From the Field to the Lab: Community Analysis and Isolation of a Highly Branched Isoprenoid Producing Diatom, *Nitzschia frustulum* (Kütz.) Grunow (Bacillariophyta)

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Abstract

Bacillariophyta (diatoms) are single-celled photosynthetic algae. Diatoms are found in most aquatic environments, and species occupy both benthic and planktonic areas. Some diatoms produce a molecule known as a highly branched isoprenoid (HBIs). HBIs are important molecules used in paleolimnology as a proxy for sea-ice reconstruction. In addition, there is evidence that HBIs can be used as a biofuel additive and have anti-cancer effects. It is not known what biological function and metabolic pathways diatoms use to produce HBIs. Through environmental and diatom community analysis, trends in species composition and environmental parameters can help us understand more about the production of HBIs. Growth analysis of isolated diatoms can further our understanding of the production of HBIs. In the present study, seven sites were selected for HBI and diatom community analysis. Common genera, including known HBI producers, match those in previous studies in other freshwater bodies of water. There are no clear trends between pH, temperature, and qualitative factors and the production of HBIs, indicating that other factors could contribute to this production, such as nutrient levels and the water conductivity. An HBI-producing diatom, *Nitzschia frustulum*, was isolated and used in growth analysis experiments. *N. frustulum* grows at an optimal pH of 7.5 to 8 at 25°C. HBIs were produced at a pH of 7 and at temperatures of 20°C and 25°C. C25 and C30 HBIs were found with varying amounts of unsaturation. The number of double bonds increased at a higher temperature. This study adds to the current understanding of the biological function of HBIs as potentially being involved in membrane fluidity.

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1. Introduction

1.1 Diatom life history

Bacillariophyta (diatoms) are single-celled photosynthetic algae that encompass two morphologically distinct groups: radial centric diatoms and elongated pennate diatoms. Diatoms are found in most eutrophic aquatic environments, both freshwater and marine, and species occupy benthic and planktonic areas (Shruti, 2016). There are an estimated 30,000 diatom species (Mann and Vanormelingen, 2013) that occur in diverse habitats, in alkaline and acidic environments, and at arctic and tropical temperatures. Diatoms are estimated to contribute 20% of global primary production and they play an important role in silica and carbon biogeochemical cycles (Falkowski et al., 1998; Treguer and De La Rocha; 2013, Smetacek, 1999).

The evolutionary origin of diatoms is complex, but two main hypotheses have emerged: 1) that diatoms arose through a primary endosymbiosis event between a non-photosynthetic eukaryotic microbe and a red alga (Parker, 2008), or 2) diatoms arose from endosymbiosis of a green alga followed by secondary endosymbiosis of a red alga from this eukaryote (Moustafa, 2009). Along with a complicated history of endosymbiosis, multiple horizontal gene transfer events (Vancaester et al., 2020), and evidence of viral genomic elements (Maumus, 2009) have contributed to chimeric genomes in diatoms.

1.2 Diatoms as bioindicators

Diatoms fill many different ecological niches and, as a result, they can be important biological indicators of water quality. Diatoms are sensitive to ecological

conditions such as pH, salinity, nutrient availability, and organic matter (Lobo et al., 2016). The first diatom indices to be produced were by Kolkwitz and Marsson (1908). This led to the use of diatoms as indicator species for detecting organic matter like wastewater run-off, sewage, and heavy metal contamination (Sabater, 2000; Sládeček, 1973). In forensic science, diatoms are used as biological indicators to show where evidence potentially originated within specific water bodies (Lobo et al., 2016; Márquez-Grant and Roberts, 2012). Biomarkers can also be used to determine historical and present geological states such as past eutrophication events in the Great Lakes region (Meyers, 2003).

1.3 Sea-ice reconstruction and highly branched isoprenoids

Paleolimnological assessments allow researchers to reconstruct historical events from various sources such as inorganic material like frustules, and organic material, including biomarkers. Biomarkers are organic substances that are often used in the medical field as signs of certain physiological issues in patients (Strimbu and Tavel, 2010). The use of biomarkers is not limited to medicine, as these tools have been used to construct phylogenetic trees to evaluate relatedness among organisms (Lundgren et al., 2022). One such historical series of events is that of Arctic sea-ice reconstruction (Kinnard, 2011). Arctic sea-ice melt has been increasing through time due to the increasing warming of the planet (Vaughan et al., 2003). Biomarkers allow researchers to look back in the geological records, revealing geological trends in deep time. Many biomarkers are slow to degrade over time and it is necessary to find a proxy that does not

go undergo extensive degradation within the targeted timeline of reconstruction (Halim et al., 2021).

One such proxy is a class of molecules known as highly branched isoprenoids (HBIs). Isoprenoids are carbon-based compounds that are diverse in structure, such as functional components of organisms like cholesterol, plant hormones, and defense mechanisms (Bukingham, 1997). Isoprenoids are made of isoprene units. Isoprenes are 5 carbon alkanes and the precursor to all isoprenoids is a molecule called isopentenyl-diphosphate. HBIs are characterized by branching that occurs along the carbon backbone. HBIs occur in different forms, such as C20, C25, and C30 HBIs (Belt et al., 2000), and can have many isomeric forms. It has been proposed that some species of diatoms use HBIs as a membrane lipid to aid in membrane fluidity (Pozzi et al., 1996). This is due to phosphate esters within acyclic isoprenoids that may be a part of membrane lipids, as these are typical of membrane lipids. HBIs can also be used as biomarkers (Belt et al., 2007).

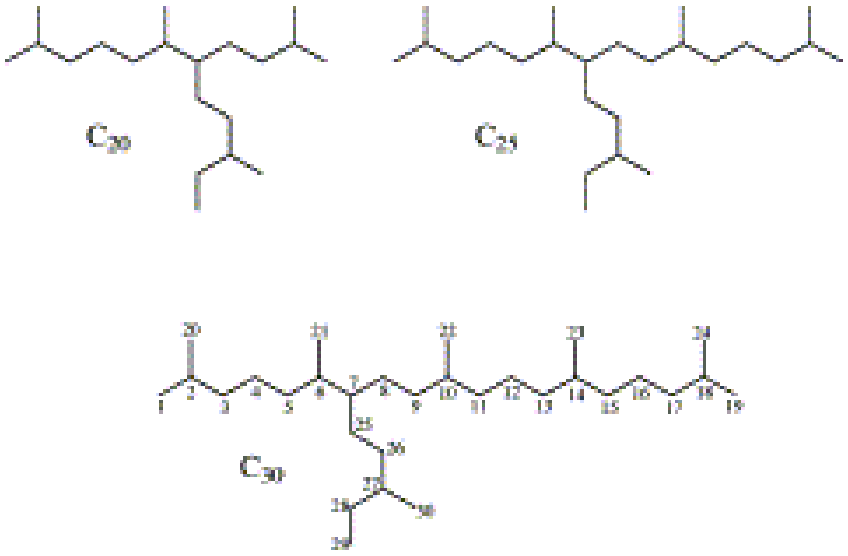


Figure 1. Basic structural forms of C_{20}, C_{25}, and C_{30} highly branched isoprenoids.

The first diatoms identified that produce HBIs were the marine diatoms *Haslea ostrearia* (Gaillon) and *Rhizosolenia setigera* (Brightwell) (Nichols and Volkman, 1988). Now, freshwater, benthic, and planktonic genera of HBI-producing diatoms are known (Belt et al., 2001, Grossi et al., 2004, and Kaiser et al., 2016). The metabolic pathways and genes that generate HBIs are unknown. But we do know that the production of HBIs requires either the mevalonate pathway (MVA) or the methylerythritol 4-phosphate pathway (MEP) to generate the precursor isopentenyl pyrophosphate (IPP), but further downstream branches of the metabolic pathway remain unstudied. Recently, HBI synthesis genes have been proposed for *Haslea ostrearia* (Athanasakoglou, 2019) and *Rhizosolenia setigera* (Ferriols, 2015).

1.4 Highly branched isoprenoids and diatom communities in freshwater environments

Using highly branched isoprenoid proxies for seasonal-sea ice reconstructions focus generally on marine environments such as the Artic and Antarctic (Massé et al., 2011; Smik et al., 2016). There are a limited number of studies in freshwater systems. These include studies in the Florida Everglades (He et al., 2016) and in places such the Adirondacks in New York (Corcoran et al., 2020). HBIs in all forms were found in these freshwater systems and the source of freshwater HBI-producing diatoms have been discovered. In laboratory cultures, *Navicula sclesvicensis* (Grunow) was found to produce C25 HBIs (Belt et al., 2001). The genus *Cyclotella* (Kutzing) has also been proposed as the source of HBIs in the eutrophic freshwater systems(Xu et al., 2006).

Freshwater diatoms communities are important primary producers that can be subject to many changes that could affect them. These include metal contamination and changes in the environment. Succession of diatoms in particular areas can be indicators of these disturbances. Diatoms such as *Achnanthidum* (Kutzing) often are the first genera to colonize newly disturbed area and relative abundance of this genera can be used to determine when this disturbance has happened (Cantonati et al., 2014). Other genera of diatoms common in freshwater environments near the Lake Superior region include genera such as *Achnanthidum*, *Cyclotella*, *Fragilaria* (Lyngbye), *Navicula* (Bory de Saint-Vincent), *and Cymbella* (Agardh), among others (Barbiero and Tuchman, 2001).

1.5 Freshwater diatom cultures and growth cultivation

Diatoms are increasingly becoming an industrial interest due to compounds that they produce. These included lipid for biofuels, vitamins, and even biosensors (Sharma et al., 2021). This has made isolating and growing diatoms in the lab increasingly appealing for easier access to these materials. Many studies on cultivation focus on already isolated diatoms from phytoplankton suppliers (Jiang et al., 2020).

Optimal cultivation conditions are known for many large scale phytoplankton cultures. For novel diatoms, cultivation methods exist (Ifuku et al., 2015; Kimura and Tomaru, 2013) but optimal growth conditions need to be assessed in order for continued growth (Kourtchenko et al., 2018). In order to cultivate benthic diatoms, a solid surface must be utilized for growth. Planktonic diatom able to use gas vacuoles to remain suspended within the water column (Lunnergren, 1979). Benthic diatoms are unable to successfully grow in these environments without a surface area to attach to. Studies often

use agar plates that have surfaces scratched on the surface for ease of attachment (Scholz, 2014).

Chapter 2. Diatom community analysis for seven freshwater sites near Lake Superior

2.1 Goals and Hypotheses for the present study

Goal: The goals of this study are 1) to determine the dominant diatom taxa at each of seven sites and 2) to explore site-specific differences in biotic and abiotic conditions, including community composition, pH, temperature, and highly branched isoprenoid presence.

Hypothesis 1: Diatoms found at nearby Lake Superior study sites will match common taxa in the region and diversity between seasons will change as productivity decreases during the winter.

