An Investigation into the Biomarker Potential of Highly Branched Isoprenoids in Northern Minnesota Lacustrine Sediments

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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August 2022

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List of Abbreviations

OM	Organic matter
HBI	Highly branched isoprenoid
$IPSO_{25}$	Ice proxy Southern Ocean with 25 carbons
IP_{25}	Ice proxy with 25 carbons
DRC	Democratic Republic of the Congo
Ma	Million years ago
MVA	Mevalonate
MEP	Methylerythritol
FPPS	Farnesyl pyrophosphate synthase
MIZ	Marginal ice zone
MN	Minnesota
7-NHD	7-hexylnonadecane
9-OHD	9-octyl-heptadec-8-ene
GC-MS	Gas Chromatography Mass Spectrometry
cm	Centimeter
µL	Microliter
µg	Microgram
mL	Milliliter
HPLC	High performance liquid chromatography
RPM	Revolutions per minute
g	Gram
GC	Gas chromatography
m	Meter
mm	Millimeter
µm	Micrometer
min	Minute

TIC	Total ion chromatogram/current
m/z	Mass to charge ratio
eV	Electron volts
LOD	Limit of detection
LOQ	Limit of quantification
^{1}H NMR	Proton nuclear magnetic resonance

Chapter 1. Introduction

1.1 Biomarkers & Sediments

Sediments contain mineral grains as well as decaying plant and animal components and other types of organic matter (OM) which accumulate at the bottom of a body of water. The composition of sediment is highly dependent on its location and sediment can act as a historical archive, containing information of various small- to large-scale changes that have occurred through time (Schnurrenberger, Russell, and Kelts 2002). Lacustrine, or lake, sediments tend to contain regionally specific information, while marine sediments contain a more global-scale record of environmental and climatic information (Pancost and Boot, 2004).

When specific organic compounds that can be extracted and identified are correlated to past or current environmental conditions or events, they are called biomarkers (Meyers 2003). Biomarkers are often used to create proxies, or molecular indicators for specific environmental conditions or events (Meyers 2003). the development of a proxy can include determining the biomarker abundance or isotopic composition in natural environments (Meyers 2003).

Useful biomarkers must be resistant to degradation in the water column and sediments, must be derived from a single identifiable source or be indicative of a known process, and must have a unique molecular structure that is easily detectable. For example, many lipids produced by microalgae are used as biomarkers to determine the source of OM contributions to sediments (Volkman 2018). However, a significant problem with the use of organic biomarkers is the possibility of alteration or degradation before collection and analysis (Cranwell 1981). Proteins and nucleic acids are degraded faster than carbohydrates, as they contain large amounts of desirable nutrients, such as nitrogen and phosphorus (Mylotte et al. 2016; Bianchi and Canuel 2014). Thus, molecules with low concentrations of nutrients, like lipids and hydrocarbons, are preferentially retained in the water column and sediments (Mylotte et al. 2016; Bianchi and Canuel 2014). Lipids are often used as biomarkers since the energy required to break

carbon-carbon bonds is relatively high and they lack nutrients beyond carbon (Bianchi and Canuel 2014).

1.2 Highly Branched Isoprenoids

Isoprenoids are branched hydrocarbons derived from isoprene subunits (Figure 1) and are commonly found in plants, animals, and bacteria. All isoprenoids are classified as prenol lipids, which is any lipid synthesized through the condensation of isoprene subunits. Prenols share structural and functional similarities to sterols, another category of lipids, which includes compounds like squalene and cholesterol (Fahy et al. 2005). Isoprenoids are classified as either "regular" or "irregular". Regular isoprenoids are acyclic, fully saturated, contain a methyl group (CH_3) branch bonded to every fourth carbon atom, and are formed by head-to-tail linkages of multiple isoprene subunits (Bianchi and Canuel 2014). Irregular isoprenoids are commonly formed from either head-to-head or tail-to-tail linkages and can have cyclic structures (Bianchi and Canuel 2014).

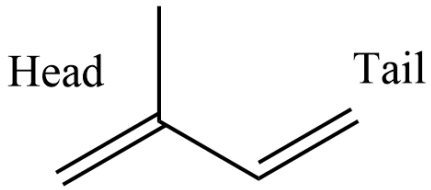

Figure 1: Individual isoprene subunit

Isoprenoid lipids have a variety of intracellular functions ranging from structural lipids contained within the cell membrane to aiding in cellular stress response (Lipko and Swiezewska 2016). However, the specific cellular location and biological functions of highly branched isoprenoids (HBIs) have not been identified although several hypotheses exist. One hypothesis is that HBIs aid in maintaining cell membrane fluidity as structural lipids, implying that HBIs are located within the plasma membrane (Rowland et al. 2001). This hypothesis is supported by reports that the degree of unsaturation in HBIs is influenced by growth temperature (Rowland et al. 2001). Optimal conditions for HBI production have not been determined, however Rowland and colleagues showed that increasing the number of *cis*-double bonds above two in an HBI molecule result in a decreased melting point of the molecule, which supports the previous hypothesis since most double bonds synthesized by biological life are *cis* (Rowland et al. 2001). An alternative, less studied hypothesis is that HBIs may function similarly to antioxidants,

which protect the cell against highly reactive radical oxygen species (Rontani et al. 2011).

HBIs are diatom-sourced, irregular isoprenoids that have been used as proxies for paleoenvironmental sea ice reconstructions (Belt et al. 2007; Belt and Müller 2013; Belt 2018) and are currently under investigation for other uses (Brown et al. 2018; Rowland et al. 2001). HBIs were first reported in 1988, where high concentrations of one specific HBI sourced from sea ice diatom communities, later termed $IPSO_{25}$ (Ice Proxy Southern Ocean with 25 carbon atoms) (Figure 2a), was identified by Nichols and colleagues (1988). This discovery led to the identification of a suite of diatom produced HBIs, such as the Arctic Sea ice proxy, IP_{25} (Ice Proxy with 25 carbon atoms) (Belt et al. 2007; Figure 2b).

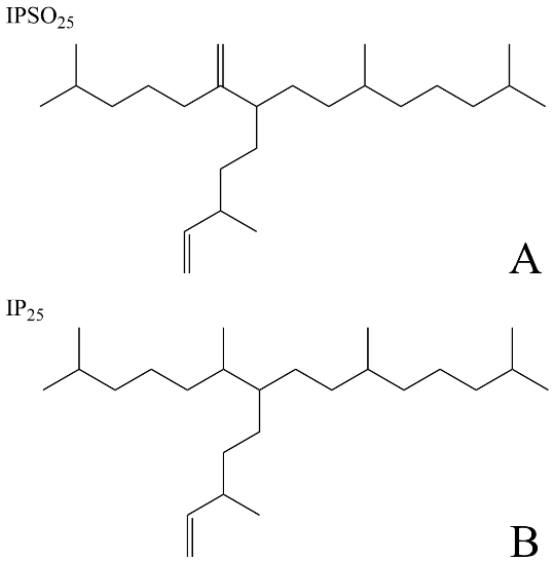

Figure 2: Structures of $IPSO_{25}$ (A) and IP_{25} (B)

Highly branched isoprenoids are mostly formed by head-to-tail condensation similar to regular isoprenoids but contain one tail-to-tail linkage, forming a large "T" shaped branch (Volkman 2018; Figure 3). HBIs are synthesized through the addition of an isoprenyl, geranyl (two isoprene subunits), farnesyl (three isoprene subunits), or geranylgeranyl (four isoprene subunits) pyrophosphate to the sixth carbon of a farnesyl

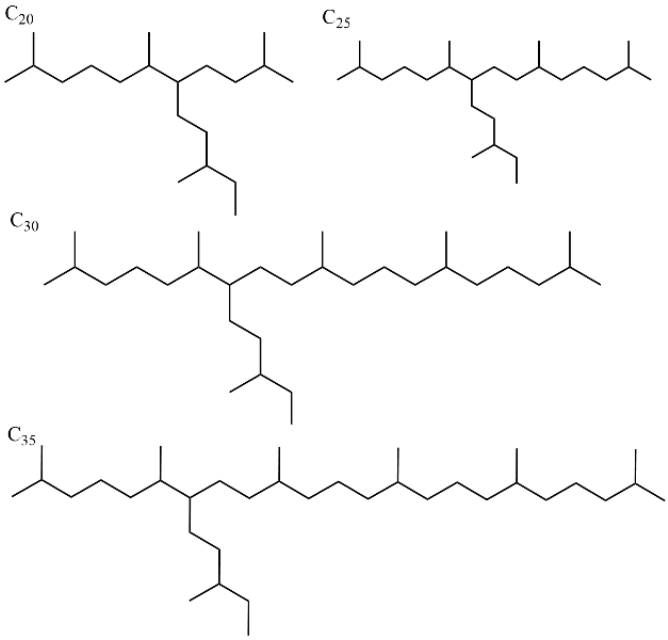

Figure 3: Parent skeletal structures of the four major types of HBI

pyrophosphate backbone, which results in C_{20}, C_{25}, C_{30}, or C_{35} HBIs, respectively (Belt et al. 2007; Ferriols et al. 2015; Figure 3). HBIs are often the dominant class of alkenes found in sediments and can also occur as alkenoic acids (Bianchi and Canuel 2014).

C_{20} HBIs are one of the least studied classes of HBI, with only one saturated and two monounsaturated isomers being characterized to date (Hird et al. 1992; Aichner et al. 2010; Corcoran et al. 2020), two additional methylated C_{20} HBIs have also been identified (a C_{21} and a C_{22}, Kenig et al. 1990). No definite source for C_{20} HBIs has been described, but C_{20} HBIs were first identified in lagoonal seagrass samples in Abu Dhabi (Kenig et al. 1990) and later in sediment samples from Lake Koucha in the Northeastern Tibetan Plateau (Aichner et al. 2010).

C_{25} HBIs, known as haslenes, are the most characterized and among the most abundant class of HBI isomers. The name haslene is derived from the diatom species *Haslea ostrearia*, that was first found to synthesize C_{25} HBIs (Volkman et al. 1994). Most haslenes occur in a range from fully saturated alkanes to penta-unsaturated alkenes (He et al. 2016; Belt et al. 2017), however, one hexa-unsaturated haslene has been identified in a laboratory culture of *H. ostrearia* (Wraige et al. 1997). Two C_{25} sea ice diatom biomarkers have been identified, a monoene (IP_{25}) and diene ($IPSO_{25}$) that are both used widely in paleoenvironmental sea ice reconstructions (Belt et al. 2007; Volkman et al. 1994). IP_{25} has been found to be produced by several diatom species in the Arctic Ocean including *Haslea ostrearia, Haslea kiellmanni, Haslea crucigeroides/spicula,* and *Pleurosigma stuxbergii* (Brown, Belt, and Cabedo-Sanz 2014), while $IPSO_{25}$ has only been found to be produced by a single species of diatom in the Southern Ocean, *Berkeleya adeliensis* (Belt et al. 2016).

