we performed the reaction at 40 °C for 22 h, neither the DMDS adducts nor the original alkenones were visible on the gas chromatograms. This indicated that reaction time was also critical to maximizing DMDS-1 yield, and that extended reaction times did not always lead to higher product yields.

Altering the amount of iodine (all previous reactions were initiated with 2.4 mg) used in the reaction, appreciably changed DMDS-1 yields. A reduction in iodine to 1.2 and 0.6 mg decreased yields of DMDS-1 adducts. Conversely, increasing the amount of iodine to 3.0, 4.0, and 6.0 mg, led to an increase in product yield. Because the molar ratio of iodine/original alkenone is 138.7 at 2.4 mg I₂ and the molar ratio of DMDS/original alkenone is over 30,000 for 200 µL DMDS, we can reasonably exclude the possibility that the optimal iodine amount is dictated by the amount of reactant alkenones. It is likely that optimal amount of iodine is mainly determined by the amount of DMDS reagent used.

As part of the initial experiments we also varied the solvents and stirring/no-stirring at constant times and temperatures. In all previous reactions, hexane was used to dissolve the reaction mixture. There was, however, no significant change in yield when hexane was switched to a different solvent (tetrahydrofuran and toluene). Not stirring the reaction while it was held at a constant temperature for an extended period of time led to a significant decrease and even the disappearance of DMDS-1. In contrast, stirring the reaction at 375 rpm, as previously described by Francis (1981), greatly increased DMDS adducts.

3.2 Optimal reaction conditions for dimethyl disulfide adduct formation

In order to determine the optimal reaction conditions, we used a DoE to first systematically vary the reaction temperature and time followed by an additional DoE where we varied the time, temperature, and iodine amounts. The results for different reaction times, temperatures, and iodine amounts are plotted in Figure 2. A cubic interpolation was applied to the data in order to map out changes in yield under varying reaction conditions. The experimental temperatures were chosen based on how easy and practical it was to maintain the reaction at the given temperature for an extended period of time. For instance, reactions at 0 °C were performed in an ice bath, while 20 °C corresponds to room temperature and experiments at 40 °C were heated using a hot plate. Reactions at 10 °C or –10 °C could produce appreciable yields, however, these temperatures were not pragmatic for most laboratory set-ups and thus were not considered in this study.

We found that the highest DMDS-1 yields occurred at different times and temperatures when the amount of iodine was changed (Figure 2). For 2.4 mg of I₂ the highest yields occurred at 250 min at 0 °C (22.6%) and 160 min at 20 °C (23.2%). When I₂ was increased to 3 mg, the optima occurred at: 220 min at 0 °C (19.8%), 250 min at 0 °C (18.2%), 280 min at 0 °C (18.5%), 310 min at 0 °C (21.2%). For 4 mg I₂ the DMDS-1 yield peaked at 160 and 250 min at 0 °C (22.2% and 21.0%, respectively), and for 6 mg I₂ at 120 min at 0 °C (19.7%). Variable yields under different conditions suggest that DMDS adduction is kinetically controlled because our desired product, DMDS-1, is transient between the original alkenones and multiply adducted alkenones. This suggests that any subtle changes in the experimental procedure can lead to significant shifts in DMDS-1 yields. Most samples were analyzed as soon as possible after the

reactions were completed and were kept frozen prior to being analyzed with the GC–FID.

However, a more detailed analysis is required to determine how quickly samples degrade at room temperature and over the course of a couple of hours.

The most consistent and highest yields occur at 0 °C. The results showed that increased amounts of iodine reduced the reaction time. An optimum was also observed at 20 °C for 2.4 mg of I₂. This agreed with the time and temperature DoE: high DMDS-1 yields occurred at low temperatures and long reaction times, and at high temperatures and short reaction times (Supplementary Figure S2). Based on these results, we hypothesize that higher temperatures and more iodine contribute to increased reaction rates (see Sections 3.4 Influence of time and temperature on reaction rates, 3.5 Influence of iodine on reaction rates).

3.3 Confirmation of dimethyl disulfide derivatives

Diagnostic mass fragments obtained by GC–MS were used to confirm the identity of DMDS-1 adducts. The mass spectra of all DMDS-1 peaks exhibited characteristically low molecular ion (M^+) peaks (Figure 3). The mass spectra of the DMDS derivatives show fragment ions associated with the loss of SCH₃ ($M^+ - 47$), CH₃SCH₂⁺ ($M^+ - 61$), and CH₃SSCH₃ ($M^+ - 94$). The spectra contain peaks that indicate fragmentation at the various carbon-carbon double bonds, for example, with the C_{37:4} Me alkenone, the double bond located at the $\Delta 7$ position was determined by locating the ketone containing fragment (m/z 173), and the corresponding alkene fragment (m/z 447). In this case, DMDS-1 adducts for each of the four double bonds on the C_{37:4} Me alkenones were not resolved by GC–MS under the experimental condition. Hence, the mass spectrum in Figure 3 represents combined mass fragments from all four possible DMDS-1 adducts. Fortunately, determining the position of any specific double bonds on the alkenone only

requires identification of two complementary molecular masses in the mass spectrum (Figure 3). Therefore, co-elution of DMDS-1 adducts for a given alkenone on the GC–MS does not pose a significant problem for conclusive structure elucidation. The entire suite of DMDS alkenone homologues (i.e., C₃₇ Me, C₃₈ Et, C₃₈ Me, and C₃₉ Me) exhibited the same fragmentation patterns described above (for more mass spectra see Supplementary Figs. S4–S11 and Supplementary Information for Dillon et al. (2016)).

3.4 Influence of time and temperature on reaction rates

In the initial time and temperature experiments, a potential inverse relationship was observed between time and temperature: local optima for DMDS-1 yields was observed at 10 min at 40 °C, 160 min at 20 °C, and 250 min at 0 °C. This could indicate that the reaction rate increased at higher temperatures. We looked at how each of these reactions evolved over time at a specified temperature, which showed that the highest DMDS-1 yields occurred just as DMDS-0 significantly decreased and DMDS-2 adducts started to form (Supplementary Figure S2).

We propose that the peak in DMDS-1 occurred just as excess DMDS began to react with additional alkenone double bonds to form DMDS-2 adducts. Similarly, if the reaction was not stopped at the right time, any excess DMDS would continue to react with available alkenone double bonds to form DMDS-3 and DMDS-4 adducts. This was validated by the presence of DMDS-3 adducts when reactions were allowed to run for longer periods of time (Supplementary Figure S1). DMDS-4 adducts, however, were not visible on the GC due to their high molecular mass. The relationship between time and temperature for optimal DMDS-1 product yields indicated that the rate at which DMDS bonds to alkenone double bonds increased at higher

temperatures. Conversely, the rate of DMDS bonding to alkenone double bonds decreased at lower temperatures.

The reactions performed by Yamamoto et al. (1991) with methyl linoleate are in agreement with our experiments: the authors observed that DMDS-1 adduct formation peaked at shorter times for 35 °C. For instance, the authors found that the initial methyl linoleate concentrations decreased rapidly from 0 to 15 min as DMDS-1 adducts began to form. Similarly, the initial alkenones and DMDS-1 adducts in our reactions decreased between 0 and 60 min when reactions were performed at 40 °C (Supplementary Figure S2).

The relationship between time and temperature for optimal DMDS-1 adducts indicated that multiple reaction conditions could produce optimal DMDS-1 yields. This agreed with the range of times and temperatures cited by previous papers (Table 1). Each of these methods generated DMDS-1 adduct yields for both fatty acids and alkenones, but with varying yields of DMDS-1 adducts. The optimal time and temperature conditions for DMDS reactions with fatty acids were not addressed in this paper and may be different from the proposed method for alkenone and DMDS reactions.