Hypothesis 2: Known highly branched isoprenoid (HBI) producing diatoms will be found at sites where HBIs are detected while pH and temperature will effect whether these diatom communities produce HBIs.

2.2 Experimental Methods

2.2.1 Study locations

Seven sites located in St. Louis and Lake Counties, MN, USA were sampled: two lakes (Island Lake and Pike Lake), three estuaries (Oliver's Landing, Woodstock Bay, and North Bay), one creek (Tischer Creek), and one nearshore pool (Two Harbors Lighthouse) (Fig. 1). Qualitative data were taken from each site and reported in Table 1.

2.2.2 Specimen and environmental sampling

Samples were collected from each site once during late spring (5/25/21– 6/16/21) and once during late fall (11/20/21–12/04/21). Algal biomass was removed from rocks at

each site except the North Bay site were wood debris and leaves were used as there was a lack of rocks present. Deionized water and a bristle brush was used to scrap algal biomass from the substrate and the resulting water was collected in glass jars and taken back to the lab for cleaning of organic material. Three run-off water samples were collected for each sample in 2 mL microcentrifuge tubes and frozen at -80 ºC for later molecular barcode analysis (R. Jan Stevensom and Loren L. Bahls, n.d.)

Environmental parameters measured include pH and temperature. The pH and temperature of the water from each sample site was measured using a handheld pH probe (VIVOSON, PH-001) and a thermometer (VWR, 61019-001) respectively. Five pH measurements were taken from the surrounding water where the rocks were taken from. Temperature measurements were measured in triplicates. All measurements were taken on the day of sampling at the same time and replicates were averaged, with the 95% confidence interval reported.

Qualitative data were also collected during sampling. Both turbidity of the water and how stable or unstable the sampling site was recorded. If the bottom of the sampling site water bed could not be seen, this site was classified as turbid. If the sites water bed's sediment was moving, this was classified as unstable.

2.2.3 Slide preparation and diatom identification

Organic material was removed from samples to expose the clear frustules of the silica cell wall using the hydrogen peroxide cleaning method (Renberg, 1990). Two cleaned samples per site (1 mL) were pipetted onto a cover slip. These were left to evaporate at room temperature (~20 ºC) overnight so that aggregation of diatoms would not occur. Pluerax fixative was used to affix coverslips onto microscope slides. Slides

were placed on a hot plate for 1–2 minutes to help cure the Pluerax and to create permanent slides. Slides were then stored at room temperature for downstream microscopy and identification.

Fixed slides were examined under a Nikon Eclipse TS2 light microscope at 100× magnification. Transects across the top and bottom of the coverslips were visually scanned until 250 frustules were observed for each sample. Each frustule was identified to genus using the Diatoms of North America website (Spaulding et al., 2021) as a guide. Frustule counts were used to calculate relative abundance at each site and to conduct downstream analyses.

2.2.4 Relative abundance and diversity data anlaysis

Relative abundance for each genera was calculated by taking the count of individuals representing the genus divided by the total number of genera found at that site. All graphs were made using the R software (R Core Team, 2022). Refraction curves were calculated and graphed using the Rarefy package in vegan (Oksanen et al., 2013). Shannon-Weiner diversity (1), Pielou's Evenness (2) and Bray-Curtis diversity (3) were all calculated in R using the vegan package (Oksanen et al., 2022).. Principal coordinate analysis was performed on the resulting distance matrix and visualized using the software.

$$1)\ H'_{max} = -\sum_{i=1}^{S} \frac{1}{S} ln \frac{1}{S}$$

$$2)\ J' = \frac{H'}{H'_{max}}$$

$$3)\ BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

2.3 Results

2.3.1 Environmental sampling

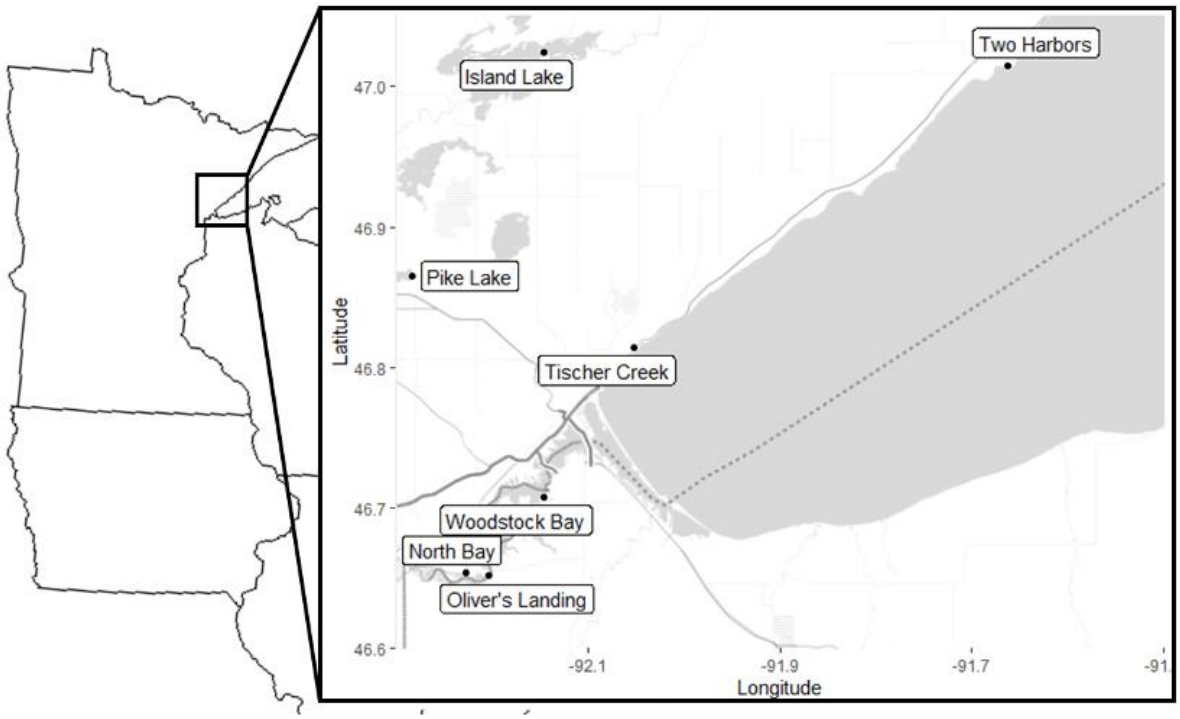


Figure 2. Sampling sites for the present study. (Hanson & Schreiner, 2022)

Table 1. Site details for the seven sampling locations.

					5/25/21 - 6/16/21		11/20/21 - 12/04/21	
Study Site	**Latitude**	**Longitude**	**Bed Stability**	**Water Clarity**	**pH**	**Temperature (°C)**	**pH**	**Temperature (°C)**
Tischer Creek	46.814395	-92.05167	Unstable	Turbid	8.41 ± 0.09	11.7 ± 0.2	8.52 ± 0.07	-1.98 ± 0.07
Two Harbors	47.01415	-91.662215	Stable	Clear	8.96 ± 0.16	19.3 ± 0.2	6.75 ± 0.05	-0.02 ± 0.11
Island Lake	47.023794	-92.145825	Stable	Turbid	8.16 ± 0.028	24.1 ± 0.2	8.12 ± 0.02	0.36 ± 0.2
Pike Lake	46.865378	-92.282971	Stable	Clear	8.91 ± 0.033	22 ± 0.2	8.21 ± 0.02	-0.025 ± 0.05
North Bay	46.653686	-92.226995	Stable	Clear	8.38 ± 0.060	20.8 ± 0.3	8.46 ± 0.03	-2.98 ± 0.08
Oliver's Landing	46.651761	-92.202993	Stable	Turbid	8.41 ± 0.022	20.6 ± 0.4	8.47 ± 0.04	-3.88 ± 0.22
Woodstock Bay	46.707653	-92.146	Unstable	Clear	9.08 ± 0.011	21.6 ± 0.2	8.12 ± 0.07	-3.2 ± 0.13

Environmental sampling was conducted during the summer and winter of 2021 (Fig. 2). During the summer time point (5/25/21–6/16/21), pH ranged from 8.16 to 9.08, and pH during the winter time point (11/20/21–12/04/21) was similar to the summer ranging from 6.75 to 8.52. The pH at the Two Harbors site during the summer was 8.96 ±

0.16 and 6.75 ± 0.05 in the winter. At Woodstock Bay the pH during the summer sampling at 9.08 ± 0.011 and 8.12 ± 0.07 during the winter.

During the summer, temperatures at surveyed sites ranged from 11.7 to 24.1℃. The lowest temperature during summer sampling was at the Tischer Creek site (11.7℃). The temperatures during winter sampling ranged between -3.88 to 0.36℃.

Tischer Creek, Island Lake, and Oliver's landing were all classified as turbid. The remaining sites were classified as being clear. Two sites, Tischer Creek and Woodstock Bay, were classified as having an unstable water bed. The five other sites were classified as being stable (Table 1).

2.3.2 Rarefaction curves

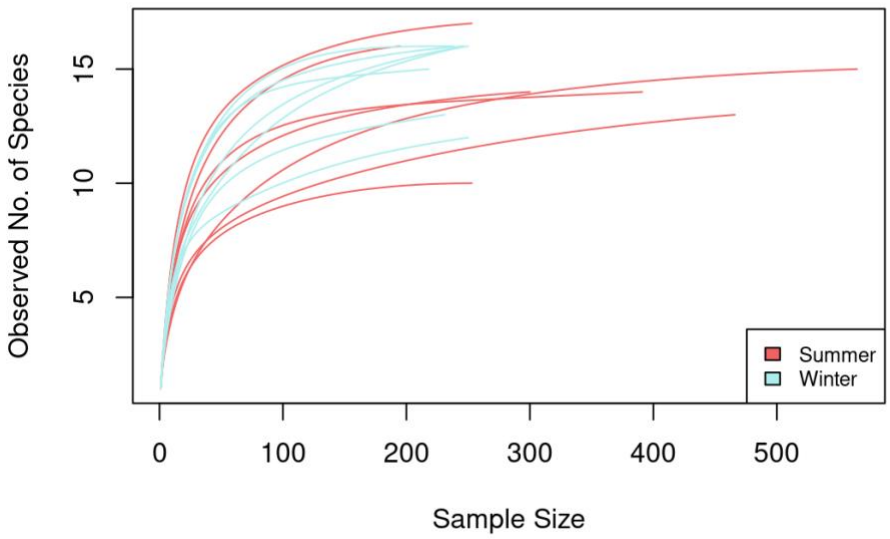
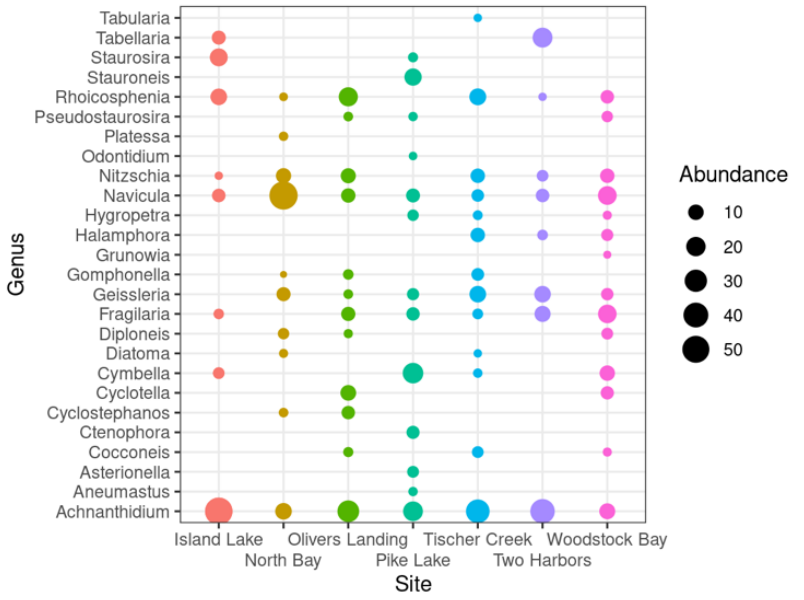


