Addressing Indigenous community concerns of lake health by assessing water quality in Constance Lake, ON and biomarker responses in freshwater mussels, *Pyganodon grandis* and *Lampsilis siliquoidea*

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Abstract

Constance Lake First Nation has had concerns regarding the health of a culturally significant lake, Constance Lake, for several years. There are several external stressors to this lake including urbanization and forestry, and as a result the community has stopped utilizing the lake for cultural and recreational purposes. To investigate these concerns, specifically regarding eutrophication and algal blooms, this study examined water chemistry and biomarker responses pertaining to energy and stress in freshwater mussels *Lampsilis siliquoidea* and *Pyganodon grandis*. The purpose was to determine current trophic status and potential for future algal blooms, as well as determine stress levels in mussels from eutrophication. Studies on glycogen levels over the winter season also provided some insight into overwintering strategies for freshwater mussels. Indigenous Knowledge from Constance Lake First Nation was used to guide this study, and tissue from the gills and foot of mussels were assayed to determine levels of glycogen and lipid peroxidation, specifically 4-hydroxynonenal. It was found that Constance Lake is still in a eutrophic state and has the potential to produce harmful cyanobacterial blooms for years to come. Glycogen and 4-hydroxynonenal levels varied between species, however it appears that the biomarker responses observed could be a result of normal life-cycle patterns and the fact that *L. siliquoidea* and *P. grandis* are tolerant species to outside stressors, suggesting that the mussels in Constance Lake are not stressed. Not only has this study responded to concerns raised by an Indigenous community, but it also has provided insight into the overwintering strategy of freshwater mussels.

Lay Summary

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The over-arching theme of this research was to connect Indigenous Knowledge with western science to be able to respond to environmental concerns. This helps to explain the diversity of life because this research responded to Indigenous community concerns through two lenses. This study also adds to our understanding of biology of freshwater mussels by evaluating two abundant species and learning more about their life histories and how they respond to certain stressors.

Acknowledgements

Water Acknowledgements

As an Ojibwe woman working with and within the water, it is customary that I acknowledge Grandmother Earth and the water that she provides to us. Women are water protectors. We carry the water from which we all come from, for nine months. We need water to survive. My familial grandmother is a survivor of residential school and with that, I am a survivor of intergenerational trauma and have grown up without culture, language, or an identity. I am thankful to have the opportunity through my career and my research to regain my culture, my language, and identity as a water protector. With that, I thank Grandmother Earth for her teachings and for the water that she provides us.

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I give thanks to Constance Lake First Nation for allowing me to conduct my research within their homelands and traditional territory and giving me the opportunity to address some of their concerns regarding a lake that is so culturally important to the community. It is also important to acknowledge that my education and the place I call home, are within the homelands and traditional territory of Fort William First Nation.

I acknowledge that part of my ancestry, paternal side (English, Irish, and French), are settlers to these lands, and I accept and carry the responsibilities that come with being a child from both an Indigenous and settler way of living, rather, Two-Eyed Seeing. Being one who has lived, walked, and studied both ways of life, I have come to adopt many friendships and allies in both worlds. By looking through two lenses, both western science perspective and Indigenous ways of knowing, I have been able to not only take what I have learned and what I have been taught to move towards more meaningful relationships with both our peoples and the land in which we live upon.

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Completing a graduate degree is not something one undertakes on their own, there are many people both in the foreground and behind the scenes who help. First and foremost, I thank my family and friends for their continued support through this long journey, whether it was through words of encouragement or coming up with ways in which to distract me from my studies, to keep me sane while juggling life, graduate work, and a full-time job. I thank my dad for teaching me how to fish, drive a boat (and let me borrow yours), learn to be mechanically inclined, and be comfortable and confident around vehicles and on the water. These skills have helped me to be more of an

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Partnership and Funding Acknowledgements

I thank Matawa First Nations Management for the opportunity to pursue higher education and skills advancement, all while being able to work a full-time job in a project management role. This role allowed me to participate in several environmental monitoring projects while learning from our Guardians and passing on my skills and knowledge to the Matawa member communities. I thank the Department of Fisheries and Oceans for their funding through the Indigenous Habitat Participation Program. This funding not only allowed me to further develop the skills and knowledge needed to assist the Matawa member communities in their environmental monitoring of fish and fish habitat, but also allowed me to have access to and collect the data which assisted in the completion of my research. My current career, both in my work and in academics, would not have been possible without the Matawa Water Futures project, funded by Global Water Futures out of the University of Saskatchewan. Through this project I was fortunate to meet, and work with Dr. Kelly Munkittrick, University of Calgary. I thank and appreciate him for his support and guidance throughout my research and being given the opportunity to work with his laboratory partners and students on the Transboundary Water Monitoring project between the Fort Smith and Fort Resolution Indigenous communities, Government of Alberta and Government of Northwest Territories.

Dedication

I dedicate this work to my mother and grandmother. They have raised me into the woman that I am today - caring, light-hearted, and persistent. These women have provided me with love, encouragement, and unwavering support throughout my life, and especially while completing my graduate degree.

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List of Abbreviations

AChE - acetylcholinesterase

BPA – Bisphenol A

CCME – Canadian Council of Ministers of the Environment

CI – condition index

CLFN – Constance Lake First Nation

CWQG – Canadian Water Quality Guidelines

EED – environmental exposure distribution

FMZ – Fisheries Management Zone

GFN – Ginoogaming First Nation

GST – glutathione-S-transferase

HNE – 4-hydroxynonenal

LPO – lipid peroxidation

LUEL – Lakehead University Environmental Laboratory

LUGL – Lakehead University Geology Lapidary

MFNM – Matawa First Nations Management

MN – micronucleus

MNRF – Ministry of Natural Resources and Forestry

MT – metallothionein

NRR – neutral red retention

PBS – phosphate buffered saline

PWQO – provincial water quality objectives

WDS – waste disposal site

WQC – water quality criteria

Chapter 1: General Introduction

1.1 Constance Lake First Nation

Constance Lake First Nation (CLFN), a member community of Matawa First Nations Management (MFNM), established in its current location (adjacent to Constance Lake on the east side) in 1943 because of Indian Affairs (now Indigenous Services Canada) amalgamating the English River First Nation and the indigenous peoples living around Pagwa (near present-day Constance Lake First Nation; Constance Lake First Nation, 2018). In 1951, a sawmill (now known as Lecours Lumber Ltd.) was established on the south side of the lake, which historically would float logs across the lake prior to being processed (Hearst Public Library, 2006). To float these logs, the outflow was dammed with a small control structure. This resulted in flooding of wetlands, more so on the west side of the lake, and community member reports state that water levels rose approximately 3 m. Current operations of Lecours Lumber produce more than 100 million feet of lumber per year. The sawmill can be seen and heard directly across the lake by Constance Lake First Nation. The tallest structures that can be seen are a woodchip storage dome and a silo/filling station for the woodchip hauling trucks. Community members have stated that during windy days, the woodchips can be seen blowing across Constance Lake. In 1975, the Ministry of Natural Resources and Forestry (MNRF) established the Calstock Waste Disposal Site (WDS), which was meant to be a temporary location for storage of residential waste. The WDS closed 38 years later in September 2018 but is yet to be fully decommissioned (M. Ladouceur, personal communication, October 7, 2019). As a result, the WDS is not properly capped (with impermeable materials) and still has the potential to leach. The WDS is located to the

southeast of Constance Lake, adjacent to Pike Lake. Pike Lake ultimately flows into Constance Lake.

Figure 1 shows the watershed detail of Constance Lake, including flow direction into and out of the lake (©Kings's Printer for Ontario, 2023). The watershed drainage area is approximately 28 km^2 and flows northwest into the Kabinakagami River. Constance Lake is relatively shallow (5 m) with a small deep point (8 m) near the outflow of the lake. Based on a shoreline assessment that I completed in September 2019, many of the inflows were blocked either by overgrowth or beaver dams. The inflow coming from Pike Lake was not visible or accessible due to a vast number of cattails (*Typha* spp.) in the bay. The southern-most inflow was visible and passable. Although flow was not measured, water movement was not clearly visible (i.e. no ripples on the surface of the water, submerged vegetation showed limited to no movement). The inflow on the west side of the lake was not visible, despite efforts to go ashore and locate using handheld GPS. Of the three inflows on the north end of the lake, only the western-most inflow was visible, although flow into Constance Lake was inhibited due to overgrowth of shoreline vegetation. The eastern-most inflow was observed, however there was no flow of water into Constance Lake. There was a beaver dam at the outflow, drastically limiting movement of water out of the lake.

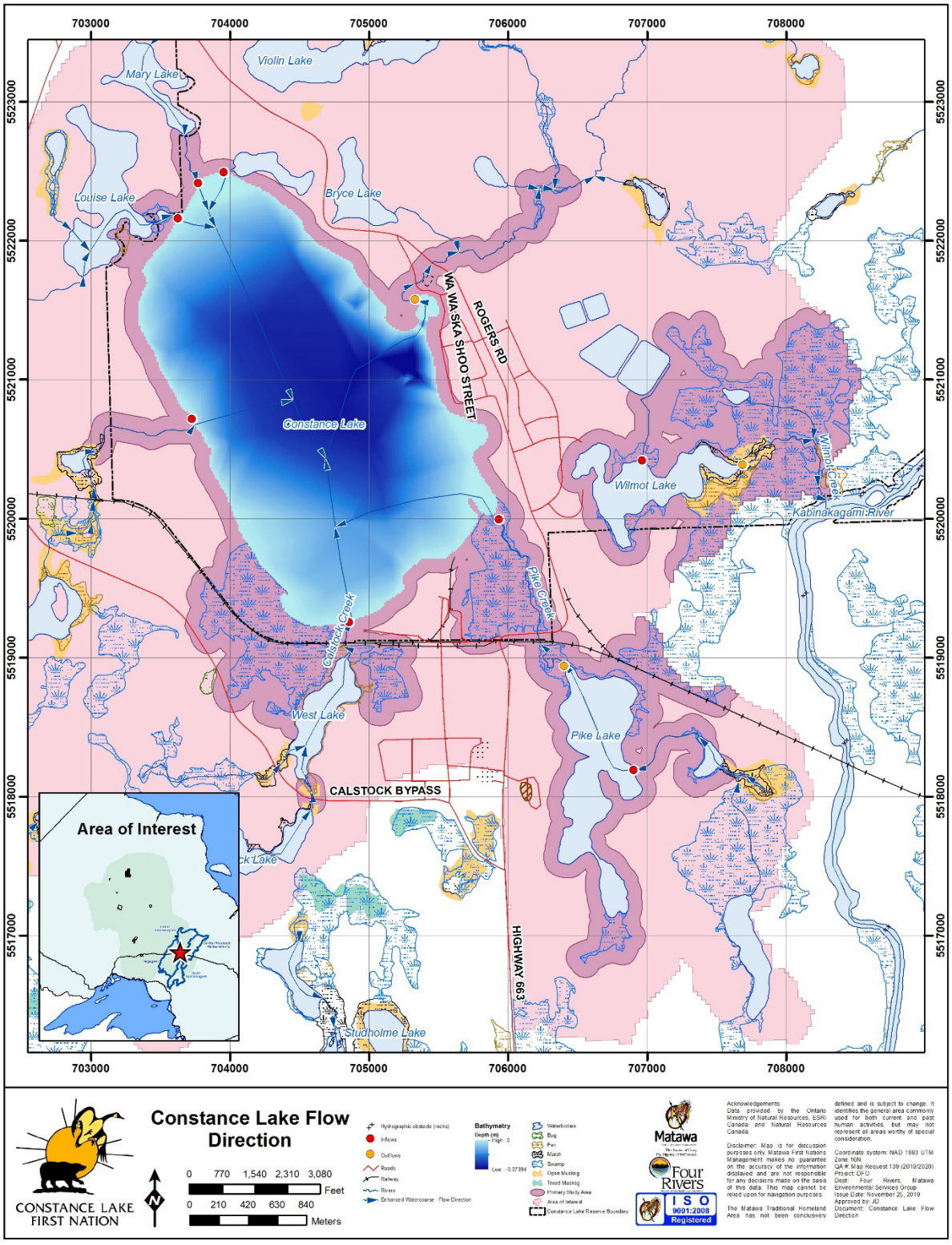

Figure 1. Map of Constance Lake flow direction as developed from the Ontario Flow Assessment Tool.

In 2010, Constance Lake First Nation experienced a total shut down of the water treatment plant due to clogging of the intake filter and contamination of the raw water storage reservoir with algae (M. Gillis, personal communication, November 13, 2019). Elevated nutrients and eutrophication have been an ongoing concern in Constance Lake for several years (Hutchison Environmental Sciences Ltd., 2014). A surface water sample taken in July 2010 at an unknown location in the lake showed the presence of *Gloeotrichia* sp., *Anabaena* sp., and *Microcystis* sp., all of which are colonial cyanobacteria associated with eutrophic conditions, and two of which are capable of producing the deadly toxin microcystin-LR (Amado & Monserrat, 2010; Carey *et al.*, 2017; Chia *et al.*, 2018). Water quality parameters provided by the water treatment plant for 2010 indicate high turbidity (average 14.65 NTU) at the beginning of April, and maximum water temperatures of 21.5°C and an average pH of 7.03 in July.

In 2013, a preliminary assessment of Constance Lake was carried out by Hutchinson Environmental Sciences Ltd on behalf of AECOM Canada Ltd to understand the drivers and impacts affecting water quality of the lake (Hutchison Environmental Sciences Ltd., 2014). In July 2013, anoxic conditions were observed below 4.5 m and it was suggested by the authors of the report that nutrients, in particular phosphorus, were potentially released into the water column providing nutrients for algal growth. Total phosphorus levels in all samples taken exceeded the Provincial Water Quality Objectives (PWQO, 0.010 $mg \cdot L^{-1}$), except for one sample in March. Samples taken in June and July ranged from 0.042 $mg \cdot L^{-1}$ to 0.051 $mg \cdot L^{-1}$, 4 to 5 times the PWQO.

Four Rivers Environmental Services Group, in partnership with Kiikenomaga Kikenjigewen Employment and Training Services, hosted a 10-day environmental

monitoring training program in Constance Lake First Nation from September 24 to October 4, 2014. Six surface water samples were taken from Constance lake on October 1, 2014, and analysis indicated that water quality parameters, except for nutrients, did not exceed PWQO or Canadian Council of Ministers of the Environment (CCME) water quality guidelines for the protection of aquatic life. Total phosphorus values in 2014 indicated that Constance Lake was in a meso-eutrophic state (0.026 – 0.036 $mg \cdot L^{-1}$). Surface water samples were collected again by Four Rivers in September 2019 for the Department of Fisheries and Oceans Indigenous Habitat Protection Program. Five surface water samples were taken, and one sample taken on the north end of the lake exceeded the PWQO for aluminum (Al, 0.075 $mg \cdot L^{-1}$), Cr (0.001 $mg \cdot L^{-1}$), iron (Fe, 0.300 $mg \cdot L^{-1}$), and lead (Pb, 0.001 $mg \cdot L^{-1}$). Sample levels for Al = 0.480 $mg \cdot L^{-1}$, Cr = 0.0011 $mg \cdot L^{-1}$, Fe = 0.883 $mg \cdot L^{-1}$, and Pb = 0.00149 $mg \cdot L^{-1}$. When comparing water quality parameters of Constance Lake to a lake with low productivity (Huebner *et al.*, 1990), it was evident that conditions in Constance Lake were deteriorated.

A meeting was held in June 2019 for the Matawa Water Futures: Developing an Indigenous-Informed Framework for Watershed Monitoring and Stewardship project. This project aimed to develop Matawa member First Nations-specific indicators and thresholds of acceptable change to water quality from an Indigenous perspective. By combining Indigenous approaches to stewardship with western water science and environmental monitoring, it is possible to link Indigenous-informed indicators of change into existing monitoring and modelling frameworks (Four Rivers, 2019). To achieve the goal of establishing Indigenous Knowledge (IK) indicators of water quality, the project adopted a Two-Eyed Seeing approach based on the Two-Row Wampum methodology

(Latulippe, 2015). This methodology is based on two ways of knowing, IK and western science, coming together to achieve a common goal. The benefits of this adopting this methodology is to combine IK with western science to guide frameworks for environmental monitoring based on what is important to the Indigenous peoples, as well as utilizing western science to further explain and quantify what the IK has already been telling us. During this meeting, members from CLFN provided anecdotal reports on historic use and condition of the lake. Constance Lake First Nation stated that community members no longer had a relationship with the lake due to fear of the condition of the water but would occasionally host catch-and-release style fishing derbies where only limited numbers of *Esox lucius (*Northern Pike) were caught. The perception of the lake by community members was that the lake is toxic from leachate from the WDS and inputs from historical forestry practices. Community members have also noticed that the water was greener in colour as opposed to blue, and children developed rashes after playing in the water from their backyards. Historical use of the lake includes harvesting activities for subsistence (*E. lucius*, *Sander vitreus (*Walleye), *Perca flavescens* (Yellow Perch), *Catostomas commersonii* (White Sucker)), recreational activities such as swimming, and was also used as a main source of clean drinking water for the community. It was also stated at this meeting that Constance Lake First Nation would like to improve the quality of water in the lake again to return to recreational activities and hopes to have walleye return to the lake for recreational fishing and subsistence purposes. Based on previous works completed in Constance Lake, as well as the concerns raised by community members, it was decided that freshwater mussels would be used as a study species, due to their noted abundance in the lake, to examine stress responses and energy

levels of two species - *Lampsilis siliquoidea* (Fatmucket), and *Pyganodon grandis* (Giant Floater). The purpose of examining the biological responses of these species is to address concerns from Constance Lake First Nation surrounding the health of the lake as it pertains to increased nutrient loading and as a more detailed study into the perceived impacts on Constance Lake.

1.2 Biology and Ecological Function of Freshwater Mussels

Unionid mussels have a unique and complex lifecycle in which the larval stage becomes a short-term obligate parasites on fish hosts (Trdan & Hoeh, 1982; Huebner *et al.*, 1990; Jansen & Hanson, 1990; Hewitt *et al*, 2019). The life cycle starts by males releasing sperm into the water column, which are taken into the incurrent syphon of the female and fertilize the ova in the gills. Fertilized eggs develop in specialized portions of the female gill demibranch called the marsupium. It is in the marsupium in which the embryos develop into glochidia. Once mature, the glochidia are released into the environment where they parasitize on the gill or fins of a fish host (Trdan & Hoeh, 1982; Jansen & Hanson, 1990; Jacobson *et al.*, 1997). There is a short transformational period, between 2-4 weeks (Hewitt *et al.*, 2019), after which the metamorphosed individual releases from its host and becomes a free-living member of the benthic community (Trdan & Hoeh, 1982; Jansen & Hanson, 1990; Jacobson *et al*., 1997; Hewitt *et al.*, 2019).

Unionids have two breeding cycles – tachytictic (short term) and bradytictic (long term) (Lewis, 1985). Tachytictic mussels spawn in the spring and embryos and larvae are brooded in the female gills until they have fully developed into glochidia. This whole

process generally takes place from late spring to late summer. Bradytictic mussels spawn in the late summer, brood the glochidia over winter, and release the glochidia in early spring (Graf & Foighil, 2000). There are certain exceptions to the duration of brooding times for both brooding types, involving climate and synchronization to seasonal hosts (Lewis, 1985; Graf & Foighil, 2000).

Mussels feed through suspension feeding (Vaughn *et al.*, 2008), deposit (suspension) feeding, and pedal sweeping. Suspension feeding is when water is brought into the mantle cavity and across the gills through the incurrent siphon. After it passes through the gills and food particles are captured by cilia, the water is then excreted through the excurrent siphon. Deposit feeding uses the inhalant siphon as a vacuum across substrates and pedal sweeping uses the foot to bring the substrate into the mantle cavity. Juvenile unionids (transformed from glochidia) are known to pedal feed until the gills and siphon have completely developed. Pedal sweeping has also been observed in smaller species from *Pisidiidae* and *Sphaeriidae* of the order Sphaeriida (Nichols *et al.*, 2005).

Once inside the mantle cavity, the gill cilia filter food items from non-food items. This is achieved with the help of cilia to pump water through an incurrent siphon into the mantle cavity. Here, ciliary action draws water over the gills. Laterofrontal cirri (group of gill cilia) then capture the food particles, frontal gill cilia send these captured food particles to a food groove on the gills where it then becomes incorporated into a strand of mucus. The mucus strand is directed to labial palps where the food particles are sorted and ingested. Any unwanted and/or undigested particles are expelled from the mantle cavity as a psuedofeces (Galbrait *et al.*, 2009). Silverman *et al.* (1997) reported that

difference in the gill cirral structure allows different species of mussels to specialize on different particle types of varying sizes. Due to their epibenthic nature, unionids can filter food from both the water column and sediment (Vaughn *et al.*, 2008). Unionids typically feed on phytoplankton, bacteria, suspended and re-suspended algae, zooplankton, and detritus (Vaughn *et al.*, 2008; Liu *et al.*, 2014; Bartsch *et al.*, 2017; Vaughn, 2018;).

1.3 Threats to freshwater mussels

More than 70% of freshwater mussels in North America are at some level of risk of extinction (Tuttle-Raycraft & Ackerman, 2018). Many studies are guided to help inform the conservation and protection of freshwater mussels as more than 7% of freshwater mussels in North America have become extinct due to numerous anthropogenic impacts which include habitat alteration (dams), urban run-off from roads, waste water treatment plant and other industrial effluent, and agricultural run-off (Haag & Staton, 2003; Galbraith *et al.*, 2009) . Despite populations being on the decline, mussels have been considered a suitable sentinel organism for monitoring pollutants in aquatic environments due to their relationship with the surrounding environment and the rapid uptake and bioaccumulation of contaminants of concern (Hayer & Pihan, 1996; Martel *et al.*, 2003).

Because they constantly filter water, freshwater mussels are exposed to everything in the water. Molluscs are quite sensitive to several contaminants and toxins compared to other invertebrates and fishes. Adult unionids may be less sensitive to some contaminants (pesticides, organic compounds, effluents) than the earlier life stages. Glochidia are among the most sensitive for metals, chlorine and ammonia (Newton *et al.*, 2003; Wang,

Ingersoll, *et al.*, 2007). The time in which glochidia are exposed directly to environmental contaminants is short, however this stage is most critical due to the unique lifecycle of unionid mussels. Because of this short time frame, exposure studies with glochidia are generally carried out over a period of 24 to 48 h (Huebner & Pynnonen, 1992). Juveniles on the other hand, are typically exposed for 96 h in acute tests and 21 – 28 days in chronic tests (March *et al.*, 2007). Juveniles can be considered representative of different life stages regarding the sensitivity to metals in a standard 4-wk chronic exposure. However, due to the longevity of North American species of unionids, there are concerns that the 4-wk exposure is only reflective of a short portion of the overall life history. To increase exposure time, researchers must overcome the challenges of maintaining juveniles in a laboratory setting. To further refine the chronic testing, researchers need to increase growth, maintain adequate food levels, and ensure proper water quality for juveniles to be able to withstand longer exposures (12-wk) (Kunz *et al.*, 2020). Using a pulsed flow-through auto-feeding approach, Kunz *et al.* (2020) were successful in increasing the survival rate of juvenile mussels over 12-wk period. The pulsed flow-through auto-feeding approach delivers a mixture of fresh food and water to juvenile mussels at timed intervals. The responses of juveniles in longer-term exposure may provide more accurate estimates into the sensitivity of other life stages of freshwater mussels.

Wang *et al.* (2013) began conducting laboratory experiments in 2006 to assess acute and chronic toxicities of specific metals, nutrients, and other contaminants on the glochidia and juveniles of freshwater mussels. Prior to 2006, there were no consensus-based methods available for conducting acute or chronic toxicity tests with the early life

stages of mussels (Wang *et al.*, 2013). A gap was identified in available data to evaluate chronic toxicity of chemicals to mussels and to compare these acute and chronic effects (Wang *et al.*, 2007a). Dr. Wang identified the lack of use of toxicity data from freshwater mussels by the United States Environmental Protection Agency (U.S. EPA) when deriving water quality criteria (WQC). As a result, Dr. Wang and his team developed standardized laboratory methods for conducting toxicity tests with freshwater mussels by comparing data from other standard test organisms to that of unionids to improve acceptability of data for regulatory purposes. To help in standardizing these methods, joint research projects were conducted to evaluate intra- and interlaboratory variability (Wang *et al.*, 2007b; Raimondo *et al.*, 2016).