3.5 Influence of iodine on reaction rates

Iodine was thought to act as a catalyst in the reaction and had a large influence on DMDS-1 yields. In the initial time and temperature reactions, the amount of iodine (2.4 mg) was kept constant. However, additional experiments showed that decreasing the amount of iodine led to a decrease in the DMDS-1 yield, and conversely an increase in iodine led to an increase in DMDS-1 adducts. The time and temperature that corresponded with the highest DMDS-1 adduct yields varied with the amount of iodine used, such that increased iodine led to shorter reaction

times at lower temperatures (Figure 2). For instance, a peak in DMDS-1 occurred at 250 min at 0 °C for 2.4 mg I₂ (22.6%). When I₂ was increased to 6 mg, DMDS-1 peaked at 120 min at 0 °C (19.7%). Based on these results, we propose that additional iodine increased the reaction rate and thus the formation of DMDS adducts. If a large excess of iodine was present in the reaction, however, iodine had the potential to further catalyze the formation of DMDS-2, DMDS-3 and DMDS-4 adducts. This could make it more difficult to stop the reaction at the appropriate time for the optimal DMDS-1 adducts and could lead to variable results. Based on our experimental results, the highest DMDS-1 yields occurred for 2.4 mg I₂ at 250 min at 0 °C and 160 min at 20 °C, and for 4 mg I₂ at 160 min at 0 °C and 250 min at 0 °C (Figure 2).

3.6 Proposed dimethyl disulfide reaction mechanism

Aspects of the reaction mechanism for DMDS adduct formation are not well understood. Previous studies for fatty acid DMDS derivatization determined that the reaction is anti-stereospecific (Buser et al., 1983) and in di-unsaturated fatty acids, DMDS addition is catalyzed by iodine and forms a sulfonium ring intermediate (Vincenti et al., 1987). The reaction mechanism proposed by Vincenti et al. (1987), was mainly relevant to fatty acids with double bonds in the Z-configuration, however, they propose that the mechanism might also hold true for double bonds in the E-configuration. A similar mechanism might also be applicable to alkenones, where double bonds are in the E-configuration (Rechka & Maxwell, 1988). The E-configuration could decrease the rate of DMDS reacting with alkenone double bonds, which would amplify the importance of iodine as a catalyst in the reaction. Alkenone double bonds are usually separated by at least three methylene groups (de Leeuw et al., 1980; López & Grimalt, 2004; Rontani et al., 2006; Dillon et al., 2016), which would reduce the likelihood of forming

cyclization products during the DMDS reaction. In comparison, when fatty acids with multiple conjugated double bonds that have one, two, or three methylene-interruptions were reacted with DMDS, they were prone to form cyclized products (Christie, 1998; Méjanelle et al., 2002; Zhu, 2003) thus complicating identification of double bond positions. Xu et al. (2001) described one instance where three methylene interrupted alkenone cyclization occurred following a reaction of Black Sea alkenones with DMDS at 40 °C for 20 h. As shown in this paper and in our recent study, Zheng et al. (2016), a reduction in the reaction time can generate DMDS-1 adducts without obvious cyclization products and facilitate the identification of double bond positions.

To account for the major controls on DMDS adduct formation we propose a potential reaction mechanism that complements the previous mechanism described by Vincenti et al. (1987), but with an additional iodonium complex that precedes DMDS addition (Figure 4). The reaction is initiated by an iodine catalyst that forms an iodonium complex with a carbon-carbon double bond. The remaining iodide anion then undergoes a substitution reaction with DMDS that generates a methanethiolate anion and thiomethyl iodine. The methanethiolate anion adds via anti-addition to the iodonium ion, which then forms a three-membered sulfonium ring intermediate. The iodide anion regenerates the iodine catalyst and forms a second methanethiolate anion. The methanethiolate anion adds via anti-addition to the three-membered ring and thereby forms the observed DMDS adduct.

3.7 Mass balance for the dimethyl disulfide reaction products with alkenones

At optimal conditions, the reactions in this study produced DMDS-1 adduct yields of $20.4 \pm 1.8\%$. The product yield did not, however, account for the 80% loss of the original sample. For instance, when reactions were performed with 2.4 mg I_2 for 250 min at 0 °C the

following adducts were observed: DMDS-0 42.0%, DMDS-1 22.6%, and DMDS-2 1.7%. This accounted for 66.3% of the original alkenones but did not account for the remaining 33.7% that was lost during the reaction.

To explain this, we propose three possible scenarios that could lead to sample loss (Figure 5; Supplementary Figure S3): (1) the reaction formed additional DMDS-3 and DMDS-4 adducts, which were not visible on the chromatographs due to their high molecular mass and low peak intensities; (2) some of the initial alkenones and products were degraded prior to running the samples on the GC–FID and/or (3) additional reactions, such as polymerization through polysulfide linkages, occurred between alkenone DMDS adducts. Further and more in-depth studies are required to test these hypotheses.

4. Conclusions

In this study, we showed that DMDS adduction onto alkenones is a kinetically controlled reaction initiated by an iodine catalyst. At lower temperatures, we were able to reduce the reaction rate and control the production of DMDS-1 adducts. We found that DMDS-1 adducts were transient alkenone products with DMDS bound to only one alkenone double bond, which was ideal for identifying double bond positions using the GC–MS. To optimize the yield of DMDS-1 we used a DoE set up to target the best reaction temperature, reaction duration, and amount of catalyst needed to initiate DMDS binding to alkenone double bonds. Most previous published work on alkenone double bond derivatization techniques attempted to derivatize all double bonds at once (Xu et al., 2001; van Soelen et al., 2014). This often resulted in high molecular weight products that were unsuited for GC–MS analysis.

We demonstrated that the optimal conditions for consistent alkenone DMDS-1 adduct formation occurred at 0 °C with yields of $20.4 \pm 1.8\%$. The reaction time also varied depending on the amount of iodine used to catalyze DMDS bonding to alkenone double bonds. The overall highest yields were observed when an iodine amount of 2.4 mg was used and the reaction was run for 160 min at 20 °C (23.2%) and 250 min at 0 °C (22.6%), and at 4 mg I₂ for 160 and 250 min at 0 °C (22.2% and 21.0%, respectively). Reactions at higher temperatures, such as 20 or 40 °C, also generated high DMDS adduct yields when performed for shorter time periods and with lower iodine amounts. DMDS adduct yield at higher temperatures, however, was generally more variable than reactions performed at 0 °C. At these high temperatures it could be more difficult to capture the point of optimal yield for transient DMDS-1 products, especially because any subtle changes in experimental conditions could lead to a shift in the peak and result in sample loss. We also observed sample degradation after we left samples frozen for two days. Lower temperatures, reaction times of 160–250 min, and I₂ amounts of 2.4–4 mg guarantee DMDS-1 adduct formation that can be applied to alkenone double bond identification. We therefore recommend performing DMDS derivatization with 4 mg of I₂, 200 µL DMDS at 0 °C for 160 min.

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Table 1. A summary of previous studies that used the DMDS optimization method, and the various conditions used to derivatize alkenones and form DMDS adducts. There are many more studies that apply the DMDS derivatization method with small modifications, most of these studies, however, relate to structural identification of fatty acids and are beyond the scope of this paper.