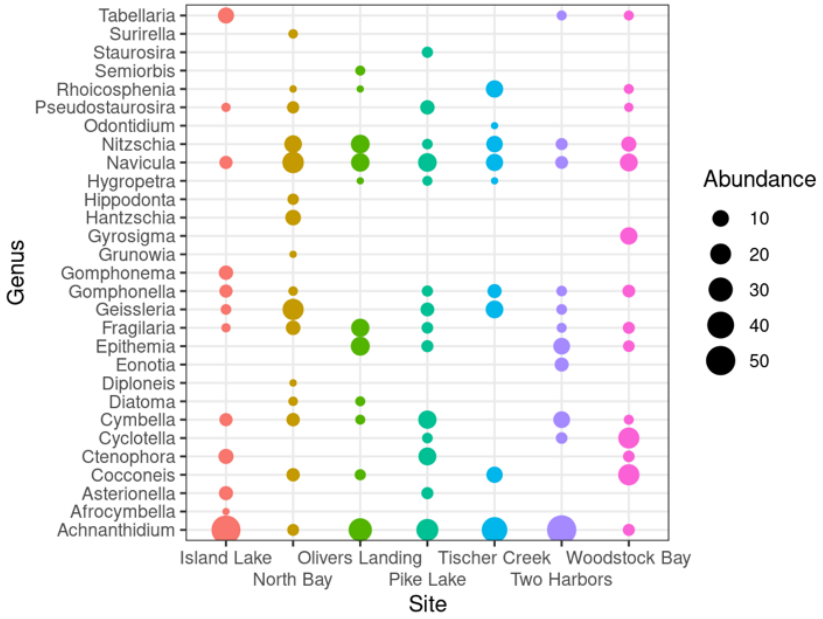
Figure 3. Rarefaction curves for the summer and winter sampling. The y-axis indicates the observed number of species at each site, and the x-axis represents the sample size at each site. Each line represents a study site. Red lines indicate that the sampling occurred during the summer, and the teal lines represent sampling during the winter.

A rarefaction curve was constructed to ensure that the full community was represented for each site, as indicated by each curve reaching an asymptote. Three of the summer sites were overcounted. This is indicated by the rarefaction curve as the lines had reached an asymptote well before counting ended. The four other summer sites had a sufficient level of counting, while all winter sites were also sufficiently counted (Fig. 3).

2.3.3 Community abundance



A)



B)

Figure 4. Relative abundance of diatoms during the summer and winter sampling periods. The y-axis represents genera that were found at the sites, and the x-axis represents each site. The size of the bubble represents the percent relative abundance, with the smallest size representing 10% relative abundance, and

the largest size representing 50% relative abundance. **A)** Bubble plot representing the summer sampling period (5/25/21– 6/16/21). **B)** Bubble plot representing the winter sampling period (11/20/21– 12/04/21).

Community sampling and analysis were conducted to understand what taxa were present at each site. There were 26 genera present during the summer sampling period. *Achnanthidium* and *Navicula* were the only two genera found at all sites during the summer. Six genera were present at only one of the sites during the summer: *Aneumastus* (Mann), *Odontidium* (Kutzing), and *Stauroneis* (Ehrenberg) were only found at Pike Lake; *Grunowia* (Rabenhorst) was found only at Island Lake; at Tischer Creek, *Tabularia* (Kutzing) was found; and species belonging to *Platessa* (Lange-Bertalot) were found only at the North Bay site. The number of genera within each site ranged from 8 to 14, with Island Lake and Two Harbors having the smallest number of genera at 8, and Woodstock Bay having 14 genera at the site is the most diverse. The majority of genera had a relative abundance of 20% to 10%. Species belonging to the genus *Achnanthidium* were the most abundant at each site with a relative abundance of 50% (Fig. 3A)

The winter sampling period retrieved 29 genera across all sites. Species belonging to *Achnanthidium* and *Navicula* were present at all sites. Eleven out of 29 genera were only present at one site during the winter: *Afrocymbella* (Krammer) and *Gomphonema* (Agardh) were only present at Island Lake; species belonging to *Diploneis* (Ehrenberg), *Grunowia*, *Hantzschia* (Grunow), *Hippodonta* (Lange-Bertalot), and *Surirella* (Turpin) were found only in North Bay; *Semiorbis* (Patrick) was found only at Oliver's Landing; *Staurosira* (Ehrenberg) was found only at Pike Lake; *Odontidium* was found only at Tischer Creek; *Eunotia* (Ehrenberg) only at the Two Harbors site; and *Gyrosigma*

(Hassall) only at Woodstock Bay. The number of genera within each site ranged from 8 at Tischer Creek to 16 at North Bay (Fig. 3B)

2.3.4 Alpha diversity analysis

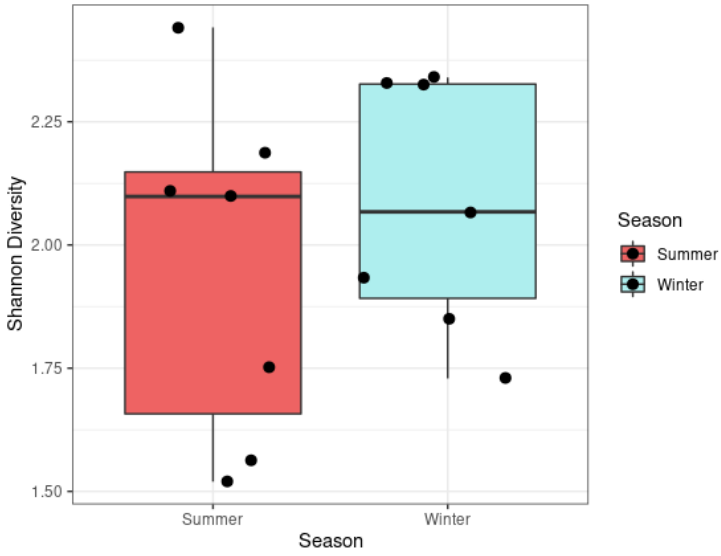


Figure 5. Shannon Diversity between seasons for all sites. The two box and whisker plots represent the range of Shannon diversity indices between summer and winter, respectively. The median, upper and lower quantiles, and min and max values are shown. The y-axis represents Shannon diversity, and the x-axis represents season. Black points represent the individual site.

Table 2. Shannon-Weiner Diversity and Pielou's Evenness for each study site across seasons.

Sites	Shannon-Weiner Diversity	Pielou's Evenness
Tischer Creek Summer	2.10	0.79
Tisher Creek Winter	1.93	0.79
Two Harbors Summer	1.75	0.68
Two Harbors Winter	1.85	0.66
Oliver's Landing Summer	2.11	0.80
Oliver's Landing Winter	2.07	0.75
North Bay Summer	1.52	0.56
North Bay Winter	2.32	0.84
Woodstock Bay Summer	2.44	0.86
Woodstock Bay Winter	2.34	0.86
Pike Lake Summer	2.19	0.79
Pike Lake Winter	2.33	0.84
Island Lake Summer	1.56	0.68
Island Lake Winter	1.73	0.67

Shannon-Weiner diversity index is a measurement of species diversity within each site that accounts for species richness and relative abundance (C.E. Shannon and W.W. Weaver, 1963). The Shannon-Weiner diversity index for each site across the summer and winter seasons is reported in Table 2. Diversity during the summer season ranged from 1.52 to 2.44 while diversity during the winter ranged from 1.73 to 2.34. No significant difference in diversity during the summer and winter seasons was detected (Fig. 4).

Pielou's Evenness is an index that infers the count of individuals for each species within a given site (Pielou, 1966). Evenness of the distribution of species at each site is measured, with 0 being no evenness and 1 being complete evenness. During the summer, evenness ranged from 0.56 to 0.86. North Bay was the least evenly distributed at 0.56 and Woodstock Bay was the most even at 0.86. The winter's evenness ranged from 0.67 to 0.86. Island Lake was the least even at 0.67 and Woodstock Bay was the most even at 0.86 (Table 2).

2.3.5 Beta-diversity analysis

A Bray-Curtis dissimilarity matrix measures how similar or dissimilar our different sites are from each other (Bray and Curtis, 1957). Bray-Curtis is measured on a scale of 0 to 1, 0 indicating that all species occur at both sites, and 1 indicating that all species are different at the two sites. A principal coordinate analysis (PCoA) of the species abundance at each site for each season, can be used to represent the Bray-Curtis data. Principle components 1 and 2 account for around the same amount of variation in each axis, 34.88% and 31.90% respectively. The Tischer Creek, Two Harbors, Oliver's Landing, and Island Lake sites are closely clustered together, indicating similarity among sites. Three sites remained separate from all the other groups: North Bay, Woodstock Bay, and Pike Lake Sites (Fig. 5). Tischer Creek and Oliver's Landing are the most similar sites with a similarity of 0.39. While North Bay compared to Pike Lake is the most dissimilar at 0.79. North Bay is the only site that consistently groups away from all other groups (Table 3).