1.3.1 Metals

Heavy metals such as aluminum (Al), chromium [IV] (Cr), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn) are thought to be an important group of pollutants in an aquatic ecosystem and can be classified as either essential (defined biological functions), such as Zn and Cu, and non-essential (without biological roles), such as Cr. It is known that there are different bioaccumulation trends for the two groups of metals based on regulating systems of metals in the organs including uptake, storage, detoxification and excretion processes (Moëzzi *et al.*, 2013). Metals occur naturally in water, however anthropogenic activities (industry, mining, agricultural practices, household waste) contribute to an increase of these compounds in the aquatic environment (Sohail *et al.*, 2016). Several studies (Falfushynska *et al.*, 2012; Sohail *et al.*, 2016; Markich, 2017; Wang *et al.*, 2017) have examined the sensitivity of glochidia to heavy metals (Cr, Cu,

Pb, Ni, Zn) and found that high levels of metals accumulated in the gills, foot, mantle and digestives glands, with the gills containing the highest metal concentrations due to their direct exposure to water pollutants in their immediate environment *(*Falfushynska *et al.,* 2012*;* Sohail *et al.*, 2016). Acute and chronic toxicity tests of these metals have shown that glochidia and newly transformed juvenile mussels are more sensitive than commonly tested cladocerans, amphipods, and fish (Wang *et al.*, 2007a; Wang *et al.*, 2007b; Wang *et al.*, 2017). From these toxicity tests, researchers reported the EC50 of Cu for both acute and chronic tests to be less than the U.S. EPA WQC, indicating that these criteria may not adequately protect the species tested (Wang *et al.*, 2007a; Wang *et al.*, 2007b). While Cu is also considered an essential metal due to its role as cofactor of mitochondrial enzymes and is a component of haemocyanin, elevated concentrations act as a potent toxicant (ionoregulatory and oxidative toxicant; Giacomin *et al.*, 2013). The acute toxicity of Cu is also known to be strongly influenced by dissolved organic carbon present in water by binding with the positively charged metal cation and reducing its bioavailability (Wang *et al.*, 2009; Giacomin *et al.*, 2013). When evaluating the toxicity of the non-essential Cr, Wang *et al.*, (2017c) reported that elevated temperatures increased the acute toxicity of Cr to *L. siliquoidea* juveniles, and temperature or nitrate increased the chronic Cr toxicity.

Metals are known to induce changes in bivalve immune function and hemocyte phagocytosis, which is one of the key immune system components in mussels (Gillis, 2012) as well as filtering behaviour (Kádár *et al.*, 2001). Long-term exposure of bivalves to heavy metals is known to increase susceptibility to diseases and may cause histological malformations, have a negative effect on clearance rates (Moëzzi *et al.*, 2013), and can induce DNA damage to living cells (Khan *et al.*, 2019). An increase of metal

concentrations can lead to a decrease in fish host populations, thus reducing the opportunity for glochidia to find a fish host and metamorphose to the juvenile larval stage (Huebner & Pynnönen, 1992).

Under optimal conditions of feeding, the adductor muscles of bivalves are typically relaxed with the valves open. Kádár *et al.*, (2001) monitored shell gape acitivty in *Anodonta cygnea* (Swan Mussel) during additions of 0.250 $mg \cdot L^{-1}$ and 0.500 $mg \cdot L^{-1}$ aluminum (Al) over a period of 45 days. It was reported that exposure to the higher concentration over 15 days caused a significant decrease in mean duration of shell opening, thus reducing and avoiding filtering. Once transferred to untreated lake water, shell gape of those animals exposed did not recover to pre-exposure levels. Taskinen *et al.* (2011) reported that the Freshwater Pearl Mussel, *Margaritifera margaritifera*, experienced low reproductive success attributable to high concentrations of Al and iron (Fe) during period of low pH (<5.0). During a 48 h exposure of free glochidia to Fe, it was suggested that Fe concentrations of 1.0 $mg \cdot L^{-1}$ or higher may considerably reduce the success of *M. margaritifera* glochidia. Exposure to Al suggested that the same concentration as for Fe also considerably decreased survival of glochidia, but after a 72 h exposure. Cope *et al.* (2008) also found that upon detection of a contaminant, Cd, at a high level (5 $\mu g \cdot L^{-1}$), *Lampsilis siliquoidea* closed its valves and ceased respiration as an avoidance response. This response was sustained only for the first 24 h of an accute test however, upon which the mussels eventually opened their valves and began respiring again.

Mussels can also obtain approximately 80% of their food by deposit feeding, siphoning food from sediment and interstitial water, and pedal-feeding directly from the

sediment. Juveniles also spend their entire portion of this life cycle embedded in the sediment. Sediment-based testing protocols for both juveniles and adults is required because water quality criterion do not take into consideration the diet of freshwater mussels or their life history and ecology (Cope *et al.*, 2008). Besser *et al.* (2015) demonstrated that existing chronic water-only toxicity tests could be adapted successfully to test the toxicity of contaminated sediments. In this study, juvenile *L. siliquoidea* were exposed to sediments known to be contaminated with Cd, Cu, Pb, Ni, and Zn. Field surveys on mussel communities were also conducted. The results provided evidence of casual relationships between sediment metal contamination and toxic effects on mussels.

Researchers investigating the trend of metal accumulation in the gills of mussels found that concentrations of metals decreased while moving downstream from the point source. It has been reported that there is a direct relationship between the concentration of metallothionein (MT) and the increase in accumulated metals moving downstream from a point source (Gillis, 2012; Gillis *et al.*, 2014b; Machado *et al.*, 2014). Machado *et al.* (2014) found that mussels that had high gill metal concentrations also had significantly lower gill water content, suggesting that there is a disturbance in the cell volume and/or with osmoregulation.

1.3.2 Effluent and Surface Run-off

Numerous studies have been undertaken to better understand the effects of municipal wastewater effluents on freshwater mussels (Gillis, 2012; Falfushynska *et al.*, 2014; Gillis, *et al*, 2014; Machado *et al.*, 2014). To help understand these effects and the stress they may case to an organism, biomarkers of exposure have been employed. The

most common biomarkers used include MT concentrations (Gagné *et al.*, 2004a; Gillis *et al*., 2014b), endpoints of lipid peroxidation (LPO) in the gills such as 4-hydroxynonenal (HNE; Gagné *et al*., 2004a; Gillis *et al.*, 2014a; Gillis *et al.*, 2014b), glutathione-S-transferase (GST) level in the digestive glands and gonads (Falfushynska *et al.*, 2014; Gillis *et al.*, 2014a; Jasinska *et al.*, 2015), vitellogenin-like proteins in the gonadal region (Falfushynska *et al.*, 2014; Gillis *et al.*, 2014a), and hemocyte viability (Gillis *et al.*, 2014a). Other biomarkers are also considered but are less commonly used. These biomarkers include antioxidant capacity against peroxyl radicals, gill protein concentration, lipid concentration, determination of levels of protein-free DNA strand breaks in the digestive gland, and ethoxyresorufin-O-deethylase activity in the post-mitochondrial supernatant of the digestive gland (Gagné *et al.*, 2004; Gillis, 2011; Falfushynska *et al.*, 2014; Gillis *et al.*, 2014a; Gillis *et al.*, 2014b; Jasinska *et al.*, 2015).

Many of these studies have taken place in the Grand River watershed in southern Ontario. The Grand River is one of the largest river in southern Ontario and receives input from 30 municipal wastewater treatment plants (Gillis *et al.*, 2014). Including the municipal wastewater effluent, the Grand River receives sources of anthropogenic runoff including agricultural, forestry, and runoff from the various roads that connect a large population of people (Gillis, 2012). These studies found that MT concentrations increased with distance from the point source, LPO and total protein levels were higher downstream, and a decrease in hemocytes but an increase in lysosomal activity downstream. The increase in LPO levels is indicative of cellular membrane damage (Gillis, *et al.*, 2014a; Gillis, et al., 2014b). These findings suggest that mussel physiology

is altered as a function of oxidative stress from chronic exposure to complex mixtures of waterborne contaminants (Machado *et al.*, 2014).

Endocrine-disrupting compounds such as estrogens are found in significant amounts in municipal wastewater effluents. Some of these compounds are known to interact with the estrogen receptor pathway in aquatic organisms and include estradiol and bisphenol A (BPA) (Gagne *et al.*, 2001). Gagne *et al.* (2001) examined the estrogenic effects of municipal wastewater effluent on *Elliptio complanate* (Eastern Elliptio) and reported a rise in vitellogenin in the hemolymph and gonad of the exposed mussels, with females responding more readily than males. Estrogen levels typically increase only during vitellogenesis, and rapidly decline once the process is completed. Based on the results of this study, it is suggested that the presence of estrogen competitors validates the reported rise in vitellogenin. Gagne *et al.* (2001) also reported that shell length growth decreased moving downstream through the study site while whole mussel growth increased, indicating weight gain. The estrogenic chemicals may be disrupting the growth of shell, with mussels limiting their shell growth in favour of soft tissue growth, suggesting that the energy required for shell growth is being redirected to support vitellogenesis. Additional endocrine disruptors include pharmaceuticals and personal care products. Falfushynska *et al.* (2014) reported similar results of elevated vitellogenesis. Falfushynska *et al.* (2014) also reported signs of oxidative injury and general stress to mussel groups who were exposed to ibuprofen, triclosan (synthetic antimicrobial agent) and estrone.

Surrogate organisms have been used in whole-effluent toxicity testing to represent responses of the aquatic community to industrial releases (McKinney & Wade, 1996).

These organisms include *Pimephales promelas* (Fathead Minnow) and *Ceriodaphnia dubia* (Water Flea). As the responses to these surrogates may not accurately reflect those of some benthic organisms, McKinney and Wade (1996) sought to compare the responses of *C. dubia* and juvenile *Anodonta imbecillis* (Paper Pondshell) to final treated effluent from six pulp and paper mills in a laboratory setting. McKinney and Wade (1996) observed that only two of six effluent samples exhibited acute survival effects to *C. dubia* while four of the six effluent samples impaired survival in the juvenile mussels. The researchers indicate that because of these tests, *C. dubia* are less sensitive than *A. imbecillis* to selected whole effluents and may be inadequate as a surrogate to represent the freshwater mussels in a benthic community.

Sodium (Na) and chloride (Cl) occur naturally in the environment, however over the past 30 years, they have been increasing due to the increased application of de-icing salts on paved surfaces (Gillis, 2011; Wang *et al.*, 2018; Gillis *et al.*, 2021). Acute and chronic toxicity tests of sodium chloride (NaCl) with *L. siliquoidea* glochidia and juveniles (Gillis, 2011; Wang *et al.*, 2018). The EC50s for glochidia ranged from 168-1597 mg $Cl \cdot L^{-1}$, with the lower EC50 of 168 mg $Cl \cdot L^{-1}$ possibly being attributed to poor quality of glochidia (77.4%) being used for the test. Gillis (2011) did not perform a chronic test with juveniles, however observed that increasing water hardness and natural river water may offer added "protection" from acute chloride toxicity. This was confirmed by Wang *et al.* (2018). In the chronic tests by Wang *et al.* (2018), the EC50s ranged from 911 to 3092 mg $Cl \cdot L^{-1}$ for different hardness of water and were reported to be half of the final acute value used for deriving the U.S. EPA WQC for Cl. Wang *et al.* (2018) concluded that juveniles were less sensitive to NaCl than glochidia, and both

studies concluded that glochidia were sensitive to NaCl. Further to the toxicity tests of NaCl, Gillis *et al.* (2021) paired laboratory toxicity tests with of winter road runoff with an assessment of the mussel populations that occupy the habitats receiving the salt-impacted runoff. The study reported that the road run-off samples were acutely toxic to early life stages of mussels in the laboratory, and that distribution, abundance and diversity were low in the immediate vicinity of the receiving waters. Gillis *et al.* (2021) also noted that while salt appears to be the driver of toxicity in melt-water samples, other contaminants (potassium, ammonia, and/or Zn) could have also contributed to the observed toxicity.

Other sources of contamination from wastewater effluent and surface water run-off include insecticides (Prosser *et al.*, 2016), pesticides (Salerno *et al.*, 2018), and pharmaceuticals (Gilroy *et al.*, 2017). Studies have been carried out on these chemicals to estimate their toxicity to various freshwater mussels. Prosser *et al.* (2016) examined the sensitivity of *Lampsilis fasciola* (Wavy-rayed Lampmussel) glochidia to neonicotinoids, an insecticide used in soil/foliar spray and seed treatments for the protection of crop plants against insect pest species. The researchers observed that all 24 and 48 h LCs were greater than the greatest concentration of exposure, indicating that *L. fasciola* glochidia are relatively insensitive to acute exposures of neonicotinoids compared to aquatic insects. The researchers noted that the lack of data on the concentration of neonicotinoids in surface water make it difficult to determine the probability of molluscs being chronically exposed at that magnitude.

Various classes of pesticides found in Ontario surface waters were studied to assess the risk posed to the different life stages of *Villosa iris* (Rainbow Mussel). Toxicity

tests were carried out for 11 identified pesticides using 48 h tests on glochidia and 28 d sub-chronic tests on juveniles. Reported effect concentrations were higher than the greatest concentrations measured in Ontario surface waters in the past 20 yr, indicating that it is unlikely that the identified pesticides are contributing to the decline of mussel populations in those water bodies (Salerno *et al.*, 2018). Gilroy *et al.* (2017) assessed the toxicity of three prevalent pharmaceuticals, amitriptyline, iopamidol, and sertraline, of caged *L. siliquoidea* in the wild. Sertraline was the most toxic chemical assessed for both acute (glochidia, LC50 = 0.6 $mg \cdot L^{-1}$) and chronic (juvenile, LC50 = 0.04 $mg \cdot L^{-1}$) tests. Iopamidol proved to be not toxic to any of the life stages of mussels investigated at concentrations up to 100 $mg \cdot L^{-1}$. Across compounds, glochidia were consistently as sensitive as juveniles, with amitriptyline LC50 of 0.3 $mg \cdot L^{-1}$ for glochidia and 0.4 $mg \cdot L^{-1}$ for juveniles. While endocrine-disrupting compounds and their effects on freshwater mussels are widely studied, they were not evaluated in this study. While all of these other threats and their effects on freshwater mussels are widely studied, they were not evaluated in this study.

1.3.3 Other Contaminants and Toxins

Leachates are a complex mixture of liquid effluent that is generated by the precipitation and penetration of water into municipal solid waste that is disposed in landfills. The leachate consists of numerous pollutants including dissolved organic matter, inorganic salts, heavy metals, and xenobiotic organic compounds. These pollutants are known to enhance oxidative and genotoxic effects in *Mytilus galloprovincialis,* Mediterranean Mussel, as they accumulate a large amount of

contaminates in their tissues (Tsarpali & Dailianis, 2012; Toufexi *et al.*, 2013). Caging methods using unionid mussels as test specimens has also been employed to detect leaching of polychlorinated biphenyls and chlorophenols from landfills (Herve *et al.*, 2002).

Tsarpali & Dailianis (2012) aimed to investigate the toxic endpoints of landfill leachate, referring to mortality, by monitoring stress indices. A neutral red retention assay (NRR) in the hemocytes of mussels was used to estimate the lysosomal membrane stability as an index of cellular damage. The micronucleus (MN) test was used to indicate organic and inorganic mutagens and clastogens. Estimations of acetylcholinesterase (AChE) activity and MT content were also analyzed. A 96 h mortality test showed that leachate concentrations greater than 0.5% (v/v) could lead to a significant increase in mussel mortality. The researchers observed a loss of lysosomal membrane integrity in the hemocytes of the exposed mussels. A significant depression of AChE activity in the gills and digestive gland was observed. The researchers also observed increased levels of MT measurements in the gills and digestive gland, as well as increased levels of nuclear abnormalities in the tissues of exposed mussles. These results show that leachate has the ability to induce cytotoxic, oxidative, neurotoxic and genotoxic effects in the tissues of mussels. Toufexi *et al.* (2013) performed a similar experiment also using *M. galloprovincialis* and came to similar conclusions. The researchers found that the results for NRR showed almost a dose-dependent lysosomal membrane impairment in hemocytes at 10% (v/v) with concentrations of 0.01, 0.1 and 1. The researchers also observed that leachate could disturb DNA integrity in hemocytes of mussels both *in vivo*

and *in vitro.* The researchers concluded that landfill leachate could enhance genotoxic effects in mussels.

Microcystins are hepatotoxins and are produced by a number of cyanobacteria genera including *Nostoc, Anabaenopsis*, *Anabaena* and *Microcystis* (Amado & Monserrat, 2010; Carey *et al.*, 2017; Chia *et al.*, 2018). Other toxins produced by cyanobacteria include neurotoxins, tumor promoters, and dermatoxins (Msagati*et al.*, 2006). Microcystins are the most frequently produced toxin by cyanobacteria and therefore, the most commonly studied (Lepoutre *et al.*, 2020).

Prepas *et al.*, (1997) conducted a study using *Anodonta grandis simpsoniana* (now *Pyganodon grandis*) to examine accumulation and elimination of cyanobacteria hepatotoxins. The results of the study suggested that freshwater mussels accumulate the toxins primarily through grazing on toxic phytoplankton and minimally through the uptake of the dissolved toxin and that the microcystin concentrations in the mussel reflected the toxin concentrations in the phytoplankton. Through analysis of the visceral mass, gill and muscle tissues it was found that 71% of the total accumulated toxin was eliminated within the first six days after being removed from the toxin source and then fed nontoxic green alga. For mussels to live in bacterial-contaminated environments and accumulate toxins with little detrimental effects, physiological adaptations must be made. To do so, mussels can biotransform toxins via GST detoxification of microcystins. Conjugation of the toxin with glutathione enhances the water solubility of the toxin for better excretion. The antioxidant enzyme activity from GST protects cells in the mussel from the oxidative stress caused by cyanobacterial toxins (Burmester *et al.*, 2012).

Gene *et al.* (2019) identified the need to characterize the toxicity of microcystins to freshwater mussels, as most studies only examined the accumulation and excretion of the toxin (Prepas *et al.*, 1997; Yokoyama & Park, 2003; Travers *et al.*, 2011; Burmester *et al.*, 2012). Gene *et al.* (2019) investigated the acute and chronic toxicity of *Microcystis aeruginosa*-associated microcystin-LR on *L. siliquoidea* glochidia and juveniles. By generating an environmental exposure distribution (EED) from microcystin-LR monitoring data from Ontario, Quebec and Alberta, the researchers were able to determine the environmental relevance of the lethal concentrations. During chronic exposure of juveniles, it was observed that microcystin-LR had a significant effect on mortality after 21 and 28 d exposure, with an LC10 of 0.45 and 1.4 $\mu g \cdot L^{-1}$ respectively. The researchers noted that the LC10 value for 28 d was similar to the Health Canada acceptable drinking water concentration of 1.5 $\mu g \cdot L^{-1}$, suggesting that chronic exposure of microcystin-LR may pose considerable risk to mussel populations. Gene *et al.* (2019) concluded that there is a major uncertainty with their conclusion as it is based on the concentrations contained in the EED being chronic exposures, and that current monitoring data do not provide insight into the persistence of peaks in microcystin concentration during harmful algal blooms.

Traditional surveys of microcystins are based on visual monitoring coupled with water sampling. The challenge with this lies withing the recommended sampling frequency of twice-per-month, as this sampling frequency leaves the possibility of missing either an entire bloom occurring between sampling periods or one that is in its early stages. This is especially true since some cyanobacteria species are known to display a daily vertical migratory cycle *(*Lepoutre *et al.*, 2020).

To identify the current trophic status of the lake, and to consider the probability of future algal blooms in Constance Lake, surface water samples were studied for nutrient levels. These levels were then taken into consideration with levels of stress on two species of freshwater mussel to determine how the quality of water was impacting the overall condition of the mussels. The objectives of this thesis were to evaluate water quality to address concerns of toxicity. To do this, I aimed to understand nutrients in the systems as well as to understand potential metal contamination. It was hypothesized that water quality in Constance Lake would be degraded by having higher nutrient levels than the reference lakes based on local knowledge provided by CLFN regarding current and historic uses of Constance Lake. Another objective was to evaluate impacts of water quality on local biota. To do this, I aimed to understand stress levels in freshwater mussels by analyzing glycogen as a quantifier for energetic status, and HNE as a quantifier of stress. It is hypothesized that glycogen levels in mussels will be lower due to limited food availability as a result of change in algae composition from eutrophication, and that HNE levels will be higher due to being in a stressed environment from degraded water quality.

Chapter 2: Water Quality in Constance Lake, ON

2.1 Introduction

Nitrogen (N) and phosphorus (P) are key elements for phytoplankton, bacteria, and other aquatic organisms in freshwater ecosystems, and are the most important nutrients in causing shifts in the productive state of ecosystems (Wetzel, 2001ab). Increased nutrient loading through internal or external sources and either natural or cultural sources is known to result in eutrophication, which creates cascading changes throughout the ecosystem (Wetzel, 2001a; Bhagowati *et al.*, 2019). Eutrophication causes reduced water clarity, oxygen depletion, fish kills and biodiversity loss and the release of sediment-bound inorganics, among other issues (Orihel *et al*., 2017). A common consequence of eutrophication is increased production and biomass of algae and phytoplankton (Conley *et al.*, 2009; Strayer, 2014; Bhagowati *et al.,* 2019). Both algae and phytoplankton are dependant on N and P (Orihel *et al.*, 2012) and other environmental conditions including high temperature and pH, and little exchange of water which ultimately results in low dissolved oxygen (Amado & Monserrat, 2010). While both nutrients are important and drivers of growth, P is considered the main limiting nutrient for lake productivity (Bormans *et al.*, 2016; Dodds & Smith, 2016; Liang *et al.*, 2020), specifically phytoplankton (Fink *et al.*, 2018), as dissolved N can be lost in the form of N_2 through microbial denitrification, in turn reducing available N in the water column (Dodds & Smith, 2016).

Phosphorus occurs in lake sediments as a variety of molecular species. Internal loading of P can happen in a variety of ways depending on which P species is present in the sediment (Song & Burgin, 2017), and is controlled largely by the oxidation-reduction cycle of iron (O'Connell *et al.*, 2020). Phosphorus can enter the water column by

diffusion or advection through pore water following numerous different mechanisms, including hydrolysis or mineralization of organic matter. Oxygen also plays an important role in the decomposition of P-containing organic matter and the redox states of elements that are associated with P in sediments, such as iron and manganese oxides. In sediment-water interfaces that are anoxic, or where aerobic respiration consumes oxygen at a greater rate than is provided by surface water, dissolution of iron hydroxides results in the release of P (Orihel *et al*., 2017).

Unionid mussels are filter feeders (Prepas *et al.*, 1997; Vaughn *et al.* 2008) and at lengths between 62-73mm can filter water at an average of 0.164 $L \cdot g^{-1} \cdot d^{-1}$ (Kim *et al.*, 2011). Due to their epibenthic nature, unionids can filter food from both the water column and sediment (Vaughn *et al.*, 2008). Unionids typically feed on phytoplankton, bacteria, suspended and re-suspended algae, zooplankton, and detritus (Bartsch *et al*., 2017; Liu *et al.*, 2014; Vaughn, 2018; Vaughn *et al.*, 2008). When nutrients are increased, there is not only an increase in algal production and biomass, but also a change in the composition of the algal community with increased toxic algae (cyanobacteria), possibly diminishing the nutritional quality of the algae (Strayer, 2014). Morehouse *et al.* (2013) found strong negative effects of excessive dietary P on the growth of *Dreissena polymorpha* (Zebra Mussel). Plath & Boersma (2001) found the same negative effects in *Daphnia* and identified behavioural and physiological mechanisms to explain this response. It was found that *Daphnia* spp. decreased feeding when presented food with excessive P, resulting in starvation of energy in individuals after P requirements were met, despite the presence of abundant food. Physiological responses to excessive P include decreased assimilation efficiency of phosphorus, inhibitory interactions with the

transport of micronutrients, and alterations of several metabolic substrates (Plath & Boersma, 2001).

This chapter evaluated the water quality in Constance Lake to address concerns of toxicity and algal blooms by members of Constance Lake First Nation. It was hypothesized that there would be some difference between the lakes addressed in this study on N and P composition, with values being elevated, or in a eutrophic state in Constance Lake, ON, compared to Fushimi Lake and McKay Lake. It was also hypothesized that heavy metals will be in exceedance of water quality guidelines in Constance Lake from forestry practices and WDS leachate compared to Fushimi Lake and McKay Lake.

2.2 Materials and Methods

2.2.1 Study sites

Due to constraints on provision and use of funding for this project, study locations were limited to road-accessible lakes that are considered priority monitoring areas in Matawa member communities and homelands.

Constance Lake is located off Highway 11 in Calstock, ON, about 32 km west of the town of Hearst, ON (coordinates 49°48’09.16” N, 84°09’24.04” W) in Ontario Fisheries Management Zone (FMZ) 3, adjacent to Constance Lake First Nation and is 460 ha (Table 1) in size (Figure 2). There are numerous known stressors impacting this lake including but not limited to limited inflow and outflow to the lake, a historical log drive that resulted in large amounts of organic material accumulating on the lakebed, and

input of wind-blown sawdust from an adjacent sawmill. There is also urban surface-water run-off from the community as roads and backyards run adjacent to the lake.

Fushimi Lake was chosen as a reference lake due to its proximity to Constance Lake (coordinates 49°49’41.73” N, 83°54’42.31” W) (Figure 2) with few known stressors impacting this lake. Fushimi Lake is located within Fushimi Lake Provincial Park in FMZ 3, which was established in 1979 and is 1030 ha (Table 1) in size. The Park consists of three zones: the Development Zone where fifty campsites are located, including facilities, staff housing and maintenance; the Historical Zone which includes an old fire tower and some archaeological sites; and the Natural Environment Zone which makes up the rest of the park. There are ten boat-only accessible campsites located on the lake. Aside from recreational fishing in the summer months, the only other known stressor comes from water discharge from the water treatment pump house (K. Wilson, personal communication, November 25, 2019).