Compounds	DMDS volume (μL)	Time (hrs)	Temperature (°C)	I ₂ (mg)	Study
Unsaturated fatty acid esters		24	20 [†]	0.1	Francis (1981) [‡]
Mono-unsaturated acetates	50	12 [*]	40	0.3	Buser et al. (1983) [‡]
Mono-unsaturated fatty acids	70-100	24	40		Dunkelblum et al. (1985) [‡]
Mono-unsaturated alcohols, aldehydes, and methyl esters	100	20	40	1.8	Leonhardt & DeVilbiss (1985) [‡]
Fatty acid methyl esters			35		Shibahara (1985)
Mono-unsaturated fatty acids	100	48	50		Nichols et al. (1986) [‡]
Di-unsaturated compound	50	40	60	0.3	Vincenti et al. (1987) [‡]
Mono-unsaturated fatty acid methyl esters	100	48	50	1.2	Scribe et al. (1988) [‡]
Dienoic esters	300	0.5	35	4	Yamamoto et al. (1991) ^{‡, §}
Tri-unsaturated compounds: alkatrienes	100	48	50	1.2	Pepe et al. (1997)
Tri-unsaturated compounds: alkatrienes	100	100	50	1.2	Pepe et al. (1997)
Unsaturated aliphatic compounds		4	20	2.4	Urbanová et al. (2004) [§]
Fatty acid methyl esters	100		35	1.3	Shibahara (2008)
Alkenones	100	20	40	1.8	Xu et al. (2001)
Alkenones		48	50		Méjanelle et al. (2002)
Alkenones	100	20	40	1.2	van Soelen et al. (2014)
Alkenones	100	2	70	3	Zhao et al. (2014) [§]
Alkenones	100	2	40	1.8	Dillon et al. (2016) [§]
Alkenones	200	2	40	2.4	Zheng et al. (2016) [§]

* Overnight = 12 hrs

[†] 20 °C = Room temperature

[‡] These methods are commonly cited as references for methods using the DMDS derivatization method.

[§] These studies derived mono-DMDS (or DMDS-1) adducts.

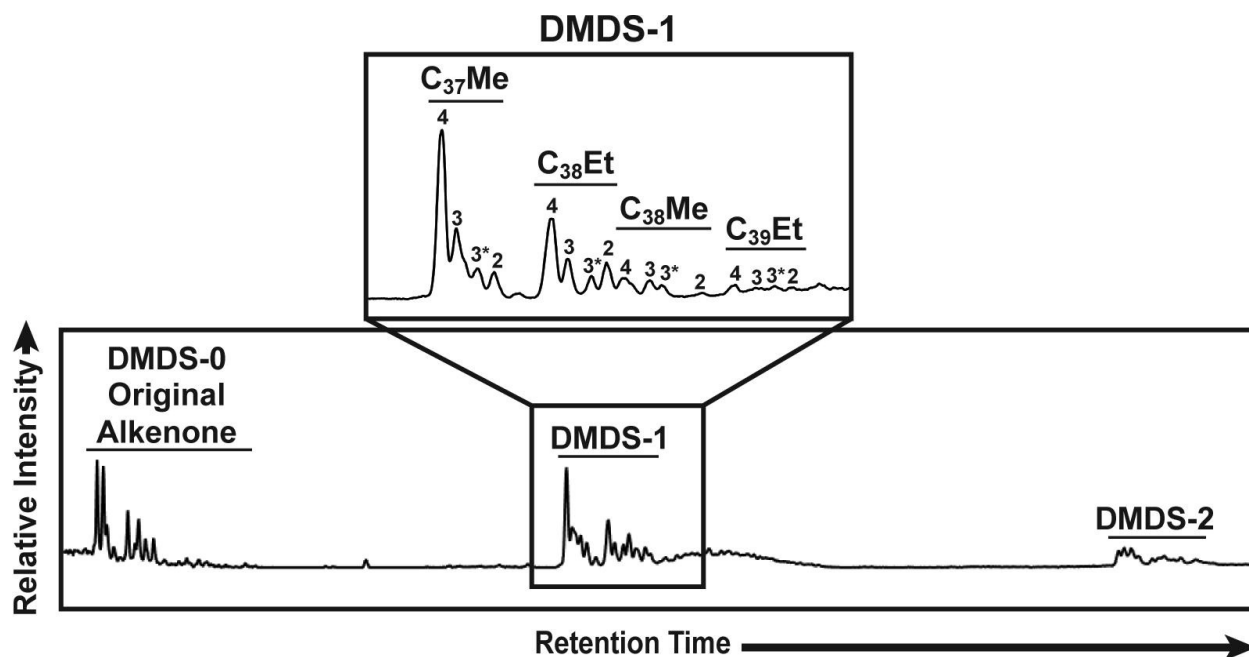


Figure 1. GC–FID results showing the original (unreacted residual) alkenone, and the DMDS adducts. DMDS-1 represents all of the mono-adducts, while DMDS-2 represents DMDS bound to two different double bonds in the alkenone. Groups of alkenones are noted and were identified for quantification purposes. The DMDS-1 peaks are split into multiple peaks based on the number of double bonds. Relative to the unreacted alkenones, the peak height might appear lower than the actual quantities that were determined using the peak area.