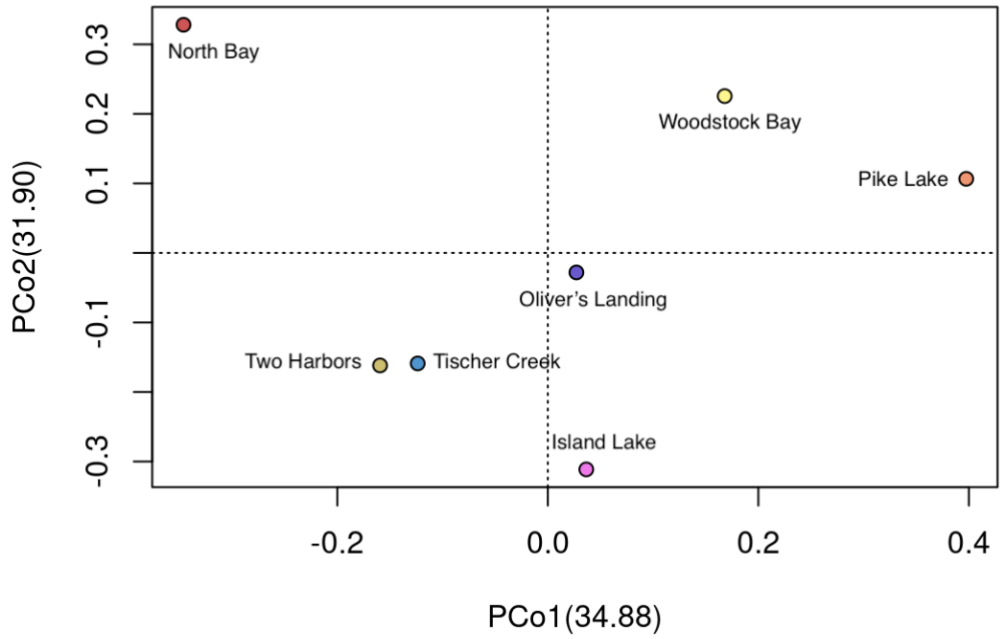


Figure 6. Principal coordinate analysis of the summer sampling period. A Bray-Curtis dissimilarity matrix was calculated for the summer sites. Principal coordinate 1 accounts for 34.88% of the variation, while principal coordinate 2 accounts for 31.90% of the variation. Each point represents a different summer site. Dashed lines represent y = 0 and x = 0.

Table 3. Beta diversity values of the diatom communities during the summer

Sites	Tischer Creek	Two Harbors	Oliver's Landing	North Bay	Woodstock Bay	Pike Lake	Island Lake
Tischer Creek							
Two Harbors	0.41						
Oliver's Landing	0.39	0.57					
North Bay	0.60	0.66	0.66				
Woodstock Bay	0.58	0.58	0.46	0.67			
Pike Lake	0.65	0.72	0.62	0.79	0.53		
Island Lake	0.40	0.50	0.51	0.75	0.69	0.6	

Legend		Similar		Dissimilar
0.20 - 0.29		0	-	1
0.30 - 0.39				
0.40 - 0.49				
0.50 - 0.59				
0.60 - 0.69				
0.70 - 0.79				
0.80 - 0.89				

During the winter, we see a similar grouping of sites with some differences. PCo1 accounts for 47.83% of the variation during the winter, while PCo2 represents around 17.84%. There are still similar groupings within the winter. Oliver's Landing and Tischer Creek diatom communities still group together as being similar, and Island Lake and Two

Harbors communities also still group together. North Bay and Woodstock Bay diatom communities are different in taxonomic composition compared to the other sites. The Pike Lake community is similar to communities found at Oliver's Landing and Tischer Creek, unlike in the summer when the Pike Lake community is dissimilar to them (Fig. 6). Two Harbors and Island Lake have the most similar communities with a Bray-Curtis value of 0.29. North Bay is grouped as the most dissimilar community to Island Lake at 0.79 (Table 4).

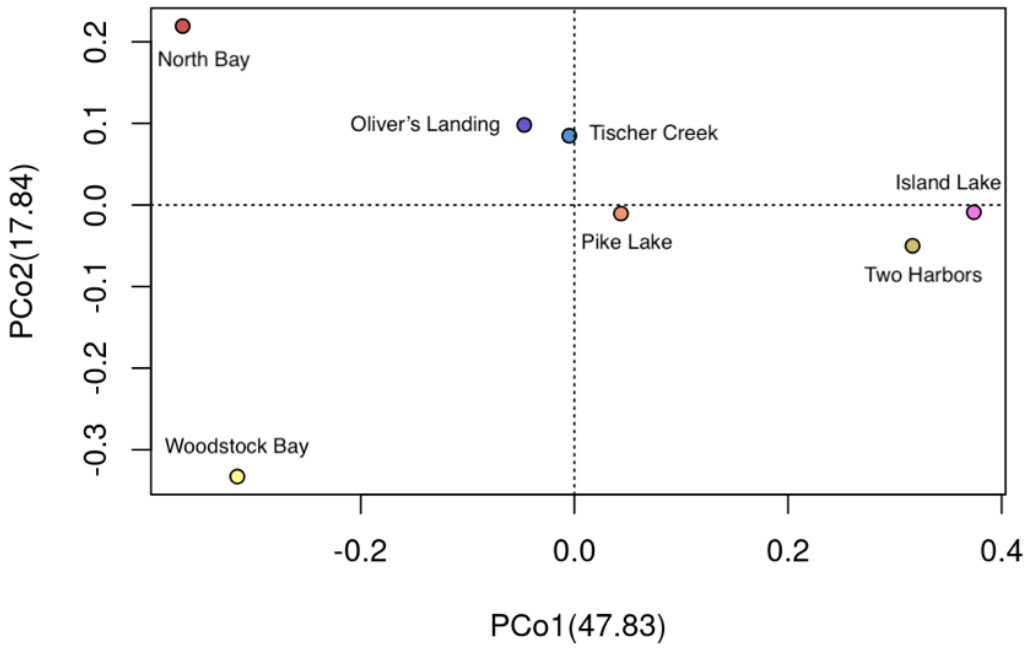


Figure 7. Principal coordinate analysis of the winter sampling period. A Bray-Curtis dissimilarity matrix was calculated for the winter sites. Principal coordinate 1 accounts for 47.83% of the variation, while principal coordinate 2 accounts for 17.84% of the variation. Each point represents a different winter site. Dashed lines represent y = 0 and x = 0.

Table 4. Beta diversity for sampled diatom communities during the winter.

Sites	Tischer Creek	Two Harbors	Oliver's Landing	North Bay	Woodstock Bay	Pike Lake	Island Lake
Tischer Creek							
Two Harbors	0.50						
Oliver's Landing	0.43	0.47					
North Bay	0.53	0.76	0.53				
Woodstock Bay	0.58	0.71	0.59	0.58			
Pike Lake	0.51	0.46	0.47	0.59	0.61		
Island Lake	0.50	0.29	0.61	0.79	0.78	0.53	

Legend		Similar		Dissimilar
0.20 - 0.29		0	-	1
0.30 - 0.39				
0.40 - 0.49				
0.50 - 0.59				
0.60 - 0.69				
0.70 - 0.79				
0.80 - 0.89				

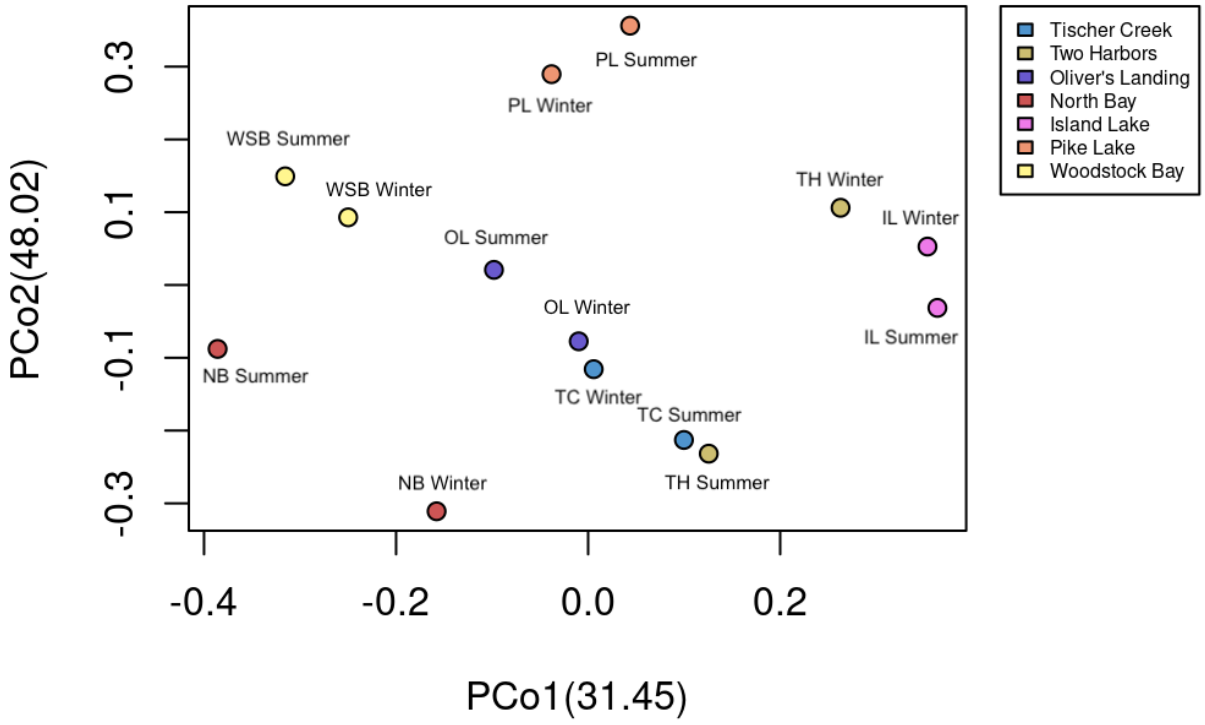


Figure 8. Principal coordinate analysis for the winter and summer sampling period. A Bray-Curtis dissimilarity matrix was calculated for winter against summer sites. Principal coordinate 1 accounts for 31.45% of the variation, while principal coordinate 2 accounts for 48.02% of the variation. Each point represents a different summer and winter site. .

Table 5. Beta diversity values of diatom communities during winter against summer

Sites	TC Summer	TH Summer	OL Summer	NB Summer	WSB Summer	PL Summer	IL Summer
TC Winter	0.29	0.52	0.35	0.58	0.53	0.61	0.44
TH Winter	0.47	0.55	0.53	0.73	0.61	0.53	0.36
OL Winter	0.51	0.57	0.41	0.62	0.44	0.57	0.59
NB Winter	0.54	0.64	0.61	0.61	0.43	0.69	0.82
WSB Winter	0.7	0.79	0.62	0.81	0.55	0.73	0.8
PL Winter	0.6	0.67	0.58	0.7	0.46	0.29	0.59
IL Winter	0.47	0.52	0.58	0.75	0.72	0.54	0.31

Legend		Similar		Dissimilar
0.20 - 0.29		0	-	1
0.30 - 0.39				
0.40 - 0.49				
0.50 - 0.59				
0.60 - 0.69				
0.70 - 0.79				
0.80 - 0.89				

A Bray-Curtis dissimilarity matrix can also be calculated to look at how similar or dissimilar sites are across the seasons. PCo1 accounts for the majority of the variation with 70.09%, while PCo2 accounts for 48.02% of the variation. Most of the communities across the seasons are clustered with each other, however, there are two sites where the summer and winter communities are not clustered together. North Bay's summer and winter diatom communities do not cluster with each other or with any other site. The Two Harbors summer and winter communities do not cluster together, but they do cluster with other sites. The Two Harbors summer community clusters with that of Tischer Creek summer site. Two Harbors winter community clusters closely with the winter community at Island Lake (Fig. 7). North Bay was the most dissimilar to Island Lake at 0.82. Tischer Creek and Pike Lake summer and winter sites are most similar at 0.29 (Table 5).