C_{30} HBIs, or rhizenes, are the second most reported HBI isomer and are named after the diatom species *Rhizosolenia setigera*. First identified by Yon and colleagues in 1982, only four distinct C_{30} HBIs have been characterized, all of which are from laboratory cultures (Belt et al. 2001; Rowland et al. 2001; Yon et al. 1982). It is likely that more C_{30} isomers are present in sediment since published reports of uncharacterized rhizenes from sediment samples exist (Massé et al. 2004; Belt et al. 2002; Rowland et al. 2001).

Other classes of diatomaceous HBIs have been reported. The presence of C_{35} HBIs has been confirmed in the Indian Ocean (Hoefs et al. 1995). Certain cyclic HBIs have been observed in culture, synthesized by specific strains of *R. setigera* (Belt et al. 2003). Bicyclic HBIs may exist, as there are reports of bicyclic molecules eluting alongside HBIs in the analysis of sapropels, however no structures have been fully characterized (Bosch et al. 1998; Rinna et al. 2002).

Substituted HBIs can also naturally occur, likely resulting from OM processing and degradation in HBI-containing sediments. In several reported instances, methyl cleavage occurred during degradation and is hypothesized to be responsible for the identification of a C_{23} HBI monoene reported in Lake Tanganyika, DRC (Simoneit, Aboul-Kassim, and Tiercelin 2000) and a C_{29} HBI reported in Northern Italy (Schwark et al. 2009). Early degradation of HBIs in sediments can result in defunctionalization, including removal of double bonds, leading to a higher prevalence of saturated HBIs in sediments (Bianchi and Canuel 2014). Polyunsaturated HBIs have been reported to undergo cyclisation and double bond isomerization reactions under specific simulated conditions aimed at replicating mildly acidic clays (Belt et al. 2000). Other substituted HBIs resulting from diagenetic activity are thiophenes, sulfur-containing aromatic HBIs, and thianes, sulfur-containing cyclic, nonaromatic HBIs (Sinninghe Damsté et al. 1989; Sinninghe Damsté et al. 2007). Novel HBIs continue to be and likely have yet to be discovered, especially in freshwater systems, where fewer studies have taken place.

1.3 Diatoms, the Source of HBIs

The first evidence of diatoms, a group of photosynthetic, eukaryotic phytoplankton, has been dated as early as the Jurassic period (145 Ma) (Sinninghe Damsté 2004) and they have since become ubiquitous throughout the world's marine and freshwater systems (Dixit et al. 1992). Individual bodies of water are likely to contain more than 100 species of diatom and over 1000 species are commonly found in North America (Dixit et al. 1992). Diatoms are classified as benthic or planktonic, indicating the aquatic region where they are found. Benthic diatoms are bound to a solid medium, while planktonic diatoms are free-floating in the water column (Dixit et al. 1992). Diatoms provide

nutrition for many organisms in the ecosystems where they are found, such as lakes, oceans, rivers, wetlands, and soils as long as there is sufficient light (Dixit et al. 1992). Diatoms are known to be the primary producers of HBIs (Belt et al. 2001) and have been reported to fix approximately one-fifth of the world's carbon dioxide (Armburst 2009).

Many genera of diatoms have been found to produce HBIs, including several species within the *Haslea* genus (Massé et al. 2004; Massé 2003). C_{25} and C_{30} HBIs have been directly sourced to specific species of diatoms, as described above (Volkman et al. 1994). Haslene biosynthesis first evolved in *Haslea* diatoms 90-93 Ma, and later independently evolved later in other diatom species (Sinninghe Damsté et al. 2004). Rhizene synthesis evolved later, emerging approximately 3 Ma in some *Rhizosolenia* species (Sinninghe Damsté et al. 2004). The source of C_{25} and C_{30} HBIs has not been definitively identified although C_{35} biosynthesis is likely to have evolved more recently than 3 Ma (Hoefs et al. 1995; Burns et al. 2010).

Isoprenoid biosynthesis is known to follow two pathways, the mevalonate (MVA) and methylerythritol (MEP) pathways (Ferriols et al. 2015; Massé et al. 2004). The specific pathway used is variable between diatom species and the specific stage of life the diatom is in. For example, *R. setigera* mainly uses the MVA pathway when synthesizing haslenes and rhizenes (Massé et al. 2004), however, at certain points in its life cycle, *R. setigera* utilizes the MEP pathway for C_{25} and C_{30} biosynthesis (Massé et al. 2003). Both pathways occur in eukaryotes, but the MVA pathway occurs in the cytoplasm of the cell, while the MEP pathway occurs in the chloroplasts (Lombard and Moreira 2011). The full synthesis pathways for HBIs have not been identified, but several potential synthesis mechanisms have been proposed and key enzymes in HBI synthesis have been identified. These enzymes include farnesyl pyrophosphate synthase (FPPS) and 3-hydroxyl-3-methylglutaryl-CoA reductase (Ferriols et al. 2015; Massé 2003; Massé et al. 2004).

The variable with the greatest impact on the class of HBI produced in cultures is the genotype of the HBI-producing diatom (Rowland et al. 2001; Ferriols et al. 2017). *R. setigera* is one example of this, as some cultured strains produce a single pentaunsaturated C_{25} while other strains of *R. setigera* produce a multitude of C_{25}, C_{30}, and monocyclic sesterpenoids and triterpenoids (Belt et al. 2003; Massé et al. 2004;

Ferriols et al. 2017). Life stage also has influence on the isomer of HBI a particular diatom produces. Auxosporulation, or sexual reproduction in diatoms, has been known to increase the degree of unsaturation, or increase the number of double bonds, in HBIs produced by *R. setigera* in culture studies (Belt et al. 2002).

1.4 Biomarker Potential of HBIs

HBIs have biomarker potential, as they are found globally (Dixit et al. 1992), are synthesized only by diatoms, and remain stable in sediments for long periods of time (Collins et al. 2013; Volkman et al. 1994). The first HBI isomer to be recognized as an OM source biomarker was a C_{20} identified in petroleum samples from Rozel Point, Utah (Yon et al. 1982). The biomarker potential was further realized when a C_{25} diene, $IPSO_{25}$, was discovered in diatom ice communities from the Antarctic Ocean (Nichols et al. 1988).

More recently, HBIs have been important in the development of paleoenvironmental sea ice proxies (Belt 2018; Belt et al. 2016). The two HBIs used as the basis for these proxies are IP_{25} and $IPSO_{25}$ (Figure 2). These HBIs are commonly found within the marginal ice zones (MIZ) of the Arctic and Antarctic Oceans (Belt et al. 2014; Belt et al. 2016; Nichols et al. 1988). Several studies support the use of these HBIs as MIZ proxies, including a study where IP_{25} was found in 44 of the 45 sediment samples taken from areas within the MIZ, was detected in lower concentrations in samples from open waters, and was not detected in areas of permanent ice cover (Belt et al. 2015). However, the proxy for Arctic ice cover could not rely on IP_{25} concentrations alone since IP_{25} was detected both in the open ocean and in the MIZ, so the index PIP_{25} was developed. PIP_{25} is defined as the ratio of the concentrations of IP_{25} to the abundance of a phytoplanktonic biomarker, brassicasterol, in each sample. Low levels of brassicasterol, a sterol synthesized by phytoplankton, are indicative of permanent sea ice coverage, while higher levels indicate ice-free conditions (Müller et al. 2009). When utilized together as PIP_{25}, areas of permanent ice can be distinguished from ice-free areas (Belt and Müller 2013). A high PIP_{25} indicates marginal ice cover, a low PIP_{25} occurs in open water, where no ice

forms (Belt and Müller 2013). However, low PIP_{25} and no presence of brassicasterol is indicative of permanent sea ice (Belt and Müller 2013).

Other HBI based proxies exist, such as the H-print, which is used to track food webs in the Arctic. The H-print is an index of the ratio of the concentration of dissolved HBIs in open water to the total HBI concentration in the livers of animals that live in the arctic (Brown et al. 2018). This proxy tracked the presence of ice algae-produced OM through the food web and identified the MIZ as an important, but indirect primary energy source for the arctic food web (Brown et al. 2018).

Another potential application for HBIs beyond their environmental biomarker uses is in cancer therapy. Specific isomers of HBI, namely C_{25} trienes and tetraenes, have been introduced to murine cancer cell lines to test their efficacy as an alternative to more harmful therapies. At high concentrations, the HBIs were found to be lethal to cancer cells while leaving healthy cells unaffected (Rowland et al. 2001). At lower concentrations, the HBIs did not produce these cytocidal effects but still produced cytostatic and antiproliferative effects (Rowland et al. 2001).

1.5 HBIs in Freshwater Environments

Only a single C_{20} (Figure 4. A) and six distinct C_{25} (Figure 4. B) HBIs have been reported from freshwater environments, since most HBI research has focused on marine environments (He et al. 2016; Aichner et al. 2010; Rowland and Robson 1990; Corcoran et al. 2020). The characterized structures of these six C_{25} HBIs include the fully saturated hydrocarbon, one monoene, one diene, two trienes, and one tetraene (Figure 4. B). These structures were identified through their chromatographic and mass spectral properties (He et al. 2016).

The locations of these freshwater studies vary greatly (Figure 5), having taken place in the Everglades National Park (He et al. 2016), Lake Koucha in the Eastern Tibetan Plateau (Aichner et al. 2010), three lakes in Northern England (Rowland & Robson 1990), and a lake in the Galápagos Islands (Zhang et al. 2011). The medium tested in these studies also vary, surface sediments, sediment cores, periphyton, and leaves and

roots of local plants all have been analyzed for the presence of HBIs (Corcoran et al. 2020; Aichner et al. 2010; He et al. 2016).