DMDS-1 Yields

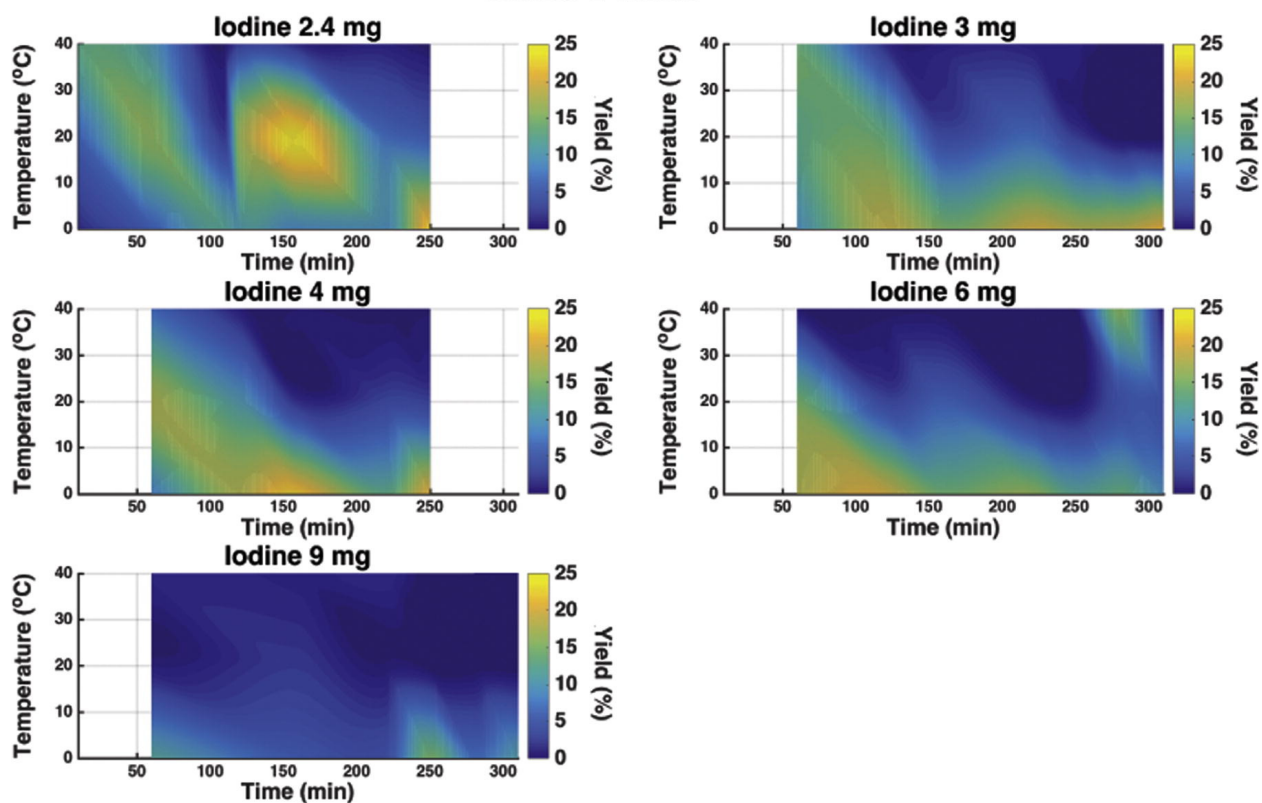


Figure 2. Contour plots for DMDS-1 yields obtained with varying amounts of iodine, time, and temperature. For I_2 2.4 mg the optimal conditions occur at: 250 min 0 °C (22.6%) and 160 min 20 °C (23.2%). At I_2 3 mg Et_2O the optimal conditions occur at: 220 min 0 °C (19.8%), 250 min 0 °C (18.2%), 280 min 0 °C (18.5%), 310 min 0 °C (21.2%). The DMDS-1 yield peaks for I_2 4 mg Et_2O at 160 min 0 °C (22.2%) and 250 min 0 °C (21.0%). Finally, for I_2 6 mg the DMDS-1 adducts peaked at 120 min 0 °C (19.7%). The low points between temperatures of 0 and 20 °C and the lack of data below 0 °C result from a lack of data. Reactions at these conditions were considered difficult and impractical to maintain, and thus were not considered in this paper.

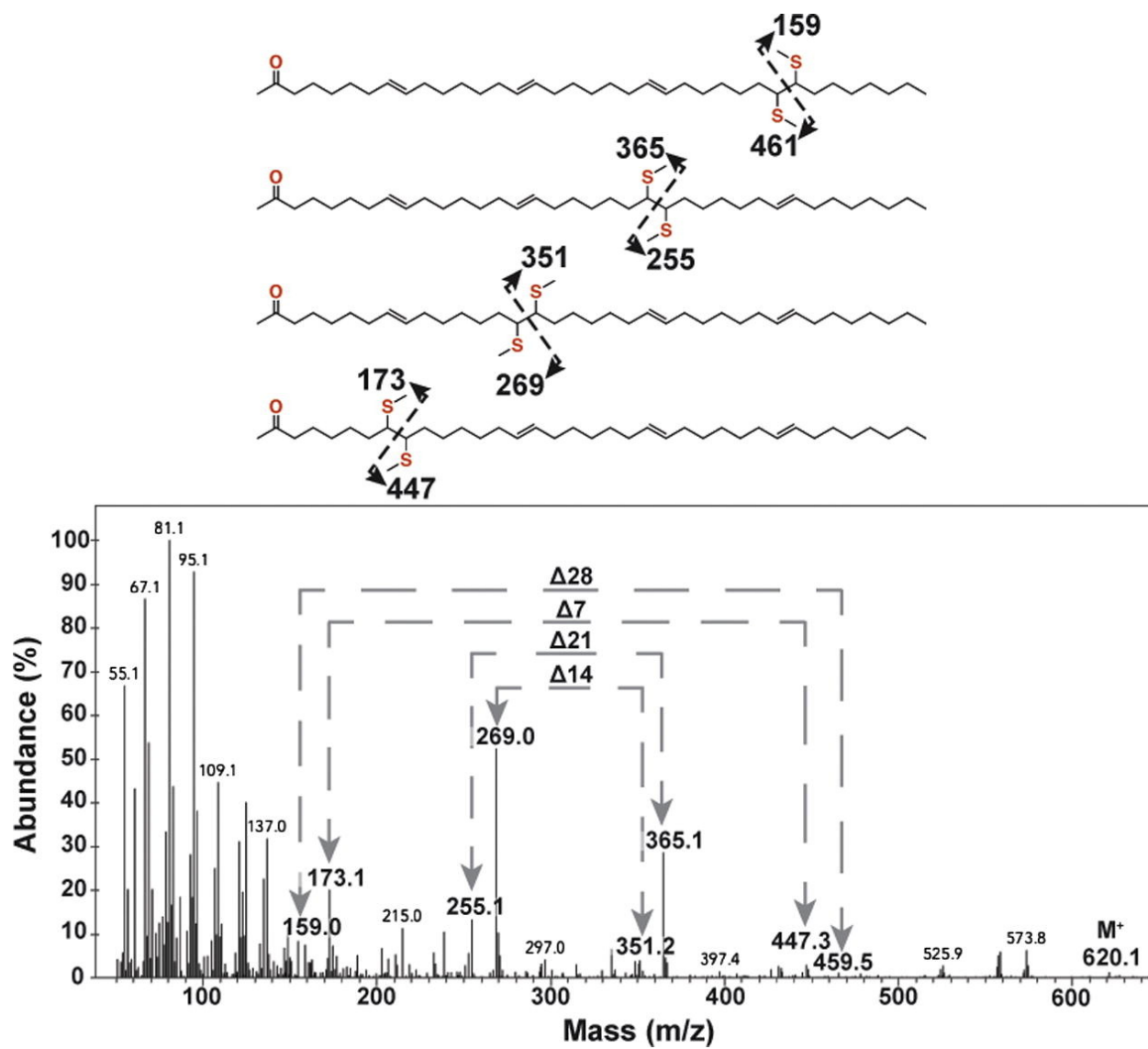


Figure 3. An example of mass spectra showing the characteristic mass fragments for the $C_{37:4}$ Me alkenone with DMDS binding. More mass spectra for alkenone DMDS-1 products can be found at Dillon et al. (2016).

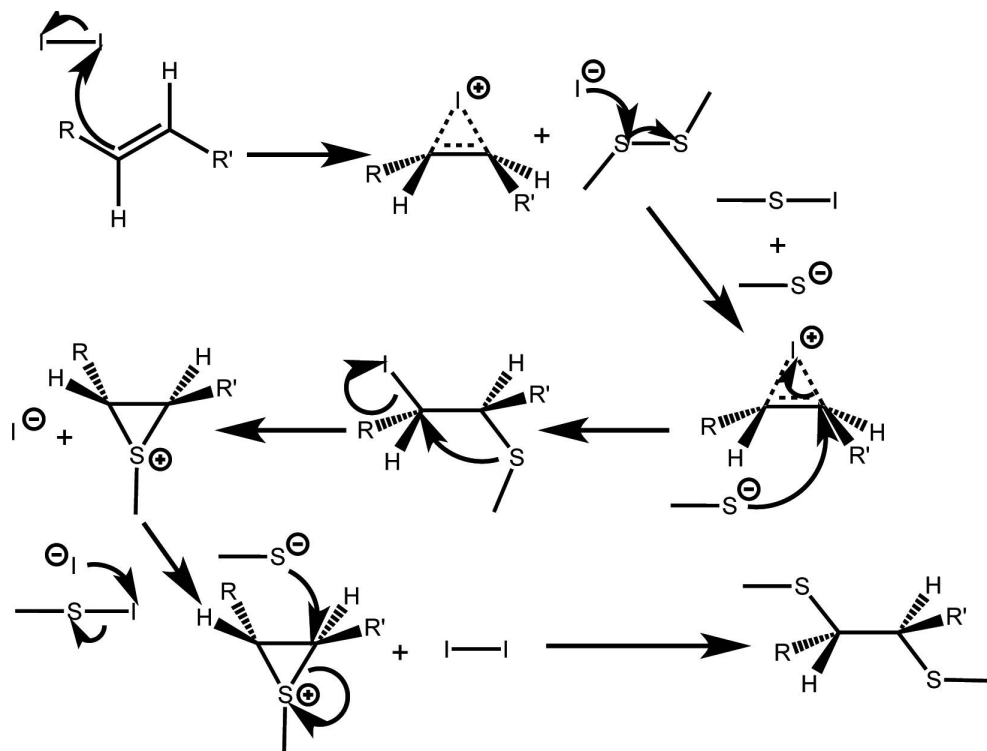


Figure 4. Proposed mechanism where the iodine catalyst forms an iodonium complex and generates thiomethyl anions which add via anti-addition to the alkene with a three-membered ring intermediate.