2.3.6 Community and highly branched isoprenoid analysis

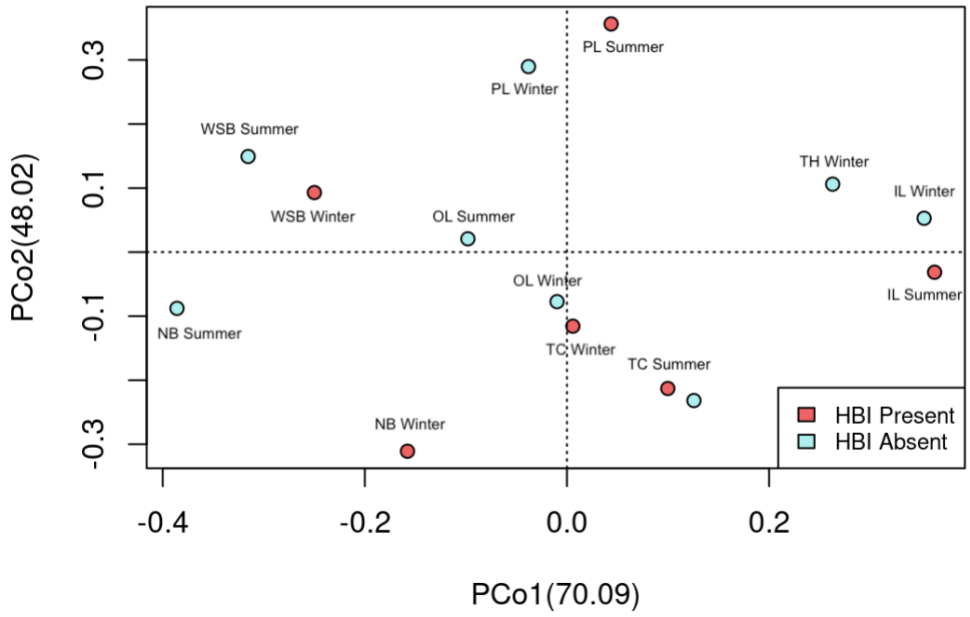
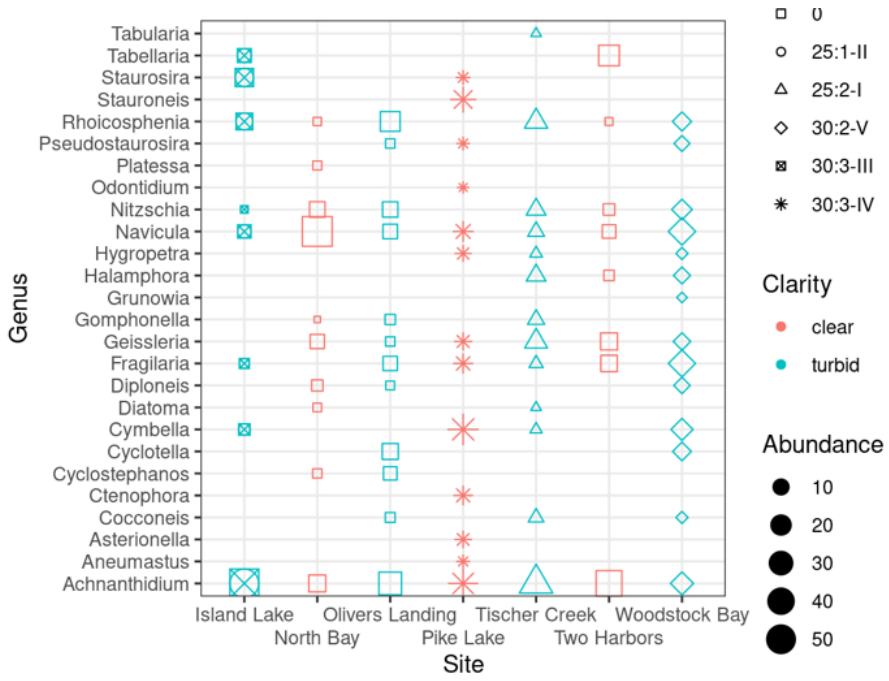


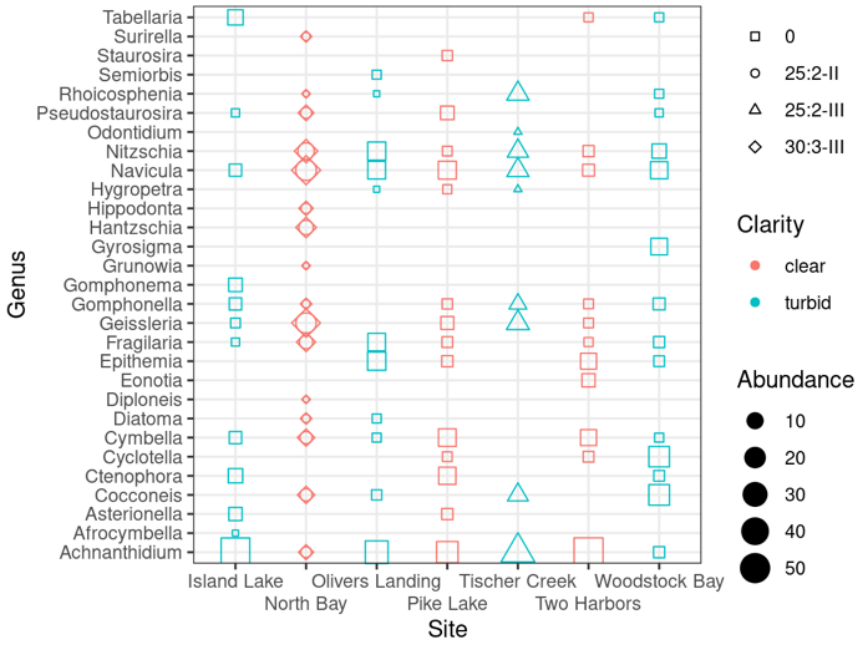
Figure 9. Principal coordinate analysis for the diatom community during the winter and summer sampling period. A Bray-Curtis dissimilarity matrix was calculated for winter against summer sites. Principal coordinate 1 accounts for 70.09% of the variation, while principal coordinate 2 accounts for 48.02% of the variation. Each point represents a different summer and winter site. Red points represent sites where HBIs were present while teal points represent sites where there were no HBIs detected. Dashed lines represent y = 0 and x = 0.

In a concurrent study of the seven sampling sites, six out of the fourteen sites across seasons had HBIs detected (Hanson, 2022). Two of these sites had HBIs detected in the summer; in three sites, HBIs were detected in the winter. Figure 8 shows a principal coordinate analysis of summer and winter diatom communities, color-coded by whether or not HBIs were detected. No clear clustering of sites was observed based on

the presence or absence of HBIs. Additionally, Tischer Creek winter and summer diatom communities clustered together (Fig. 8).



A)



B)

Figure 10. Relative abundance during the summer and winter sampling periods. The y-axis represents the genera found at each site, and the x-axis represents each site. The size of the bubble is scaled to indicate the percent relative abundance, with the smallest size representing around 10% relative abundance, and the largest size representing 50% relative abundance. The red bubbles represent clear water. The teal bubbles represent turbid water. Different symbols signify different HBIs detected. **A)** Bubble plot representing the summer sampling period (5/25/21– 6/16/21). **B)** Bubble plot representing the winter sampling period (11/20/21–12/04/21).

During the summer, species belonging to the genus *Cymbella* were found only at sites where HBIs were detected. Species of *Cymbella* occur at Island Lake, Pike Lake, Tischer Creek, and Woodstock Bay. Island Lake and Pike Lake have an occurrence of C30 HBIs, but C25 HBIs were detected at Tischer Creek and Woodstock Bay.

Cymbella only occurred during the summer months. In the winter, no genus was exclusively found where HBIs were detected. Two sites during the winter had HBIs: C30 and C25 HBIs occurred at North Bay, and C25 HBIs were found at the Tischer Creek site.

Qualitative factors of bed stability and water clarity were recorded for each site (Table 1) to examine patterns of co-occurrence with HBIs.

2.4 Discussion

Genera that are present during both seasons are common genera found near the Laurentian Great Lakes. Dominant genera include *Achnanthidum, Navicula,* and *Cyclotella* (Barbiero and Tuchman, 2001) Sampling sites during the summer and winter have a large and reliable abundance of *Achnanthidum* and *Navicula* as these show up at

all sites. (Fig. 3). Relatively rare genera including *Odontidium* also show up in our sites along with the Great Lakes regions

North Bay, Woodstock Bay and Pike Lake have the most different genera in the summer sampling (Fig. 3). North Bay is the largest outlier among the three, and groups considerable away from Oliver's Landing, Tischer Creek, Two Harbors, and Island Lake. North bay is the only site during the summer with a large abundance of *Navicula* present at around 50% relative abundance. *Platessa, Diatoma,* and *Cyclostephanos* all show up at North Bay and only one other site. These two factors are most likely to explain the high dissimilarity of the communities between North Bay and the other sites. North Bay is one of the sites that was oversampled during the summer sampling at over 500 for a sampling size (Fig. 2). However, North Bay was sufficiently sampled during the winter sampling point, and it still groups away from the other points during this time period.

During the winter sampling period, North Bay and Woodstock Bay still have the most different genera among the sampling sites as they group away from each other (Fig. 6). Compared to the summer, Woodstock Bay is the largest outlier among the sampling sites being considerably away from the other six. *Cyclotella, Cocconeis* (Ehrenberg), and *Gyrosigma* occur at Woodstock Bay at higher relative abundances than other sites. Pike Lake during the winter is similar to three other groups. During the summer, Pike Lake was different showing a shift in the compostion of diatom communities there.

The diversity between the summer and winter samplings are very similar to each other. North Bay and Two Harbors are the only sites where the summer and winter samplings do not group together (Fig. 7). During the summer, Shannon-Weiner diversity was not significantly different to the winter (Fig. 4). The present study is similar to

findings by Virta et al (2020) and showed alpha diversity of freshwater ecosystems is stable, even though community composition changes seasonally(?).

pH does not seem to affect the diversity of our sites in any way. When comparing sites with higher pH to sites with lower pH, Shannon-Weiner Diversity and Pielou's Evenness do not differ between the two seasons. Studies looking at freshwater lakes in the Adirondack Mountains in New York, however, show that as pH increased, the diversity of diatoms also increased (Charles, 1985). A possible explanation for this is that pH measurements were only taken on one day, and a temporal measurement could yield a more accurate picture of the pH profile at our sites.