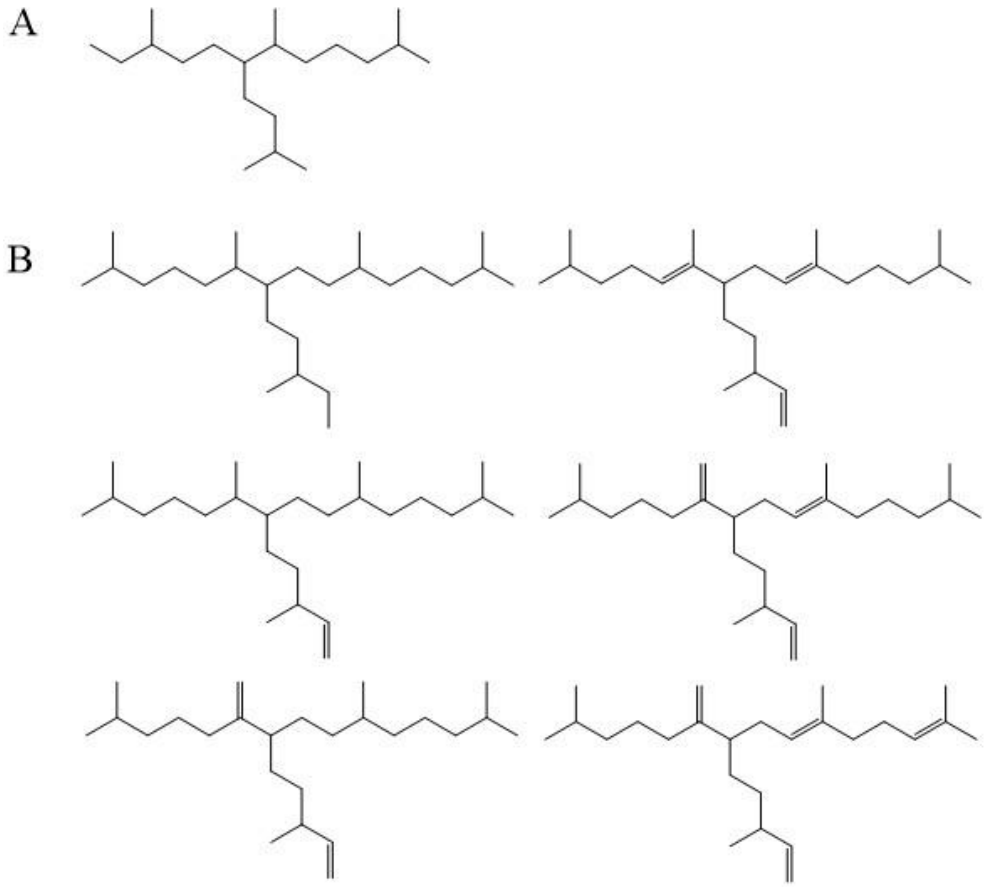

Figure 4: The structures of the structurally characterized freshwater HBIs. (A) The C_{20} structure. (B) The C_{25} structures

No previous study has focused on source identification or identification of the environmental conditions present in the sediments where freshwater HBIs are found. However, one study used stable isotope analysis (δ^2H) of HBIs to model precipitation patterns, reporting that HBIs may be good indicators of how lake systems change over time (Corcoran et al. 2020). Other studies have correlated nutrient availability or changes in the algal/ diatom communities to changes in the suite of HBIs in freshwater systems (Aichner et al. 2010; He et al. 2016). No freshwater studies have directly attempted to correlate specific sediment HBIs with specific diatom species.

Figure 5: Sites of previous freshwater HBI studies are marked (Aichner et al. 2010; Brooks et al. 1977; Corcoran et al. 2020; He et al. 2016; McKirdy et al. 2010; Yon, D. A. 1981; Zhang et al. 2011). Lighter points indicate studies focused on coastal areas, while darker points indicate inland studies

However, one study has identified several species of diatom present alongside HBIs in a marine sediment core from a Florida Bay, near the Everglades (Xu et al. 2006). The species identified include *Nitzschia semirobusta* and *Fragilaria synegrotesca*, though the HBIs had stronger correlations with the abundances of *Cyclotella distinguenda* and *Cyclotella litoralis* (Xu et al. 2006; He et al. 2016).

1.6 Goals and Hypotheses of this Study

This study will focus on the Duluth, MN and Lake Superior North Shore area, as no freshwater HBI studies have been performed in this area or the greater Laurentian Great Lakes, and HBIs have been observed in preliminary samples taken from sites in this area (Figure 6). In addition, Lake Superior is warming faster than the air temperature and has resulted in a decrease in annual ice coverage (Austin and Colman 2008). Ice cover data for the Great Lakes only spans the timeframe of the mid 1960's to present day since most data used to reconstruct Lake Superior ice conditions is obtained through satellite imaging (Austin and Colman 2008; Assel et al. 2003). This does not allow for

comparison of current ice conditions to pre-anthropogenic ice conditions. This lack of historical data could be solved by using biomarkers, like IP_{25}, which has been previously shown to be effective in estimating the extent of paleoenvironmental sea ice (Belt 2018; Belt et al. 2016; Belt and Müller 2013). One goal of this study is to identify the presence of and partially characterize the HBI suite at each study site. Along with this, this study aims to correlate the HBIs found at each of the study sites (Figure 6) to specific freshwater diatom species also identified at each site.

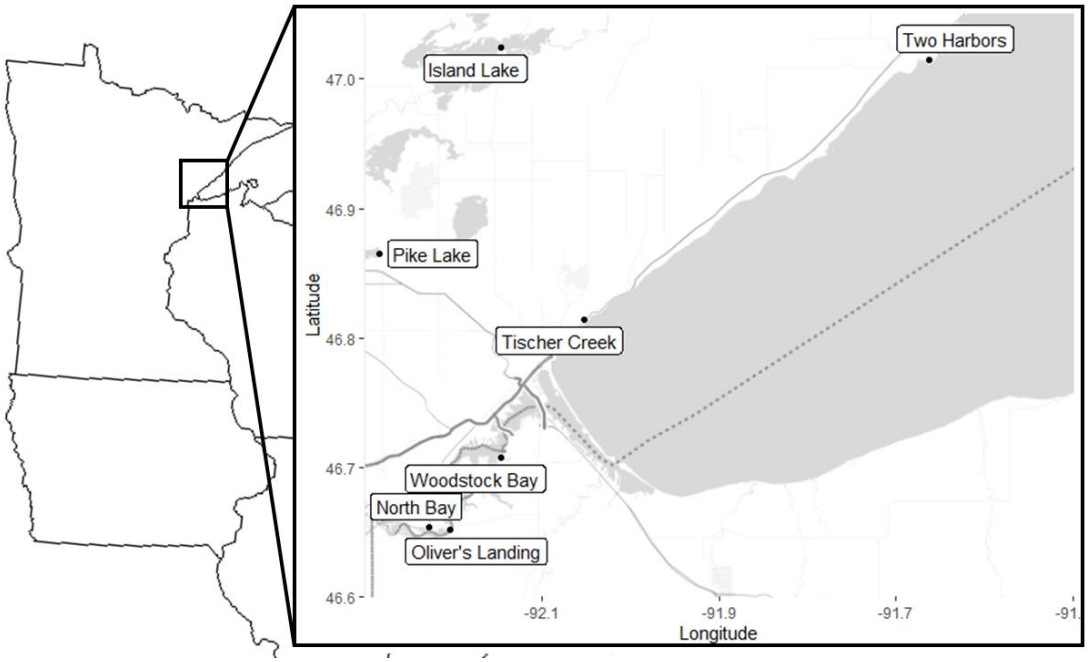

Figure 6: The sites sampled for the present study

Objective 1: **Identify the presence of HBIs and characterize the suites of HBIs produced at each study site.**

Hypothesis 1: C_{25} HBIs are the most common class of HBI produced in freshwater environments (He et al. 2016; Rowland & Robson 1990). Therefore, it is expected that C_{25} HBIs will be present in the highest concentrations, though C_{20} and C_{30} HBIs are also expected in lower concentrations.

Objective 2: **Determine whether cultured Lake Superior freshwater diatoms synthesize HBIs identical to those observed in sediments.**

Hypothesis 2: Lake Superior diatoms are hypothesized to produce HBIs and HBIs have been observed in sediments in preliminary studies of the study area. Thus, cultured diatoms collected from these study sites are hypothesized to produce HBIs identical to those found in sediments.

Chapter 2. Methods

2.1 Sample Collection

Samples were collected in the summer and winter of 2021. Summer sampling dates are as follows: Tischer Creek samples were collected on May 25; Two Harbors samples were collected on June 2; Pike Lake and Island Lake samples were collected on June 8; while North Bay, Oliver's Landing, and Woodstock Bay samples were collected on June 16. Winter sampling dates are as follows: Tischer Creek, Two Harbors, Pike Lake, and Island Lake samples were collected on November 20; North Bay, Oliver's Landing, and Woodstock Bay samples were collected on December 4. The coordinates of each study site (Figure 6) as well as water temperature readings were recorded. When water temperature was measured, the thermometer was allowed to equilibrate with the air temperature between measurements. After recording water temperature, surface sediment samples were collected into labeled, combusted glass jars. This was repeated for a total of 5 samples at each study site. Samples were placed in a cooler for transit to the lab.

2.2 Freeze Drying Sediment Samples

Sediment samples were frozen until solid at -20℃. Once solid, the lids of the jars were removed and replaced with lint-free kimwipes secured over the jars with rubber bands. Samples were then arranged in the Labconco Freezone 6 freeze dry system and freeze dried at -45℃; ≤ 100 mbar for ca. 3 days, or until all water was removed.

2.3 Homogenization of Dry Sediments

After samples were removed from the freeze drier, lids were returned to the proper jars. The samples were then individually placed in a solvent-rinsed steel vessel with ball bearings and then sealed. The vessel was then placed into a ball mill for ca. 1 minute, or until sediment appeared homogeneous. Homogeneous samples were then returned to the sample jars. The vessel and ball bearings were cleaned after each sample, first with water then methanol to ensure no OM was left on the vessel. This process was repeated for each sample.

2.4 Total Organic Extraction

Extraction followed the protocol of Belt et al (2012) with minor updates. Briefly, both 7-hexylnonadecane (7-NHD) (Figure 7) and 9-octyl-heptadec-8-ene (9-OHD) (Figure 7) were obtained for use as internal standards, allowing HBI quantification using gas chromatography-mass spectrometry (GC-MS) analysis of sediment samples. These two compounds were chosen since they do not naturally occur in sediments and have similar structures to HBIs. 7-NHD was used to determine the extraction efficiency, while 9-OHD was used to quantify HBIs present in each sample.