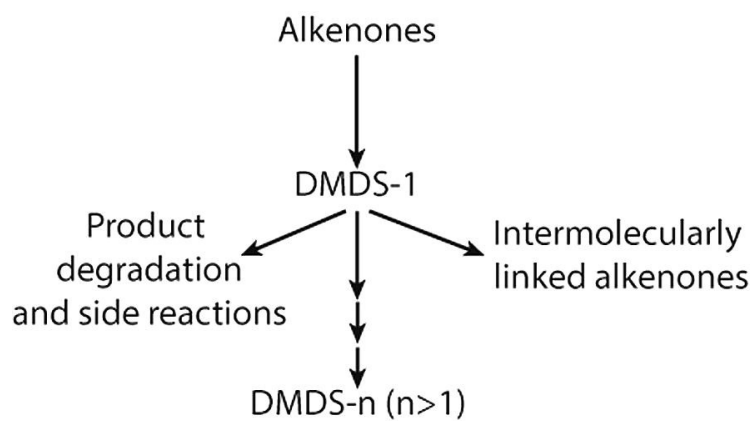


Figure 5. Potential reactions that the original alkenones can undergo when exposed to DMDS and an iodine catalyst. After the DMDS-1 adducts are formed, the products can (1) react with DMDS to form DMDS-n adducts, (2) undergo side reactions or product degradation or (3) form intermolecular linkages with other DMDS adducts.

Supplementary Information

Table S1. A summary of the reactions performed in this study and the DMDS adduct yields.

Iodine (mg)	Time (min)	Temperature (°C)	DMDS-0 (%)	DMDS-1 (%)	DMDS-2 (%)
2.4	10	0	29.56	0.71	0.00
2.4	10	0	38.58	1.14	0.00
2.4	35	0	34.92	2.19	0.00
2.4	45	0	37.46	5.18	0.00
2.4	60	0	90.38	3.16	0.00
2.4	60	0	34.52	4.63	0.00
2.4	60	0	34.99	7.71	0.00
2.4	75	0	30.18	6.14	0.00
2.4	90	0	36.76	10.16	0.00
2.4	110	0	26.38	11.96	0.90
2.4	110	0	22.56	10.29	0.78
2.4	120	0	51.08	10.41	0.00
2.4	160	0	30.02	8.39	0.49
2.4	160	0	16.16	15.30	6.96
2.4	180	0	14.55	14.62	7.40
2.4	220	0	26.87	9.07	1.73
2.4	220	0	13.60	8.32	4.64
2.4	250	0	42.00	22.55	1.73
2.4	10	20	33.59	6.67	0.00
2.4	10	20	26.34	5.32	0.00
2.4	30	20	45.98	8.47	0.47
2.4	35	20	15.78	10.12	1.29
2.4	45	20	7.20	9.95	2.43
2.4	60	20	21.12	24.26	5.24
2.4	60	20	8.11	9.18	2.14
2.4	60	20	3.85	6.13	1.75
2.4	75	20	0.00	4.34	4.11
2.4	90	20	0.00	2.26	2.55
2.4	110	20	1.85	0.00	1.26
2.4	120	20	6.73	16.34	10.92
2.4	120	20	2.24	4.83	3.87
2.4	160	20	13.79	23.21	9.01
2.4	210	20	3.08	2.91	2.54
2.4	220	20	2.67	6.75	7.48
2.4	250	20	1.28	5.39	7.07
2.4	20	30	19.02	7.68	0.44
2.4	45	35	7.47	7.15	2.94
2.4	10	40	27.95	16.73	1.88

2.4	10	40	18.44	10.91	1.00
2.4	35	40	0.00	2.63	3.45
2.4	60	40	11.95	19.45	8.82
2.4	60	40	0.00	2.99	2.57
2.4	60	40	0.00	0.00	0.83
2.4	110	40	0.00	0.00	0.27
2.4	120	40	2.26	2.35	4.56
2.4	160	40	2.73	1.33	2.71
2.4	220	40	2.77	0.83	1.91
2.4	250	40	2.67	0.00	0.00
2.4	30	50	3.59	6.01	3.47
2.4	120	50	0.64	0.95	1.73
2.4	210	50	1.01	0.33	0.00
2.4	30	75	0.95	1.60	1.87
2.4	120	75	0.00	0.00	0.00
2.4	210	75	53.32	0.00	0.00
3	60	0	59.39	10.24	0.20
3	60	0	42.57	7.65	0.51
3	120	0	52.18	18.56	0.95
3	160	0	37.56	12.47	3.58
3	160	0	27.52	16.44	2.78
3	220	0	27.24	15.28	1.20
3	250	0	49.19	20.70	1.44
3	250	0	18.20	12.57	2.89
3	250	0	27.70	19.28	4.84
3	250	0	19.43	20.12	3.50
3	280	0	27.97	18.49	3.46
3	310	0	19.62	21.15	7.11
3	60	20	14.36	24.53	8.80
3	120	20	5.19	13.27	9.93
3	160	20	3.50	10.15	9.22
3	160	20	0.83	0.26	1.29
3	220	20	1.27	6.63	9.53
3	250	20	1.25	4.95	7.23
3	250	20	0.00	0.00	0.38
3	280	20	0.69	0.00	0.13
3	310	20	0.95	0.00	2.73
3	60	40	5.38	13.72	9.46
3	60	40	2.13	0.74	3.28
3	120	40	2.66	1.56	3.94
3	160	40	2.56	0.93	2.96
3	220	40	2.76	0.87	1.77

3	250	40	2.59	0.00	1.07
3	250	40	0.00	0.72	2.49
3	280	40	0.00	0.00	0.94
3	310	40	0.00	0.00	0.32
4	60	0	60.58	7.67	0.00
4	120	0	36.51	17.66	1.07
4	160	0	29.77	22.17	4.38
4	220	0	20.89	13.06	5.06
4	250	0	26.52	20.97	3.54
4	60	20	8.34	17.39	8.36
4	120	20	2.75	10.27	8.62
4	160	20	1.48	1.68	2.67
4	220	20	1.12	3.01	5.62
4	250	20	1.00	2.80	5.40
4	60	40	2.61	6.47	7.98
4	120	40	2.55	0.73	1.86
4	160	40	2.65	0.37	1.93
4	220	40	2.80	0.00	1.54
4	250	40	2.84	0.26	1.08
6	60	0	59.24	18.78	0.64
6	60	0	30.40	16.74	2.83
6	120	0	27.41	19.73	2.65
6	160	0	21.49	21.81	0.00
6	160	0	7.13	10.31	4.39
6	160	0	4.82	6.90	4.81
6	220	0	13.10	18.50	0.00
6	220	0	4.13	7.24	10.65
6	250	0	9.73	13.45	4.23
6	250	0	62.06	10.84	0.00
6	250	0	11.63	12.07	5.08
6	280	0	52.66	11.61	0.30
6	280	0	11.82	15.32	8.41
6	310	0	40.64	6.91	0.00
6	310	0	8.93	11.57	6.66
6	60	20	5.58	14.77	10.86
6	120	20	1.23	3.29	5.68
6	160	20	3.41	10.28	8.25
6	220	20	1.37	0.79	1.24
6	250	20	1.02	0.58	2.01
6	250	20	1.41	0.00	2.99
6	280	20	1.58	4.14	4.70