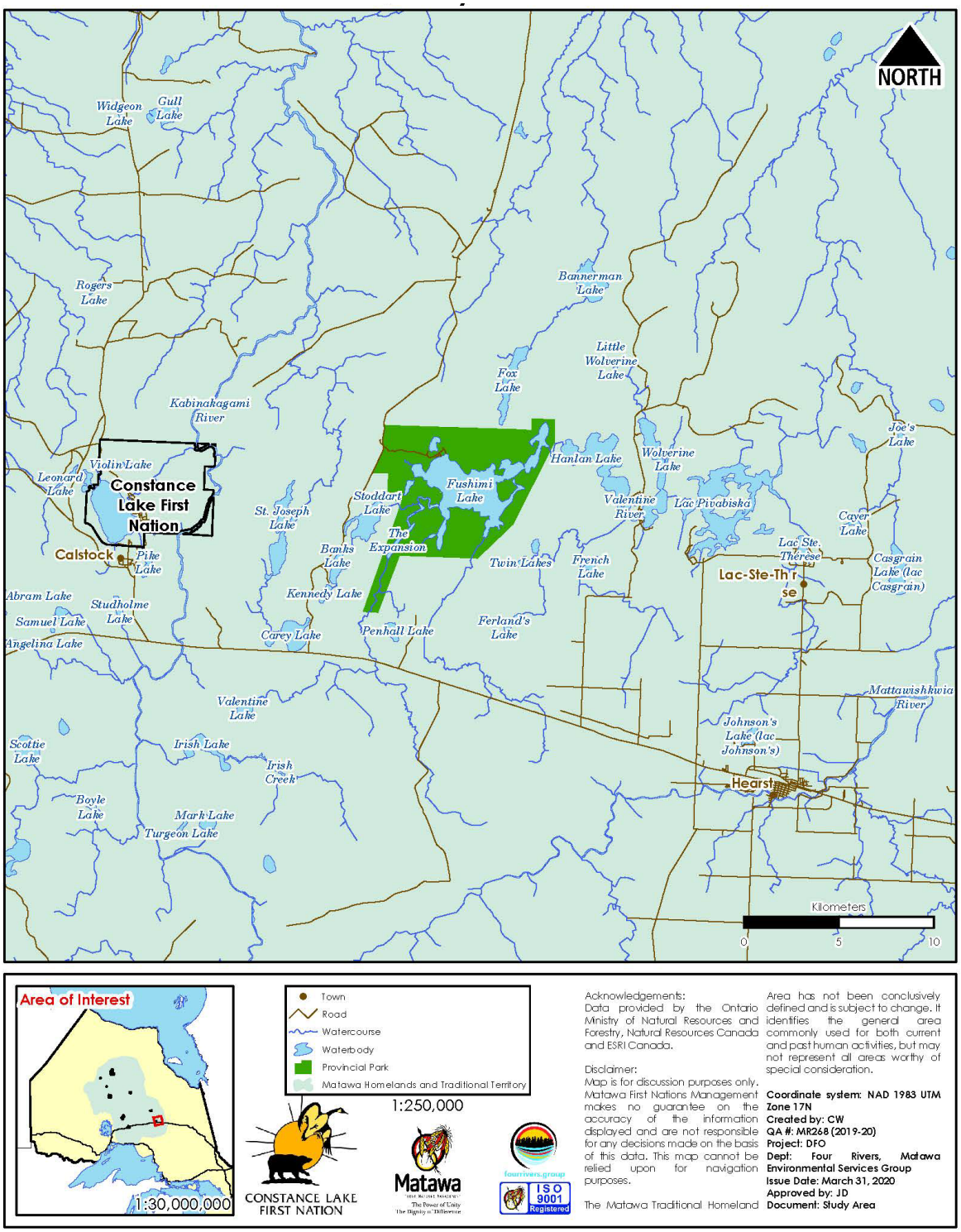

Figure 2. Map showing locations of Constance Lake, ON and Fushimi Lake, ON.

Another reference site that has been sampled is McKay Lake (coordinates 49°36’57.85” N, 86°26’41.88” W), located southeast of the Town of Longlac, ON in

FMZ 7 (Figure 3). McKay Lake is 3035 ha (Table 1) in size. Aside from year-round recreational fishing, the potential impacts to this site are unknown. McKay Lake was chosen as it is within the homelands of Ginoogaming First Nation (GFN) and Long Lake # 58 First Nation (LL58), both member communities of MFNM. Ginoogaming FN and LL58 have historic ties to McKay Lake as there are burial grounds on numerous islands within the lake, as well as pictographs located on the northeastern shores. Members of both communities frequent McKay Lake for fishing and harvest *S. vitreus*, *Salvelinus namaycush* (Lake Trout), and *Coregonus clupeaformis* (Whitefish).

Table 1. Comparison of Constance Lake, Fushimi Lake, and McKay Lake relative to size (ha) and maximum depth (m).

Lake	**Area (ha)**	**Max. Depth (m)**
Constance Lake	460	8
Fushimi Lake	1030	10
McKay Lake	3035	60

A suitable reference site is one where impacts are low, and disturbance is minimal. Reference sites should share natural habitat features with test sites, and be similar in biogeographic and hydrological variability (Omernik, 1995). For this study, there were constraints on which lakes could be used as controls, as the sites sampled were required to be within the Matawa member community traditional homelands and identified by community members as priority areas for environmental monitoring. Fushimi Lake and McKay Lake are considered suitable reference lakes as there is currently no urban development near these lakes. Personal communications with the superintendent of the park for Fushimi Lake and community members from GFN have confirmed that there are no known non-natural inputs of nutrients or contaminants into

the system. Both lakes fall within the traditional homelands of CLFN and GFN and have been identified as priority areas for environmental by these communities.

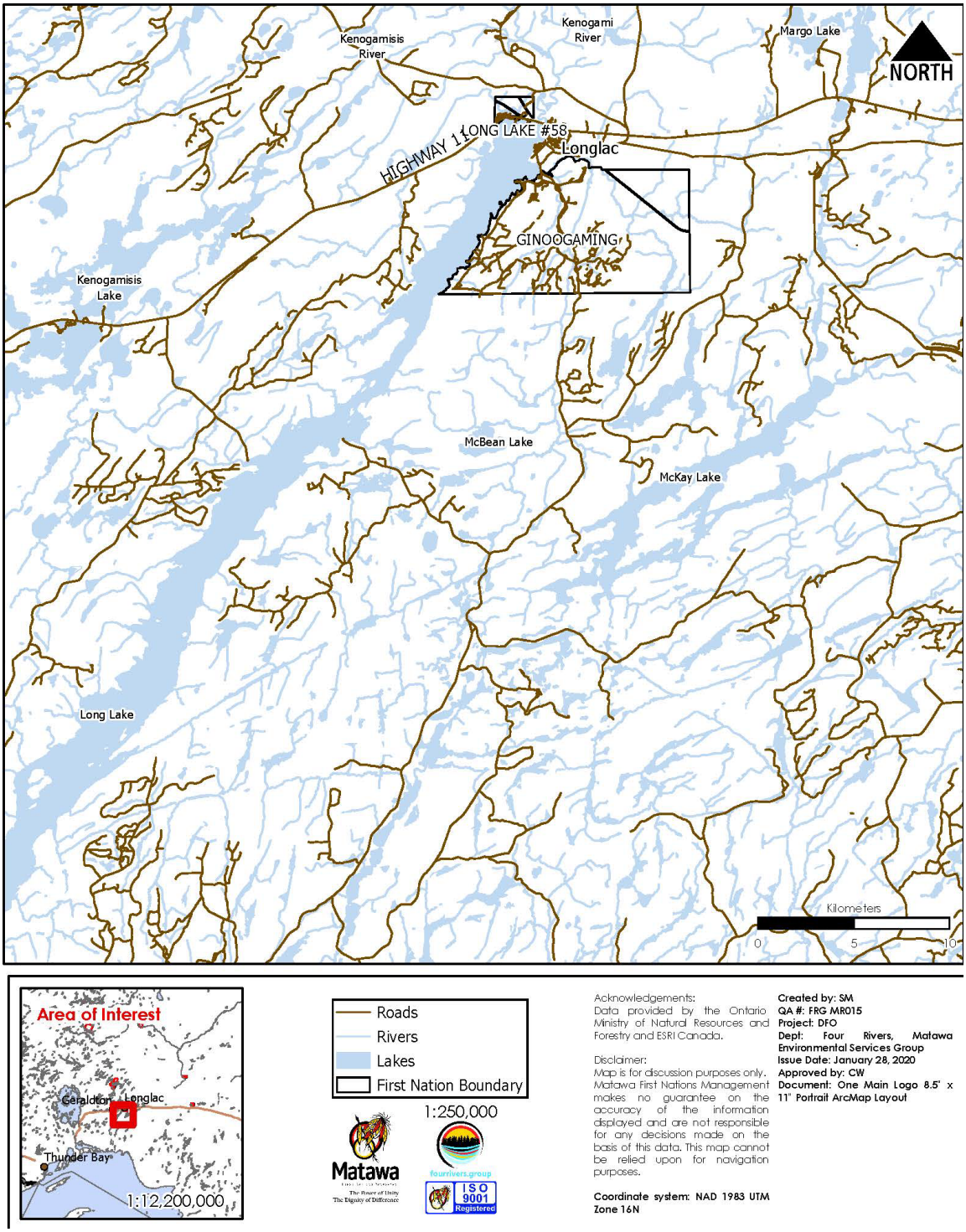

Figure 3. Map showing location of McKay Lake, ON.

2.2.2 Surface water sample collection

In August 2020, I collected surface water samples from Constance Lake (n = 4), McKay Lake (n = 3) and Fushimi Lake (n = 1). The samples were collected with a Kemmerer bottle two metres below surface where lake depth permitted and collected into sample bottles provided by the Lakehead University Environmental Laboratory (LUEL). No preservatives were used. Where lake depth did not allow for samples to be collected at two metres, the sample was collected at one metre, and where lake depth did not allow for one metre, a surface grab sample was collected. Samples were placed into a cooler with ice and transferred to a refrigerator until the field team returned to Thunder Bay, ON. Parameters analysed include anions, nutrients, chlorophyll α (chl α), general water chemistry (alkalinity, conductivity, pH, dissolved organic carbon) and total metals. Water quality profiles were also recorded with a YSI 600QS unit. Water quality profiles include the collection of temperature, pH, specific conductance, and dissolved oxygen at 1 m depth increments from water surface to depth of lakebed.

The same sampling methodology was carried out in October 2020 (CL n = 4, ML n = 3, FL n = 3), March 2021 (CL n = 5, ML n = 3, FL n = 4) and June 2021 (CL n = 5, ML n = 3, FL n = 4) (Figure 4, Figure 5, Figure 6). In March 2021, Four Rivers replaced the YSI 600QS with a YSI ProDSS unit measuring the same parameters at the same depth interval. This unit was used for the June 2021 sampling event at all three lakes, where the 2020 surface water methodology was also followed.

Surface water samples were collected at minimum from two sites in each lake. The first location being that of the initial mussel collection and the second being in the deepest spot of the lake. Deep spots of the lake were determined first by desktop analysis of available bathymetry data and confirmed in-field using a Garmin EchoMAP Plus 45cv

depth finder with GT20 transducer. Water quality profile data were also collected at these sites, and included any additional areas where mussels were collected, as well as other sample locations identified from desktop analyses of each lake.

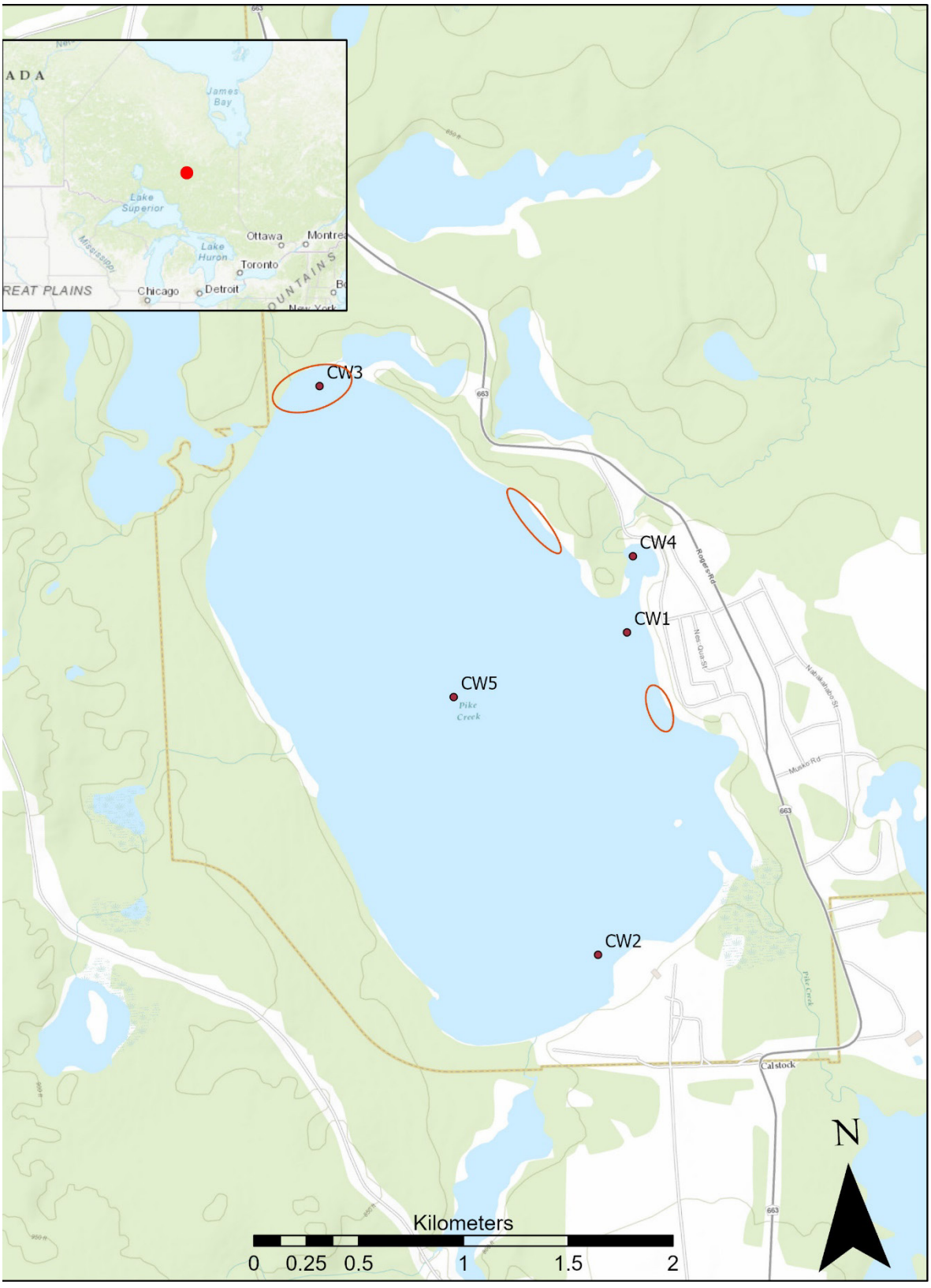

Figure 4. Water chemistry sample locations and mussel collection locations (orange ovals) in Constance Lake, ON.

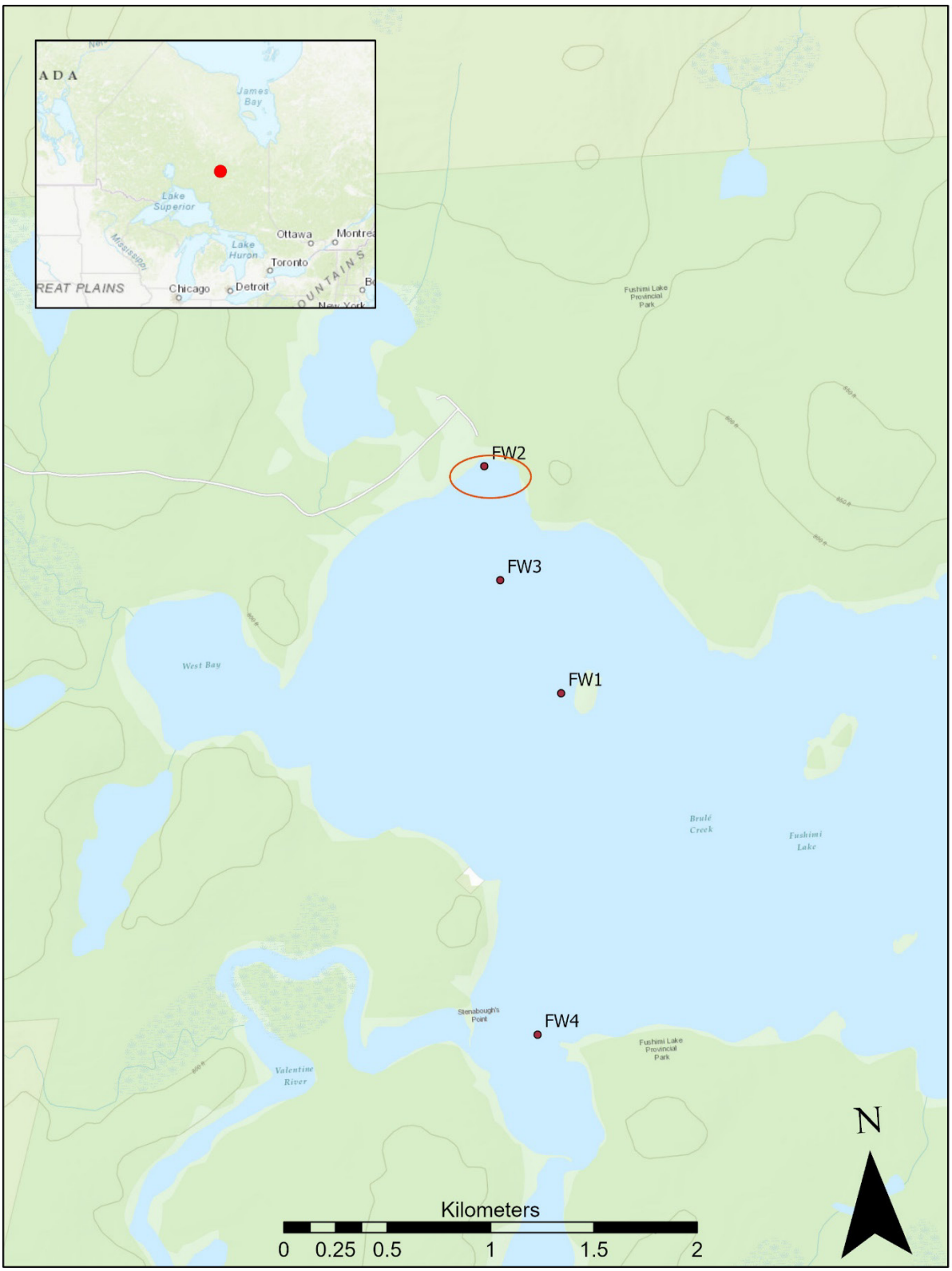

Figure 5. Water chemistry sample and mussel collection locations (orange oval) in Fushimi Lake, ON.

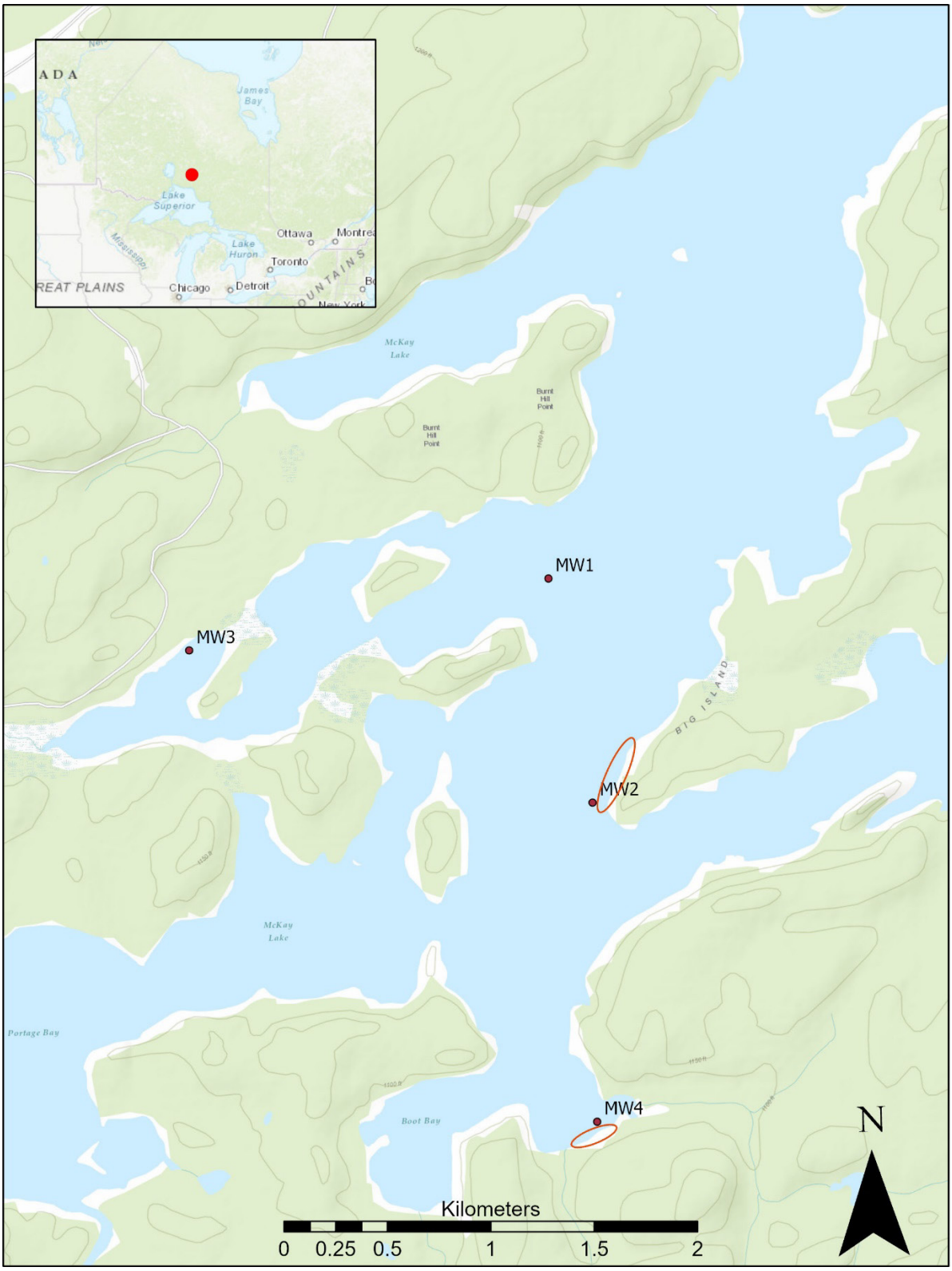

Figure 6. Water chemistry sample and mussel collection locations (orange ovals) on McKay Lake, ON.

2.2.3 Data analysis

To evaluate possible interactions between lakes and across seasons for my three response variables of interest (i.e. N, P, and chl α), a factorial ANOVA was used. It was hypothesized that there would be some difference between lakes on N and P composition, with values being elevated, or in a eutrophic state in Constance Lake, ON, compared to Fushimi Lake and McKay Lake. The null hypothesis would be that there is no difference between nutrient levels in either lake across seasons.

Due to funding limitations under the Matawa Water Futures project, and challenges with equipment, the number of, and access to, sample sites was limited. Equipment challenges included outboard failure and a loss of a propeller. As a result, the water sampling methodology became an unbalanced design. COVID-19 also posed challenges in access sampling sites as travel was restricted and special permission from both the community and MFNM were required. These permissions, along with available funding, only allowed for a one-day access to each lake during each sampling event. This restricted the number of samples being collected in conjunction with equipment failures.

2.3 Results

Constance Lake is seasonally eutrophic (Table 2). This is based on comparing the results for P and N to the Canadian Water Quality Guidelines (CWQG) for the protection of aquatic life, as described by the Canadian Council of Ministers of the Environment (Wetzel, 2001ab; CCME, 2004). No other parameters measured, including heavy metals exceeded any of the CWQG. The CCME Guidelines state that a body of water is considered eutrophic when P levels are between 0.035 – 0.1 $mg \cdot L^{-1}$, and Wetzel (2001a)

states that eutrophic N levels are between 0.500 – 1.50 $mg \cdot L^{-1}$. All samples in August 2020 for P and N indicate that Constance Lake is in a eutrophic state. All samples in March 2021 for N, and three samples in June 2021 for P, also indicate a state of eutrophy in Constance Lake. Fushimi Lake is not considered eutrophic, apart from nitrogen levels in June 2021 (0.592 – 0.644 $mg \cdot L^{-1}$). McKay Lake is also not considered eutrophic, with levels of both N and P below the threshold for eutrophic conditions. Nutrient data for Fushimi and McKay Lakes can be found in Table 3 and Table 4.

Table 2. Surface water quality results for Constance Lake, ON. Analytical parameters represented in the table include total N, total P and chlorophyll α for purposes of determination of trophic status in Constance Lake. Missing samples are denoted by "x", and non-detected samples are denoted by "—". Bold and red indicate eutrophic level of lake productivity.