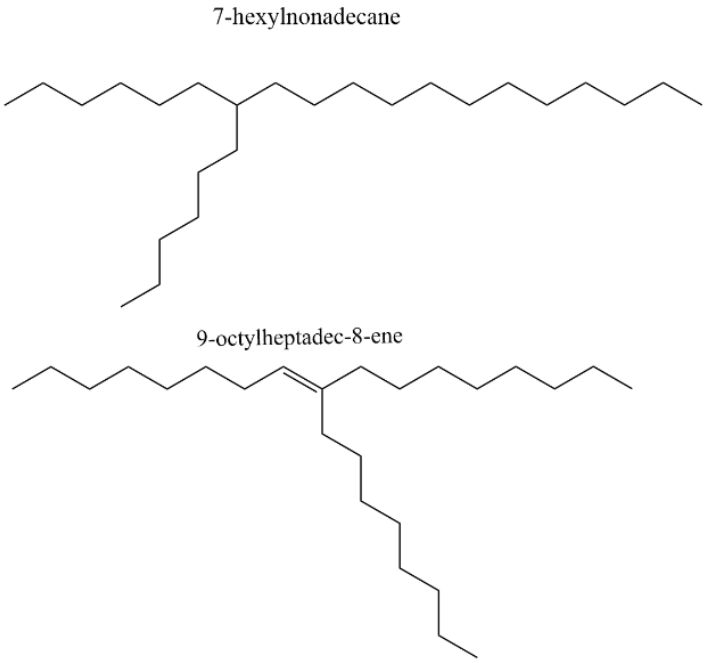

Figure 7: Structures of the internal standards 7-hexylnonadecane (7-NHD) and 9-octylheptadec-8-ene (9-OHD)

One gram of dried and homogenized sediment was transferred to tared, threaded test tubes. Enough 5% potassium hydroxide in 9:1 methanol: water to cover sediment by ca. 1 cm was added to each test tube. 100 µL of 0.1 µg mL^{-1} of 7-NHD was then added to each sample as an extraction efficiency standard. Samples were then placed in a heating block at 75°C for 1 hour. Samples were then removed from the heating block and allowed to cool to room temperature. Ca. 1 mL of HPLC grade hexane was added to each test tube. Samples were capped and vortexed for 30 seconds and centrifuged at 2500 RPM for 2 minutes and 30 seconds. The hexane layer is then transferred to fresh combusted test tubes via combusted Pasteur pipet. This liquid-liquid extraction was repeated for a total of 3 extractions. The solvent was then removed under a gentle stream of N_2 (25°C) until dry. If necessary, samples are then stored in a refrigerator.

2.5 Sample Cleanup

Samples were reconstituted by addition of ca. 1 mL hexane and vortexed briefly. The chromatography column was then prepared by placing a combusted Pasteur pipet in a test tube clamp and a small amount of combusted glass wool was used to plug the bottom of the pipet. Deactivated silica (ca. 0.5g; 60-200 µm) stored under hexane was pipetted into the column and settled by tapping the side of the column. The solid phase was then rinsed with ~3mL hexane before any sample was pipetted onto the column. The sample was then transferred to the column and the hydrocarbon fraction was eluted with hexane (~6mL) into a combusted 7mL scintillation vial. The remaining fractions (4:1 hexane: dichloromethane, 1:1 dichloromethane: methanol, methanol) were eluted into combusted 7mL scintillation vials. Solvent was then removed by a gentle stream of N_2 (25°C).

2.6 Gas Chromatography Mass Spectrometry

Hexane fractions were reconstituted in 100 µL of 0.1 µg mL^{-1} 9-OHD (ionization standard) and vortexed for 30 seconds. Samples were then pipetted into GC vials with 250 µL inserts. Samples were loaded into an Agilent 7890A GC coupled to a 5975 series mass selective detector fitted with an Agilent HP-5ms (30m x 0.25mm x 0.25 µm) column. 1 µL of each sample was injected into the GC via auto-splitless injection (300°C) with helium carrier gas (1mL min^{-1} constant flow). The total ion chromatogram (TIC; m/z 50-500 Daltons) was collected at 70eV. The GC oven was ramped from 40-300°C at a rate of 5°C min^{-1} and held at 300°C for 10 minutes.

2.7 QA/QC and HBI Identification

Before each sample set, two instrument blanks were run using the same method as the samples to ensure no contaminants from previous runs remained within the GC column. Throughout the course of the project three calibration curves of each internal standard were created, each when new dilutions of the internal standards were made, to ensure quantitative accuracy. Chromatograms of each sample were analyzed in Total Ion Current (TIC) mode and were quantified in relation to the 9-OHD internal standard (Figure 8).

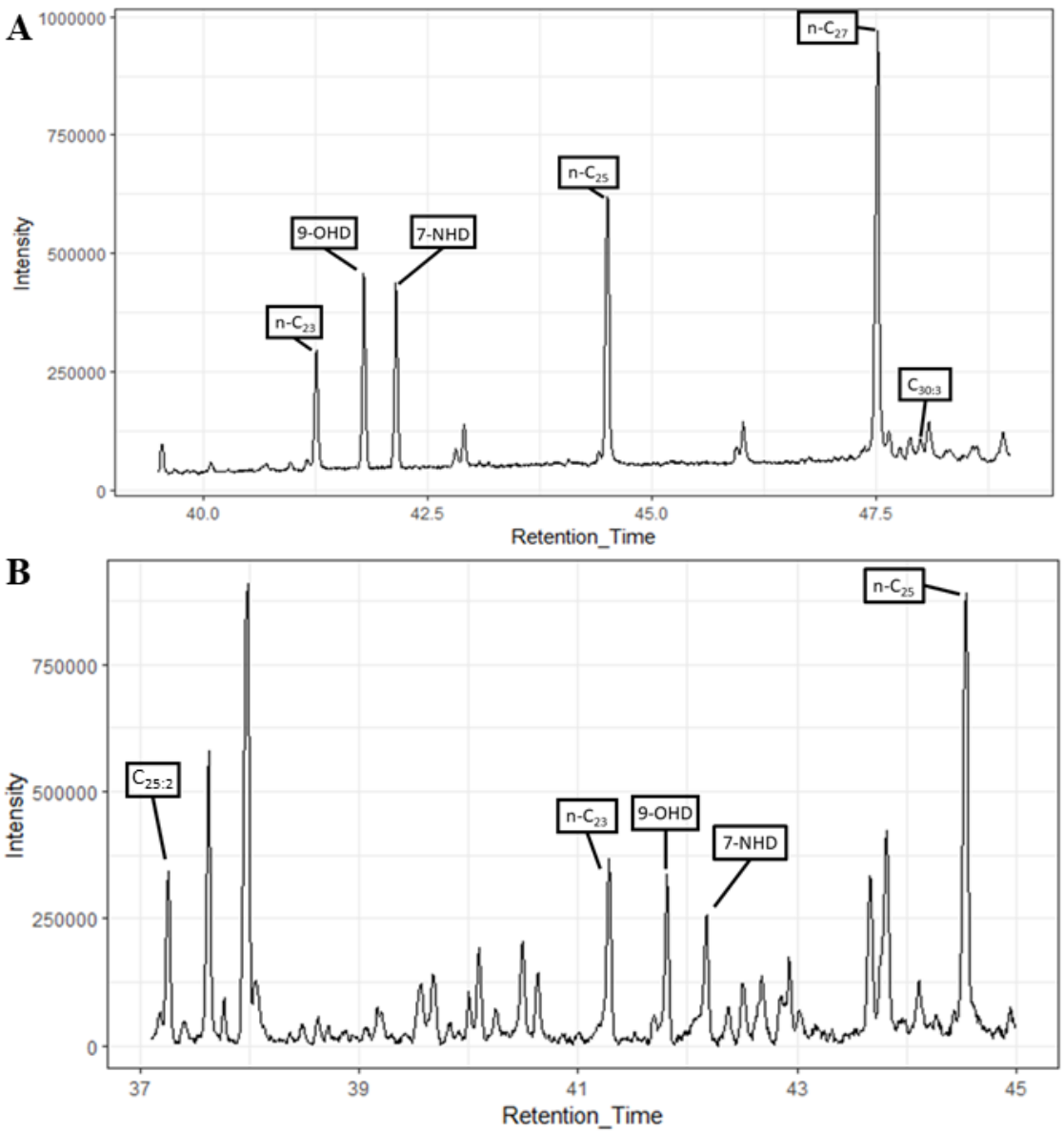

Figure 8: A) Partial chromatogram of the IL $C_{30:3}$ HBI (Figure 10, III) in relation to n-alkanes and internal standards (9-OHD and 7-NHD). B) Partial chromatogram of the TC $C_{25:4}$ HBI (Figure 10, I) in relation to n-alkanes and internal standards (9-OHD and 7-NHD)

The limit of detection (LOD) was determined by finding the ratio of the standard deviation of the lowest concentration standard to the slope of the calibration curve. This ratio was then multiplied by 3.3, resulting in a calculated LOD of 0.01 µg. The LOQ was determined by multiplying the ratio by 10, resulting in an LOQ of 0.03 µg. These values coincide with previously reported detection limits (Belt et al. 2012). Extraction efficiency was calculated based upon the area of the 7-NHD internal standard in relation to a 7-

NHD calibration curve. Extraction efficiencies of less than 90% were rerun to achieve efficiencies above 90% indicating more complete extraction.

Both 9-OHD and 7-NHD were employed to calculate and validate, respectively, the concentrations of observed HBIs. 9-OHD is widely used to quantify HBIs (Belt et al. 2014). 7-NHD has been found to aid in the consistency of HBI quantification calculations, especially when concentrations are validated with multiple laboratories (Belt et al. 2014).

Kováts retention index was calculated by taking the logarithm of the retention time of the HBI peak of interest and subtracting that by the logarithm of the retention time of the n-alkane peak just before the HBI (Kissin et al. 1986). This value is divided by the logarithm value of the retention time of n-alkane peak immediately after the HBI peak of interest, less the logarithm of the retention time of the n-alkane used in the previous step (Kissin et al. 1986).

HBI peaks were identified based upon their relation to n-alkane peaks and more characteristic mass spectral fragments. C_{25} HBIs elute before the C_{23} n-alkane (Figure 8, B), while C_{30} HBIs elute after the C_{27} n-alkane peak (Figure 8, A). The important fragments for all HBIs are the parent ion, which indicates the size and degree of unsaturation of the HBI, and the peak at m/z of 69, indicating the isoprene subunit.

2.8 Single-strain Culture Treatment

Diatoms were sampled from sediments of the Two Harbors study site on May 25, 2021. These diatoms grown and isolated into single strains of *Nitzschia* and *Fragilaria* diatoms on agar plates, then transferred to Erlenmeyer flasks for growth studies. The samples are grown in media enriched in silica (modified from Chon and Pickett-Heaps, 1988), in a Percival environmental growth chamber under 12-hour dark/light cycles for a total of 25 days. After growth curves were calculated, samples were transferred to the Large Lakes Observatory where they were placed in a refrigerator until analysis.

To analyze the single-strain samples, the Erlenmeyer flasks were first sonicated to remove the diatoms from the solid substrate of the glass. The media was then filtered

onto a preweighed glass fiber filter with a 0.7 µm pore size. The samples were then wrapped into an aluminum foil capsule and frozen for 12 hours, or until completely frozen. Samples were then transferred to the Labconco Freezone 6 freeze dry system and were freeze dried until all moisture was removed (ca. 24 hours). Samples were then reweighed to determine the amount of dry biomass present in each sample. After dry biomass was determined, samples were saponified, extracted, and analyzed in the same manner as the environmental samples.