6	310	20	2.88	3.84	3.13
6	60	40	3.38	0.61	2.92
6	120	40	2.59	0.00	1.25
6	160	40	2.70	0.00	1.03
6	220	40	2.61	0.26	0.86
6	250	40	2.58	0.25	0.97
6	250	40	0.00	0.00	1.41
6	280	40	0.00	0.00	0.74
6	310	40	0.00	0.00	0.14
9	60	0	0.00	11.87	8.96
9	160	0	3.61	5.42	6.07
9	220	0	1.50	4.04	5.51
9	250	0	29.19	15.23	1.69
9	250	0	9.49	12.90	8.96
9	280	0	30.09	7.15	0.00
9	280	0	7.59	9.07	5.55
9	310	0	17.39	19.05	4.03
9	310	0	2.42	2.54	2.57
9	60	20	0.00	0.73	0.37
9	160	20	0.00	2.28	0.35
9	220	20	0.00	0.50	0.55
9	250	20	1.16	0.00	0.89
9	280	20	1.04	0.00	2.38
9	310	20	0.85	0.00	0.94
9	60	40	0.00	1.20	2.03
9	160	40	0.00	0.98	2.40
9	220	40	0.00	1.44	3.39
9	250	40	0.00	0.00	0.37
9	280	40	0.00	0.00	0.35
9	310	40	0.00	0.00	0.55

Note: All reactions at 20 °C were run at room temperature, which typically varied from 22 to 23 °C. Reactions that were performed at 0 °C typically varied between 1 to 3 °C.

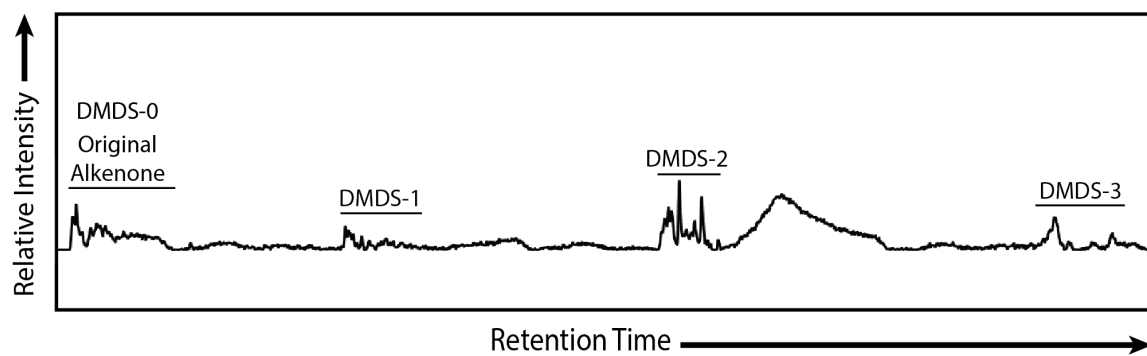


Figure S1. GC–FID results showing the original alkenone, and the DMDS adducts: DMDS-1, DMDS-2, and DMDS-3.

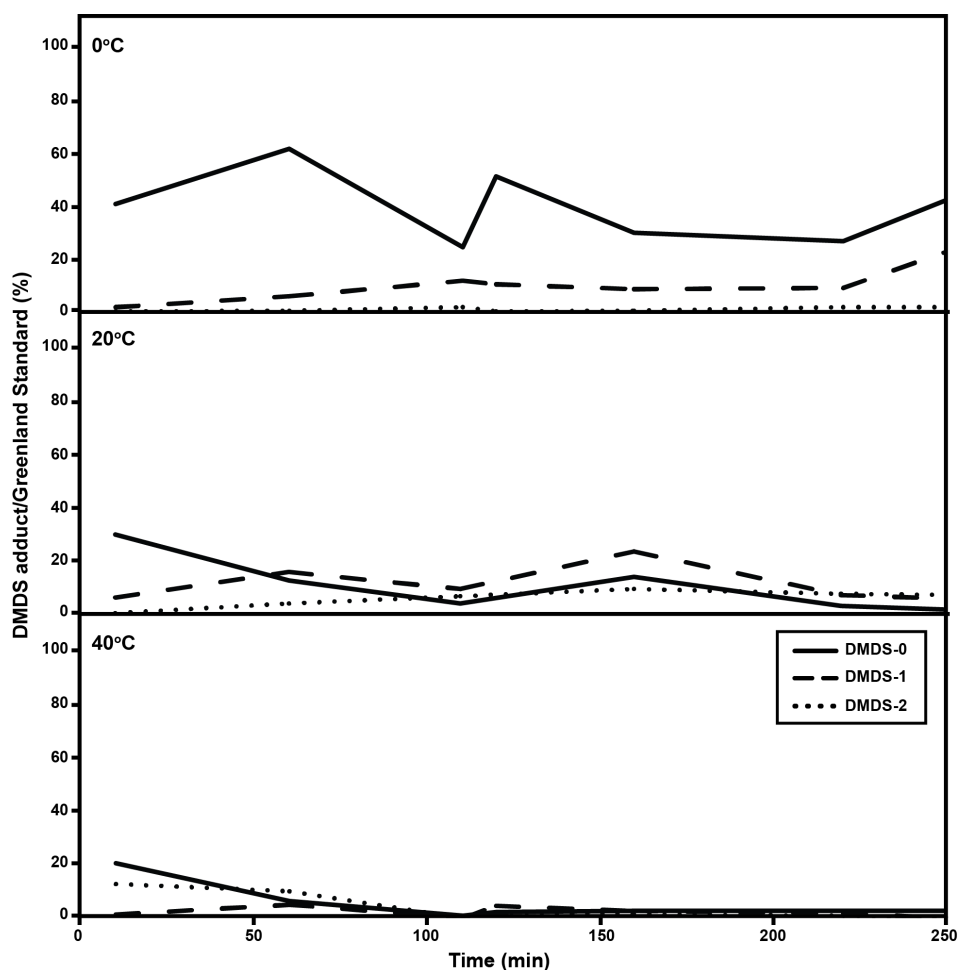


Figure S2. The graphs depict the progression of the DMDS reaction from DMDS-0 to DMDS-2 at I_2 2.4 mg for different temperatures. The original alkenones start to decrease as DMDS-1 forms; similarly DMDS-1 starts to decrease as DMDS-2 begins to form. This process occurs at a faster rate at higher temperatures and decreases at lower temperatures.