Analytical Parameter	**Month**	**Analytical Results – Constance Lake**					**Units**	**Detection Limit**
		CW1	**CW2**	**CW3**	**CW4**	**CW5**		
Total Nitrogen	Aug-20	**0.626**	**0.559**	**0.559**	**0.551**	x	mg/L	0.015
	Oct-20	0.337	0.286	0.326	0.363	x		
	Mar-21	**0.559**	**0.557**	**0.527**	**0.544**	**0.537**		
	Jun-21	0.266	0.281	0.302	0.247	0.34		
Total Phosphorus	Aug-20	**0.051**	**0.053**	**0.052**	**0.051**	x	mg/L	0.005
	Oct-20	0.018	0.016	0.015	0.016	x		
	Mar-21	0.025	**0.036**	0.028	0.031	0.027		
	Jun-21	0.033	**0.04**	**0.045**	**0.039**	0.034		
Chlorophyll α	Aug-20	14.7	14.7	14.9	6	x	µg/L	0.2
	Oct-20	7.9	7.05	6.5	6.7	x		
	Mar-21	--	2.2	2.9	0.7	0.4		
	Jun-21	5.3	5.1	5.6	6.9	5.7		

Table 3. Surface water quality results for Fushimi Lake, ON. Analytical parameters represented in the table include total N, total P and chlorophyll α for purposes of determination of trophic status in Fushimi Lake. Missing samples are denoted by "x", and non-detected samples are denoted by "—". Bold and red indicate eutrophic level of lake productivity.

Analytical Parameter	Month	Analytical Results - Fushimi Lake				Units	Detection Limit
		FW1	FW2	FW3	FW4		
Total Nitrogen	Aug-20	x	0.507	x	x	mg/L	0.015
	Oct-20	0.4	0.455	0.393	x		
	Mar-21	**0.601**	**0.644**	**0.592**	**0.623**		
	Jun-21	0.463	0.352	0.368	0.342		
Total Phosphorus	Aug-20	x	0.022	x	x	mg/L	0.005
	Oct-20	0.011	--	0.011	x		
	Mar-21	0.01	0.017	0.012	0.018		
	Jun-21	0.028	0.031	0.023	0.033		
Chlorophyll α	Aug-20	x	2.29	x	x	µg/L	0.2
	Oct-20	4	3.9	4.3	x		
	Mar-21	0.5	0.8	--	1.6		
	Jun-21	2.4	3.9	2.4	2.7		

Table 4. Surface water quality results for McKay Lake, ON. Analytical parameters represented in the table include total N, total P and chlorophyll α for purposes of determination of trophic status in McKay Lake. Non-detected samples are denoted by "—".

Analytical Parameter	Month	Analytical Results - McKay Lake			Units	Detection Limit
		MW1	MW2	MW3		
Total Nitrogen	Aug-20	0.29	0.408	0.285	mg/L	0.015
	Oct-20	--	0.006	0.011		
	Mar-21	0.314	0.322	0.441		
	Jun-21	0.228	0.23	0.273		
Total Phosphorus	Aug-20	0.014	0.015	0.015	mg/L	0.005
	Oct-20	0.005	--	0.006		
	Mar-21	0.025	0.009	0.017		
	Jun-21	0.019	0.019	0.029		
Chlorophyll α	Aug-20	1.3	1.3	1.1	µg/L	0.2
	Oct-20	2.7	2.7	1.3		
	Mar-21	1.2	0.9	9.8		
	Jun-21	1.8	1.8	2.9		

It is evident that there is a significant effect of lake ($F_{2,30} = 72.91$, $p = <0.0001$), seasonality ($F_{3,30} = 80.15$, $p = <0.0001$), and some interactive effect of lake and seasonality ($F_{6,30} = 4.83$, $p = 0.001$) on total nitrogen values in Constance Lake, Fushimi Lake and McKay Lake (Figure 7). A Tukey's post-hoc test revealed highly significant pairwise differences between Constance Lake and McKay Lake ($p = <0.0001$), Constance Lake and Fushimi Lake ($p = 0.013$), and Fushimi Lake and McKay Lake ($p = <0.0001$) for levels of N. There are some significant differences between seasonality overall, with all months except for August 2020 and March 2021, and October 2020 and June 2021, having significantly different levels of N. Constance Lake has significant seasonality differences of N levels from McKay Lake for August 2020 ($p = <0.0001$), October 2020 ($p = 0.032$), and March 2021 ($p = 0.011$). There is no seasonality difference between

Constance Lake and Fushimi Lake within season (i.e. August 2020), however there is significant differences between the lakes across seasons (i.e. Constance Lake June 2021 and Fushimi Lake August 2020), and within the lakes across seasons (i.e August and October 2020 in Constance Lake). Fushimi Lake has significant seasonality differences of N levels from McKay Lake for August 2020 (p = 0.0298), October 2020 (p = <0.0001), March 2021 (p = <0.0001), and June (p = 0.007).

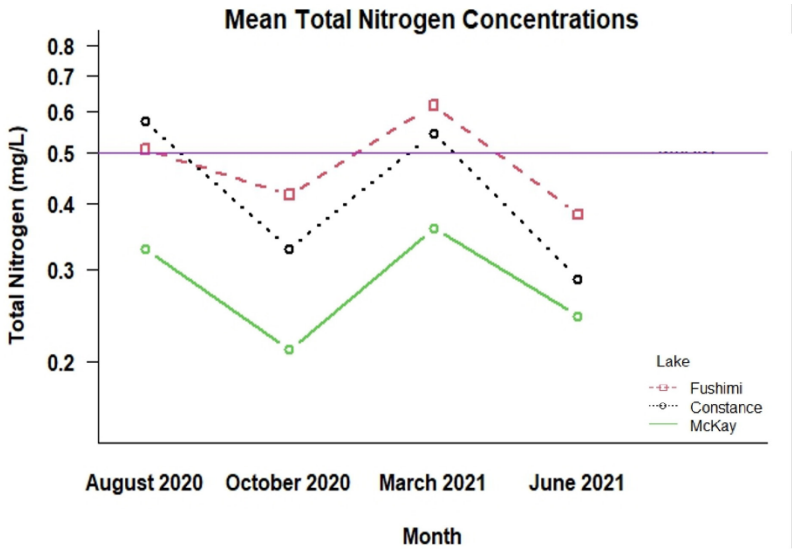

Figure 7. Plot of mean total nitrogen values ($mg \cdot L^{-1}$) for Constance Lake, ON, Fushimi Lake, ON, and McKay Lake, ON. Line at 0.50 $mg \cdot L^{-1}$ indicates N trigger range for eutrophication.

There is also a significant effect of lake ($F_{2,30}$ = 93.85, p = <0.0001), seasonality ($F_{3,30}$ = 60.69, p = <0.0001), and an interactive effect of lake and seasonality ($F_{6,30}$ = 8.93, p = <0.0001) on total P values in Constance Lake, Fushimi Lake and McKay Lake (Figure 8). A Tukey's post-hoc test revealed highly significant pairwise differences between Constance Lake and Fushimi Lake (p = <0.0001), and Constance Lake and McKay Lake (p = <0.0001) for levels of P, while there are no significant differences

between Fushimi Lake and McKay Lake. There are some significant differences between seasonality overall with all months, except for August 2020 and June 2021, having significantly different levels of P. Constance Lake has significant seasonality differences of P levels from Fushimi Lake in August 2020 (p = <0.0001) and March 2021 (p = 0.003). Constance Lake has significant seasonality differences of P levels from McKay Lake for August 2020 (p = <0.0001), October 2020 (p = 0.045), March 2021 (p = 0.011) and June 2021 (p = 0.0005). There is no significant difference in seasonality between Fushimi Lake and McKay Lake.

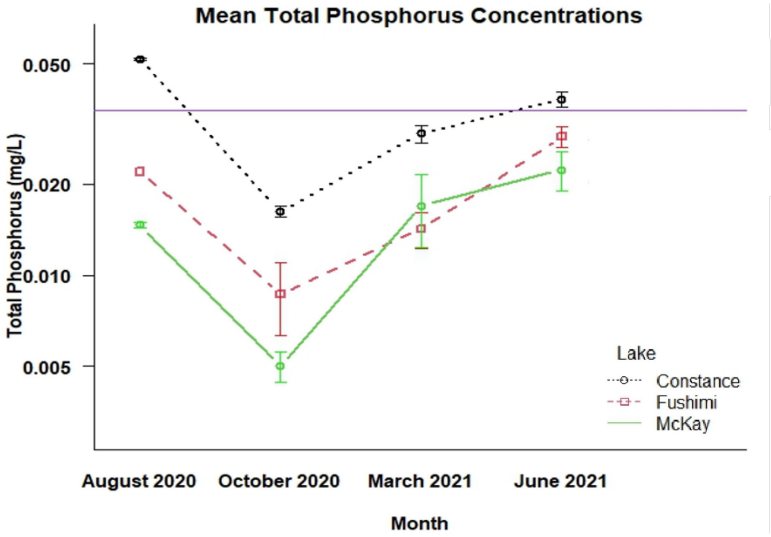

Figure 8. Plot of mean total phosphorus values ($mg \cdot L^{-1}$) for Constance Lake, ON, Fushimi Lake, ON, and McKay Lake, ON. Line at 0.035 $mg \cdot L^{-1}$ indicates P trigger range for eutrophication.

The same holds true for values of chl α. There is some effect of lake ($F_{2,30}$ = 18.80, p = <0.0001), some effect of seasonality ($F_{3,30}$ = 10.72, p = <0.001), and an interactive effect of lake and seasonality ($F_{6,30}$ = 7.68, p = <0.001) (Figure 9). A Tukey's post-hoc test revealed pairwise differences between Constance Lake and Fushimi Lake (p

= 0.037) and Constance Lake and McKay Lake (p = 0.019) for chl α levels. There are some significant differences between seasonality overall with all months, except for August and October 2020, August 2020 and June 2021, and October 2020 and June 2021, having significantly different levels of chl α. Constance Lake and McKay Lake only had siginficant seasonality differences of chl α levels within the month of August 2020 (p = 0.016), whereas the rest of the differences is between the lakes across seasons (i.e. Constance Lake June 2021 and Fushimi Lake August 2020), and within the lakes across seasons (i.e August and October 2020 in Constance Lake).

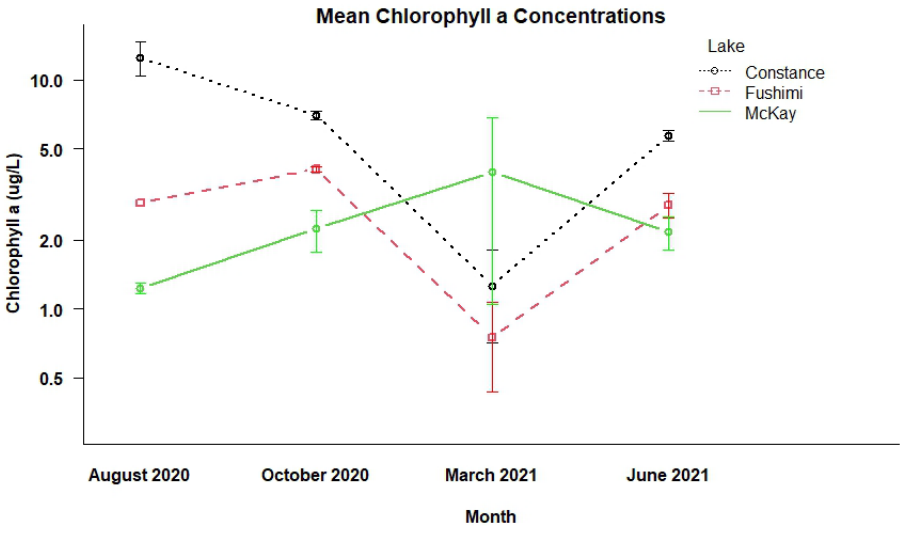

Figure 9. Plot of chlorophyll α values ($\mu g \cdot L^{-1}$) for Constance Lake, ON, Fushimi Lake, ON, and McKay Lake, ON.

2.4 Discussion

It was hypothesized that nutrients and metal concentrations would be highest in Constance Lake. This hypothesis was partially accepted, due to the fact that P and chl α were highest in Constance Lake, with N being the highest in Fushimi Lake. Metal

concentrations were not of concern as neither of the metal parameters analyzed exceeded any guidelines. Of all three nutrient parameters analyzed, N was highest in October 2020, March 2021, and June 2021 in Fushimi Lake compared to Constance Lake and McKay Lake. While the sediment chemistry is unknown for Fushimi Lake, increased levels of N, especially in March, could be attributed to several factors. It is known that microbial fixation of nitrogen gas in sediments is a major source of N to a system. Fushimi is considered a polymictic lake, and N concentrations are also dependant on stratification. During the winter when ice is covering the lake, concentrations of nitrate decrease with depth, where ammonia is evenly distributed to depth (Wetzel, 2001a). This could possibly explain the higher nitrogen levels in Fushimi in March, given that the surface water sample was collected 2 m below ice surface and total N was analyzed, which is a sum of all the forms of N. It is now known that Constance Lake is seasonally eutrophic, and a symptom of eutrophication is low dissolved oxygen. Under anoxic conditions in eutrophic lakes, nitrate rapidly becomes denitrified to nitrogen gas. This is potentially the cause of lower N in Constance Lake compared to Fushimi Lake.

Phosphorus, and chl α were highest in Constance Lake, compared to Fushimi Lake and McKay Lake especially in August 2020, however there was an elevation of chl α in McKay Lake in March 2021. The elevated mean of chl α in McKay Lake is likely due to sampling error. This was verified in the raw analytical data supplied from the lab (Table 2, Table 3, and Table 4). There was one sample which had unusually high levels of chl α, and when considering the location of where the sample was collected, it is possible that the ice auger used to open the ice stirred up sediments, which were captured in the sample. Variation in all parameters can be attributed to the varying sample size across

season for each of the lakes. The data are confirming what the anecdotal reports from Constance Lake First Nation community members are saying and previous surface water sampling (Hutchison Environmental Sciences Ltd, 2014), that Constance Lake is in an altered state from what the community members previously know and have previously experienced in terms of recreational activities and physical observations. It is known that phosphorus serves an important role in controlling primary production of a lake, and controls cyanobacteria bloom formation (Strayer, 2014; Yan et al., 2017). Hutchison Environmental Sciences Ltd. (2014) identified that P and N levels in sediment samples exceeded the Severe Effect Level for aquatic organisms as defined by the Ontario Provincial Sediment Quality Guidelines, with sediment cores being composed of nutrient-rich, soft, organic-rich sediment down to depths of at least 50 cm. Sediment-bound P is known to play a significant role in internal P-loading of a lake for several years depending on the loading history and the capacity of the sediment to retain P (Søndergaard *et al.*, 2013; Orihel *et al.*, 2017b; O'Connell *et al.*, 2020).

Søndergaard *et al.* (2013) conducted a study on six eutrophic and iron-rich lakes that were continuously sampled over a span of 21 y. The purpose of their study was to determine changes in the seasonal pattern of P release from sediment post external nutrient loading reductions. They determined that P is released from the sediment for many years after external loading is reduced, and that seasonal uptake or release of P occurs from the sediment surface where P is retained during the winter months (October to April) and released again in the summer (May to August), or productive period. This is reflective of the situation in Contance Lake, Fushimi Lake, and McKay Lake, where P levels are seen to increase from March 2021 to June 2021, suggesting that the majority of

P in the systems is from internal loading from sediment-bound P. This does not however, explain the continuous increase from October 2020 to March 2021, however, the anoxic conditions in Constance Lake determined through water quality profiling during those months, plays a part in the release of sedmiment-bound P (Orihel *et al.*, 2017; Liang *et al.*, 2020).

Studies have found that neither N or P alone can describe trophic levels or algal biomass (Dodds & Smith, 2016; Filstrup & Downing, 2017; Liang *et al.*, 2020; Bennett *et al.*, 2021), and that reducing P can help to control eutrophication and algal blooms (Bormans *et al.*, 2016; Fink *et al.*, 2018; O'Connell *et al.*, 2020). It has been found that P accumulates faster than N in freshwater systems (Yan *et al.*, 2016) and microbial processes such as denitrification an anaerobic ammonium oxidation can convert inorganic N to N_2 gas, removing N from the water column (Dodds & Smith, 2016; McCarthy *et al.*, 2016). This phenomenon likely explains why N levels decrease during productive months (March to June 2021) where P levels are increasing. There are also fundamental differences that exist between N and P biological processes such as transformations of N through N fixation, nitrification, and denitrification (Yao *et al.*, 2018) that were not considered in this study, as only total N was considered for analysis. It is also possible that the decrease in N during productive months could be attributed to the presence of a cyanobacterial bloom in Constance Lake during the time of sampling, as a reduction in N tends to favour N_2-fixing cyanobacteria (van Gerven *et al.*, 2019). The above processes are also likely to explain the elevated N levels in Fushimi Lake compared to the other lakes, suggesting that these microbial processes were happening at a much slower rate in

Fushimi Lake, possibly due to colder water temperatures as Fushimi Lake is a larger, deeper lake.

Several studies have also identified chl α, in combination with N and P, as an intuitive reflection of trophic level within lakes (Du *et al.*, 2019; Liang *et al.*, 2020; Bennett *et al.*, 2021). Chlorophyll α levels reflect algal biomass (BoQiang *et al.*, 2013; Søndergaard *et al.*, 2013; Smith *et al.*, 2016; Liang *et al.*, 2020) and chl α levels and total P levels are closely linked (BoQiang *et al.*, 2013) as primary producers are one of the first ecological indicators to respond to an increase of nutrients (Bennett *et al.*, 2021). Elevated chl α levels during the productive months (March and June 2021) correspond to increases in P during the same timeframe. Since chl α levels are directly linked with P, this describes why there are differences between Constance Lake and Fushimi Lake and McKay Lake, as these lakes have less P compared to Constance Lake.

2.5 Conclusion

It can be concluded that Constance Lake is in fact different from Fushimi Lake and McKay Lake, with P land chl α levels being higher in Constance Lake across seasons, and N being lower in Constance Lake compared to Fushimi Lake across seasons. Neither lake exceeding values for heavy metals when compared to the CWQG for protection of aquatic life. With respect to chl α, Constance Lake was only different, with higher levels, from McKay Lake in August 2020, and no other season, nor different from Fushimi Lake across seasons. Based on the several discrepancies reported in literature, what is happening in Constance Lake, Fushimi Lake, and McKay Lake could be attributed to the small sample size and limited timeframe in which observations from this

research occurred. A power analysis was not completed to determine the number of samples required for meaningful statistical analysis, however the small sample sizes from each lake (as described in Chapter 2.2.2) and limited time frame of sampling, are not sufficient to develop definitive conclusions on the status of each of these lakes.

With limited exchange of water in the system due to altered inflows and outflows, combined with internal nutrient inputs from the forestry industry and unknown external nutrient inputs, it would take a significant amount of work to help CLFN establish a relationship with the lake again. Further investigations into the inflows and outflows, as well as the other lakes within the watershed, should be undertaken to identify and understand all the stressors and impacts to Constance Lake, and determine where the nutrient input is coming from, whether it is external loading, internal loading, or both. Sediment sampling through grab samples and cores should be considered to determine the depth of organic matter on the lake bottom. This in turn could be used to determine the full potential of nutrient loading into the system and used to predict the potential for future cyanobacterial blooms (Yang *et al.*, 2020). Further agal sampling during productive months to identify the species and life stages of cyanobacteria present to get a better prediction for blooms (Bhagowati & Uddin Ahamad, 2019). With water chemistry parameters well within the limits of the CCME CWQG for protection of aquatic life, regular water quality and sediment monitoring should be implemented to continue to determine trends of P levels and P internal loading. Not only will regular monitoring detect changes to the water quality and the amount of sediment-bound P, but it would allow for CLFN to remain informed on the status of a lake that is so culturally important to the community.

Bormans *et al.* (2016) provide and compare some additional physical methods of controlling internal phosphorus loading. These methods include hypolimnetic aeration and oxygenation which works well in lakes with a large hypolimnetic area (>15 m), has the most beneficial impact and has the potential to increase fish habitat and zooplankton density. The second method is hypolimnetic withdrawal which works well in small, deep, stratified systems, which decreases water level and has the potential to cause adverse impacts on downstream water quality. The last method involved sediment dredging, which is only feasible in small systems, and has the potential to expose unwanted toxic substances such as heavy metals, release of sediment-bound contaminants, and also has the potential to disturb sediment bacterial communities and fauna. Hutchison Environmental Sciences Ltd. (2014) suggested the prevention of anoxia through hypolimnetic aeration, however due to the large, flat-bottomed nature of Constance Lake, several treatment points would be required to help improve circulation of the bottom water. Additionally, Phoslock® has also been suggested by Hutchison Environmental Sciences Ltd. (2014) as a means of inactivating the sediment-bound phosphorus. Phoslock® has been applied to several different types of lake globally (Zeller & Alperin, 2021). While these treatments have been successful in reducing P in the water column, other intended impacts such as change of location of sediment oxic-anoxic boundary, ecological toxicity from the leaching of lanthanum, and bioaccumulation of lanthanum within the ecosystem have also been reported. This method is also only useful if the sole source of P is coming from internal loading.

There are several options that can be applied to Constance Lake to decrease productivity and return to a sustainable and balanced equilibrium, however, the source of

P must first be determined. Further discussions should be had with CLFN to determine what a realistic end goal for Constance Lake would look like, and what level of relationship the community would like to rebuild with the lake again.

Chapter 3: Stress Responses of Freshwater Mussels

3.1 Introduction

Ecosystems around the world have experienced major modifications through anthropogenic activities, which typically have negative effects on the native flora and fauna (Fritts *et al.*, 2015a). To help monitor how these changes impact the fauna, biomarkers have been used to evaluate the stress caused by such activities (Gagné *et al.*, 2002; Fritts *et al.*, 2015a; Blaise *et al.*, 2017). Glycogen is an indicator of mussel health because it is the principal carbohydrate storage compound in bivalves and is a useful marker for the energetic status of an organism (Beggel *et al.*, 2017; Hornbach *et al.*, 2019) as it assesses the amount of available energy the mollusc has for survival, growth and development, and reproduction (Said & Ezzat Nassar, 2022). Varying environmental conditions can affect glycogen levels including nutrients, and temperature (Fritts *et al.*, 2015; Payton *et al.*, 2016; Hornbach *et al.*, 2019). Glycogen levels can react quickly to these changes in the environment and can be connected to the nutritional condition, varying types of stress, life cycle stages, and sexual maturity (Vodáková & Douda, 2019) as glycogen levels have been shown to decrease under stressful conditions (Fritts *et al.*, 2015b).

Another indicator of mussel health is 4-hydroxynonenal (HNE). This protein is a by-product of lipid peroxidation, resulting from oxidative stress. 4-hydroxynonenal is a major aldehyde that is derived from the decomposition of peroxidation products of omega-6 polyunsaturated fatty acids (Zhang & Jay Forman, 2016), and affects the cell cycle by regulating signalling for apoptosis, differentiation, and gene expression (Singhal *et al.*, 2015). When the production of reactive oxygen species (ROS) becomes out of

balance due to exposure to pathogens or toxins, HNE forms adducts, or addition products (i.e. addition of two or more distinct molecules that result in a single reaction product) with proteins, nucleic acids and lipids causing dysfunction of biological molecules (Zhang & Jay Forman, 2016).

Studies suggest that mussel physiology is altered as a function of oxidative stress from chronic exposure to contaminants (Gillis *et al.*, 2014b; Machado *et al.*, 2014). While adult unionids may be sensitive to contaminants and environmental changes (Newton *et al.*, 2003), some species, such as *L. siliquoidea* and *P. grandis*, appear to be more tolerant (Pip, 2006).

The objective of this study was to evaluate stress in local freshwater mussels due to degraded water quality. It was hypothesized that mussels in Constance Lake will have lower glycogen levels than Fushimi Lake and McKay Lake during winter months due to limited food availability from algal blooms, and higher HNE protein levels during summer months due to the eutrophic condition of the lake. It was hypothesized that mussels in Fushimi Lake and McKay Lake will have higher glycogen levels during winter months as a result of steady food supply, and lower HNE protein levels in the summer months due to lower inputs of nutrients and external stressors.

3.2 Materials and Methods

3.2.1 Study sites

Study site methodology follows the approach as described in Chapter 2.2.2. Locations of mussel collections from each lake can be found in Figure 4, Figure 5, and Figure 6. Mussels in Constance Lake were collected from the north shore in a sandy area,

and two locations on the east shore, just north of the community and adjacent to the community, in more rocky substrates. Mussels in Fushimi were collected from the north shore in sandy substrates near a public beach, and mussels in McKay Lake were collected from a sandy beach on an island near the middle of the lake, as well as in a sandy bay to the southeast.

3.2.2 Mussel collection and dissection

Mussels were collected in October 2020 and in June 2021 using either a clam rake or a combination of a 500µm d-frame dipnet with an underwater viewing box. The clam rake was used to rake a ~1 m long radius in a half circle. Once the half circle was completed, the person using the rake moved outside the circle and began raking again in the same fashion. Using the viewing box, mussels were located and then swept by foot into the d-net.

My field partner and I waded into the water roughly waist deep and walked to and from shore and down the length of the beach until the required number of specimens was collected. During sampling, the specimens were placed in a container filled with lake water until they could be brought to shore and placed in a 20 L Dewar of liquid nitrogen (provided by Lakehead University Chemical Store) for preservation. Mussels were placed individually in a nylon stocking with a label and zip-tied to keep each mussel separate before placing in the Dewar. A length of 4 mm blue multi-braided polypropylene line was tied to each stocking, then tied to the handle of the Dewar to allow for easier extraction. Once transported back to Lakehead University, the mussel-filled stockings were pulled from the Dewar and stored in a -80°C freezer.