Chapter 3. Results

3.1 Environmental Experiments

Surface sediment and periphyton samples collected during the summer and winter of 2021 were analyzed and yielded evidence of eight distinct highly branched isoprenoids in five of the seven study sites (Table 1, Figure 9). The concentrations of the observed HBIs coincide with previously reported concentrations in surface sediments, which typically range from 0.01-23 μg g^{-1} of dry sediment and have been reported in concentrations as high as 40 μg g^{-1} of sediment (Rowland and Robson, 1990; He et al. 2016; Kaiser et al. 2016). Molecular ion peaks were present in all identified HBIs, which allows for the determination of the number of double bonds for each HBI. Water temperatures ranged from 11.7℃ – 24.2℃ in the summer months and ranged from -3.1℃ – -1.9℃ in the winter.

Table 1: Location, type, and concentration of HBIs extracted from sediment and periphyton samples. Concentrations of NQ indicated that the corresponding HBI were observed with too low of a S/N to quantify. Superscripts in the Type of HBI column indicate distinct positional isomers

Location	Label in Figure 10	Type of HBI	Concentration (μg g^{-1} of dry sediment)	Season	Water Temperature (℃)	Type of Sample
Tischer Creek	I	$25:2^1$	0.9	Summer	11.7	Sediment
Island Lake	II	25:1	NQ	Summer	24.0	Sediment
Island Lake	III	$30:3^1$	0.2	Summer	24.2	Sediment
Pike Lake	IV	$30:3^2$	NQ	Summer	22.2	Periphyton
Woodstock Bay	V	30:2	0.2	Summer	21.6	Sediment
North Bay	VI	$30:3^3$	0.1	Winter	-3.1	Sediment
North Bay	VII	$25:2^2$	NQ	Winter	-3.0	Sediment
Tischer Creek	VIII	$25:2^3$	NQ	Winter	-1.9	Sediment

Evidence of distinct C_{25} HBIs were observed at Island Lake, North Bay, and two at Tischer Creek (Figure 10, HBI II, VII, I & VIII, respectively), one of which has been previously reported and was detected in the highest concentration (Figure 10, HBI I; Kaiser et al. 2016, Grossi et al. 2004). None of the other C_{25} HBIs (HBI II, VII, VIII) were present in a quantifiable abundance.

Four distinct C_{30} HBIs were observed across Island Lake, Pike Lake, Woodstock Bay, and North Bay (HBI III, IV, V, VI, respectively). One of the four C_{30} HBIs was not quantifiable.

Twenty-six diatom genera were observed across the seven study sites, and 18 of these 26 genera were observed at multiple sites (Figure 11). Every genus of diatom was present at a site where HBIs were observed; however, *Achnanthidium* and *Navicula* diatoms were present at every study site, regardless of whether HBIs were observed at the study site (Figure 11). The genera *Asterionella*, *Aneumastus*, *Ctenophora*, *Cyclotella*, *Cymbella*, *Diatoma*, *Grunowia*, *Hygropetra*, *Odontidium*, *Platessa*, *Stauroneis*, *Staurosira*, and *Tabularia* were only identified at sites where HBIs were observed (Figure 11).

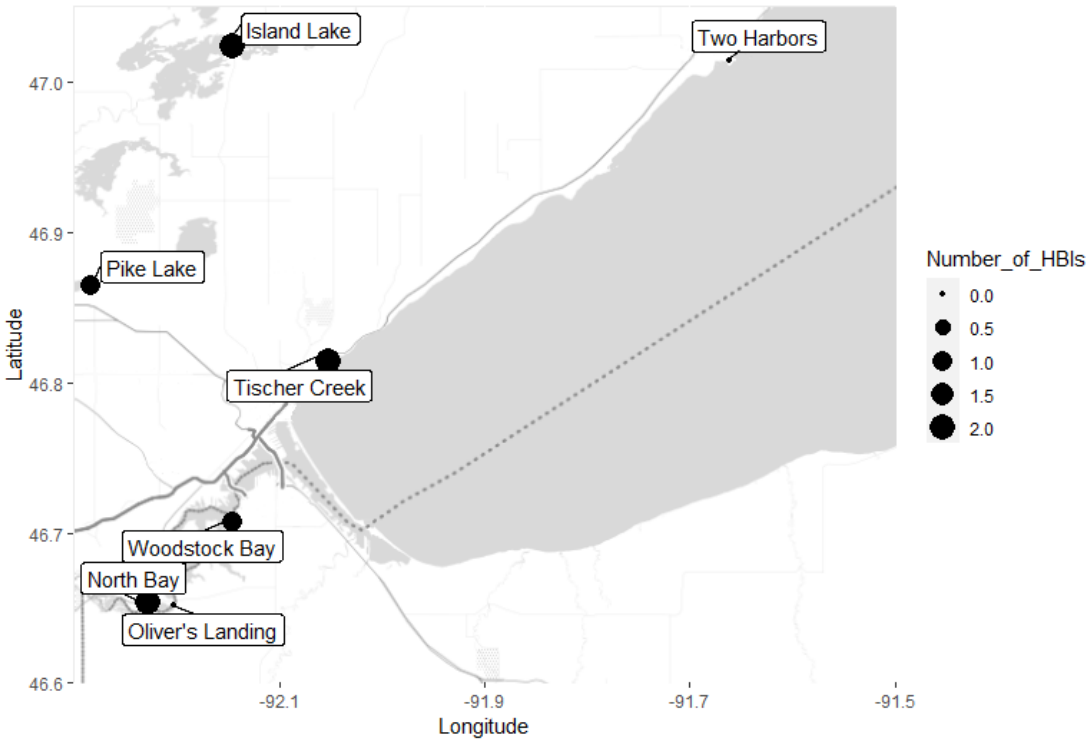

Figure 9: Map of the 6 study sites sampled. The size of the point corresponds to the number of distinct HBIs observed across all samples at the study site

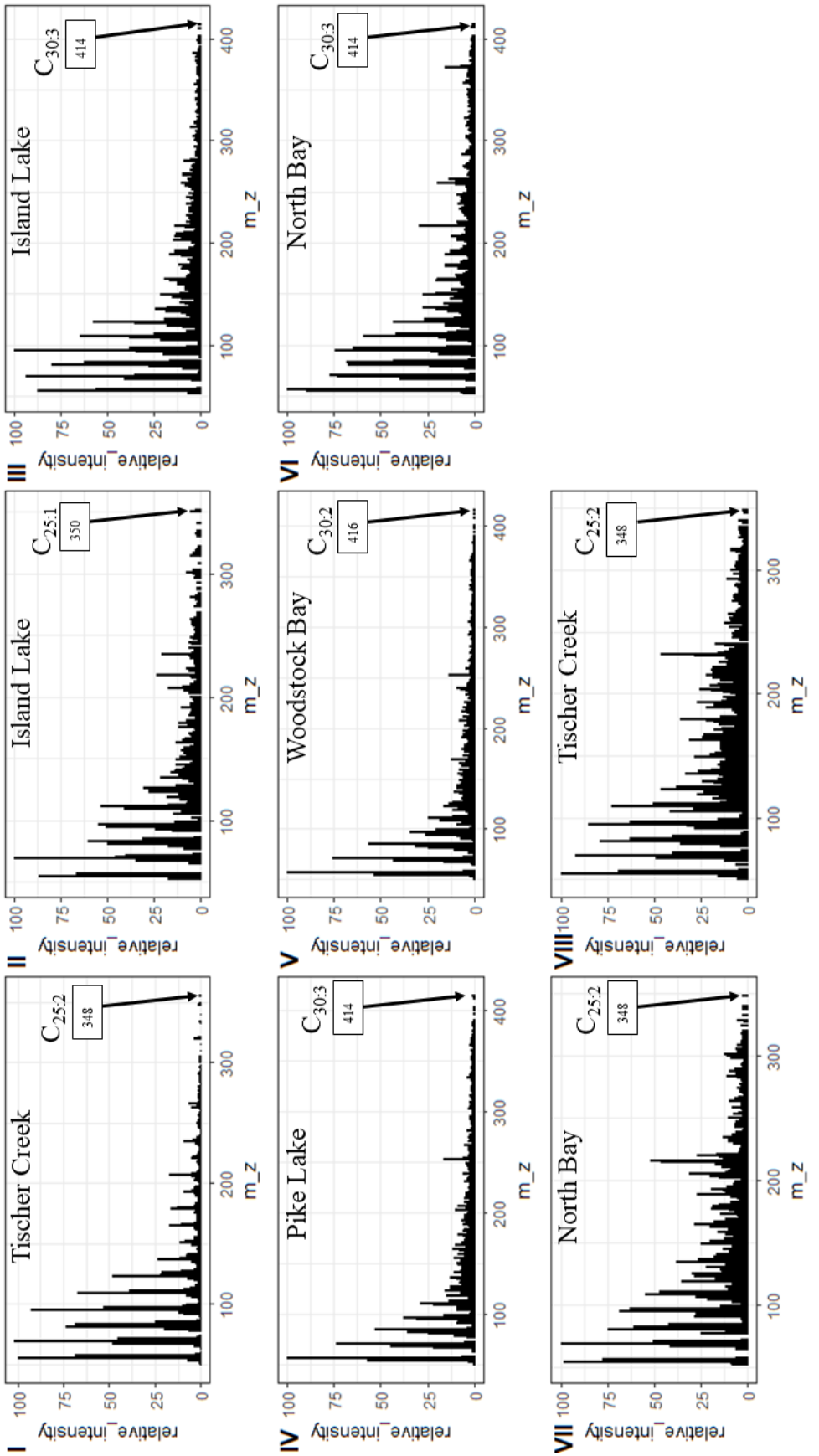

Figure 10: Mass spectra of the five HBIs identified in Summer 2021 samples. The mass spectra have parent ions at m/z (I) 414, (II) 350, (III) 414, (IV) 348, (V) 416, (VI) 414, (VII) 248, (VIII) 248