Temperature also did not seem to affect the diversity of the diatom communities. Winter and spring sites were not different in Shannon's-diversity (Fig. 4; Table 2) and only two sites summer and winter sampling were not similar to each other (Fig. 8). Community diversity in other freshwater studies was shown to decrease with increasing temperatures (Delgado et al., 2020).

This indicates that at the present studies sites, there are more factors that could contribute to the difference found in sites. Nutrients such as nitrogen, phosphorus, and iron, along with the conductivity of freshwater ecosystems, can shape diatom communities (Virtanen and Soininen, 2012). Other factors such as high water velocity decrease planktonic diatom diversity and enrich benthic diatoms (Soininen, 2005). In future studies, nutrient availability, conductivity, and water velocity should all be considered as possible variables to study.

There was no clear pattern in diversity between sites where highly branched isoprenoids were detected and absent. Although six sites between summer and winter had

HBIs, only 2 sites clustered together. The Tischer Creek summer and winter site had HBIs that were detected, and they clustered together as being similar. This is most likely due to this being the only site where HBIs were found across seasons. As previously mentioned, other factors could possibly contribute to the differences in sites. This could also be true for what controls the production of HBIs.

Qualitative factors such as turbidity and water clarity, also did not affect when HBIs occurred. HBIs occurred at both turbid and clear sites, along with stable and unstable sites. This suggests that these factors do not control HBI production. Although environmental factor are hypothesized to control the production of HBIs, it is not necessarily known what environmental factors. For example, there are differences in what isomeric forms of HBIs are distributed within surface sediment, and differences in the mean concentration of these, however it is not known why (Smik et al., 2016). There are studies that focus on HBI-producing diatoms in laboratory cultures and under what conditions they produce HBIs. *Haslea ostrearia* was grown *in situ* under varying environmental conditions such as the temperature increased, the number of double bonds in C25 HBIs also increased (Belt et al., 1996).

Temperature, pH and qualitative factors have no effect on alpha diversity, and on if HBIs occur or not. Other factors could have an effect, such as nutrient availability and velocity of the water, but species composition at each site could also have an effect. *Cymbella* was the only genus that was detected only when HBIs were also detected during the summer sampling. In a study of the occurrence of HBIs in the Florida everglades, *Cymbella* was only found where C25 HBIs occurred (He et al., 2016). He et al. also found a high correlation between the occurrence of *Cyclotella* and C25 HBIs,

contrary to the present study, in which *Cyclotella* predominated at sites where HBIs were not found.

Other genera of diatoms are known to produce HBIs. For example, species of *Navicula* (Belt et al., 2001), *Fragilaria*, and *Nitzschia* (Hanson, 2022) are all known to produce HBIs. These genera show up at most of our sites. Because only certain species of diatoms produce HBIs within a genus, sites with no HBIs present also have the occurrence of these diatoms as well.

2.5 Conclusion

Diatom communities at the seven study sites represent those found within the literature. Alpha diversity does not change between the summer and winter seasons, but some sites show clear differences. North Bay and Woodstock Bay both are dissimilar from each other and the other sites in both summer and winter diatom communities, probably due to the species composition, rather than environmental conditions. Despite differences in the literature, temperature and pH are not shown to affect diversity between the seasons. This suggests that there are other factors that differentiate sites, such as nutrients, conductivity, and velocity of the water.

Factors such as pH, temperature, turbidity, and water clarity, do not affect whether HBIs are found at our sites or not. It is more likely that certain species have the greatest effect. These include species within *Cymbella, Navicula, Fragilaria,* and *Nitzschia*.

Chapter 3. Growth optimization and identification of a highly branched isoprenoid producing diatom, *Nitzschia frustulum*.

3.1 Goals for the present study

Goal: The two main goals for this present study are 1) to isolate HBI-producing diatoms from Lake Superior or surrounding freshwater systems and determine the optimal growth conditions for these diatoms, and 2) to better understand the conditions when HBIs are produced by the isolated diatoms. This will facilitate experimentation on HBI-producing diatoms, such as transcriptomics and further manipulations of environmental conditions to determine the enzymatic pathway leading to HBI production.

Hypothesis 1: Isolated diatoms will grow at an optimal pH of 8 and temperature of 20°C.

Hypothesis 2: *Nitzschia frustulum* will produce HBIs at a pH and temperature that is not optimal for growth.

3.2 Experimental Methods

3.2.1 Isolation of *Nitzschia frustulum*

Nitzschia frustulum (Grunow) was isolated from the Two Harbors Lighthouse nearshore pool (Lat. 47.01415, Long. -91.662215) in September of 2020. Temperature and pH were measured using a handheld thermometer (VWR, 61019-001) and a pH probe (VIVOSUN, PH-001). Rock samples were collected from the study site and stored in an environmental growth chamber (Percival, E36LX4) with a 12h day/night cycle at 20°C (standard conditions) until they were processed. Modified freshwater media (MF) (Cohn and Pickett-Heaps, 1988) was used. Autoclaved filter paper (11.0 cm, Whatman,

1541-110) as placed on cooling agar plates and removed once agar plates were fully solidified. This created a rough surface on the agar plates for benthic diatom enrichment.

For the inoculation of plates, 10 µl of water surrounding sampled rocks was pipetted onto agar plates. Pipetting was done to spread out algal species to ensure that potential diatom colonies would not be outcompeted by surrounding algae species. Five milliliters of MF media was placed on each plate to prevent colonies from desiccation. Plates were incubated in an environmental growth chamber under standard conditions at pH 7 until visible colonies formed.

Colonies were picked with a 20 µl pipette tip and examined using a Nikon Eclipse TS2 light microscope at 40x to confirm if diatoms were present. Diatom colonies were transferred to a new rough surface agar plate and incubated under standard conditions until colonies were visible. Once the colonies were absent of green algae, they were transferred to 125 ml Erlenmeyer flasks with MF media and passaged every three weeks to ensure continuous growth of the culture.

3.2.2 Identfication of *Nitzschia frustulum*

A morphologic approach was used to identify *Nitzschia frustulum* by identifying common frustule features (De Tommasi et al., 2017). Samples were cleaned of all organic material to expose precise details of frustules, including striae and fibulae. Diatoms were placed in a 10 ml culture tube (Basix, 14-d55-413) with 5.25% sodium hypochlorite for three hours. After the diatoms settled to the bottom, the bleach supernatant was removed. The cultures were washed 5 times with deionized water until the sample reached a neutral pH (Carr et al., 1986). Diatoms were examined under light microscopy at 100x to

determine the length and width, along with striae and fibulae density for morphologic identification (Spaulding et al., 2021).

3.2.3 Growth Optimization Assays

Growth curves were conducted in 10 ml glass culture tubes (Basix, 14-d55-413) with 5 ml of MF media, in triplicate for each time point. All cultures were inoculated with 1.5 x 10^3 cells/ml. The cultures were incubated in an environmental growth chamber set at a 12-hour day/night cycle and counted every two days until the stationary phase was reached. Each tube was vortexed and sonicated to break up diatom aggregates and subsequently counted using a hemocytometer (Hausser Scientific, 426587). Concentrations in cells/ml were recorded and doubling time was calculated graphically to determine specific growth rate and carrying capacity. Temperatures of 5°C, 10°C, 15°C, 20°C and 25°C and pH of 6, 6.5, 7, 7.5, and 8 were tested.

3.2.4 Highly Branched Isoprenoid Identification in Nitzschia frustulum

Nitzschia frustulum was grown in 150 ml Erlenmeyer flasks (n = 5) in 125 ml of MF media. To test for HBI production at different pH ranges, MF media was corrected to the corresponding pH. For the temperature assays, Erlenmeyer flasks were placed in an environmental growth chamber that was set to the corresponding temperature. Cultures were incubated until stationary phase was reached, between 20 days and 25 days.

Samples were sonicated to separate aggregated diatoms that formed on the glass surface of the Erlenmeyer flask. The resulting media was filtered through a glass fiber filter with a 0.7 µm pore size, and samples were wrapped in an aluminum foil capsule

and freeze dried. Freeze dried samples underwent lipid extraction and were analyzed via GC/MS for HBI structure and abundance (Belt et al., 2012).

3.2.5 Data Analysis

Growth curves were visualized using the R software (R Core Team, 2022) along with Microsoft Excel(Microsoft Corporation). A logistical curve was fit to each growth curve, to calculate carrying capacity. Using the GrowthCurver R package (Sprouffske and Wagner, 2016) and the K-value in the logistical growth equation, carrying capacity can be found. Specific growth rate (Eq. 1) and doubling time (Eq. 2) (Zhang et al., 2018) were calculated in Microsoft Excel and also estimated from the growth curves. A one-way analysis of variance (ANOVA) was used to test for significant differences in specific growth rate, doubling time, and carrying capacity among treatments. Tukey's post-hoc analysis (Haynes, 2013) was performed if the ANOVA exposed any significant differences. The Tukey's post-hoc analysis was used to determine significant differences in mean values among groups.

$$1)\ \mu = \frac{(lnN_{t2} - lnN_{t1})}{\triangle\, t}$$

$$2)\ dt = \frac{ln2}{\mu}$$

3.3 Results

3.3.1 Study location and isolation of *Nitzschia frustulum*

Diatoms collected at the Two Harbors study site were isolated. The water temperature at the time of isolation was 17.2 ± 0.3°C and the average pH was 8.82 ±

0.03. The isolated species was 35–45 µm long and 3–3.5 µm in width. Stria density was approximately 32 striae/10 µm while fibulae density was approximately 11 fibulae/10 µm. Sequencing of the COI, ITS and 18S genes confirmed the identity of *N. frustulum* genetically.

Figure 11. Isolated *Nitzschia frustulum* at 100x magnification.

3.3.2 Growth optimization assay

Growth experiments were performed to test the optimal pH and temperature conditions for *Nitzschia frustulum*. The pH assays were run for 24 days until the stationary phase was reached. For all pH's, the lag phase lasted approximately 10 days and cell counts were not statistically different during this time. The cultures reached exponential growth phase at 10 days for all pH conditions. *Nitzschia frustulum* grown at pH 6 and 6.5 reached stationary phase faster than when grown at other pH's, with stationary phase occuring on day 18 (Fig. 11). The different specific growth rates and doubling times at pH 6 and 6.5 (Fig. 12; Table 6) were not significantly different. *Nitzschia frustulum* grown at pH 7, 7.5 and 8 reached the stationary phase around 20 days. The carrying capacity at pH 7.5 and 8 were $2.6 \times 10^5 \pm 2.9 \times 10^4$ cells/ml and 2.5 x

$10^5 \pm 1.1 \times 10^4$ cells/ml respectively. The carrying capacities of pH 7.5 and 8 were significantly different than those of pH 6, 6.5, and 7 at $9.5 \times 10^4 \pm 3.5 \times 10^4$ cells/ml, $1.5 \times 10^5 \pm 3.1 \times 10^4$ cells/ml, and $1.7 \times 10^5 \pm 3.6 \times 10^4$ cells/ml (Fig. 13; Table 6)

In the 5°C, 10°C, 15°C conditions, there was limited to no growth of *N. frustulum*. The only measurable growth of *N. frustulum* occurred at 20ºC and 25ºC. There was no difference in the length of lag phase between 20ºC and 25ºC, however the cell count at this time was differed, as *N. frustulum* grown at 25ºC had a higher cell count then *N. frustulum* grown at 20ºC, with a difference of 1.0×10^5 cells/ml. For *N. frustulum* grown at 20ºC, the exponential growth phase ended at around 20 days, but the exponential phase for the cultures grown at 25ºC ended on day 18 (Fig. 11B). There was a significant difference in both specific growth rate, doubling time, and carrying capacity between 20ºC and 25ºC treatments. *Nitzschia frustulum* grown at 25ºC had a faster specific growth rate (0.30 ± 0.002 d^{-1}) and doubling time (2.31 ± 0.02 days) (Fig. 14) and a significantly higher carrying capacity ($2.91 \times 10^5 \pm 1.61 \times 10^3$ cells/ml) when compared to 20ºC (Fig. 15; Table 6).