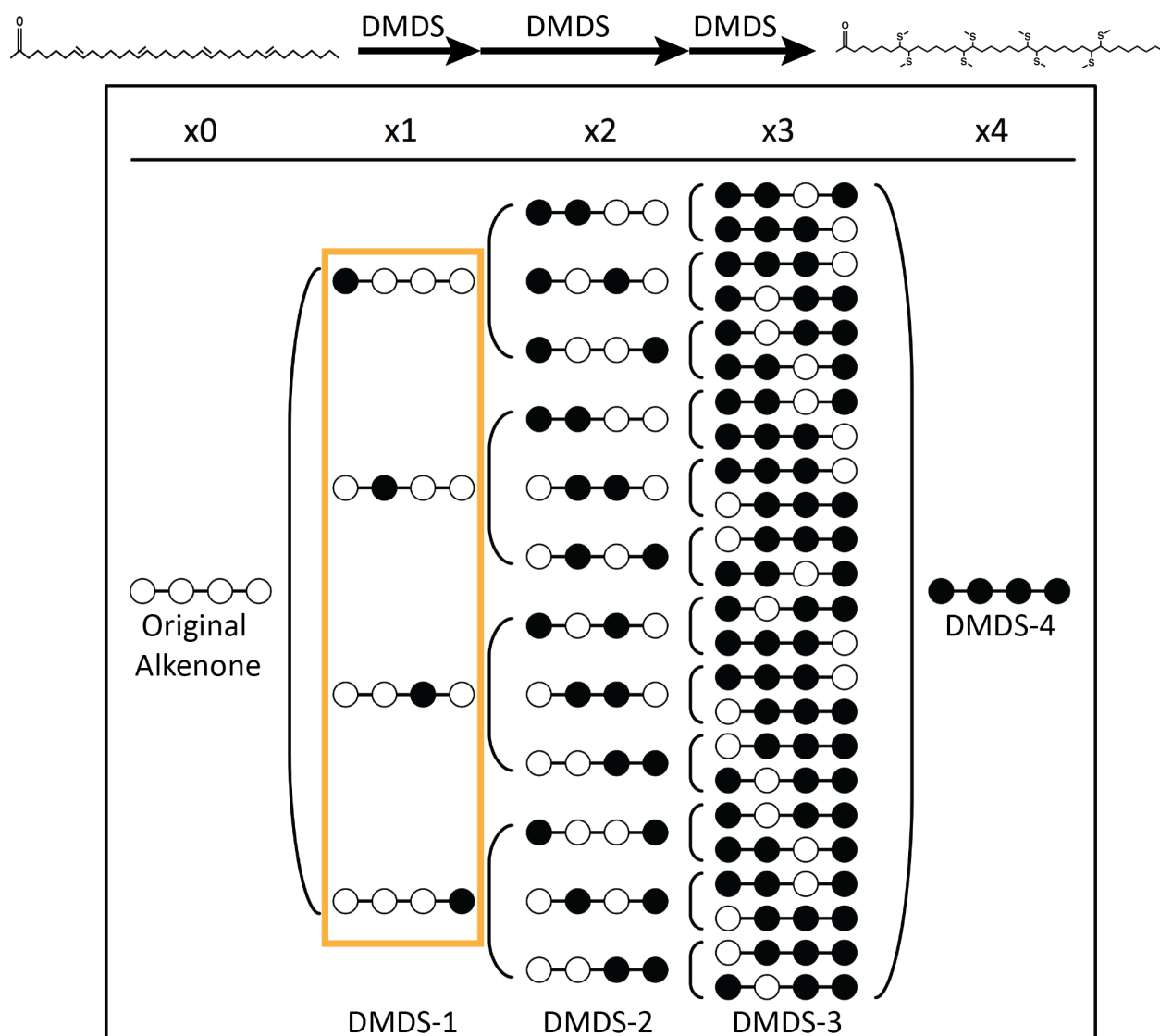


Figure S3. This figure represents DMDS adduct formation as the reaction progresses. Each dot represents an alkenone double bond, where the initial alkenone is represented by empty dots. Reaction with DMDS produces a combination of products based on where DMDS binds to the alkenone double bonds, represented by the black dots. Further reactions with DMDS lead to additional DMDS bonding with other double bonds in the alkenone. This eventually produces a molecule where all four double bonds have undergone DMDS bonding.

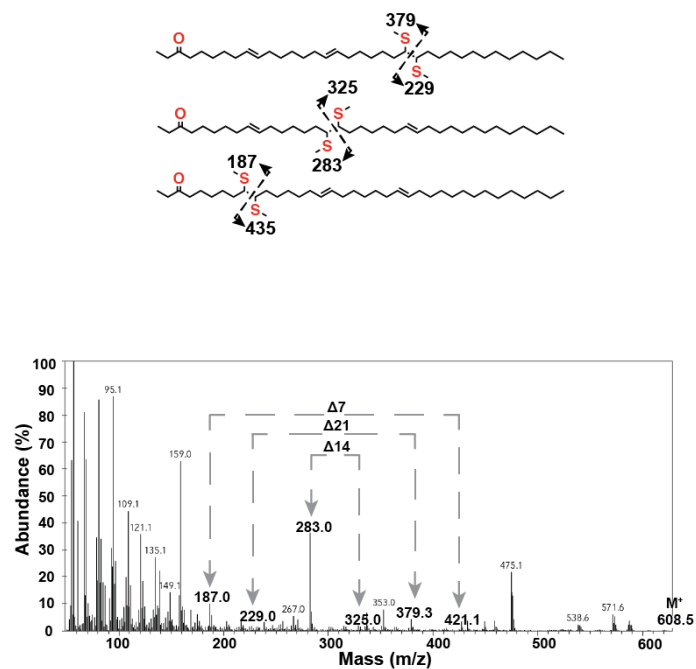


Figure S4. Mass spectrum of the $C_{38:3}$ Et DMDS derivative.

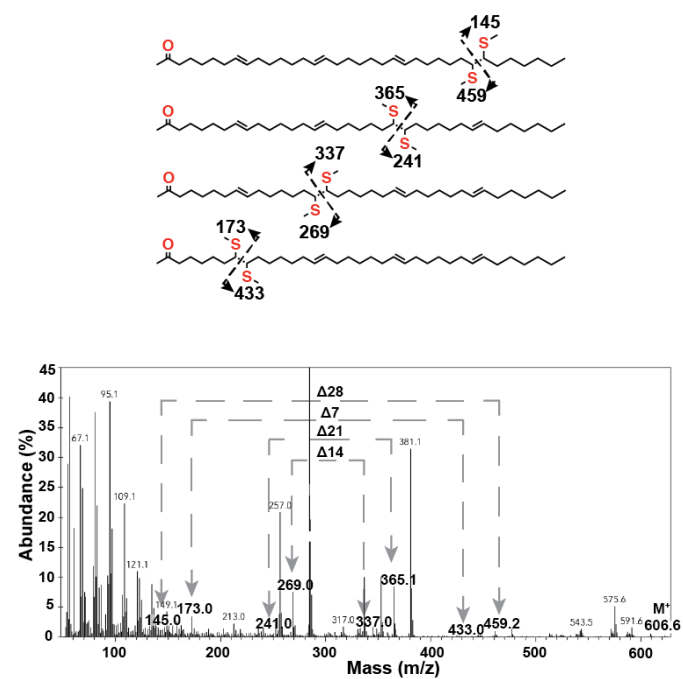


Figure S5. Mass spectrum of the $C_{38:4}$ Me DMDS derivative.

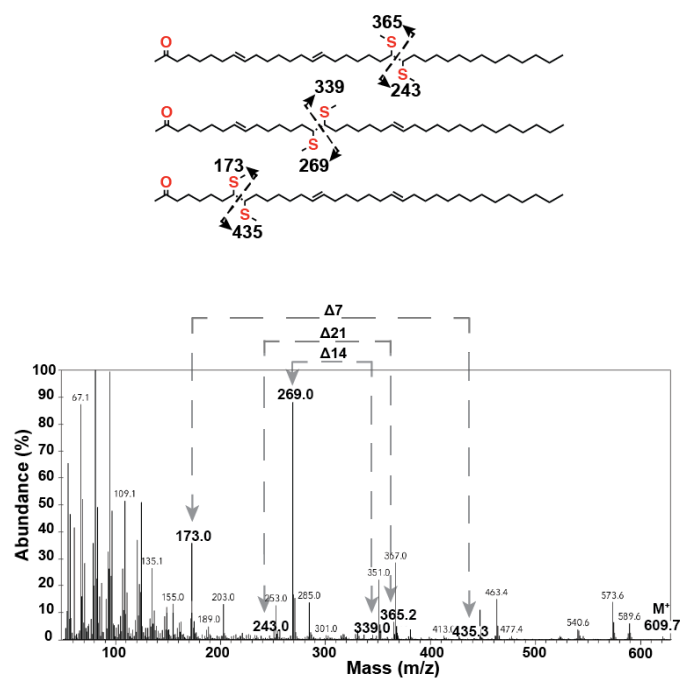


Figure S6. Mass spectrum of the $C_{38:3}$ Me DMDS derivative.