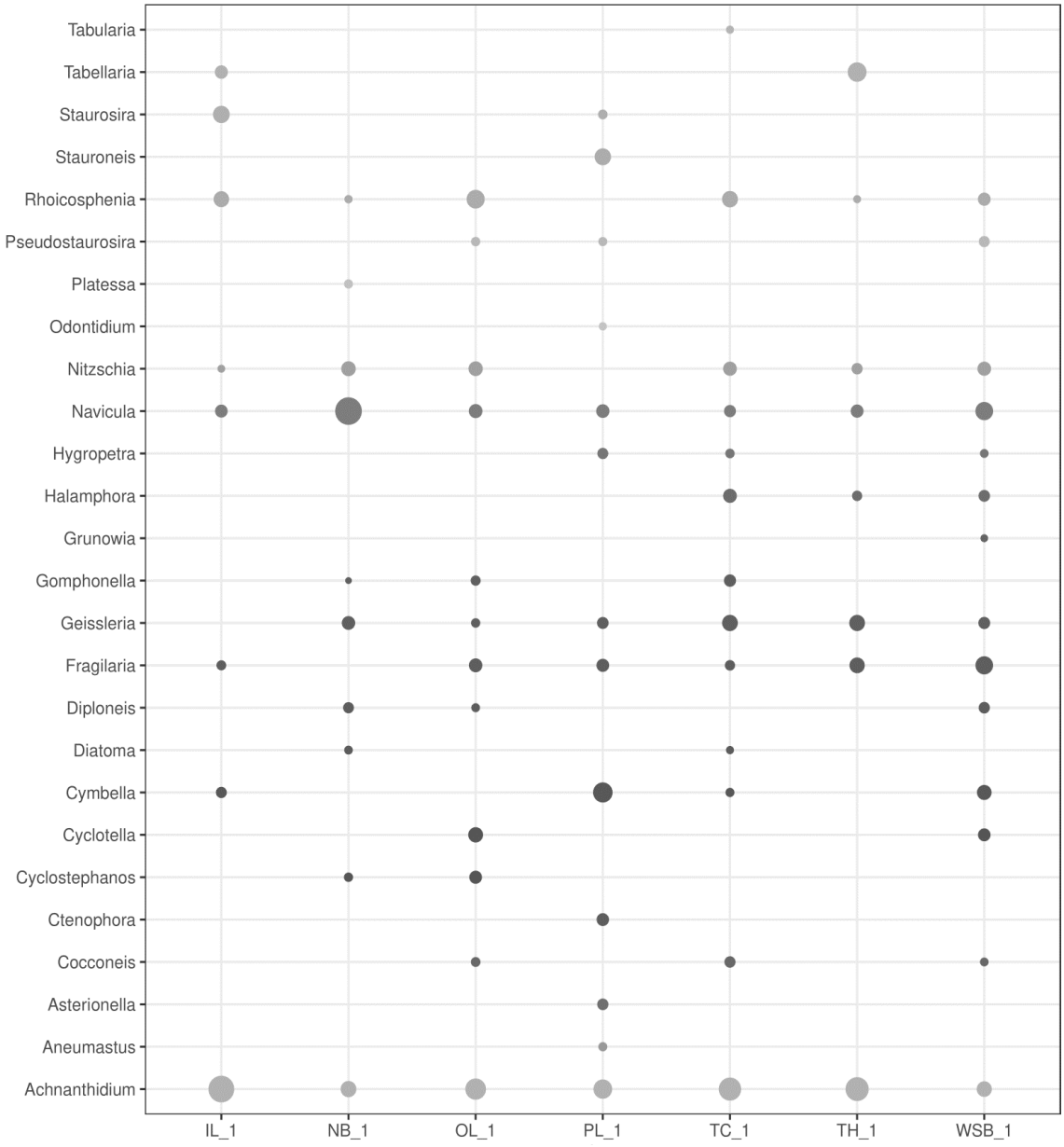

Figure 11: The locations where diatom genera were observed in the Summer of 2021. The size of each point corresponds to the relative abundance of this diatom at each site. The locations are listed are abbreviations for the following sites (from left to right): Island Lake, North Bay, Oliver's Landing, Pike Lake, Tischer Creek, Two Harbors, and Woodstock Bay

3.2 Single-strain Culture Experiments

HBIs were identified in both *Nitzschia* and *Fragilaria* diatoms (Figure 12). These diatoms were collected from Two Harbors, isolated, and grown at multiple temperatures (20℃ and 25℃) and levels of acidity (pH values of 6, 6.5, 7, 7.5, 8). Although *Nitzschia* and *Fragilaria* diatoms were isolated from a single study site, both of these genera were present at six of the seven study sites, and one or both of these genera were present at all study sites (Figure 11). *Nitzschia* was present at Island Lake, North Bay, Oliver's Landing, Tischer Creek, Two Harbors, and Woodstock Bay; *Fragilaria* was present at Island Lake, Oliver's Landing, Pike Lake, Tischer Creek, Two Harbors, and Woodstock Bay (Figure 11). HBIs were detected at temperatures of 20℃ and 25℃ and a pH of 7. Three distinct HBIs were identified in *Nitschia*, while one distinct HBI was identified in *Fragilaria* (Figure 12).

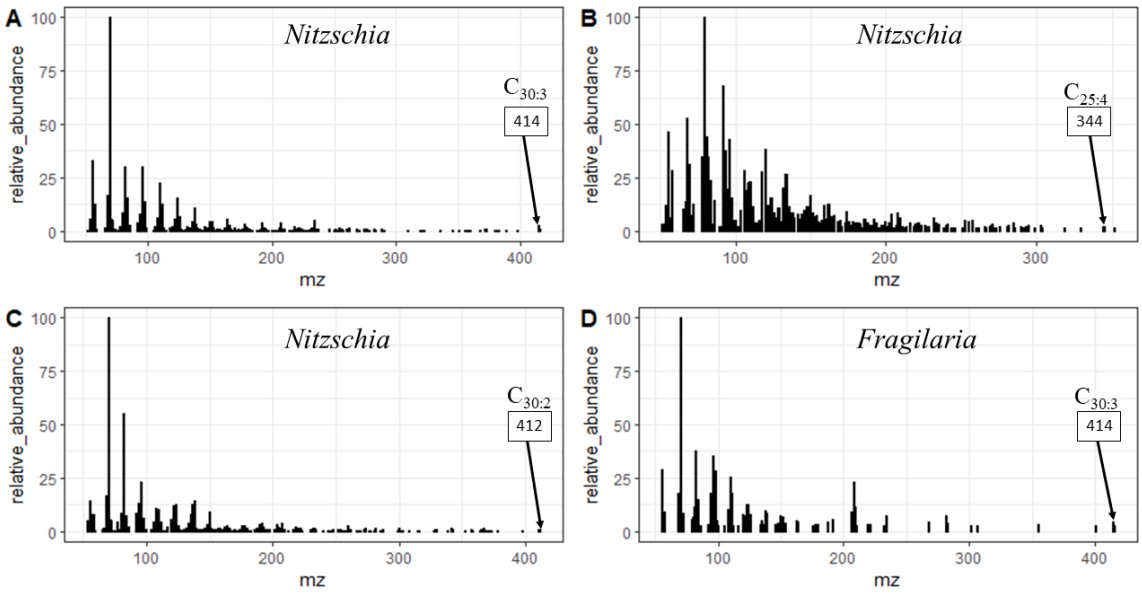

Figure 12: Mass spectra of the HBIs observed in the single-strain culture experiments. A-C were observed from Nitzschia diatoms, while D was observed in Fragilaria diatoms. The spectra had parent ions of 414 (A), 344 (B), 412 (C), and 414 (D)

Of the HBIs identified from *Nitschia*, the C_{30} HBIs had three and five degrees of unsaturation (HBI A and C, respectively), while one was a $C_{25:4}$ (HBI B). A single $C_{30:3}$ HBI (HBI D) was observed from *Fragilaria* diatoms. The $C_{25:4}$ HBI (HBI B) was observed in a concentration of 48.4 µg/g of dry biomass. None of the other culture HBIs were able to be quantified, as the internal standard was acquired after they were extracted

and analyzed. The $C_{30:3}$ HBIs (Fig 12; A & D) may be the same molecule, however further testing must be performed to verify this relationship.

Chapter 4. Discussion

4.1 Novel HBIs Found

The data collected from the present study has shown 12 distinct HBIs (Figures 10 and 12; Table 1), including HBIs identified from 5 of the 7 study sites (Figure 10) and HBIs produced directly from two common freshwater diatom genera, *Nitzschia* and *Fragilaria* (Figure 12). The environmental HBI identified in the summer at Tischer Creek (HBI I) has likely been previously described (Grossi et al. 2004; Kaiser et al. 2016). The identification of HBI I, as previously described, is based upon relative retention times and mass spectral qualities. Although HBI I eluted before the n-C_{23} alkane, which is common for C_{25} HBIs, the retention index of this HBI is distinctly different from the previously reported HBI (Grossi et al. 2004; Kaiser et al. 2016). Grossi et al reported the Kováts retention index value for this HBI as 2074, while the retention index of HBI I in the present study was found to be 2161 (Kissin et al. 1986; Grossi et al. 2004). This is distinctly different, as a retention index of 2161 typically correlates with $C_{25:4}$ HBIs (Grossi et al. 2004; Belt et al. 2000). This could be a result of a difference in GC-MS methods; however, this is unverifiable as the methods used in these studies is not reported. This difference could also indicate that HBI I is distinct and is not the HBI identified previously (Belt et al. 2000). The mass spectra of HBI I does contain similar important fragments as the previously identified HBI (Figure 13). These fragments include a base peak at m/z 69 and major fragments at m/z 207, 268, and the parent ion at 348 (Figure 13).

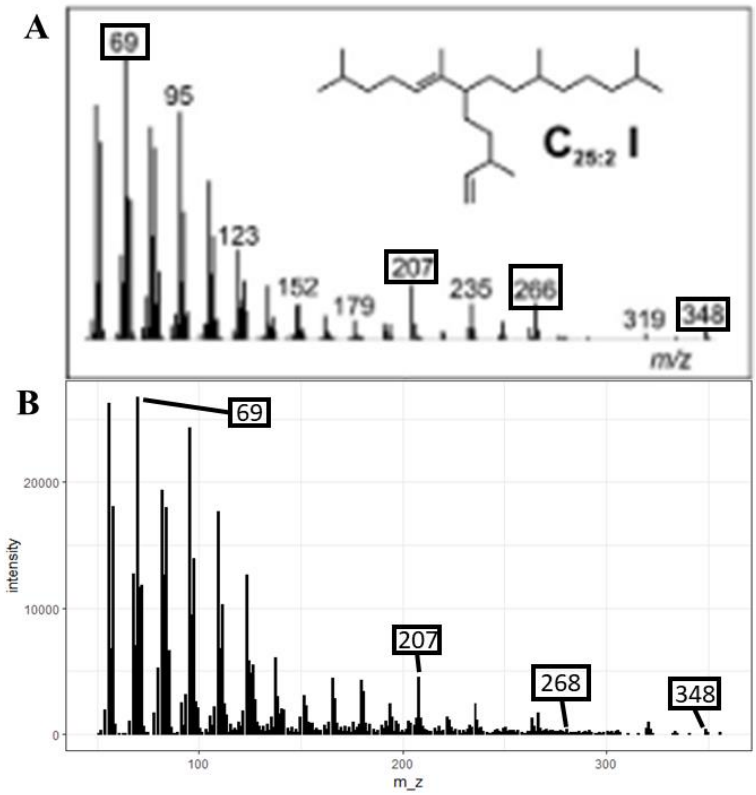

Figure 13: Mass spectra of $C_{25:2}$ HBI observed by Kaiser et al. 2016 (A) and in Tischer Creek (B). Highlighted boxes indicate shared major ions

The appearance of these common fragments suggests that HBI I is the previously identified molecule (Grossi et al. 2004; Kaiser et al. 2016) that has previously been reported in the Florida Everglades; however, additional analysis, such as proton nuclear magnetic resonance (^{1}H NMR), must be performed to confirm the locations of the double bonds in HBI I. Apart from HBI I, all other HBIs identified in this study differ in the number or position of double bonds from HBIs previously reported in the literature, potentially due to the lack of studies in the region. No other HBI identified in the present study have been reported in the literature based upon the chromatographic retention time and major fragments in the mass spectra.