A greater number of mussels was collected in October 2020, which posed challenges with extraction of the nylon stocking from the Dewar due to the lines becoming severely tangled during transportation. As a result, a new method of preservation and storage during transport was used during the June 2021 sampling event. Upon collection of the mussels, the mussels were individually placed in nylon stockings and zip-tied, placed in a container and liquid nitrogen poured over top until they stopped bubbling. The mussels were then held in a YETI cooler over dry ice until they could be returned to Lakehead University for final storage in a -80°C freezer until dissection.

Mussels were thawed over ice in batches of 10. Thawing took on average ~2 hrs. Similar to the methods in Gillis *et al.* (2014), external parameters of shell length, height, and width was determined with digital calipers (± 0.01 mm). Once all measurements were collected, soft tissues were dissected into gills and mantle. The tissues were places in pre-labelled Whirl-paks and placed in a freezer at -4°C until the batch was completed. Once the batch was complete, the samples were returned to the -80°C freezer, with another batch of 10 mussels taken out to thaw. This process was repeated until all mussels were measured and dissected. Tissues remained in the -80°C freezer until assays were ready to be completed.

When assays were ready to begin, tissues from the Whirl-Paks were thawed, and 0.004 ± 0.001 g of mantle tissue for the glycogen assay, and 0.1 ± 0.001 g of gill tissue for the HNE assay were separated into pre-labelled cryovials for the respective assays.

3.2.3 Mussel Ageing

Upon thawing the mussels, I noted that several of the valves were damaged from the rapid freezing in liquid nitrogen. Specifically, *P. grandis* was more susceptible to shell damage than *L. siliquoidea* due to their shells being so thin. For *L. siliquoidea*, the left valve was used to make a shell cross-section at the umbo. I used an Isomet rock saw from the Lakehead University Geology Lapidary (LUGL) laboratory to create my cross-sections. A second cut was made to create a thinner slice of shell, making it easier to view under a dissecting scope. Once all valves were cross-sectioned, I used 400 grit lapping compound and a glass plate (provided by LUGL) to polish the cross-sections. After polishing, each cross-section was viewed at both 25x and 40x magnification on a Wild M3C dissecting scope. To determine age, the winter "rings" of the valve were counted. Blind counts were independently conducted by me, the researcher, and Dr. Carney, thesis supervisor. Where discrepancies in age were found, the cross-sections were again blind counted until a consensus on age was reached.

3.2.4 Glycogen assay

Glycogen levels were analyzed to evaluate the nutritive status and stress level of *P. grandis* and *L. siliquoidea* as a result of trophic status in Constance Lake, Fushimi Lake, and McKay Lake. Naimo *et al.* (1998) developed an analytical method for measuring low concentrations of glycogen in unionid mussels. The same method was used for this study as described below. Approximately 8 mg of mantle tissue from 154 mussels (Table 5) were dissected and placed into 2 mL centrifuge tubes. The samples were then frozen at -80°C until the assay could be completed.

Table 5. Total n for the glycogen assay for each *P. grandis* and *L. siliquoidea* as collected in October 2020 and June 2021.

Sample n for Glycogen

	Constance Lake		Fushimi Lake		McKay Lake	
Month	*Pyganodon grandis*	*Lampsilis siliquoidea*	*P. grandis*	*L. siliquoidea*	*P. grandis*	*L. siliquoidea*
October 2020	12	20	3	15	16	--
June 2021	8	26	5	29	17	3

Samples were thawed prior to beginning the assay. During this time, I prepared a set of 6 aqueous glycogen calibration standards by serially diluting 300 µL of 20 $mg \cdot L^{-1}$ stock glycogen standard (MilliporeSigma™ Calbiochem™ Glycogen, Molecular Biology Grade, from Mussel). 300 µL of stock glycogen standard was pipetted into a 2 mL centrifuge tube containing 300 µL double distilled water (DDW) and then vortexed, creating a standard of 10 $mg \cdot L^{-1}$. 300 µL of the 10 $mg \cdot L^{-1}$ standard was pipetted into another 2 mL centrifuge tube containing $mg \cdot L^{-1}$ of DDW and then vortexed, creating a 5 $mg \cdot L^{-1}$ standard. This process was repeated to create standards of 2.5, 1.25 and 0.625 $mg \cdot L^{-1}$. All other reagents used in this assay were supplied by Lakehead University Chemistry Store.

To begin the assay, 250 µL of standard was placed into a 2 ml centrifuge tube. I added 100 µL of 30% aqueous potassium hydroxide (KOH, w/v) to each tube of standards and to 34 samples, and heated the vials for 20 min in an Isotemp (Fisher Scientific, model #2001) at 100°C. After heating, I vortexed the vials for 30 s and placed on crushed ice for 5 min. I added 150 µL of 95% ethanol (EtOH) to the samples, vortexed for 5 s then heated on the Isotemp for an additional 5 min at 100°C. Standards were not completed at this time as there was not enough volume in the centrifuge tube. During heating, caps of the centrifuge tubes began popping open due the pressure of the boiling

EtOH. One sample was lost completely as the pressure of the cap opening caused it to dislodge from the Isotemp. Standards were re-done in an additional batch. The samples were cooled and pipetted into 2 mL cryovials. The standards were also pipetted into cryovials, EtOH added, and then both standards and samples were placed in a water bath at 100°C for 15 min to complete the glycogen extraction from the tissues. Once the water bath was complete, the cryovials were stored at -80°C until analysis could take place. The next batch of 40 samples was assayed following the same procedure for adding KOH. After vortexing, the samples were pipetted into 2 mL cryovials where EtOH was added and the cryovials placed in a hot water bath for 15 min. These samples were then placed in the -80°C freezer. The remaining 26 samples were assayed in the same manner.

For analysis, the contents of each vial were washed into a 10 mL test tube with 5 consecutive rinses of 1000 µL of DDW. I diluted the standards and samples with DDW to a common volume (6600 µL) and vortexed for 15 s. To ensure common volumes were reached, a test tube was filled with 6600 µL DDW and placed next to a test tube containing standard or sample. Using a pipette, a single 2 mL aliquot of solution was placed into another test tube and 100 µL of 80% aqueous phenol (v/v) added. A 5 mL aliquot of reagent-grade concentrated H_2SO_4 was immediately added by directing the stream of acid to the liquid surface. As a result of the exothermic reaction, Parafilm was used to cover the tubes for vortexing. The tubes were vortexed for 10 s and the parafilm immediately removed. The samples remained at room temperature for 30 min to obtain maximum colour development. Absorbance was determined at 490 nm on a spectrophotometer (Biorad SmartSpec Plus). Each of the standards were placed into a new labelled test tube after each absorbance was read, with the remaining liquid of

standards also kept. For the samples themselves, only the remaining liquid was kept. Each of these tubes was wrapped in parafilm and stored at room temperature.

3.2.5 4-Hydroxynonenal Assay

To determine levels of 4-HNE in the mussel gill tissue, an OxiSelect HNE Adduct Competitive ELISA kit (Cedarlane Laboratories) was used. Prior to preparing the ELISA plate, I prepared 1 L of phosphate buffered saline (PBS, pH 7.4) following the recipe found in Appendix 2 – Phosphate Buffered Saline.

A 96-well plate was prepared in two parts. On Day 1, 100 µL of 1:1 Ratio 10 $\mu g \cdot mL^{-1}$ HNE conjugate: 1x conjugate diluent was added to each well on the plate. The purpose of this is to coat the wells of the plate to avoid false positive response of the conjugated anti-HNE antibody. The plate was then covered with Parafilm and placed in the fridge at 4°C overnight. While the plate was incubating, cryovials containing ~100 mg gill tissue from 101 samples (Table 6) were removed from the -80°C freezer, placed into a Styrofoam cooler with ice, and transported to the Lakehead University Paleo DNA laboratory. Here, tissues were transferred to autoclaved 2 mL centrifuge tubes. I filled the centrifuge tubes with 0.5 mL PBS and a metal homogenizing ball, and using a ball homogenizer, pulsated the homogenizer 50 sec^{-1} for 2 min and returned to ice. The centrifuge tubes were placed in a centrifuge at 10,000 min^{-1} for 20 min at 4°C. Once complete, the centrifuge tubes were placed in a centrifuge tube tray, placed back over ice in the Styrofoam cooler, and transported back to the laboratory for the assay to begin.

Table 6. Total n for the HNE assay for each *P. grandis* and *L. siliquoidea* as collected in October 2020 and June 2021.

Sample n for HNE

	Constance Lake		**Fushimi Lake**		**McKay Lake**	
Month	*Pyganodon grandis*	*Lampsilis siliquoidea*	*P. grandis*	*L. siliquoidea*	*P. grandis*	*L. siliquoidea*
October 2020	12	12	3	12	11	--
June 2021	7	11	5	12	13	3

A conjugate diluent, HNE conjugate and a 1:1 ratio of HNE conjugate: conjugate diluent, reagent was prepared. To prepare the conjugate diluent, I diluted the conjugate diluent to 100x in PBS. Phosphate buffered saline was added to a 15 mL Falcon Tube to a total volume of 4.95 mL. I then added a total of 50 µL of the 100x conjugate diluent to the same first tube. The HNE conjugate was then diluted to 1.0 $mg \cdot mL^{-1}$. I added PBS to a second 15 ml Falcon Tube to a total volume of 4.95 ml. A total of 50 µL of the 1.0 $mg \cdot mL^{-1}$ HNE Conjugate was then added to the same second tube. To prepare the 1:1 ratio reagent, I poured one Falcon Tube into the other, vortexed for 15 s.

On Day 2, the 96-well plate was washed by tapping out the liquids that sat overnight in the plate onto large Kimwipes. Each well was filled with 250 µL of PBS using an auto-repeater pipette, then tapped out and thoroughly blotted onto Kimwipes to remove as much liquid as possible. This step was repeated a second time. The pipette was cleaned with distilled water and dried thoroughly before using again. I then filled each well with 200 µL of "Assay Diluent" from the HNE kit, using the auto-repeater pipette, and covered the plate with parafilm and left it at room temperature for one hour. The pipette was washed with distilled water and thoroughly dried before using again. While the assay diluent was sitting, I prepared a 1x wash buffer and HNE-BSA standard by diluting the 10x wash buffer with 1x deionized water. I added 450 mL of deionized water

to a beaker, added 50 mL of 10x wash buffer and stirred to homogeneity. For the standard, I labelled 1.5 mL tubes 1 to 9. The HNE-BSA standard solution was thawed and vortexed, and I filled each tube with assay diluent according to Table and kept them on ice. I then filled the tubes according to Table and vortexed each tube, changing pipette tips before filling the next tube, while keeping the tubes on ice.

Table 4. Volumes for preparing HNE-BSA Standard solution as per HNE ELISA Kit Step by Step Instructions (provided by Erin Hayward).

Standard Tube	Assay Diluent (µL)
1	320
2	200
3	200
4	200
5	200
6	200
7	200
8	200
9	200

Table 5. Volumes for preparing HNE-BSA Standard solution as per HNE ELISA Kit Step by Step Instructions (provided by Erin Hayward).

Standard Tube	1 mg/mL HNE-BSA Standard (µL)
1	80
2	200 from Tube 1
3	200 from Tube 2
4	200 from Tube 3
5	200 from Tube 4
6	200 from Tube 5
7	200 from Tube 6
8	200 from Tube 7
9	0

To begin the assay, I tapped out the assay diluent from the plate onto Kimwipes and added 50 µL of standard 1 in duplicate to the appropriate wells. This step was repeated for all 9 standards, changing the pipette tip between each standard. I added 50 µL of supernatant from each sample to the appropriate wells in triplicate being mindful not to get any lipid on the pipette tip or in the well. During this process, all samples were kept on ice. The 96-well plate was placed in the Biotek Synergy H1 microplate reader to shake at 250 rpm for 10 minutes, during which the left-over samples were placed back in the -80°C freezer for later use. While the plate was shaking, I prepared the dilute anti-HNE antibody by diluting the anti-HNE antibody to a ratio of 1:1000 with assay diluent. I added 9.99 mL of assay diluent to a 15 ml Falcon Tube and added 10 µL of anti-HNE antibody to the same Falcon Tube, capped, and vortexed for 15 s. To continue with the assay, I added 50 µL of the dilute anti-HNE antibody to each well, covered the plate with parafilm and kept it at room temperature. The plate was placed in the microplate reader to

shake for one hour at 250 rpm. While the plate was shaking, I prepared the dilute secondary antibody-HRP conjugate by diluting the secondary antibody to a ratio of 1:1000 with assay diluent. I added 9.99 mL of assay diluent to a 15 mL Falcon Tube, then added 10 µL of anti-HNE antibody to the same tube, capped, and vortexed for 15 s. To continue the assay, I washed the plate by tapping out the supernatant/dilute anti-HNE antibody mixture onto Kim wipes and filled each well with 250 µL of 1x wash buffer using an auto-repeater pipette. The wash buffer was then tapped out onto Kimwipes and repeated twice more. The pipette was washed with distilled water and dried thoroughly before using again. The dilute secondary antibody was vortexed, and each well was filled with 100 µL of dilute secondary antibody. The plate was placed back in the microplate reader to shake for one hour at 250 rpm. During this time, the substrate solution was removed from the fridge. Once the plate finished shaking, the dilute secondary antibody mixture was washed from the plate by rinsing each well with 250 µL of 1x wash buffer three times using an auto-repeater pipette. The pipette was washed with distilled water and dried thoroughly before using again. Once washed, I filled each well with 100 µL of substrate solution using the auto-repeater pipette, covered with parafilm and placed the plate in the microplate reader to shake for eight minutes at 250 rpm. The pipette was washed with distilled water and dried thoroughly before using again. Once completed, I filled each well with 100 µL of stop solution using the auto-repeater pipette. The 96-well plate was immediately placed back into the microplate reader and absorbance was read at 450 nm.

3.2.6 Data analysis

To determine the energetic status and stress response of *P. grandis* and *L. siliquoidea*, a two-way factorial ANOVA, with a log transformation of the glycogen and HNE concentration values, was used to determine differences between Constance Lake, Fushimi Lake, and McKay Lake during season (October 2020 and July 2021) for glycogen and HNE content. It was hypothesized that there will be an effect of the lake and an interactive effect of lake and season on the energetic status and stress response of *P. grandis* and *L. siliquoidea*, causing energetic status to decline, and stress responses (HNE) to increase.

3.3 Results

3.3.1 Mussel Aging

A total of 184 freshwater mussels, *L. siliquoidea* and *P. grandis*, were collected from Constance Lake, McKay Lake, and Fushimi Lake during three sampling events in August and October 2020, and June 2021. A full breakdown by species and lake can be found in Table 7 in Appendix 5.3 Mussel Samples. The maximum age of *L. siliquoidea* is 31 yo, the minimum age is 2 yo, and the average age is 11.5 yo. A full breakdown of ages, by lake, can be found in Table 8 in Appendix 5.3 Mussel Samples.

3.3.2 Glycogen

There was a significant difference in *L. siliquoidea* glycogen concentrations between months ($F_{1,88} = 4.58$, $p = 0.035$) and between lakes ($F_{2,88} = 7.47$, $p = 0.001$), though the interaction between these terms was not significant (Figure 11). A Tukey's

post-hoc test revealed significant difference (p = 0.035) between October 2020 and June 2021, with October having had higher mean glycogen levels than June 2021 by 0.002 $\mu g \cdot mL^{-1}$. The same post-hoc test also revealed significant differences (p = 0.002) between Fushimi Lake and Constance Lake, where Constance Lake had higher mean glycogen levels than Fushimi Lake by 0.004 $\mu g \cdot mL^{-1}$. There was significant difference (p = 0.046) between McKay Lake and Fushimi Lake, with McKay Lake having had higher mean glycogen levels than Fushimi Lake by 0.006 $\mu g \cdot mL^{-1}$. There were no significant differences between McKay Lake and Constance Lake, as there were no *L. siliquoidea* collected from McKay Lake in October 2020.

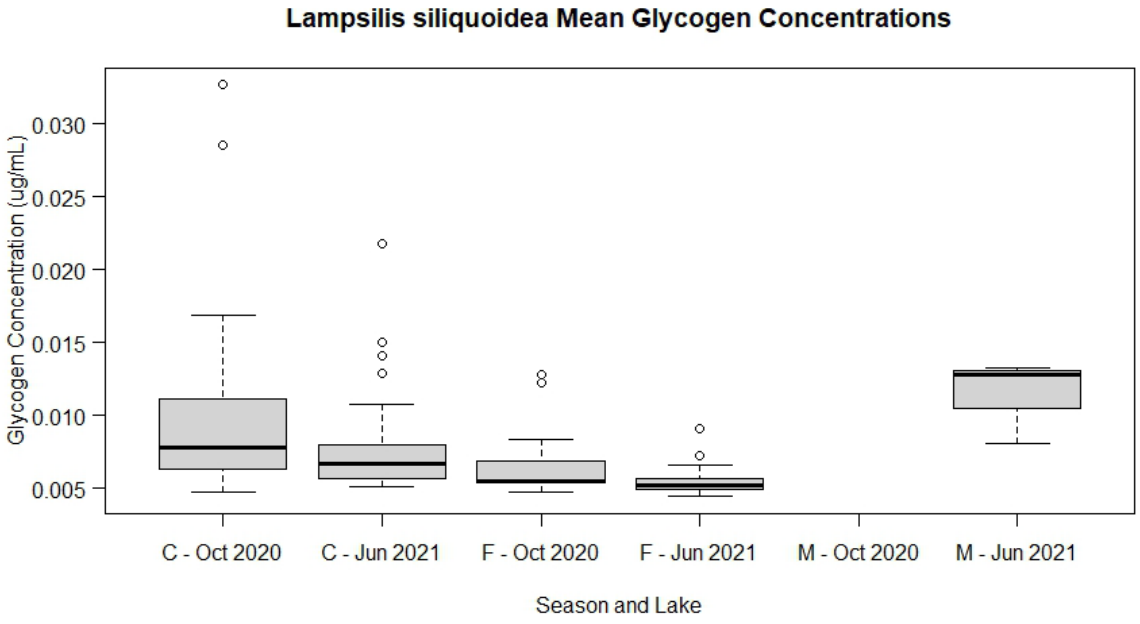

Figure 10. Boxplots of mean glycogen concentration for *L. siliquoidea* in Constance Lake (C), Fushimi Lake (F), and McKay Lake (M) during October 2020 and June 2021.

There was a significant difference in *P. grandis* glycogen concentrations between months ($F_{1,55}$ = 21.94, p = <0.0001) and between lakes ($F_{2,55}$ = 5.22, p = 0.008), though the interaction between these terms was not significant (Figure 12). A Tukey's post-hoc

test revealed significant differences (p = <0.0001) between October 2020 June 2021, with October having had lower mean glycogen levels than June 2021 by 0.140 $\mu g \cdot mL^{-1}$. The same post-hoc test also revealed a significant difference (p = 0.039) between Fushimi Lake and Constance Lake, where Fushimi Lake had lower mean glycogen levels than Constance Lake by 0.123 $\mu g \cdot mL^{-1}$. There was also a significant difference (p = 0.016) between McKay Lake and Constance Lake, where McKay Lake had lower mean glycogen levels than Constance Lake by 0.094 $\mu g \cdot mL^{-1}$. There was no significant difference in mean glycogen levels between McKay Lake and Fushimi Lake.

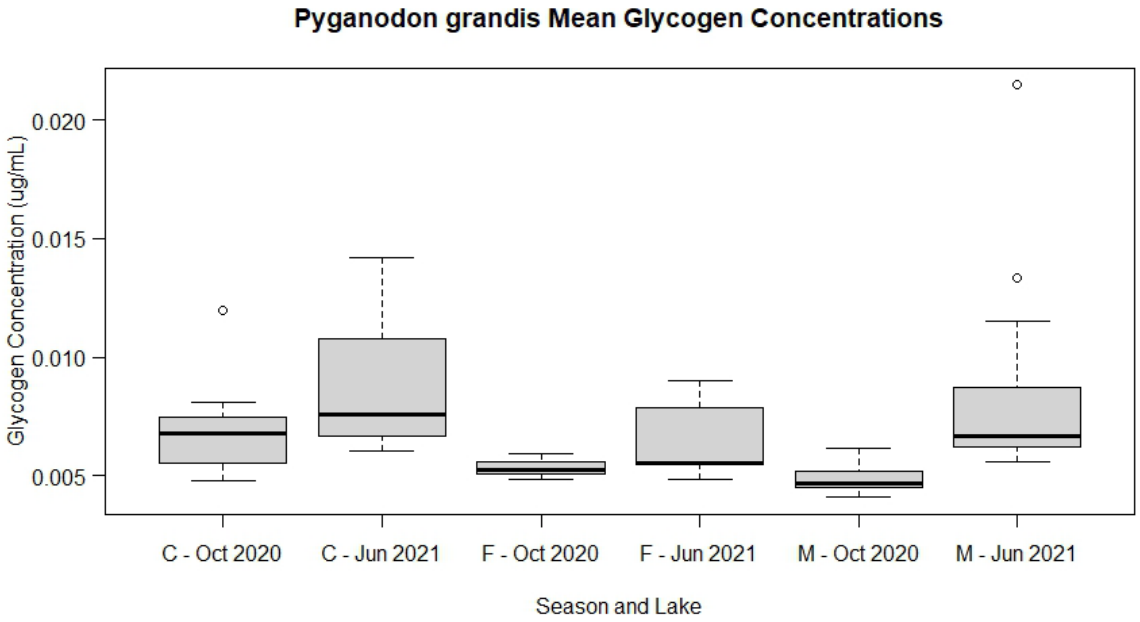

Figure 11. Boxplots of mean glycogen concentration for *P. grandis* in Constance Lake (C), Fushimi Lake (F), and McKay Lake (M) during October 2020 and June 2021.

3.3.3 4-Hydroxynonenal

There was a significant difference in *L. siliquoidea* HNE concentrations between months ($F_{1,36}$ = 16.78, p = 0.0002) and between lakes ($F_{2,36}$ = 7.69, p = 0.002), though the

interaction between these terms was not significant (Figure 13). A Tukey's post-hoc test revealed significant difference ($p = 0.0002$) between October 2020 and June 2021, with October having had lower mean HNE concentrations than June 2021 by 0.630 $\mu g \cdot mL^{-1}$. The same post-hoc test also revealed significant differences ($p = 0.003$) between Fushimi Lake and McKay Lake, where McKay Lake had lower mean HNE concentrations than Fushimi Lake by 1.081 $\mu g \cdot mL^{-1}$. There was no significant difference between Fushimi Lake and Constance Lake. There were also no significant differences between McKay Lake and Constance Lake, as there were no *L. siliquoidea* collected from McKay Lake in October 2020.

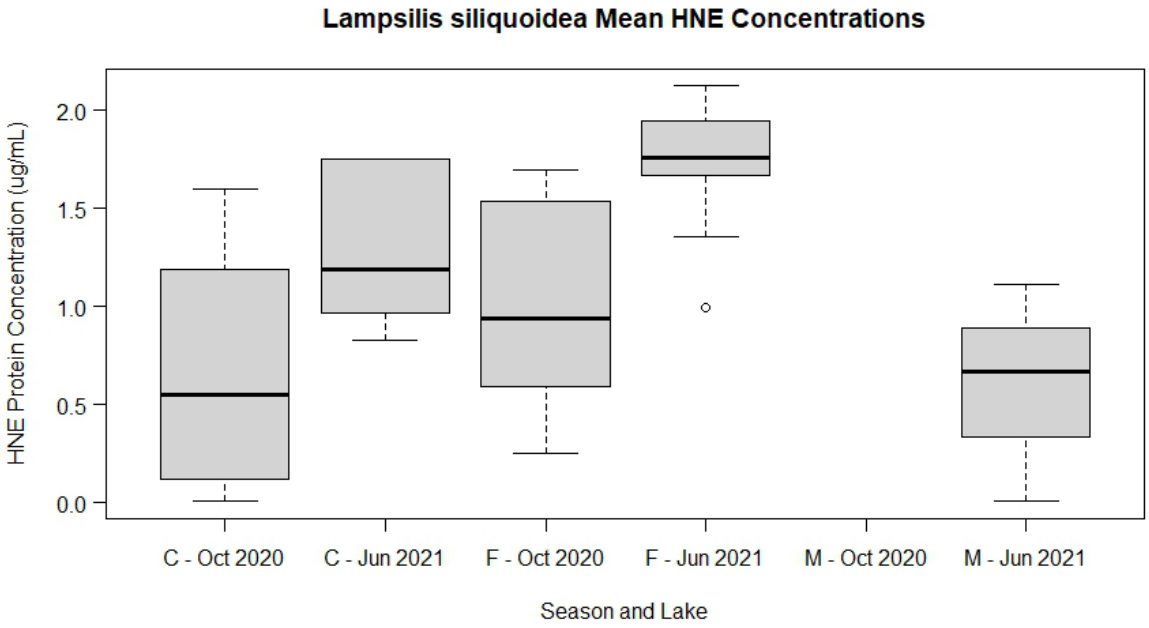

Figure 12. Boxplots of mean HNE protein concentrations *L. siliquoidea* in Constance Lake (C), Fushimi Lake (F), and McKay Lake (M) during October 2020 and June 2021.