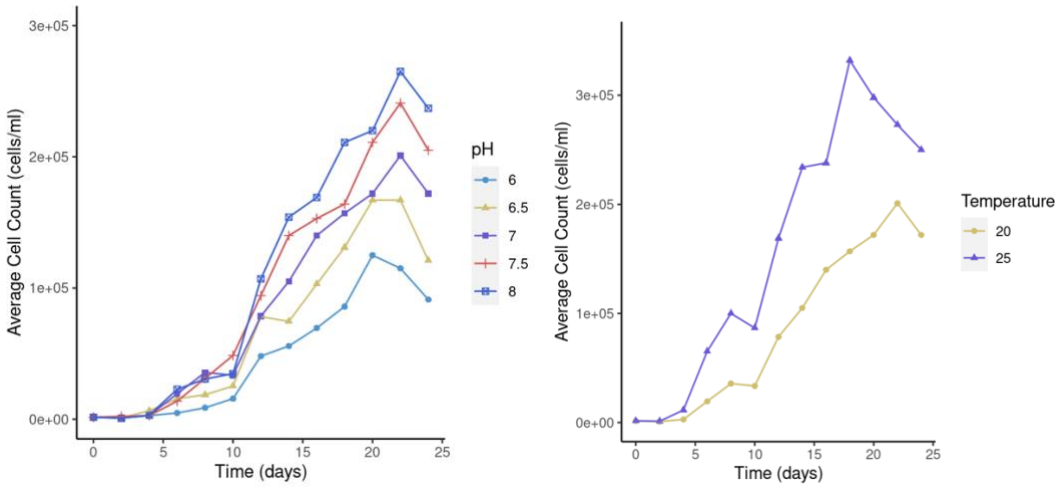
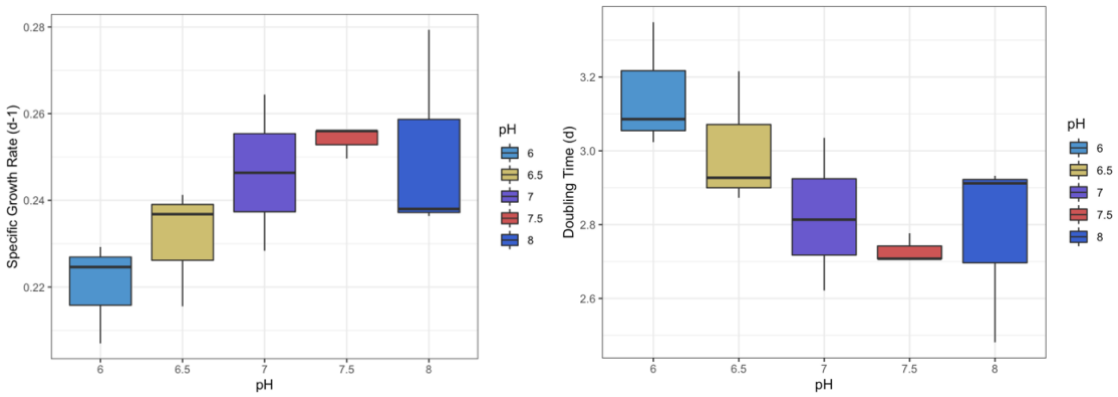


Figure 12. pH and temperature growth curves for *Nitzschia frustulum*. The y-axis indicates average cell count (cells/ml) and the x-axis represents time in days. **A)** Lines indicate the average number of cells counted every two days. pH 6, 6.5, 7, 7.5, and 8 are graphed. **B)** Lines indicate the average number of cells counted every two days. There was no growth for the 5°C, 10°C, and 15°C trials. Each line represented 20°C and 25°C (gold and blue, respectively).

Table 6. Growth metrics for pH and temperature growth curves.

Condition	ific Growth Rate (	Doubling Time (d)	Carrying Capacity (cells/ml)	HBIs Identified
pH				
6	0.22 ± 0.013	3.15 ± 0.19	9.50E+04 ± 3.51E+04	ND
6.5	0.23 ± 0.016	3.01 ± 0.21	1.54E+05 ± 3.14E+04	ND
7	0.25 ± 0.020	2.82 ± 0.23	1.66E+05 ± 3.55E+04	C30:2
7.5	0.25 ± 0.004	2.73 ± 0.05	2.58E+05 ± 2.88E+04	ND
8	0.25 ± 0.028	2.77 ± 0.29	2.50E+05 ± 1.11E+04	ND
Temperature				
20	0.25 ± 0.020	2.82 ± 0.23	1.66E+05 ± 3.55E+04	C30:3
25	0.30 ± 0.002	2.31 ± 0.02	2.91E+05 ± 1.61E+03	C25:4



A) **B)**

Figure 13. Specific growth rate and doubling time of *N. frustulum* grown at varying pH. The median, upper and lower quantiles, and min and max values are shown. There is no significant difference between specific growth rates, and between doubling times. **A)** The y-axis represents a specific growth rate at a different pH. The x-axis represents a different pH. **B)** The y-axis represents the doubling time for each pH condition, while the x-axis represents a different pH.

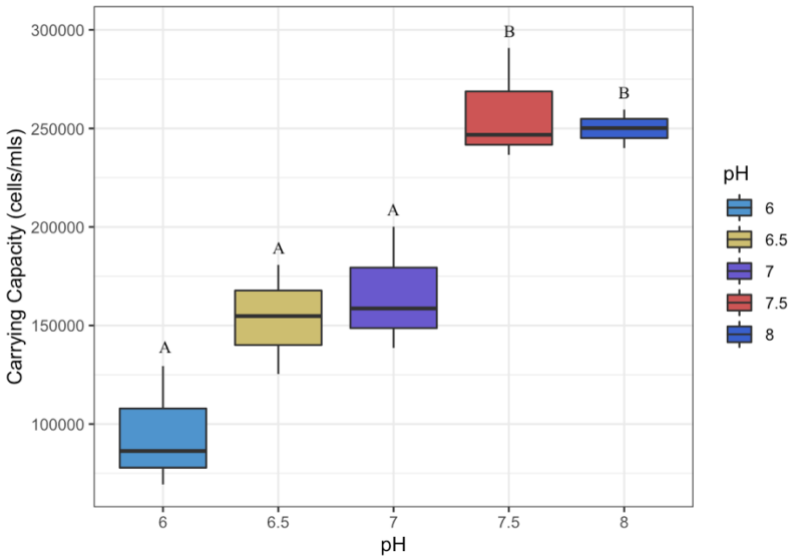


Figure 14. Carrying capacity of the population for each pH condition. Significant differences are indicated by letters. Shared letters indicate no significant difference and different letters represent a significant difference. The y-axis signifies the carrying capacity for each pH while the x-axis represents a different pH condition.

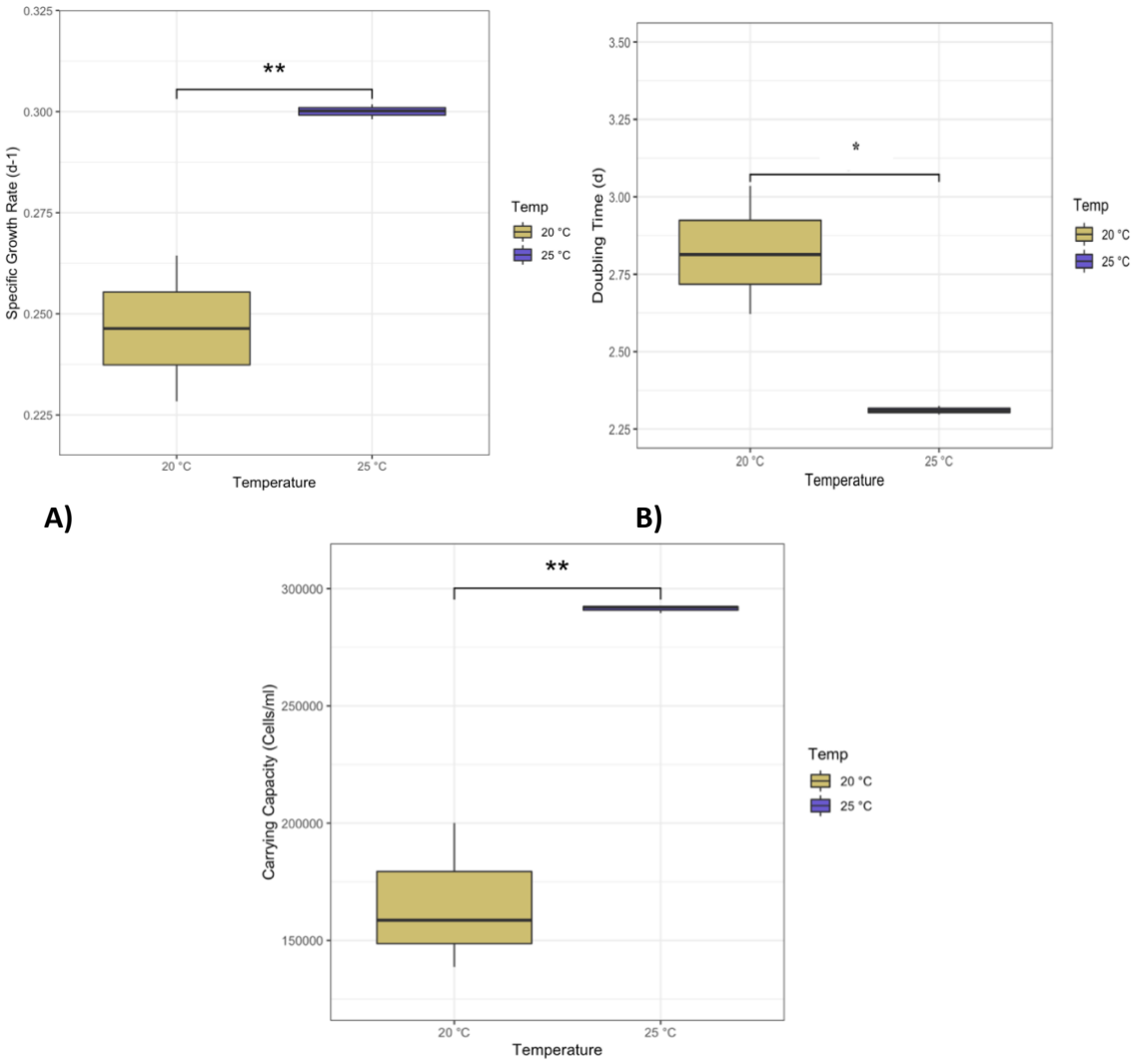


Figure 15. Specific growth rate and doubling time for *N. frustulum* grown at two temperatures. A) The y-axis represents a specific growth rate for different temperatures, and the x-axis represents temperature. Asterisks represent the significance level (** = p< 0.001). **B)** The y-axis represents the doubling time for different temperatures, and the x-axis represents temperature. The asterisk represents the significance level (* = p< 0.01). **C)** The y-axis represents the carrying capacity for each temperature and the x-axis represents different temperatures. The asterisk represents the level of significance (** = p< 0.001).