All HBIs Identified in the present study are either C_{25} or C_{30} HBIs, no C_{20} or C_{35} HBIs were observed. C_{20} HBIs were originally thought to occur in younger, coastal sediments (Hird et al. 1992) until C_{20} HBIs began to be discovered farther inland in regions such as Lake Koucha and the Adirondack Mountains, where C_{20} HBI abundance dominated other observed HBIs (Aichner et al. 2010; Corcoran et al. 2020). However,

other sediment studies have observed no C_{20} HBIs and a dominance of C_{25} HBIs (Belt et al. 2017; Belt et al. 2000). Thus, the lack of C_{20} HBI isomers observed in the present study makes sense as few C_{20} HBI isomers have been observed and their source diatom has not yet been identified, especially when compared to the prevalence of C_{25} HBI isomers in the literature. C_{35} HBIs have only been identified in one study of Indian Ocean sediments, so it is not surprising that these HBIs were not observed in Northern Minnesota lake sediments.

In general, single strain cultures had a higher degree of unsaturation than the sedimentary HBIs, evidenced by the presence of only tri- and tetraenes in cultures and the presence of di- and trienes in sediments. This trend has been previously observed, where HBIs observed in diatom cultures were found to have higher degrees of unsaturation than were found in sediments (Rowland and Robson 1990; Belt et al. 2017). One of the potential explanations for this is that acyclic isoprenoids, such as HBIs, are initially synthesized as poly-unsaturated lipids (Quinn et al. 1989 in Rowland et al. 2001). In addition, double bonds are removed during early degradation in sediments, which results in more saturated sedimentary hydrocarbons (Bianchi and Canuel 2014).

Several of the identified HBIs contain the same number of carbons and double bonds. These include four $C_{30:3}$ HBIs and three $C_{25:2}$ HBIs (Figures 10 and 12, Table 2). The presence of multiple HBIs of the same size and level of unsaturation can be explained by positional or geometric isomerism, especially since the fragmentation patterns are similar and identical parent ions are observed at m/z 414 and 348 (for $C_{30:3}$ and $C_{25:2}$ HBIs, respectively; Table 2) (Belt et al. 2001). This indicates that these HBIs are likely distinct isomers of each other, which vary only in the location of the double bonds. Overall, trienes are the most common across the observed environmental and cultured HBIs. This corresponds well to reports of HBIs in the Florida Everglades and a Florida sediment core, where di- and trienes were the most prevalent HBIs (He et al. 2016). In other freshwater environments, di- and monoenes dominate (Aichner et al. 2010; Corcoran et al. 2020; Xu et al. 2006).

Table 2: Summary of the important mass spectral fragments from all $C_{25:2}$ and $C_{30:3}$ HBIs reported in the present study and previously reported in the literature. Bolded ions indicate the parent ion of the molecule

Source	Type of HBI	Major ions
Tischer Creek - Summer	$C_{25:2}$	69, 95, 123, 137, 151, 207, 235, 266, 268, **348**
North Bay	$C_{25:2}$	69, 95, 123, 137, 151, 179, 205, 215, 266, 288, **348**
Tischer Creek - Winter	$C_{25:2}$	69, 77, 95, 109, 123, 137, 149, 179, 203, 215, 266, **348**
Belt et al. 2012	$C_{25:2}$	69, 137, 207, 235, 266, 320, **348**
Kaiser et al. 2016	$C_{25:2}$	69, 95, 123, 152, 207, 235, 266, **348**
Grossi et al. 2004	$C_{25:2}$	69, 95, 123, 152, 179, 207, 235, 266, **348**
He et al. 2016	$C_{25:2}$	69, 95, 123, 151, 165, 207, 235, 266, 320, **348**
Xu et al. 2006	$C_{25:2}$	235, 266, 219, **348**
Xu et al. 2006	$C_{25:2}$	207, 235, 266, 319, **348**
Xu et al. 2006	$C_{25:2}$	207, 266, 291, 320, **348**
Island Lake	$C_{30:3}$	69, 95, 123, 137, 151, 207, 267, 341, 386, **414**
Pike Lake	$C_{30:3}$	69, 71, 95, 123, 151, 169, 207, 253, 267, 334, **414**
North Bay	$C_{30:3}$	57, 69, 95, 123, 137, 151, 169, 189, 207, 262, 357, **414**
Nitzschia	$C_{30:3}$	69, 95, 123, 151, 163, 179, 207, 261, 371, **414**
Fragilaria	$C_{30:3}$	69, 81, 95, 109, 123, 151, 163, 177, 208, **414**

4.2 HBI Concentrations

HBIs identified in environmental samples were found in concentrations ranging from 0.1 to 0.9 µg g^{-1} of dry sediment (Table 1). One marine study found sedimentary concentrations of IP_{25}, a sedimentary C_{25} HBI, ranging from 0.06 to 1 µg g^{-1} of sediment (Belt et al. 2014). In general, other studies have reported concentrations in sediments ranging from 0.01 to 40 µg g^{-1} of dry sediment (He et al. 2016; Kaiser et al. 2016). The concentrations of HBIs presented in the current study thus fit within previously reported values but are on the lower end of the range. This may be due to the lower salinity and nutrient levels in the study sites when compared to the Florida Everglades and marine environments like the Baltic Sea. In addition, a wide diversity of C_{25} HBIs have been correlated to the increased microbial complexity of periphyton samples in the Florida Everglades (He et al. 2016). This is not observed in the present study, though one of the more complex HBIs was observed in the periphyton of Pike Lake (HBI IV).

In the cultured $C_{25:4}$ HBI observed in *Nitzschia* (Figure 12 B) a concentration of 48.4 µg g^{-1} dry biomass was observed, which is low compared to previously reported concentrations of 5670, 110, and 390 µg g^{-1} dry biomass in C_{25} HBIs from *Berkeleya rutilans* (Brown et al. 2014). There are a couple notable differences between the previous reported concentrations and the present study, however. These differences include different growth conditions, growth media, and unsaturation levels of the observed HBIs. The cultures of *B. rutilans* were grown under 14:10 light-dark cycles and in saltwater media, where the *Nitzschia* diatoms from the current study were grown in 12:12 light-dark cycles and in freshwater media (Brown et al. 2014). In addition, two $C_{25:2}$ and one $C_{25:3}$ HBIs were quantified from *B. rutilans*, while a single $C_{25:4}$ HBI was quantified in *Nitzschia* (Brown et al. 2014). These differences may explain some of the variation between the present study and the previous report by Brown and colleagues. However, the concentration of the $C_{25:4}$ HBI is still low compared to the C_{25} HBIs identified previously.

4.3 Temperature and Seasonal Comparison

The elevated presence of C_{25} HBIs in Florida Everglades freshwater sediments have been proposed as a biomarker for anthropogenically enhanced nutrient levels (He et al. 2016). No correlations to anthropogenic changes can be made in the present study. However, there is potential for HBIs to be used as biomarkers for temperature, as HBIs found in the warmer, summer months were generally larger and were found in higher abundance than those observed in the winter. For example, two $C_{25:2}$ HBIs were observed in Tischer creek (HBI I & VIII), one in each season. The HBI observed in the summer (HBI I) was found in higher abundance than the HBI observed in the winter (HBI VIII), potentially indicating a preference for HBI synthesis in the summer. However, environmental conditions for photosynthesis are more favorable in the summer, which would lead to an increase in diatom biomass.

The HBI identified in the summer at Tischer Creek (HBI I) may have been present in both the summer and winter samples of the Tischer Creek study site, though the mass spectra of the winter sample (HBI VIII) contained a substantial amount of noise and

therefore additional testing, such as ^{1}H NMR, must be performed to confirm this relationship.

The pH measurements in the environmental samples ranged from the most acidic value of 8.17 at Island Lake, to the most alkaline of 9.08 in Woodstock Bay (Table 1). This does not correlate with a trend in concentrations, as the two sites with observed HBI concentrations of 0.2 µg g^{-1} dry sediment, Island Lake and Woodstock Bay (Table 1), showed the widest variety of pH values. In addition, Tischer Creek, which had the highest individual concentration of HBIs (0.9 µg g^{-1} dry sediment) (Table 1) had pH readings averaging to 8.42, which is encompassed by two of the sites with the lowest HBI concentrations, North Bay and Pike Lake, had observed pH averages of 8.38 and 8.91 (Table 1), respectively. Temperature also did not correlate with HBI concentration, as Tischer Creek (0.9 µg g^{-1} dry sediment, HBI I) (Table 1) had the lowest observed temperature in the summer, at 11.7°C, and Island Lake (0.2 µg g^{-1} dry sediment, HBI III; NQ, HBI II) (Table 1) had the warmest temperature in the summer, at 24.2°C. Thus, the environmental conditions do not correlate with the concentrations of HBIs observed at each site. However, increasing temperature shows a general trend towards increasing unsaturation level in the summer samples (Table 1), with the exception of HBI II (Figure 10; Table 1), which was observed in non-quantifiable concentrations in Island Lake. This trend has been previously observed in cultures of *H. Ostrearia*, where increase in growth temperature was correlated with unsaturation level (Rowland et al. 2001).

The HBI suite produced by a single diatom is greatly affected by a variety of factors including culture temperature and life stage (Rowland et al. 2001; Belt et al. 2002). Cultures of *R. setigera* have been shown to increase unsaturation as a result of increasing growth temperature (Rowland et al. 2001). This effect was observed in *Nitzschia* cultures from this study, where the increase from 20°C to 25°C yielded a change from a $C_{30:3}$ HBI to a $C_{30:4}$ HBI. *H. ostrearia* has also been shown to increase HBIs unsaturation levels after auxosporulation (Belt et al. 2002). This could also be true for the cultured diatoms in the present study, though life stage of diatoms was not recorded before harvesting.

4.4 HBI Producing Diatoms

Only one of the seven HBI studies from freshwater regions report the presence of C_{25} HBIs (He et al. 2016). The remaining distinct C_{25} HBIs have been reported from marine environments. We have shown that the freshwater diatoms *Fragilaria* produced a C_{30} HBI and *Nitzschia* produced both C_{25} and C_{30} HBIs. The presence of HBIs observed across Northern Minnesota and other freshwater regions provides further evidence that diatoms are the sole source of HBIs in aquatic environments.