There was a significant difference in *P. grandis* HNE concentrations between lakes ($F_{2,44} = 13.69$, $p = <0.0001$), though month and the interaction between these terms was not significant (Figure 14). A Tukey's post-hoc test revealed significant difference (*p*

= 0.018) between Fushimi Lake and Constance Lake, where Fushimi Lake had higher mean HNE concentrations than Constance Lake by 0.412 $\mu g \cdot mL^{-1}$. Constance Lake was also significantly different (p = 0.014) from McKay Lake, such that Constance Lake had higher HNE concentrations than McKay Lake by 0.294 $\mu g \cdot mL^{-1}$. Fushimi Lake and McKay Lake were also significantly different (p = 0.00003) from each other such that Fushimi Lake had higher HNE concentrations than McKay Lake by 0.706 $\mu g \cdot mL^{-1}$.

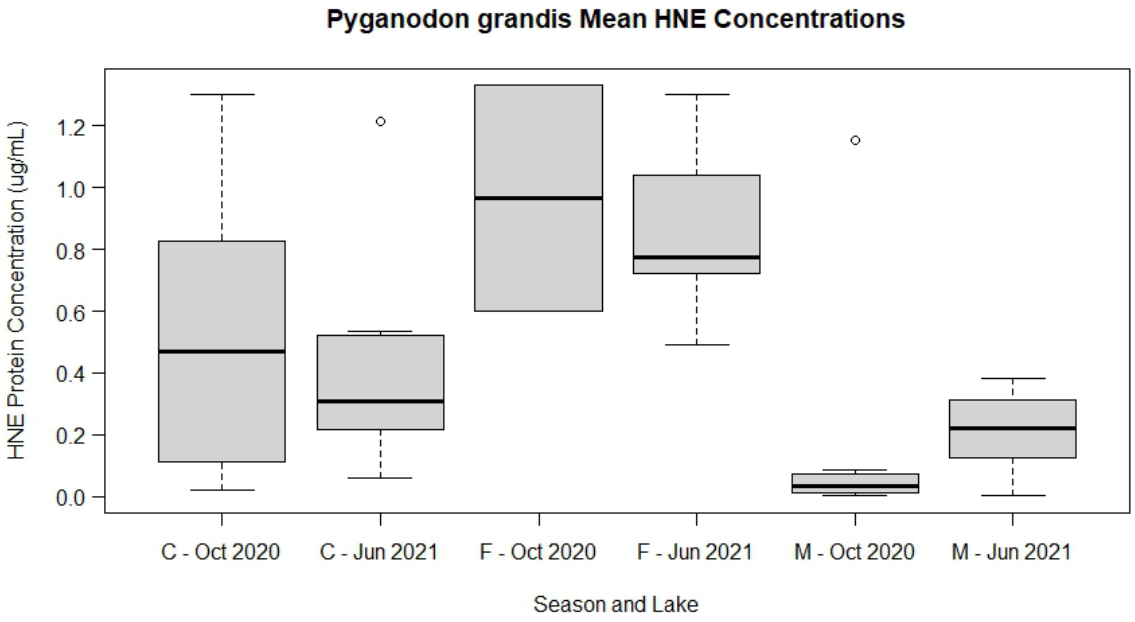

Figure 13. Boxplots of mean HNE protein concentrations for *P. grandis* in Constance Lake (C), Fushimi Lake (F), and McKay Lake (M) during October 2020 and June 2021.

3.4 Discussion

It was hypothesized that mussels in Constance Lake would have lower glycogen levels than Fushimi Lake and McKay Lake during winter months due to limited food availability and higher HNE protein levels during winter months due to the eutrophic condition of the lake. This hypothesis was accepted, however it only held true for one species – *P.* grandis. Mean glycogen and HNE concentrations vary between *L. siliquoidea*

and *P. grandis* and between seasons and across lakes. Mean glycogen levels in *L. siliquoidea* are lower in June 2021 and elevated in October 2020 across all lakes, with McKay Lake having the highest mean concentration. The reason for McKay having more elevated concentrations than Constance Lake and Fushimi Lake can be attributed to the small sample size of *L. siliquoidea* for that species (n = 3). The fact that there were no *L. siliquoidea* collected in McKay Lake during October 2020 is attributed to the differences and variability in McKay compared to the other lakes. It can potentially be predicted from Constance Lake and Fushimi Lake that glycogen concentrations in McKay Lake for October 2020 would also be elevated compared to June 2021 based on the observed trend. It is thought that female mussels would have lower glycogen levels than males due to partitioning energy production to brood their glochidia (Hornbach *et al.*, 2021). Since several female *L. siliquoidea* were gravid, separate statistical analyses were carried out between male and female *L. siliquoidea* to determine if brooding females had an effect on the overall mean glycogen levels. Mean glycogen concentrations followed the same pattern as males and females separately, that the combined sample did, with mean glycogen levels being higher in October 2020 than June 2021.

Lurman *et al.* (2014) found that *Anodonta anatina* (Duck Mussel) may have an energy-saving mechanism in winter temperatures that allows for a thermodynamically driven reduction in energy requirements, and that it correlates to seasonal food supply. When comparing the change in glycogen levels to nutrient levels in each of the lakes, an increase in P levels over the winter months corresponds to an increase in glycogen levels for *P. grandis*. As greater levels of P indicate greater food availability (Fink *et al.*, 2018), the increase in glycogen levels could be attributed to this. *Pyganodon grandis* and *L.*

siliquoidea both have a bradytictic life cycle. Sex was not determined for *P. grandis* unless glochidia was present in the gill, which only 5 of the total sample size were brooding.

The opposite holds true for HNE concentrations. Concentrations of HNE increase from October 2020 to June 2021 across all lakes for *L. siliquoidea.* While there is a difference in HNE protein concentrations for *L. siliquoidea* over winter, there is no significant difference in stress levels across lakes. Given that glycogen is also a measure of stress, it makes sense to compare both glycogen and HNE together. For *L. siliquoidea* it was noted that glycogen levels decreased over winter. It was seen that HNE levels for *L. siliquoidea* are increasing over the winter months, possibly due to a lack of preferred food, or lack of food in general, due to being burrowed in the substrate over winter and having valves closed.

Pyganodon grandis differs from *L. siliquoidea* again across all lakes as HNE protein concentrations decrease from October 2020 to June 2021. The higher concentrations of HNE in Fushimi Lake compared to the other lakes for *P. grandis* is possibly attributed to the low sample size, however, when relating glycogen levels to HNE levels, it was noted in *L. siliquoidea* that a lower glycogen level, or decrease in glycogen over winter, was related to higher levels of stress. Constance Lake and McKay Lake both had higher levels of glycogen than Fushimi for *P. grandis*, therefore it is suggested that this is the reasoning behind the increased levels of stress in Fushimi Lake compared to Constance Lake and McKay Lake. While there is a difference in HNE protein concentrations for *P. grandis* over winter, there is no significant difference in stress levels between Constance Lake and Fushimi Lake. The lack of differences across

lakes for both species suggests that the mussels are not stressed due to eutrophication, but there is some factor of stress involved from overwintering as a result of being burrowed in the substrate with valves closed for the duration of the winter (Lurman *et al.*, 2014).

Hayward (2019) conducted a study determining the influence of wastewater discharges from a sewage lagoon on biomarkers of exposure in freshwater mussels, using HNE as an indicator of lipid peroxidation. Levels of HNE in mussels from this study ranged from 12.41 ųg·mL^{-1} to 22.11 ųg·mL^{-1}. Elevated levels of lipid peroxidation have also been identified in other studies looking at the effects of contamination on freshwater mussels (Gagné *et al.*, 2011; Gillis, *et al.*, 2014b; Machado *et al.*, 2014). Given that these studies show much higher levels of lipid peroxidation than in this present study, it can be assumed that water quality is not impacting the stress levels of *L. siliquoidea* and *P. grandis* in Constance Lake, Fushimi Lake, or McKay Lake.

3.5 Conclusion

By coupling both males and females of each species, *L. siliquoidea* and *P. grandis* together in the analysis, and by analyzing each species separately, it is evident that the energy levels and stress levels vary with the time of year. A species may have less energy throughout the winter, dependent on sex and if the female is brooding. A species may be stressed due to having to burrow into the sediment to avoid being frozen during the winter months and having to close their valves for that duration in order to stay burrowed. To get a better understanding of this phenomenon, it is suggested that the study be redone with only males to control for the confounding factor of reproductive condition. Given that *L. siliquoidea* is sexually dimorphic, it is recommended that this species be used

solely for further study as only visual observation is needed to determine sex counts compared to sacrificing specimens to find the right number of males. Even though *L. siliquoidea* is a tolerant species to change in its environment, this species is abundant and has been used in several studies as an indicator species (Tuttle-Raycraft *et al.*, 2017; Gene *et al.*, 2019; Wollman, 2019).

Researchers are beginning to look at cold water tolerances during overwinter studies to begin to understand the basic biology of freshwater mussels and overwintering strategies. These studies tend to look at behaviour and metabolism and have yet to dive into how these behaviors affect energy and stress levels. While not fully addressing the correlation between patterns of behaviour and the latter metrics, this study has shown preliminary findings on overwintering strategies of freshwater mussels regarding energy levels and stress.

Chapter 4: General Discussion and Conclusion

Water is, and always has been at the heart of Indigenous land use and stewardship efforts (Four Rivers, 2019). Josephine Mandamin, Water Walker, has stated that "That's our responsibility, our role, and our duty, to pass on the knowledge and understanding of water, to all people, not just Anishinabe, but people of all colours". Indigenous knowledge is knowledge that has been collected and developed orally over several generations based on learned experiences of the world around us. While demonstrating value, some people are still under the belief that western science is a higher standard than Indigenous Knowledge since the former is objective and quantifiable, whereas the latter in anecdotal and imprecise. Several scholars (Mistry & Berardi, 2016; Hopkins *et al.*, 2019; Abu *et al.*, 2020; Reid *et al.*, 2021) have seen the benefit of what both ways of knowing can bring to the table, and have started to adopt a "Two-Eyed Seeing" approach based on the "Two Row Wampum" methodology. This methodology is based on a historical peace and friendship treaty which depicts Indigenous peoples travelling in a canoe alongside settlers travelling in a ship in common waters. They are respecting each other and travelling the river of life together with non-interference (Latulippe, 2015). This methodology builds the basis for new relationships between western science and Indigenous Knowledge. One such example of this is the study conducted by Hopkins *et al.* (2019) adopting the "Two-Eyed Seeing" approach and working with the McMurray Métis to understand freshwater mussel health in the Lower Athabasca River. The researchers aimed to facilitate partnerships and create safe, ethical spaces across diverse knowledge systems to address questions posed by the McMurray Métis regarding freshwater mussel health in a locally relevant and culturally appropriate way.

This thesis has also adopted the "Two-Eyed Seeing" approach and has acknowledged the knowledge coexistence and complementarity of Indigenous Knowledge and western science. Through various community gatherings and events, Four Rivers (2023) has been provided with general comments of what community members consider to be indicators of water quality. These indicators are based on Indigenous knowledge and consist of smell ("clean", or no smell), taste ("clean", no gases or metallic taste), and colour of water (tea coloured or blue). Community members have also stated that excessive algae growth is indicative of poor water quality. While the western analyses of water quality conducted in this study show that there is no contamination in Constance Lake from heavy metals or leachate, there is still the concern regarding high nutrient inputs and the potential for future cyanobacterial blooms to happen, as there are high levels of P in Constance Lake which is known to cause eutrophication and is consistent with local community observations.

Responses of freshwater mussels to nutrient inputs has long been studied (Pip, 2006; Gillis, 2012; Gillis *et al.*, 2014b; Strayer, 2014). For the species of mussels in this study, it appears that the glycogen and stress response levels in all lakes are following natural life-cycle variations (breeding cycles, food availability) and not showing a stress response to outside stressors such as heavy metal, leachate contamination, or eutrophication. These species may be tolerant and resilient to stressors (Pip, 2006) and may not show any stress response to P, N, and chlorophyll α outside of normal life cycle patterns. Several studies tend to focus efforts on effects of wastewater (Gagne *et al.*, 2001; Falfushynska *et al.*, 2014; Gillis *et al.*, 2014a; Gillis, *et al.*, 2014b; Gillis *et al.*, 2017; Machado *et al.*, 2014) and heavy metals (Giacomin *et al.*, 2013; Markich, 2017b;

Khan *et al.*, 2019; Wang *et al.*, 2020), specific toxins such as endocrine disruptors (Gagne & Blaisé, 2003; Gagné *et al.*, 2004b; Gagné *et al.*, 2010), and thermal stress (Falfushynska *et al.*, 2016; Payton *et al.*, 2016; Fluharty *et al.*, 2023; Fogelman *et al.*, 2023), however there is limited information on the overwintering strategy of freshwater mussels. The information that is currently available refers to survivorship, behaviour, and oxygen consumption (Cvetanovska *et al.*, 2021; Lurman *et al.*, 2014; Thyrring *et al.*, 2019; Xia *et al.*, 2021). This study provides a preliminary insight into glycogen levels (energy stores) over the winter season. Additional works for *L. siliquoidea*, *P. grandis*, and other species should include more sampling closer to ice-out and ice-in conditions on lakes where *P. grandis* and *L. siliquoidea* are present to better determine glycogen levels, as well as sampling during winter when ice is present to obtain mid-season data. This information is valuable as it provides additional insights into the basic biology of the species and can complement research that's already being undertaken on overwintering strategies.

Overall, Constance Lake First Nation now has additional information to assist in management decisions and deciding on future monitoring of waters that are of cultural importance to them.

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Appendix 1 – R Code

1.1 Water Quality

The purpose of this analysis is to compare the monthly (August & October 2020, March & June 2021) chlorophyll α concentrations in each of the lakes Constance, Fushimi, & McKay).

```
rm(list=ls())

library (car)

library (nortest)


clpa.dat <- read.table("lake_clpa.csv", fill=TRUE, header=TRUE, quote="", sep=",", encoding="UTF-8")

clpa.dat

clpa.dat$month<-factor(clpa.dat$month, labels=c("August", "June", "March", "October"))


attach(clpa.dat)

n <- tapply(clpa.dat$clpa_ugL, clpa.dat[,-3], length)

n


op<-par(no.readonly = TRUE)

par(mar=c(4,4,1,1), lwd=2, font.axis=2, font.lab=2)

par(op)

boxplot(clpa_ugL ~ lake + month, xlab = 'Month', ylab = 'Chlorophyll a (ug/L)', cex.axis=0.75)

xbar<-tapply(clpa_ugL, clpa.dat[,1:2], mean)

s <- tapply(clpa_ugL, clpa.dat[,1:2], sd)

sem <- s/sqrt(n)


op<-par(no.readonly = TRUE)
```

```
par(mar=c(4,4,1,1), lwd=1, font.axis=2, font.lab=2)

with(clpa.dat,
    interaction.plot(month, lake, clpa_ugL, log="y",
                type = "b", lwd=1, pch = c(21,22), col = c(1:10),
                xlab = "Lake", bty="l", las=1,
                ylab= "Chlorophyll a (ug/L)",
                ylim = range(clpa.dat$clpa_ugL)))

par(op)

anova1.an<-aov(clpa_ugL~month + lake + month*lake)
summary(anova1.an)

op<-par(no.readonly = TRUE)
par(mfrow=c(2,2))
plot(anova1.an)
par(op)

anova2.an<-aov(log10(clpa_ugL)~month*lake)
summary(anova2.an)

op<-par(no.readonly = TRUE)
par(mfrow=c(2,2))
plot(anova2.an)
par(op)

leveneTest(anova2.an)
```

```
ad.test(resid(anova2.an))


op<-par(no.readonly = TRUE)
par(mar=c(6,6,3,1), lwd=1, font.axis=1, font.lab=1)


with(clpa.dat,
    interaction.plot(month, lake, clpa_ugL,
                log="y", type = "b", lwd=1, pch = c(21,22), col = c(1:10),
                xlab = "Month", bty="l", las=1,
                ylab= "Chlorophyll a (ug/L)",
                trace.label= "Lake",
                ylim = range(clpa.dat$clpa_ugL)))


par(op)


TukeyHSD(anova2.an, "month")
TukeyHSD(anova2.an, "lake")


library (car)
library (nortest)


clpa.dat <- read.table("lake_clpa.csv", fill=TRUE, header=TRUE, quote="", sep=",",
encoding="UTF-8")
clpa.dat
clpa.dat$month<-factor(clpa.dat$month, labels=c("August", "June", "March",
"October"))


n <- tapply(clpa.dat$clpa_ugL, clpa.dat[,-3], length)
n
```

```
op<-par(no.readonly = TRUE)
par(mar=c(4,4,1,1), lwd=2, font.axis=2, font.lab=2)


par(op)
boxplot(clpa_ugL ~ lake + month, xlab = 'Month', ylab = 'Chlorophyll a (ug/L)',
cex.axis=0.75)
par(op)


xbar<-tapply(clpa_ugL, clpa.dat[,1:2], mean)
s <- tapply(clpa_ugL, clpa.dat[,1:2], sd)
sem <- s/sqrt(n) #n is estimated above


op<-par(no.readonly = TRUE)
par(mar=c(4,4,1,1), lwd=1, font.axis=2, font.lab=2)


with(clpa.dat,
    interaction.plot(month, lake, clpa_ugL, log="y",
                type = "b", lwd=1, pch = c(21,22), col = c(1:10),
                main = "Chlorophyll a Interactions",
                xlab = "Month", bty="l", las=1,
                ylab= "Chlorophyll a (ug/L)",
                trace.label= "Lake",
                ylim = c(0.3,15)))
arrows(1:4, xbar[1,]+sem[1,], 1:4,xbar[1,]-sem[1,], col = "black", angle=90, code=3,
length=.05)
arrows(1:4, xbar[2,]+sem[2,], 1:4,xbar[2,]-sem[2,], col = "red", angle=90, code=3,
length=.05)
arrows(1:4, xbar[3,]+sem[3,], 1:4,xbar[3,]-sem[3,], col = "green", angle=90, code=3,
length=.05)
```

```
clpa1.an<-aov(clpa.dat$clpa_ugL~clpa.dat$lake + clpa.dat$month +
clpa.dat$lake*clpa.dat$month)

summary(clpa1.an)


leveneTest(clpa1.an)

ad.test(resid(clpa1.an))

bartlett.test(resid(clpa1.an), clpa.dat$lake)


op<-par(no.readonly = TRUE)

par(mfrow=c(2,2))

plot(clpa1.an)

par(op)


attach(clpa.dat)

clpa.lm<-lm(clpa_ugL~lake*month, contrasts=list(lake=contr.sum, month=contr.sum))

anova(clpa.lm)


Anova(clpa.lm, type = 'III')
```

1.2 Glycogen

The purpose of this analysis is to compare the monthly (October 2020 & June 2021) glycogen concentrations in each of the lakes Constance, Fushimi, & McKay.

```
rm(list=ls())

library(carData)

library (nortest)

library (lattice)

library (car
```

```
glyls.dat<- read.table("230826_lampsilis_glycogen.csv", header = TRUE, sep=",")
glyls.dat



glyls.dat$month<-as.factor(glyls.dat$month)
glyls.dat$lake<-as.factor(glyls.dat$lake)
is.factor(glyls.dat$month)

attach(glyls.dat)
n <- tapply(glyls.dat$month, glyls.dat[,c(1,2)], length)
n

options(graphics.record = TRUE)
op<-par(no.readonly = TRUE)
par(mar=c(4,4,4,1))
boxplot(log10(conc +1) ~ month*lake,
        xaxt = "n",
        xlab = "Season and Lake",
        ylab = "Glycogen Concentration (ug/mL)",
        yaxt = "n",
        main ="Lampsilis siliquoidea Mean Glycogen Concentrations",
        font.main = 4)
axis(side = 1,
     at = 1:6,
     labels = c("C - Oct 2020",
                "C - Jun 2021",
                "F - Oct 2020",
```

```
            "F - Jun 2021",
            "M - Oct 2020",
            "M - Jun 2021"))
axis(side = 2, las = 2, mgp = c(3, 0.75, 0))

dotplot(conc ~ month|lake)
par(op)

glyls.aov<-aov(log10(conc + 1) ~ month*lake)
anov1<-(summary(glyls.aov))
anov1
glyls2.lm<-lm(log10(conc + 1)~month*lake)
summary(glyls2.lm)
anova(glyls2.lm)

glyls2.lm$resid
ad.test(glyls2.lm$resid)
leveneTest(glyls2.lm)

Tukeyls<-TukeyHSD(glyls.aov)
Tukeyls
plot(Tukeyls, las=1) #this is one way to plot it

xbar<-tapply(conc, glyls.dat[,1:2], mean)
s <- tapply(conc, glyls.dat[,1:2], sd)
sem <- s/sqrt(n)

op<-par(no.readonly = TRUE)
```

```
par(mar=c(4,4,1,1), lwd=1, font.axis=2, font.lab=2)
glyls.dat$month<-factor(glyls.dat$month, labels=c("October 2020", "June 2021"))
with(glyls.dat,
    interaction.plot(month, lake, conc, log="y",
                type = "b", lwd=2, pch = c(21,22), col = c(1:10),
                main = "Mean Glycogen Concentrations",
                xlab = "Month", bty="l", las=1,
                ylab= "Glycogen Concentration (ug/mL)",
                trace.label= "Lake",
                ylim = c(0.01,0.1)))
box(col = "black")
arrows(1:4, xbar[1,]+sem[1,], 1:4,xbar[1,]-sem[1,], col = "black", angle=90, code=3,
length=.05)
arrows(1:4, xbar[2,]+sem[2,], 1:4,xbar[2,]-sem[2,], col = "red", angle=90, code=3,
length=.05)
arrows(1:4, xbar[3,]+sem[3,], 1:4,xbar[3,]-sem[3,], col = "green", angle=90, code=3,
length=.05)


with(glyls.dat,
    interaction.plot(lake, month, conc, log="y",
                type = "b", lwd=2, pch = c(21,22), col = c(1:10),
                main = "Mean Glycogen Concentrations",
                xlab = "Month", bty="l", las=1,
                ylab= "Glycogen Concentration (ug/mL)",
                trace.label= "Lake",
                ylim = c(0.01,0.1)))
```

1.3 HNE

The purpose of this analysis is to compare the monthly (October 2020 & June 2021) HNE concentrations in each of the lakes Constance, Fushimi, & McKay.

```
rm(list=ls())
library(carData)
library (nortest)
library (lattice)
library (car)

hnepg.dat<- read.table("230826_pyganodon_hne.csv", header = TRUE, sep=",")
hnepg.dat

hnepg.dat$month<-as.factor(hnepg.dat$month)
hnepg.dat$lake<-as.factor(hnepg.dat$lake)
is.factor(hnepg.dat$month)

attach(hnepg.dat)
n <- tapply(hnepg.dat$month, hnepg.dat[,c(1,2)], length)
n

options(graphics.record = TRUE)
op<-par(no.readonly = TRUE)
par(mar=c(4,4,4,1))
boxplot(log10(conc +1) ~ month*lake,
      xaxt = "n",
      xlab = "Season and Lake",
      ylab = "HNE Protein Concentration (ug/mL)",
      yaxt = "n",
```

```
      main ="Pyganodon grandis Mean HNE Concentrations",
      font.main = 4)
axis(side = 1,
    at = 1:6,
    labels = c("C - Oct 2020",
               "C - Jun 2021",
               "F - Oct 2020",
               "F - Jun 2021",
               "M - Oct 2020",
               "M - Jun 2021"))
axis(side = 2, las = 2, mgp = c(3, 0.75, 0))

dotplot(conc ~ month|lake)
par(op)

library(agricolae)

hnepg.aov<-aov(log10(conc + 1) ~ month*lake)
anov1<-(summary(hnepg.aov))
anov1
hnepg2.lm<-lm(log10(conc + 1)~month*lake)
summary(hnepg2.lm)
anova(hnepg2.lm)
hnepg.aov$resid
ad.test(hnepg.aov$resid)
leveneTest(hnepg.aov)

Tukeyls<-TukeyHSD(hnepg.aov)
```

```
Tukeyls
plot(Tukeyls, las=1) #this is one way to plot it


hnepg.dat$month<-factor(hnepg.dat$month, labels=c("October 2020", "June 2021"))
with(hnepg.dat,
    interaction.plot(month, lake, conc, log="y",
                type = "b", lwd=2, pch = c(21,22), col = c(1:10),
                main = "Mean Glycogen Concentrations",
                xlab = "Month", bty="l", las=1,
                ylab= "Glycogen Concentration (ug/mL)",
                trace.label= "Lake",
                ylim = c(0.1,21)))
box(col = "black")
arrows(1:4, xbar[1,]+sem[1,], 1:4,xbar[1,]-sem[1,], col = "black", angle=90, code=3,
length=.05)
arrows(1:4, xbar[2,]+sem[2,], 1:4,xbar[2,]-sem[2,], col = "red", angle=90, code=3,
length=.05)
arrows(1:4, xbar[3,]+sem[3,], 1:4,xbar[3,]-sem[3,], col = "green", angle=90, code=3,
length=.05)


with(hnepg.dat,
    interaction.plot(lake, month, conc, log="y",
                type = "b", lwd=2, pch = c(21,22), col = c(1:10),
                main = "Mean Glycogen Concentrations",
                xlab = "Month", bty="l", las=1,
                ylab= "Glycogen Concentration (ug/mL)",
                trace.label= "Lake",
                ylim = c(0.1,21)))
```

Appendix 2 – Phosphate Buffered Saline

To make 1000 mL of phosphate buffered saline (pH 7.4) I utilized the recipe as found at https://www.aatbio.com/resources/buffer-preparations-and-recipes/pbs-phosphate-buffered-saline . Required components were on hand from Lakehead University Chemistry Store or Fisher Scientific. The following table are exact quantities of components used:

Component	Required Amount	Amount Used
Distilled water	800 mL	800 mL
Sodium chloride	8 g	8.0002 g
Potassium chloride	0.2 g	0.2002 g
Sodium phosphate dibasic	1.44 g	1.4400 g
Potassium phosphate monobasic	0.245 g	0.2443 g

I used a magnetic stirrer to thoroughly mix all components. Sodium hydroxide tablets were used to adjust the pH of the solution to ~7.4, and then the solution was bulked to 1000 mL. An Oakton PCTSTestr^{TM}50 was used to measure pH. For quality control, the PCTSTestr was tested using pH 6.86 buffer solution @ 25°C. The unit read a pH of 6.75 at 22.7°C.