Standard deviations of all points were calculated for each growth curve. For both pH and temperature, there is a high amount of variability for each average cell count (Fig. 16). This is confirmed by the carrying capacity of pH 7.5 and 8 still being significantly different from pH 6, 6.5 and 7. In the temperature assays, the variation at each average cell count is also high for 20ºC and 25ºC (Fig. 15B).

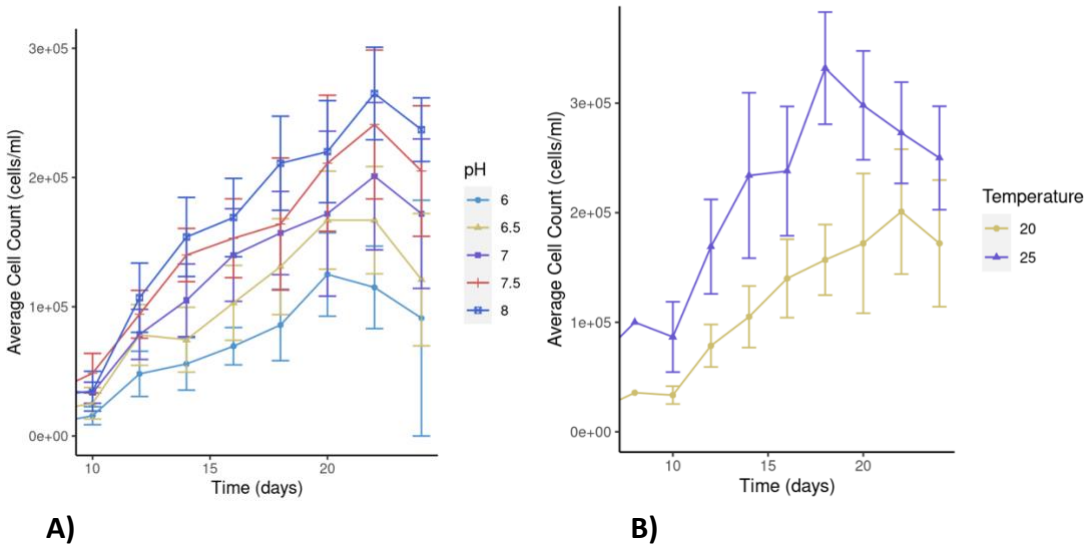


Figure 16. Impact of varying pH and temperature on *Nitzschia frustulum* growth. The y-axis represents the average cell count and the x-axis represents the time in days. Error bars represent the standard deviation of each data point. **A)** Lines represent the average cell count every two days for pH 6, 6.5, 7, 7.5, and 8. The x-axis represents days 10 through 24 to increase resolution of the exponential phase. **B)** There was no growth of *N. frustulum* at 5 °C, 10 °C, and 15 °C. The x-axis represents days 9 through 24.

3.3.3 Highly branched isoprenoid identification

Nitzschia frustulum was grown at different pH and temperature conditions to determine the condictions when HBIs are produced. Three different HBIs were identified from seven conditions that were tested. In cultures grown at pH 7, a C30 HBI was identified with 2 double bonds. There were no HBIs produced by *N. frustulum* grown at pH 6, 6.5, 7.5 and 8. At 20ºC, a C30 HBI was produced with 3 double bonds and a C25 HBI was produced at 25°C with 4 double bonds (Fig. 17; Table 6).

3.4 Discussion

3.4.1 Optimal growth conditions for the freshwater diatom *Nitzschia frustulum*

Nitzschia frustulum was found to grow optimally at a pH of 7.5 to 8. There was no significant difference between the specific growth rate and doubling time for the range of pH tested, however the carrying capacities differed. Diatoms grown at pH 8 and 7.5 had a significantly higher carrying capacity than at pH 6, 6.5 and 7. Even though pH 6 and 6.5 reached the stationary phase faster than pH 7.5 and 8, the carrying capacity of pH 6 and 6.5 were 9.50 x 10^4 ± 3.51 x 10^4 cells/ml, 1.54 x 10^5 ± 3.14 x 10^4 cells/ml respectively. This indicates that pH 6 and 6.5 are not optimal pH's. Other species within the *Nitzschia* genus have been grown in laboratory cultivations. Jiang *et al.* grew *Nitszchia sp.* at 20 – 23°C and at pH 7.5 to 7.9 (Jiang et al., 2020).The *Nitszchia frusutulum* in our study was similar to what was grown in this study, however *Nitszchia sp.* did come from a supplier whereas in the present study they were naturally isolated.

In marine environments, diatom biomass is usually higher at pH of 8. Biomass decreases after growth at pH 8.5–9.5 in certain diatom species (Pedersen and Hansen, 2003). In laboratory studies, *Chaetoceros muelleri* (Lemmermann) had a lower biomass at pH 6.8 compared to pH 7.4– 7.9 (Thornton, 2009). The specific growth rate of *Nitzschia frustulum* at a given pH differed, from $0.22 \pm 0.01\ d^{-1}$ at pH 6, to $0.25 \pm 0.03\ d^{-1}$ at pH at 8. This is similar to other laboratory experiments conducted on the marine diatom *Skeletonema costatum*, in which growth rates in pH ranging from 6.5 to 8.5 did not change significantly (Taraldsvik and Myklestad, 2000). The Two Harbors site from which *Nitzschia frustulum* was isolated had a pH of 8.82 ± 0.03, higher than what was tested in the laboratory experiments. It is possible that the optimal pH for *Nitzschia frustulum* may be higher, and more growth curves need to be done to confirm this.

The optimal temperature for the growth of *Nitzschia frustulum* was 25°C. There was a significant difference in both specific growth rate, doubling time, and carrying capacity between 20°C and 25°C. At 25°C, *N. frustulum* grew faster and had more biomass. The growth of diatoms has been shown to have an exponential increase as temperature increases, as diatom specific growth rate, division time, and carrying capacity all increase in a variety of species of marine diatoms (Montagnes and Franklin, 2001). Faster specific growth rate, doubling time, and carrying capacity also match other species in *Nitzschia*. In laboratory studies, two freshwater *Nitzschia* spp. were found to have faster growth rates and more biomass as temperature increases (Admiraal, 1977). Both the studies by Admiraal and Franklin showed *Nitzschia* growth at temperatures below 20°C and even at 4°C (Admiraal, 1977 and Franklin, 2001). Further studies in the freshwater diatom *Nitzschia palea* show that, although the optimal temperature for

growth was 30°C, *N. palea* still grows at temperatures below 18°C (Vitug and Baldia, 2014). *Nitzschia frustulum*, however, does not show growth below 20°C, potentially owing to a stress response in changing temperatures during inoculation of culture tubes. By acclimating *N. frustulum* before inoculation, cultures might be able to withstand the stress of changing conditions.

3.4.2 Highly branched isoprenoid production in *Nitzschia frustulum*

The present study reports the first recording of HBI production by a diatom species in the genus *Nitzschia*. *Nitzschia frustulum* produces both C25 and C30 HBIs with varying amounts of double bonds. Species belonging to the genera *Haslea, Navicula, Rhizosolenia, Pleurosigma,* and *Berkeleya* have all been reported to produce C25 HBIs (Belt et al., 1996, 2001a, 2001b; Brown et al., 2014; Volkman et al., 1994). Only the marine diatom *Rhizosolenia setigera* has reportedly produced a C30 HBI (Volkman et al., 1994). Like *R. setigera*, the freshwater diatom *N. frustulum* produces both C25 and C30 HBIs with varying degrees of saturation. Other studies have found that some members of the *Nitzschia* genus do not produce HBIs (Brown et al., 2014).

Nitzschia frustulum produces C30 HBIs at pH 7 and 20°C and C25 HBIs at 25°C. There are a limited number of *in situ* studies of environmental conditions that contribute to the production of HBIs, most of which are focused on the marine diatom *Haslea ostrearia.* Rowland et al. (2001) found that as temperature increased, the number of double bonds within the C25 HBIs increased. At 25°C, four double bonds were present, at 15°C three double bonds were present while at 5°C two double bonds were present. The temperatures that were tested in the present study were 20° C and 25° C, but *N.*

frustulum produced HBIs at these temperatures. The number of double bonds present within the C25 HBI produced at 25°C was 4 which is the same as that in *H. ostrearia*. All temperature assays were conducted at pH 7. This could potentially explain why HBIs were detected at pH 7, while there were none at other pH's. This suggests that pH is important in the actual production of HBIs, while temperature is important in determining how many double bonds are present within the resulting HBI molecule.

Highly branched isoprenoids have been previously suggested to be membrane lipids ((Pozzi et al., 1996). As temperature increases, membrane fluidity increases. Inversely if temperatures decrease, the fluidity of the membranes also decreases. To create a more fluid membrane at colder temperatures, unsaturated membrane lipids and cholesterol pack into the phospholipid bilayer to improve membrane fluidity (Raffy and Teissié, 1999)

3.5 Conclusion

Nitszchia frustulum was isolated from a freshwater ecosystem. *Nitzschia frustulum* is the first species identified within the genus to produce HBIs. The optimal growth conditions for *N. frustulum* are 25°C and a pH between 7.5 and 8. C25 and C30 HBIs were produced by *N. frustulum* at pH 7 at 25°C, 20°C respectively. This is the second diatom and first freshwater diatom reported to produce both C25 and C30 HBIs under laboratory conditions.

Because there was no growth at 5°C, 10°C and 15°C, more studies should be done to confirm if HBIs are produced by *N. frustulum* at these temperatures. By doing so, more evidence could be added to support that temperature is more important than pH in HBI

synthesis and to support the hypothesis that HBIs are membrane lipids that aid in membrane fluidity.

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