Multiple genera of diatom have been found to produce the same distinct HBIs, as is the case with the $C_{25:2}$ HBI from Tischer Creek (HBI I; Table 1), which has been reported in cultures of *Pseudosolenia calcar-avis*, *Pleurosigma strigosum*, and *H. ostrearia* (Grossi et al. 2004; Kaiser et al. 2016). The unique aspect of the current relationship between HBI I (Figure 10) and those previously reported is that the previous reports of this $C_{25:2}$ HBI have only occurred in marine diatoms, with the current study the first to identify this molecule in a freshwater system. Potential sources of HBI I (Figure 10) could include *Nitzschia*, *Fragilaria*, or *Cymbella*, as all of these genera were observed at Tischer Creek and have been found or hypothesized, in the case of *Cymbella*, to produce HBIs. In addition, many diatom species have been observed to produce several distinctly different HBIs. Some of these species include, *R. setigera*, *H. ostrearia*, and *P. calcar-avis* (Belt et al. 2001; Grossi et al. 2004; Kaiser et al. 2016; Massé et al. 2004). In the present study, three distinct HBIs were observed from laboratory cultures of *Nitzschia*, adding to the previously mentioned genera.

In the present study, three HBIs have been directly correlated to *Nitzschia* diatoms (Figure 12, HBI A-C), while one HBI has been directly correlated to *Fragilaria* diatoms (Figure 12, HBI D). This is the first report of HBIs directly synthesized by both *Nitzschia* and *Fragilaria* diatoms, marking the seventh and eighth genera of diatom shown to synthesize these lipids (Kaiser et al. 2016). Other diatoms are suspected to produce HBIs in environmental samples. Although *Nitzschia* or *Fragilaria* were present at each study site, there are relatively high abundances of *Ctenophora*, *Cymbella*, *Stauroneis*, and *Staurosira* diatoms at Pike Lake, where a $C_{30:3}$ HBI was observed (Figure 11). *Cymbella* was observed in the highest abundance in Pike Lake and was also observed at Island

Lake, Tischer Creek, and Woodstock Bay where C_{25} and C_{30} HBIs were observed (Table 1, Figure 11). In addition, *Cymbella* was only present at sites where HBIs were observed (Figure 11). These factors indicate that *Cymbella* could be responsible for HBI synthesis at these sites. This is the first report of hypothesized HBI production by the *Cymbella* genus.

Chapter 5. Conclusions & Future Work

This study of Highly Branched Isoprenoids is the first to occur in the Laurentian Great Lakes. HBIs are commonly used as sea-ice proxies in marine environments where HBIs are indicative of marginal ice zones (Belt and Müller 2013). This study aims to develop a similar ice proxy for Lake Superior. Nonpolar extracts from sediments at seven study sites and individual strains of *Nitzschia* and *Fragilaria* were analyzed via GC-MS to determine the concentration and suites of HBIs produced.

Lake Superior sediments and diatoms yielded evidence of up to twelve distinct HBIs (Figure 10, 12). Eleven of these HBIs are novel molecules (HBI II-VIII, HBI A-D), while one (HBI I) is likely to have been previously identified (Kaiser et al. 2016; Grossi et al. 2004). The concentration of sedimentary HBIs ranged from 0.1 μg g^{-1} dry sediment (HBI VI) to 0.9 μg g^{-1} dry sediment (HBI I). All of the identified HBIs were C_{25} or C_{30} HBIs, no C_{20} or C_{35} HBIs were identified in sediments or in single-strain culture experiments.

For the first time common Lake Superior diatoms, *Fragilaria* and *Nitzschia*, were directly observed to produce HBIs (Figure 12). *Fragilaria* was found to produce a single $C_{30:3}$ HBI, while *Nitzschia* was found to produce three distinct HBIs, one $C_{30:3}$, one $C_{30:2}$, and one $C_{25:4}$. One of these HBIs, HBI D, was observed at 48.4 μg g^{-1} dry biomass. In addition, the diatom, *Cymbella*, was only found at sites where HBIs were observed. This may indicate that *Cymbella* is responsible for some HBI production, which would be the first time this diatom has been reported to produce HBIs.

This initial study of the area around Lake Superior is one of only two freshwater HBI studies to have identified the presence of C_{25} HBIs. Five of the seven study sites showed evidence of HBIs, which ranged in concentration from below the limit of quantification to 0.9 μg g^{-1} of dried sediment. In these sediments, there was a general trend toward higher HBI unsaturation levels with increasing temperature.

Future HBI studies in this region should focus on elaborating on the correlation between temperature and unsaturation level by selecting a single site and sampling multiple times throughout the year. This would allow for statistical analysis and also allow for additional variables to be studied, such as total nutrient levels (C, N, O, P) and

turbidity. This would help determine if there are variables other than temperature affecting unsaturation level.

Additional single-strain culture experiments on *Fragilaria* and *Nitzschia* should be conducted, replicating growth conditions of previous HBI culture studies, such as Brown et al. (2014) and Brown and Belt (2016), to get a more accurate comparison between the HBI concentrations produced by *B. rutilans* and *Pleurosigma intermedium*, respectively. Single-strain culture experiments on *Cymbella* should also be studied since this genus of diatom was only found at sites where HBIs were observed and has not been previously reported to produce HBIs.

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Appendix 1, Standard Operating Procedures (SOPs)

Sediment sampling procedure

Materials:

- Pre-labeled, Combusted sampling Jars
- Teflon-lined, solvent-rinsed lids
- Sediment collection tool
- Thermometer

Procedure:

1. Record the coordinates of the sampling site
2. Record the water temperature on the lid of the combusted sampling jar
    a. allow thermometer to equilibrate with air between measurements
3. Use the sediment collection tool to scoop sediment into combusted sampling jar, fasten lid of jar
    a. clean off sediment collection tool with water between samples
4. Repeat process for each sample

Sediment freeze drying and homogenization

Materials:

- Labconco Freezone 6 freeze dry system
- Rubber bands
- lint-free wipes
- Ball mill
- solvent-rinsed, steel vessel with threaded lid
- solvent-rinsed, steel ball bearings
- Methanol (MeOH)

Procedure:

1. Remove lids of sediment samples and replace with lint-free wipes fastened with rubber bands
2. Place samples in Labconco Freezone 6 freeze dry system at -45°C; ≤ 100 mbar for ca. 3 days, or until all water was removed
3. Once samples are dry, prepare the vessel, ball bearings, and ball mill for use
4. transfer the dry sediment from one sample to the vessel, add in ball bearings, and secure the threaded lid
5. Secure the vessel in the ball mill and run for 1-2 minutes, or until sample is homogenous
6. Remove the vessel from the ball mill and transfer back to corresponding sample jar, resecure jar lid
7. Wash the vessel, lid, and ball bearings first with water, then with MeOH and allow to fully dry
8. Repeat with remaining samples

Single-strain culture filtration

Materials:

- Filtration apparatus
- Vacuum Pump
- Sonicator
- Solvent-rinsed tweezers
- Milli-Q water (MQW)
- Glass fiber filters (0.7 µm pore size)
- Combusted aluminum foil

Procedure:

1. Assemble, label, and weigh aluminum foil packets containing glass fiber filters
2. Assemble filtration apparatus with pre-weighed glass fiber filter and connect vacuum pump
3. Sonicate samples until all diatoms are suspended
4. Filter sample, rinsing sides of funnel with MQW
5. remove filter and wrap filter in pre-weighed and labelled foil packet
6. Rinse the filtration apparatus and repeat with remaining samples

Single-strain culture freeze drying and weighing

Materials:

- Labconco Freezone 6 freeze dry system
- Analytical Balance

Procedure:

1. Place sample packets in Labconco Freezone 6 freeze dry system at -45°C; ≤ 100 mbar for ca. 3 days, or until all water was removed
2. Remove samples from freeze dry system and weigh on analytical balance to determine amount of dry biomass

HBI extraction

Materials:

- 20 mL scintillation vials
- 7 mL glass vials
- 250 µL GC insert
- Combusted glass wool
- Combusted glass Pasteur pipettes (150 mm, 3 mL volume)
- Deactivated chromatography-grade silica (60-200 µm)
- 7-hexylnonadecane (7-NHD)
- Potassium hydroxide (KOH)
- Methanol (MeOH)
- HPLC-grade n-hexane
- Methylene chloride (DCM)
- Milli-Q water (MQW)

Procedure:

1. Weigh out 1g of freeze-dried sediment into a clean, pre-labelled 20mL scintillation vial capped with a solvent-rinsed polypropylene screw cap
    a. Include a procedural blank of an empty vial
    b. For single-strain samples, place whole glass fiber filter in scintillation vial
2. Add 0.1 µg (100 µL of 0.1 µg mL^{-1}) of the 7-NHD internal standard to each scintillation vial
3. Add ca. 2 mL KOH (5%, MeOH/H_2O (9:1), or sufficient volume to cover sediments with an excess of ca. 1 mL above the sediment surface), vortex for 10 seconds, and heat at 70°C for 1 hour
4. Add ca. 2 mL hexane to cooled contents of each vial, vortex mix (20 seconds) and centrifuge for 2 minutes at 2500 rpm
5. Collect the hexane layer with a combusted glass Pasteur pipette into 7 mL glass vials
    a. Repeat extraction twice more with further aliquots of hexane, combining the hexane extracts into the same 7mL vial
6. Remove hexane by gentle stream of N_2 (25°C) until dry
7. Re-dissolve dry extracts in ca. 0.5 mL hexane and transfer into a small-scale chromatography column made of a combusted glass Pasteur pipette, containing a plug of combusted glass wool and deactivated chromatography grade silica (ca. 0.5 g).
8. Elute the column with 6 mL (each) of hexane, hexane-DCM (4:1 v/v), DCM-MeOH (1:1 v/v), and MeOH
9. Collect each fraction into combusted, pre-labeled 7 mL glass vials
10. Remove solvent by gentle stream of N_2 (25°C) until dry
11. Store in laboratory refrigerator (????) until ready for analysis

GCMS analysis

Materials:

- 2mL glass GC vial and cap
- 9-octyl-heptadec-8-ene (9-OHD)

Operating Conditions:

An Agilent 7890A GC coupled to a 5975 series mass selective detector fitted with an Agilent HP-5ms (30m x 0.25mm x 0.25µm) column was used, along with a 1µL auto-splitless injection (300°C) with helium carrier gas (1mL min^{-1} constant flow). Total ion current (TIC; m/z 50-500 daltons) with an electron voltage of 70eV. The GC oven was heated from 40-300°C at 5°C min^{-1} and held at 300°C for 10 minutes. A 10-minute solvent delay was set.

Procedure:

1. Reconstitute hexane fractions in 100 µL of 9-OHD internal standard (0.1 µg mL^{-1}) and vortex for 30 seconds
2. Transfer samples to combusted, pre-labeled GC vials with 250 µL inserts and secure caps

3. Transfer to gas chromatography auto sampler and run method