Appendix 3 – OxiSelect HNE Adduct ELISA Protocol

Product Manual

OxiSelect™ HNE Adduct Competitive ELISA Kit

Catalog Number

STA-838	96 assays
STA-838-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Lipid peroxidation is a well-defined mechanism of cellular damage in animals and plants. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural bi- products of lipid peroxidation. Oxidative modification of lipids can be induced *in vitro* by a wide array of pro-oxidant agents and occurs *in vivo* during aging and in certain disease conditions. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage.
These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress.

Both MDA and HNE have been shown to be capable of binding to proteins and forming stable adducts, also termed advanced lipid peroxidation end products. These modifications of proteins by MDA or HNE can cause both structural and functional changes of oxidized proteins. Specifically, 4-HNE can react with lysine, histidine or cysteine residues in protein to form adducts.

Cell Biolabs' OxiSelect™ HNE Adduct Competitive ELISA Kit is an enzyme immunoassay developed for rapid detection and quantitation of HNE protein adducts. The quantity of HNE adduct in protein samples is determined by comparing its absorbance with that of a known HNE-BSA standard curve.
Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown protein samples.

Assay Principle

First, an HNE conjugate is coated on an ELISA plate. The unknown HNE protein samples or HNE- BSA standards are then added to the HNE conjugate preabsorbed ELISA plate. After a brief incubation, an anti-HNE polyclonal antibody is added, followed by an HRP conjugated secondary antibody. The content of HNE protein adducts in unknown samples is determined by comparison with a predetermined HNE-BSA standard curve.

Related Products

1. STA-305: OxiSelect™ Nitrotyrosine ELISA Kit
2. STA-310: OxiSelect™ Protein Carbonyl ELISA Kit
3. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
4. STA-811: OxiSelect™ Methylglyoxal (MG) Competitive ELISA Kit
5. STA-813: OxiSelect™ N^{ε}-(carboxyethyl) lysine (CEL) Competitive ELISA Kit
6. STA-816: OxiSelect™ N^{ε}-(carboxymethyl) lysine (CML) Competitive ELISA Kit

7. STA-817: OxiSelect™ Advanced Glycation End Products (AGE) Competitive ELISA Kit
8. STA-832: OxiSelect™ MDA Adduct Competitive ELISA Kit

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Protein Binding Plate (Part No. 231001): One strip well 96-well plate (8 x 12).
2. Anti-HNE Antibody (1000X) (Part No. 283801): One 10 µL vial of anti-HNE antibody.
3. Secondary Antibody, HRP Conjugate (1000X) (Part No. 231704): One 20 µL vial.
4. Assay Diluent (Part No. 310804): One 50 mL bottle.
5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
7. Stop Solution (Part. No. 310808): One 12 mL bottle.

Box 2 (shipped on blue ice packs)

1. HNE-BSA Standard (Part No. 283803): One 250 µL vial of 1 mg/mL HNE-BSA in PBS.
2. HNE Conjugate (Part No. 283802): One 50 µL vial of HNE conjugate at 1.0 mg/mL in PBS.
3. 100X Conjugate Diluent (Part No. 281603): One 300 µL vial.

Materials Not Supplied

1. Protein samples such as purified protein, plasma, serum, cell lysate
2. 1X PBS
3. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
4. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
5. Multichannel micropipette reservoir
6. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Upon receipt, aliquot and store the Anti-HNE Antibody, HNE-BSA Standard, HNE Conjugate and 100X Conjugate Diluent at -20ºC to avoid multiple freeze/thaw cycles. Store all other kit components at 4ºC.

Preparation of Reagents

- HNE Conjugate Coated Plate:

  Note: The HNE Conjugate coated wells are not stable and should be used within 24 hrs after coating. Only coat the number of wells to be used immediately.

  1. Immediately before use, prepare 1X Conjugate Diluent by diluting the 100X Conjugate Diluent in 1X PBS. Example: Add 50 µL to 4.95 mL of 1X PBS.
  2. Immediately before use, prepare 10 µg/mL of HNE Conjugate by diluting the 1.0 mg/mL HNE Conjugate in 1X PBS. Example: Add 25 µL to 2.475 mL of 1X PBS.
  3. Mix the 10 µg/mL of HNE Conjugate and 1X Conjugate Diluent at 1:1 ratio and add 100 µL of the mixture to each well and incubate overnight at 4ºC. Remove the HNE Conjugate coating solution and wash twice with 1X PBS. Blot plate on paper towels to remove excess fluid. Add 200 µL of Assay Diluent to each well and block for 1 hr at room temperature. Transfer the plate to 4ºC and remove the Assay Diluent **immediately before use**.

- 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.
- Anti-HNE Antibody and Secondary Antibody: Immediately before use, dilute the Anti-HNE antibody 1:1000 and Secondary Antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve

Prepare a dilution series of HNE-BSA standards in the concentration range of 0 to 200 µg/mL by diluting the HNE-BSA Standard in Assay Diluent (Table 1).

Standard Tubes	1 mg/mL HNE-BSA Standard (µL)	Assay Diluent (µL)	HNE-BSA (µg/mL)
1	80	320	200
2	200 of Tube #1	200	100
3	200 of Tube #2	200	50
4	200 of Tube #3	200	25
5	200 of Tube #4	200	12.5
6	200 of Tube #5	200	6.25
7	200 of Tube #6	200	3.13
8	200 of Tube #7	200	1.56
9	0	200	0

Table 1. Preparation of HNE-BSA Standards

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each HNE sample including

unknown and standard should be assayed in duplicate.

2. Add 50 µL of unknown sample or HNE-BSA standard to the wells of the HNE Conjugate coated plate. If needed, unknown samples may be diluted in 1X PBS containing 0.1% BSA before adding. Incubate at room temperature for 10 minutes on an orbital shaker.
3. Add 50 µL of the diluted anti-HNE antibody to each well, incubate at room temperature for 1 hour on an orbital shaker.
4. Wash 3 times with 250 µL of 1X Wash Buffer with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
5. Add 100 µL of the diluted Secondary Antibody-HRP Conjugate to all wells and incubate for 1 hour at room temperature on an orbital shaker. Wash the strip wells 3 times according to step 4 above.
6. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well. Incubate at room temperature for 2-30 minutes on an orbital shaker.

   Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
7. Stop the enzyme reaction by adding 100 µL of Stop Solution to each well. Results should be read immediately (color will fade over time).
8. Read absorbance of each well on a microplate reader using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical HNE Adduct Competitive ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.

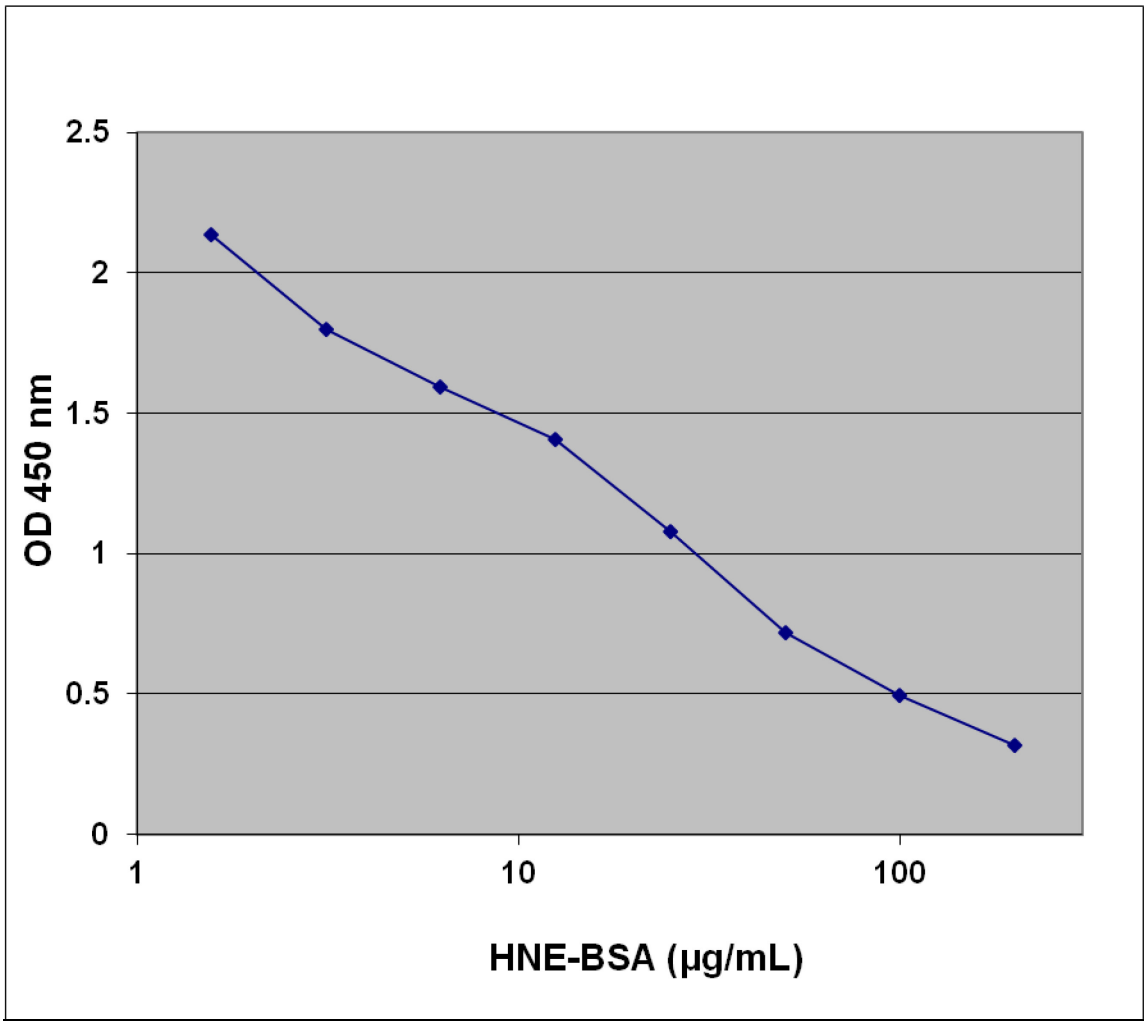

Figure 1: HNE-BSA Competitive ELISA Standard Curve.

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Appendix 4 – Microplate Reader Protocols

4.1 Shake Protocol

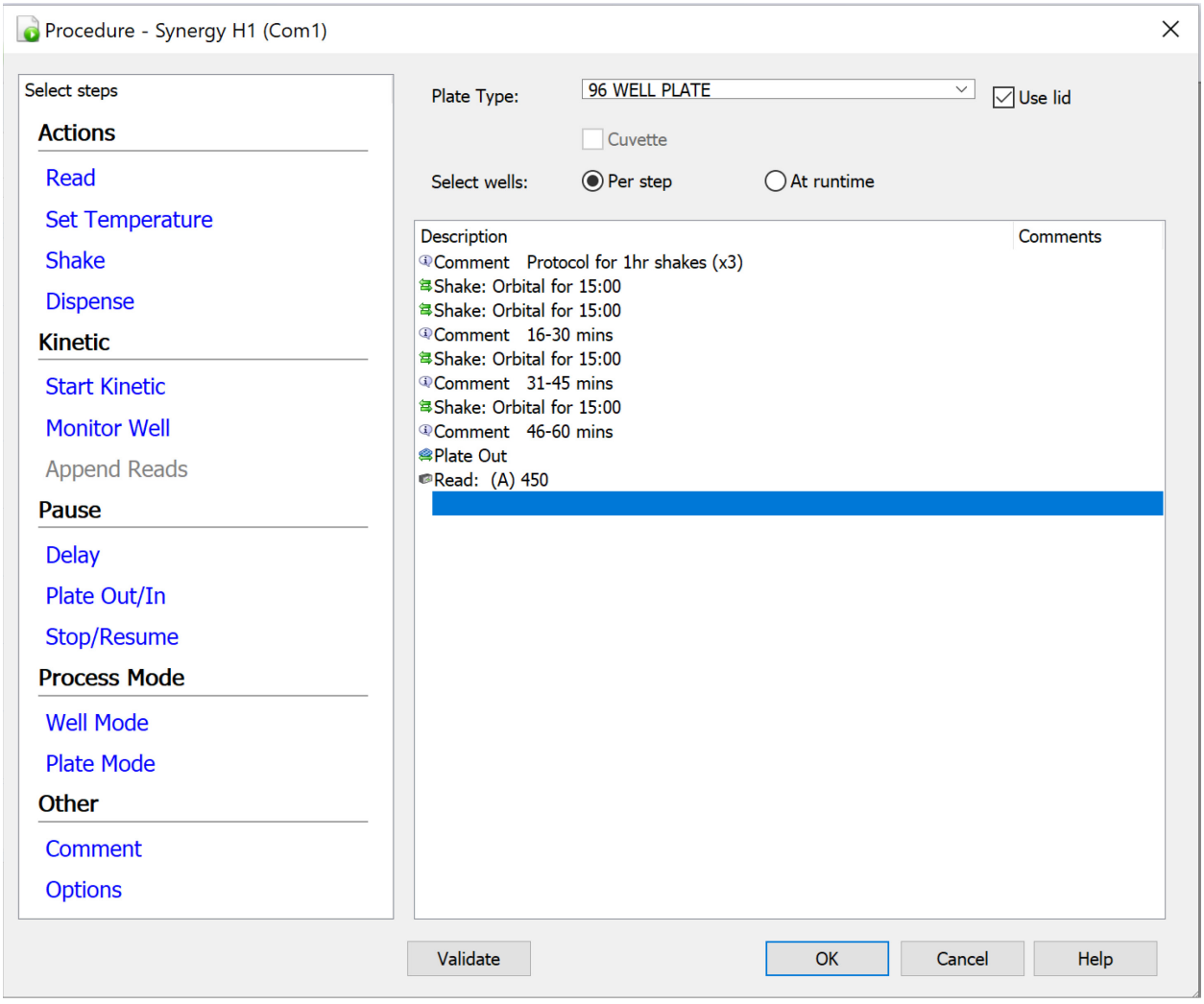

Figure 14. One-hour Shake protocol for the BioTek Gen5 microplate reader. Maximum 15 m cycles allowed from plate reader settings, therefore comments added so researcher knows elapsed time and time remaining.

4.2 Colour Change Protocol

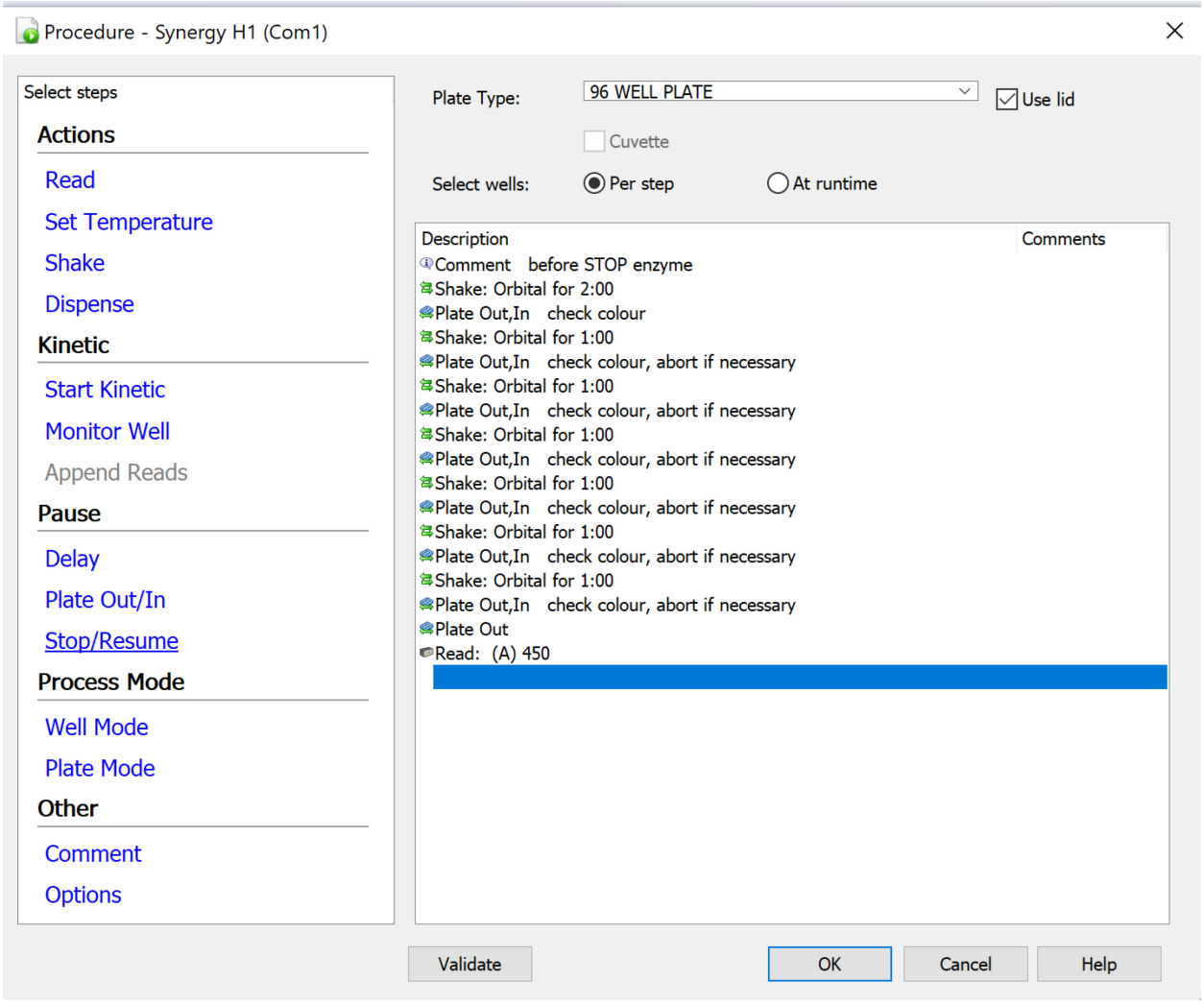

Figure 15. Colour shake protocol for the BioTek Gen5 microplate reader. Separate 1 m cycles added to allow for immediate observation of colour change within the wells.

Appendix 5 – Raw Data

5.1 Mussel Samples

Table 7. Total number of each species of freshwater mussel collected from each lake, Constance, McKay, and Fushimi, during each of the three sampling events in August and October 2020, and June 2021.

	Lake					
	Constance Lake		McKay Lake		Fushimi Lake	
Month	*Pyganodon grandis*	*Lampsilis siliquoidea*	*P. grandis*	*L. siliquoidea*	*P. grandis*	*L. siliquoidea*
Aug-20	--	2	8	--	5	5
Oct-20	12	20	17	--	3	16
Jun-21	8	27	17	4	5	35

Table 8. Ages for each *L. siliquoidea.*

Constance Lake			
Sample ID	Age	Sample ID	Age
2008CL01	13	2106CL03	12
2008CL02	17	2106CL04	15
2010CL01	19	2106CL06	18
2010CL02	22	2106CL07	13
2010CL03	15	2106CL08	13
2010CL04	15	2106CL09	13
2010CL05	22	2106CL10	6
2010CL06	8	2106CL11	9
2010CL07	12	2106CL20	25
2010CL15	15	2106CL22	16
2010CL16	22	2106CL23	8
2010CL17	10	2106CL24	13
2010CL18	15	2106CL25	9
2010CL19	14	2106CL26	10
2010CL20	12	2106CL27	11
2010CL21	12	2106CL28	22
2010CL22	11	2106CL29	10
2010CL23	14	2106CL30	11
2010CL24	8	2106CL31	13
2010CL25	9	2106CL32	13
2010CL31	21	2106CL33	6
2010CL32	13	2106CL34	6
2106CL01	28	2106CL35	8
2106CL02	21		

Fushimi Lake			
Sample ID	Age	Sample ID	Age
2008FL03	9	2106FL10	31
2008FL04	11	2106FL11	13
2008FL05	6	2106FL12	12
2008FL09	4	2106FL13	8
2008FL10	10	2106FL15	18
2010FL01	8	2106FL16	17
2010FL02	10	2106FL18	11
2010FL03	8	2106FL19*	11
2010FL05	11	2106FL20	27
2010FL08	5	2106FL22	8
2010FL09	8	2106FL23	7
2010FL10	6	2106FL24	8
2010FL11	9	2106FL25	9
2010FL12	6	2106FL26	5
2010FL13	5	2106FL27	4
2010FL14	2	2106FL28	5
2010FL15	4	2106FL29	5
2010FL16	4	2106FL30	12
2010FL17	6	2106FL31	8
2010FL18	6	2106FL32	7
2010FL19	7	2106FL33	15
2106FL01	9	2106FL34	11
2106FL02	6	2106FL35	6
2106FL04	10	2106FL36	9

2106FL06	14	2106FL37	7
2106FL07	17	2106FL38	8
2106FL08	12	2106FL39	11
2106FL09	17	2106FL40	8

McKay Lake	
Sample ID	Age
2106ML08	10
2106ML09	13
2106ML11	22

Average: 11.5
Max: 31
Min: 2

5.2 Glycogen Absorbance and Concentration Calculations

Table 9. Tabulation of calculations for glycogen concentrations for the first 96 samples based on absorbance levels. Absorbance levels are found in Rows A – H and columns 1 – 12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.289	0.941	0.289	0.428	0.412	0.208	0.569	0.258	0.266	0.210	0.493	0.844
B	0.434	0.358	0.332	0.370	0.588	1.022	0.508	1.081	0.387	0.595	0.526	0.433
C	0.575	0.609	0.514	0.570	0.276	0.517	0.458	1.453	1.548	1.261	0.955	0.254
D	0.499	0.409	0.769	0.359	0.458	0.446	0.278	0.385	0.467	0.370	0.465	0.408
E	0.811	0.581	0.962	0.266	0.450	0.722	0.323	0.252	0.539	0.425	0.459	0.734
F	0.303	0.299	0.330	0.560	1.001	0.423	0.894	0.384	0.241	0.292	0.328	0.275
G	0.359	0.229	0.190	0.359	0.222	0.297	0.282	0.352	0.356	0.295	0.274	0.215
H	0.233	0.418	0.202	0.888	0.595	0.299	0.857	0.241	0.504	0.307	0.298	0.321

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass	Conc (ug/g ww)	Stdev	CV%	Y Coeff	1.2422
Std1	S1	2.998	5	0.69897	N/A	N/A			X Coeff	1.5804
Std2	S2	1.608	2.5	0.39794001	N/A	N/A				
Std3	S3	0.894	1.25	0.09691001	N/A	N/A				
Std4	S4	0.551	0.625	-0.20412	N/A	N/A				
Std5	S5	0.484	0.313	-0.5044557	N/A	N/A				
Std6	S6	0.41	0.156	-0.8068754	N/A	N/A				
Std7	S7									
Std8	S8									
Std9	S9									

Sample	Well Code	Abs	Conc (ug/mL)	Log Conc	Mass (g)	Conc (ug/g ww)	abs Stdev	abs CV%	Conc (ug glycogen)
2008CL01		0.289	0.249379962	-0.6031384	0.0097	25.70927441			0.012469
2010CL01		0.941	0.644785612	-0.1905847	0.0090	71.64284578			0.032239