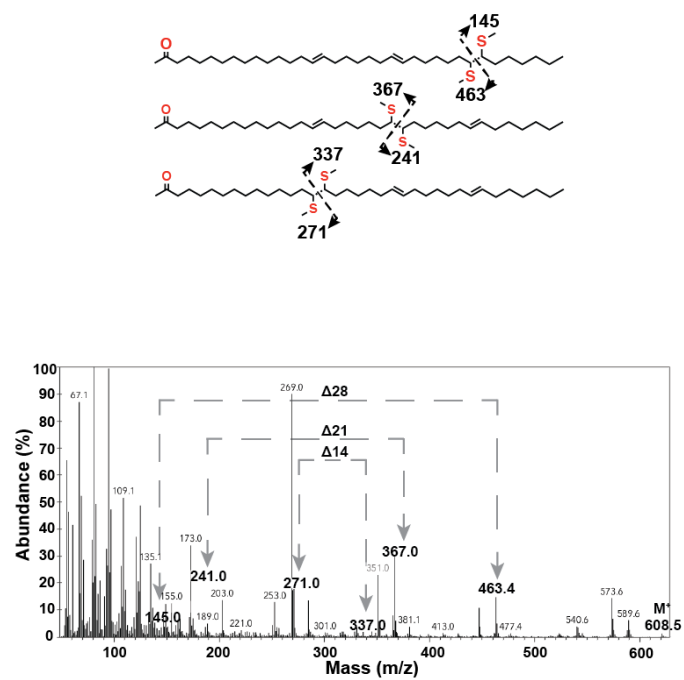


Figure S7. Mass spectrum of the $C_{38:3^*}$ Me DMDS derivative.

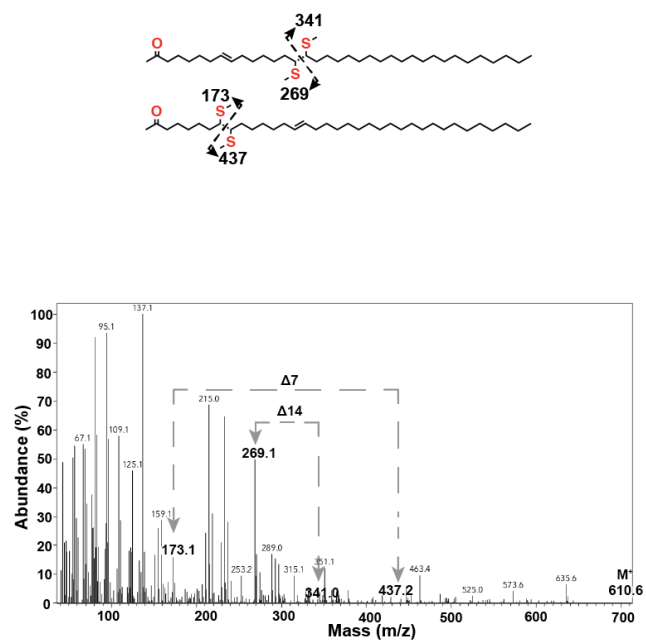


Figure S8. Mass spectrum of the $C_{38:2} \text{ Me}$ DMDS derivative.

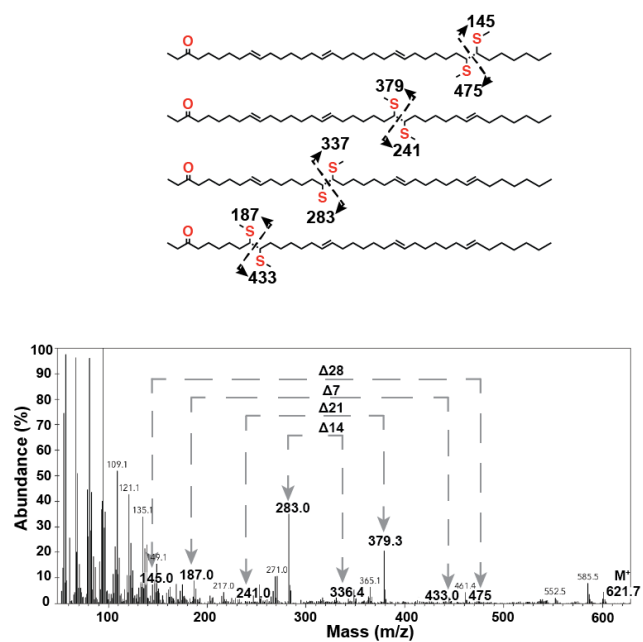


Figure S9. Mass spectrum of the $C_{39:4} \text{ Me}$ DMDS derivative.

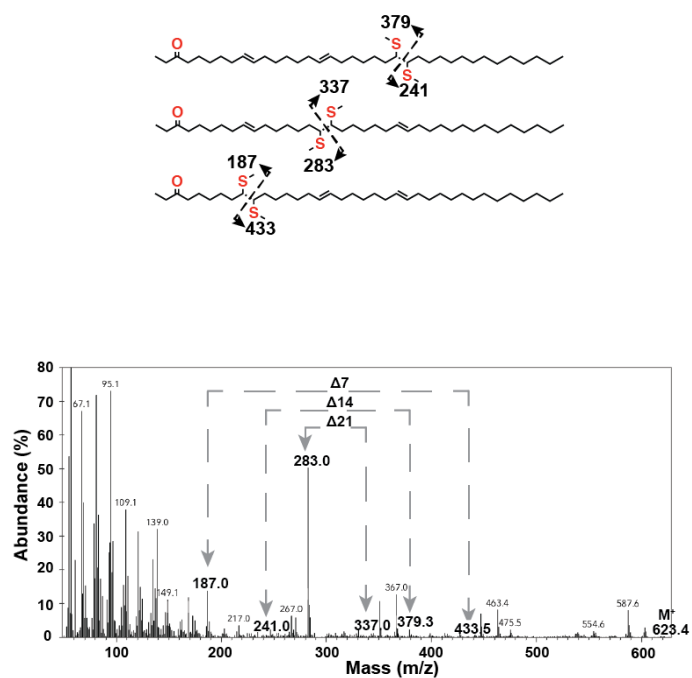


Figure S10. Mass spectrum of the $C_{39:3}$ Et DMDS derivative.

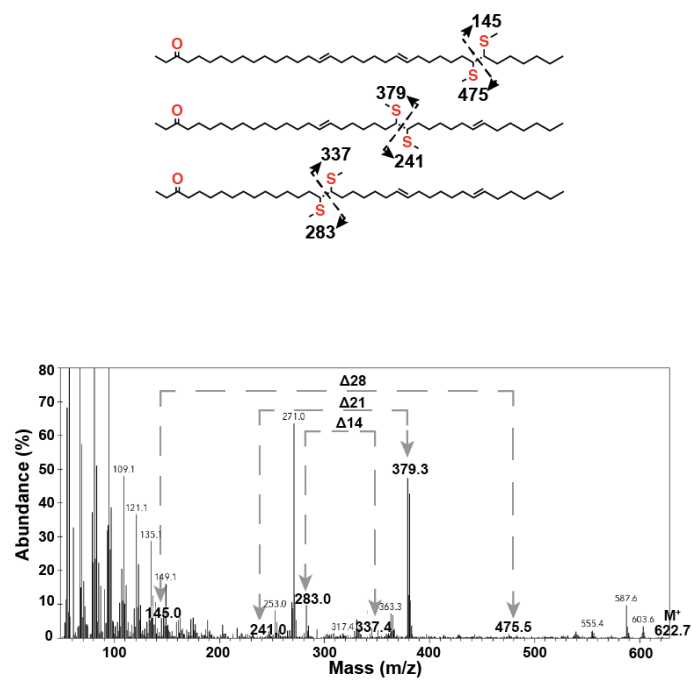


Figure S11. Mass spectrum of the $C_{39:3^*}$ Et DMDS derivative.