2010CL02		0.289	0.249379962	-0.6031384	0.0091	27.4043914	0.012469
2010CL03		0.428	0.305361283	-0.515186	0.0087	35.098998	0.015268
2010CL04		0.412	0.298325208	-0.52531	0.0088	33.9005918	0.014916
2010CL05		0.208	0.221619876	-0.6543913	0.0085	26.07292653	0.011081
2010CL06		0.569	0.375000538	-0.4259681	0.0084	44.64292118	0.01875
2010CL07		0.258	0.238367075	-0.6227537	0.0078	30.55988137	0.011918
2010CL08		0.266	0.241161667	-0.6176917	0.0082	29.40995933	0.012058
2010CL09		0.210	0.222266601	-0.6531258	0.0093	23.89963457	0.011113
2010CL10		0.493	0.335693397	-0.4740572	0.0090	37.29926637	0.016785
2010CL11		0.844	0.559807189	-0.2519615	0.0090	62.20079877	0.02799
2010CL12		0.434	0.308042386	-0.5113895	0.0092	33.48286802	0.015402
2010CL13		0.358	0.275753724	-0.5594786	0.0084	32.82782434	0.013788
2010CL14		0.332	0.265503259	-0.5759301	0.0093	28.54873749	0.013275
2010CL15		0.370	0.280617274	-0.5518856	0.0090	31.17969711	0.014031
2010CL16		0.588	0.385526436	-0.4139458	0.0082	47.01541903	0.019276
2010CL17		1.022	0.725551401	-0.1393318	0.0090	80.61682238	0.036278
2010CL18		0.508	0.343110546	-0.4645659	0.0091	37.70445564	0.017156
2010CL19		1.081	0.79067955	-0.1019995	0.0096	82.36245308	0.039534
2010CL20		0.387	0.2876545	-0.5411288	0.0095	30.27942101	0.014383
2010CL21		0.595	0.38947844	-0.4095166	0.0085	45.82099295	0.019474
2010CL22		0.526	0.352227767	-0.4531764	0.0087	40.48595018	0.017611
2010CL23		0.433	0.307593906	-0.5120223	0.0085	36.18751836	0.01538
2010CL24		0.575	0.378293081	-0.4221716	0.0090	42.03256453	0.018915
2010CL25		0.609	0.397504399	-0.4006581	0.0096	41.40670819	0.019875
2010CL26		0.514	0.346123092	-0.4607694	0.0089	38.89023504	0.017306
2010CL27		0.570	0.375547298	-0.4253354	0.0089	42.19632564	0.018777
2010CL28		0.276	0.244701025	-0.6113642	0.0082	29.84158841	0.012235
2010CL29		0.517	0.347639269	-0.4588712	0.0089	39.06059203	0.017382

2010CL30		0.458	0.319004278	-0.4962035	0.0080	39.87553475	0.01595
2010CL31		1.453	1.359514843	0.13338395	0.0080	169.9393553	0.067976
2010CL32		1.548	1.561332204	0.19349532	0.0086	181.5502563	0.078067
2106CL01		1.261	1.027769493	0.01189572	0.0084	122.3535111	0.051388
2106CL02		0.955	0.65807267	-0.1817261	0.0091	72.31567801	0.032904
2106CL03		0.254	0.236981946	-0.6252847	0.0087	27.23930417	0.011849
2106CL04		0.499	0.33864082	-0.4702607	0.0090	37.62675773	0.016932
2106CL06		0.409	0.297024107	-0.5272083	0.0084	35.36001269	0.014851
2106CL07		0.769	0.501859479	-0.2994179	0.0094	53.38930624	0.025093
2106CL08		0.359	0.27615578	-0.5588459	0.0085	32.48891534	0.013808
2106CL09		0.458	0.319004278	-0.4962035	0.0090	35.44491978	0.01595
2106CL10		0.446	0.31347542	-0.5037965	0.0089	35.22195729	0.015674
2106CL11		0.278	0.245415106	-0.6100987	0.0088	27.8880802	0.012271
2106CL12		0.385	0.286817515	-0.5423943	0.0093	30.84059306	0.014341
2106CL13		0.467	0.323214822	-0.4905087	0.0096	33.66821059	0.016161
2106CL14		0.370	0.280617274	-0.5518856	0.0089	31.53003078	0.014031
2106CL15		0.465	0.322274368	-0.4917742	0.0088	36.62208729	0.016114
2106CL16		0.408	0.296591668	-0.5278411	0.0090	32.95462983	0.01483
2106CL17		0.811	0.533528571	-0.2728423	0.0083	64.28055077	0.026676
2106CL18		0.581	0.381614533	-0.4183751	0.0081	47.11290526	0.019081
2106CL19		0.962	0.664818526	-0.1772969	0.0077	86.34006832	0.033241
2106CL20		0.266	0.241161667	-0.6176917	0.0092	26.21322462	0.012058
2106CL21		0.450	0.315307643	-0.5012655	0.0082	38.45215154	0.015765
2106CL22		0.722	0.468643745	-0.3291572	0.0089	52.65660053	0.023432
2106CL23		0.323	0.262044528	-0.5816249	0.0098	26.73923753	0.013102
2106CL24		0.252	0.236292403	-0.6265502	0.0092	25.68395689	0.011815
2106CL25		0.539	0.358962726	-0.4449506	0.0091	39.4464534	0.017948
2106CL26		0.425	0.304029495	-0.5170843	0.0091	33.40983457	0.015201
2106CL27		0.459	0.319469394	-0.4955707	0.0091	35.10652685	0.015973

2106CL28	0.734	0.476909352	-0.3215642	0.0093	51.2805755	0.023845
2106CL29	0.303	0.254518919	-0.5942799	0.0094	27.0764808	0.012726
2106CL30	0.299	0.253039934	-0.5968109	0.0089	28.43145329	0.012652
2106CL31	0.330	0.264730728	-0.5771956	0.0093	28.46566964	0.013237
2106CL32	0.560	0.370115378	-0.4316629	0.0090	41.12393087	0.018506
2106CL33	1.001	0.703688429	-0.1526196	0.0098	71.80494177	0.035184
2106CL34	0.423	0.303144864	-0.5183498	0.0090	33.6827627	0.015157
2106CL35	0.894	0.602110265	-0.220324	0.0097	62.07322321	0.030106
2008FL01	0.384	0.286399937	-0.5430271	0.0089	32.17976821	0.01432
2008FL02	0.241	0.232535625	-0.6335105	0.0094	24.73783239	0.011627
2008FL03	0.292	0.25047236	-0.6012402	0.0083	30.17739276	0.012524
2008FL04	0.328	0.263960444	-0.5784611	0.0091	29.00664225	0.013198
2008FL05	0.275	0.244344764	-0.611997	0.0093	26.27363055	0.012217
2008FL06	0.359	0.27615578	-0.5588459	0.0098	28.17916127	0.013808
2008FL07	0.229	0.228505407	-0.6411035	0.0096	23.80264661	0.011425
2008FL08	0.190	0.215883368	-0.6657808	0.0088	24.53220092	0.010794
2008FL09	0.359	0.27615578	-0.5588459	0.0089	31.02873937	0.013808
2008FL10	0.222	0.226186783	-0.6455328	0.0093	24.32115945	0.011309
2010FL01	0.297	0.252303668	-0.5980764	0.0087	29.00042156	0.012615
2010FL02	0.282	0.246849525	-0.6075677	0.0089	27.73590167	0.012342
2010FL03	0.352	0.273353652	-0.5632751	0.0085	32.15925313	0.013668
2010FL04	0.356	0.274951368	-0.5607441	0.0088	31.24447362	0.013748
2010FL05	0.295	0.251569543	-0.5993419	0.0085	29.59641685	0.012578
2010FL06	0.274	0.243989022	-0.6126297	0.0096	25.41552311	0.012199
2010FL07	0.215	0.223891685	-0.649962	0.0087	25.73467645	0.011195
2010FL09	0.233	0.229840992	-0.6385725	0.0085	27.04011666	0.011492
2010FL10	0.418	0.300944534	-0.5215135	0.0083	36.25837754	0.015047
2010FL11	0.202	0.219690966	-0.6581878	0.0083	26.46879114	0.010985
2010FL12	0.888	0.596869688	-0.2241205	0.0093	64.1795364	0.029843

2010FL13		0.595	0.38947844	-0.4095166	0.0507	7.682020514	0.019474
2010FL14		0.299	0.253039934	-0.5968109	0.4246	0.595948974	0.012652
2010FL15		0.857	0.570511282	-0.2437358	0.0089	64.1023912	0.028526
2010FL16		0.241	0.232535625	-0.6335105	0.0080	29.06695306	0.011627
2010FL17		0.504	0.341116764	-0.4670969	0.0089	38.32772624	0.017056
2010FL18		0.307	0.256006549	-0.5917489	0.0548	4.671652358	0.0128
2010FL19		0.298	0.252671533	-0.5974437	0.0087	29.04270491	0.012634
2106FL01		0.321	0.261282061	-0.5828904	0.0086	30.38163496	0.013064
	Max	1.548	1.561332204	0.19349532	0.4246	181.5502563	
	Min	0.19	0.215883368	-0.6657808	0.0077	0.595948974	
	Mean	0.485322917	0.366584271	-0.4789149	0.014127	40.58765673	
	Median	0.4105	0.297674657	-0.5262592	0.0089	33.1822322	
	StDev	0.273201969	0.216760305	0.17286887	0.042804	26.40798712	
	%CV	56.29282263	59.12973421	-36.095949	302.9935	65.06408415	

Table 10. Tabulation of calculations for glycogen concentrations for the last 77 samples based on absorbance levels. Absorbance levels are found in Rows A – H and columns 1 – 12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.252	0.549	0.307	0.217	0.393	0.296	0.496	0.433	0.333	0.647	0.284	0.177
B	0.309	0.314	0.650	0.295	0.300	0.248	0.155	0.226	0.216	0.187	0.259	0.255
C	0.269	0.188	0.244	0.203	0.238	0.330	0.326	0.291	0.195	0.242	0.203	0.281
D	0.578	0.239	0.458	0.204	0.320	0.153	0.139	0.153	0.264	0.308	0.180	0.242
E	0.184	0.381	0.270	0.186	0.274	0.200	0.097	0.175	0.225	0.918	0.479	0.448
F	0.439	0.354	0.816	0.337	0.892	0.571	0.914	0.625	0.453	0.318	0.391	0.662
G	1.251	0.328	0.391	0.440	0.428							
H												

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass	Conc (ug/g ww)	Stdev	CV%	Y Coeff	1.2422
Std1	S1	2.998	5	0.69897	N/A	N/A			X Coeff	1.5804
Std2	S2	1.608	2.5	0.39794001	N/A	N/A				
Std3	S3	0.894	1.25	0.09691001	N/A	N/A				
Std4	S4	0.551	0.625	-0.20412	N/A	N/A				
Std5	S5	0.484	0.313	-0.5044557	N/A	N/A				
Std6	S6	0.41	0.156	-0.8068754	N/A	N/A				
Std7	S7									
Std8	S8									
Std9	S9									

Sample	Well Code	Abs	Conc (ug/mL)	Log Conc	Mass (g)	Conc (ug/g ww)	abs Stdev	abs CV%	Conc (ug glycogen)
2106FL02		0.252	0.236292403	-0.6265502	-0.3903	-0.605477663			0.011815
2106FL03		0.549	0.364230967	-0.4386231	-0.0744	-4.896093011			0.018212
2106FL04		0.307	0.256006549	-0.5917489	-0.3357	-0.7625089			0.0128
2106FL05		0.217	0.224545041	-0.6486965	-0.4242	-0.52939821			0.011227
2106FL06		0.393	0.290180135	-0.5373323	-0.2472	-1.17409496			0.014509
2106FL10		0.296	0.251936338	-0.5987092	-0.3468	-0.726516907			0.012597
2106FL11		0.496	0.337163888	-0.4721589	-0.1350	-2.497601685			0.016858
2106FL12		0.433	0.307593906	-0.5120223	-0.2044	-1.504653737			0.01538
2106FL13		0.333	0.265890369	-0.5752974	-0.3094	-0.859354664			0.013295
2106FL14		0.647	0.420132699	-0.3766135	0.0435	9.653965511			0.021007
2106FL15		0.284	0.247569875	-0.6063022	-0.3587	-0.690124243			0.012378
2106FL16		0.177	0.211832904	-0.6740066	-0.4622	-0.458340477			0.010592
2106FL17		0.309	0.256753622	-0.5904834	-0.3337	-0.769345804			0.012838
2106FL18		0.314	0.258630854	-0.5873197	-0.3287	-0.786856275			0.012932
2106FL19*		0.650	0.421973072	-0.3747153	0.0473	8.929171029			0.021099
2106FL20		0.295	0.251569543	-0.5993419	-0.3478	-0.723374099			0.012578

2106FL21		0.300	0.253408873	-0.5961782	-0.3428	-0.739298606	0.01267
2106FL22		0.248	0.234919331	-0.6290812	-0.3942	-0.595997031	0.011746
2106FL23		0.155	0.205150648	-0.6879271	-0.4828	-0.424939212	0.010258
2106FL24		0.226	0.227508815	-0.6430018	-0.4155	-0.54756359	0.011375
2106FL25		0.216	0.224218125	-0.6493293	-0.4251	-0.527434108	0.011211
2106FL26		0.187	0.214941825	-0.6676791	-0.4527	-0.474760642	0.010747
2106FL28		0.259	0.23871462	-0.622121	-0.3834	-0.622615177	0.011936
2106FL29		0.255	0.237327472	-0.624652	-0.3873	-0.612735479	0.011866
2106FL30		0.269	0.242218065	-0.6157935	-0.3736	-0.648377975	0.012111
2106FL31		0.188	0.215255215	-0.6670463	-0.4518	-0.47644855	0.010763
2106FL33		0.244	0.233554237	-0.6316123	-0.3981	-0.586734167	0.011678
2106FL34		0.203	0.220011281	-0.657555	-0.4375	-0.502832625	0.011001
2106FL35		0.238	0.231521455	-0.6354088	-0.4039	-0.573232814	0.011576
2106FL37		0.330	0.264730728	-0.5771956	-0.3125	-0.847233438	0.013237
2106FL38		0.326	0.263192403	-0.5797267	-0.3165	-0.831481596	0.01316
2106FL39		0.291	0.250107697	-0.6018729	-0.3518	-0.711007408	0.012505
2106FL40		0.195	0.217461781	-0.6626171	-0.4452	-0.488507701	0.010873
2008ML01		0.242	0.232874667	-0.6328778	-0.4000	-0.582182178	0.011644
2008ML02		0.203	0.220011281	-0.657555	-0.4375	-0.502832625	0.011001
2008ML03		0.281	0.246490136	-0.6082005	-0.3617	-0.681457295	0.012325
2008ML04		0.578	0.379950177	-0.4202733	-0.0403	-9.422626385	0.018998
2008ML05		0.239	0.231859019	-0.634776	-0.4029	-0.575451089	0.011593
2008ML06		0.458	0.319004278	-0.4962035	-0.1772	-1.800257853	0.01595
2008ML07		0.204	0.220332064	-0.6569223	-0.4366	-0.504665579	0.011017
2008ML08		0.320	0.260901659	-0.5835232	-0.3226	-0.808692725	0.013045
2010ML01		0.153	0.204553724	-0.6891926	-0.4846	-0.422074518	0.010228
2010ML02		0.139	0.200423607	-0.6980511	-0.4976	-0.402758286	0.010021
2010ML03		0.153	0.204553724	-0.6891926	-0.4846	-0.422074518	0.010228
2010ML04		0.264	0.240459962	-0.6189572	-0.3785	-0.635301717	0.012023

2010ML06	0.308	0.256379813	-0.5911162	-0.3347	-0.76591564	0.012819
2010ML07	0.180	0.212760829	-0.6721083	-0.4593	-0.463180554	0.010638
2010ML08	0.242	0.232874667	-0.6328778	-0.4000	-0.582182178	0.011644
2010ML09	0.184	0.214004388	-0.6695773	-0.4556	-0.469747809	0.0107
2010ML10	0.381	0.285150846	-0.5449253	-0.2598	-1.097686101	0.014258
2010ML11	0.270	0.242571225	-0.6151607	-0.3726	-0.651041505	0.012129
2010ML12	0.186	0.21462889	-0.6683118	-0.4537	-0.473081257	0.010731
2010ML13	0.274	0.243989022	-0.6126297	-0.3686	-0.661861338	0.012199
2010ML14	0.200	0.219051735	-0.6594533	-0.4404	-0.497390906	0.010953
2010ML15	0.097	0.188526899	-0.7246267	-0.5361	-0.351663825	0.009426
2010ML16	0.175	0.211216537	-0.6752721	-0.4641	-0.455153566	0.010561
2010ML17	0.225	0.227177584	-0.6436345	-0.4165	-0.545500779	0.011359
2106ML01	0.918	0.623536758	-0.2051379	0.4184	1.490292828	0.031177
2106ML02	0.479	0.328915456	-0.4829157	-0.1540	-2.135811032	0.016446
2106ML03	0.448	0.314390197	-0.502531	-0.1881	-1.671036706	0.01572
2106ML04	0.439	0.310294612	-0.5082258	-0.1979	-1.567689604	0.015515
2106ML05	0.354	0.274151346	-0.5620096	-0.2879	-0.952383073	0.013708
2106ML06	0.816	0.537429421	-0.2696786	0.2678	2.007199618	0.026871
2106ML07	0.337	0.267444463	-0.5727664	-0.3053	-0.875942544	0.013372
2106ML08	0.892	0.600358314	-0.2215895	0.3788	1.585025604	0.030018
2106ML09	0.571	0.376094856	-0.4247026	-0.0486	-7.737343241	0.018805
2106ML10	0.914	0.619913445	-0.2076689	0.4122	1.503751886	0.030996
2106ML12	0.625	0.406879639	-0.390534	0.0163	24.89230762	0.020344
2106ML13	0.453	0.316688834	-0.4993672	-0.1827	-1.733586503	0.015834
2106ML14	0.318	0.260142518	-0.5847887	-0.3246	-0.801310974	0.013007
2106ML15	0.391	0.289335802	-0.5385978	-0.2493	-1.160769705	0.014467
2106ML16	0.662	0.429415536	-0.3671222	0.0623	6.893447899	0.021471
2106ML17	1.251	1.012903824	0.00556821	1.0185	0.99453278	0.050645
2106ML18	0.328	0.263960444	-0.5784611	-0.3145	-0.83930001	0.013198

2106ML19		0.391	0.289335802	-0.5385978	-0.2493	-1.160769705	0.014467
2106ML20		0.440	0.310747029	-0.507593	-0.1968	-1.578630263	0.015537
2106ML21		0.428	0.305361283	-0.515186	-0.2098	-1.455315868	0.015268
	Max	0.65	1.012903824	0.00556821	1.018472	24.89230762	
	Min	0.097	0.188526899	-0.7246267	-0.5361	-9.422626385	
	Mean	0.357051948	0.291215468	-0.5600785	-0.26886	-0.216752149	
	Median	0.296	0.251936338	-0.5987092	-0.34677	-0.622615177	
	StDev	0.208666688	0.124305519	0.1320341	0.254194	3.763387793	
	%CV	58.44154871	42.6850676	-23.574213	-94.5439	-1736.263201	

5.4 HNE Absorbance and Concentration Calculations

Table 11. Tabulation of calculations for HNE concentrations for Plate #1 based on absorbance levels. Absorbance levels are found in Rows A – H and columns 1 – 12. Blanks (A1, B1), Standards (A2-10, B2-10), assayed in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.241	0.141	0.134	0.116	0.121	0.128	0.145	0.142	0.138	0.166	0.172	0.18
B	0.213	0.117	0.136	0.119	0.134	0.117	0.258	0.142	0.136	0.166	0.154	0.168
C	0.141	0.144	0.159	0.144	0.164	0.234	0.146	0.169	0.138	0.204	0.163	0.182
D	0.147	0.15	0.16	0.133	0.142	0.138	0.123	0.137	0.134	0.161	0.138	0.195
E	0.154	0.145	0.129	0.134	0.13	0.141	0.159	0.154	0.134	0.138	0.172	0.147
F	0.131	0.177	0.153	0.135	0.141	0.148	0.143	0.106	0.17	0.128	0.14	0.146
G	0.141	0.209	0.165	0.159	0.13	0.133	0.129	0.126	0.155	0.158	0.166	0.155
H	0.121	0.167	0.186	0.16	0.182	0.196	0.171	0.117	0.144	0.146	0.16	0.147

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass	Conc (ug/g ww)	Stdev	CV%
Std1	S1	0.129	200	2.30103	N/A	N/A	0.016971	13.15547
Std2	S2	0.135	100	2	N/A	N/A	0.001414	1.047566
Std3	S3	0.1175	50	1.69897	N/A	N/A	0.002121	1.805379
Std4	S4	0.1275	25	1.39794001	N/A	N/A	0.009192	7.209716
Std5	S5	0.1225	12.5	1.09691001	N/A	N/A	0.007778	6.34953
Std6	S6	0.2015	6.25	0.79588002	N/A	N/A	0.079903	39.65413
Std7	S7	0.142	3.13	0.49554434	N/A	N/A	0	0
Std8	S8	0.137	1.56	0.1931246	N/A	N/A	0.001414	1.032273
Std9	S9	0.166	0	#NUM!	N/A	N/A	0	0

Y Coeff	0.1593
X Coeff	-0.0156

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass (g)	Conc (ug/g ww)	abs Stdev	abs CV%	Conc (ug protein)
10CL01		0.163	0.579189277	-0.2371795	0.1003	5.774569063	0.012728	7.808541	0.028959
10CL02		0.174	0.114206891	-0.9423077	0.1001	1.140927978	0.008485	4.876598	0.00571

10CL03	0.144	9.566855862	0.98076923	0.1007	95.00353388	0.004243	2.946278	0.478343
10CL04	0.147	6.144146161	0.78846154	0.1003	61.25768855	0.004243	2.88615	0.307207
10CL05	0.1595	0.970911147	-0.0128205	0.1005	9.660807432	0.000707	0.443327	0.048546
10CL06	0.1385	21.5443469	1.33333333	0.1	215.443469	0.007778	5.616011	1.077217
10CL07	0.153	2.534230736	0.40384615	0.1002	25.29172391	0.015556	10.16755	0.126712
10CL15	0.186	0.019429496	-1.7115385	0.1001	0.194100861	0.067882	36.49583	0.000971
10CL16	0.1345	38.8815518	1.58974359	0.1	388.815518	0.016263	12.09179	1.944078
10CL23	0.153	2.534230736	0.40384615	0.1	25.34230736	0.022627	14.78916	0.126712
10CL31	0.136	31.15944715	1.49358974	0.1	311.5944715	0.002828	2.079726	1.557972
10CL32	0.1825	0.032570207	-1.4871795	0.1002	0.325051962	0.030406	16.6606	0.001629
06ML08	0.1505	3.665241237	0.56410256	0.1012	36.21779879	0.017678	11.74596	0.183262
06ML09	0.1885	0.013433993	-1.8717949	0.1007	0.133406091	0.009192	4.876598	0.000672
06ML10	0.1425	11.93776642	1.07692308	0.1019	117.1517803	0.016263	11.41295	0.596888
10FL01	0.161	0.778082488	-0.1089744	0.0993	7.835674604	0.022627	14.0543	0.038904
10FL02	0.141	14.896249	1.17307692	0.0999	149.1116016	0.016971	12.03586	0.744812
10FL03	0.1345	38.8815518	1.58974359	0.101	384.9658594	0.000707	0.52573	1.944078
10FL05	0.1355	33.54602186	1.52564103	0.1014	330.8286179	0.007778	5.74035	1.677301
10FL09	0.1445	8.886238163	0.94871795	0.1001	88.77360802	0.00495	3.425431	0.444312
10FL10	0.151	3.404483879	0.53205128	0.1008	33.77464166	0.011314	7.492522	0.170224
10FL11	0.13	75.5448961	1.87820513	0.1002	753.9410788	0.033941	26.10856	3.777245
10FL12	0.152	2.937302791	0.46794872	0.1003	29.28517239	0.025456	16.74727	0.146865
10FL13	0.133	48.51739067	1.68589744	0.1	485.1739067	0.007071	5.316592	2.42587
10FL14	0.156	1.627565448	0.21153846	0.0997	16.32462837	0.022627	14.50475	0.081378
10FL15	0.1465	6.614740641	0.82051282	0.1002	66.01537566	0.000707	0.482667	0.330737
10FL16	0.131	65.17823025	1.81410256	0.1015	642.1500517	0.014142	10.79552	3.258912
10ML01	0.188	0.014462934	-1.8397436	0.1003	0.144196752	0.029698	15.79707	0.000723
10ML02	0.1755	0.091524731	-1.0384615	0.1008	0.907983443	0.014849	8.461107	0.004576
10ML03	0.1595	0.970911147	-0.0128205	0.1002	9.689732005	0.000707	0.443327	0.048546
10ML04	0.156	1.627565448	0.21153846	0.1	16.27565448	0.03677	23.57023	0.081378

10ML06		0.1645	0.464158883	-0.3333333	0.1007	4.609323569	0.044548	27.08069	0.023208
10ML07		0.15	3.945970609	0.59615385	0.1004	39.30249611	0.029698	19.79899	0.197299
10ML08		0.1215	264.8969288	2.42307692	0.1003	2641.046149	0.006364	5.237828	13.24485
10ML12		0.1495	4.248201698	0.62820513	0.1	42.48201698	0.007778	5.202792	0.21241
10ML14		0.152	2.937302791	0.46794872	0.1025	28.6566126	0.008485	5.582422	0.146865
10ML15		0.163	0.579189277	-0.2371795	0.1001	5.786106663	0.004243	2.602847	0.028959
10ML16		0.151	3.404483879	0.53205128	0.1005	33.87546148	0.005657	3.746261	0.170224
	Max	0.1885	264.8969288	2.42307692	0.1025	2641.046149			
	Min	0.1215	0.013433993	-1.8717949	0.0993	0.133406091			
	Mean	0.152605263	18.7813424	0.4291498	0.100432	186.9553449			
	Median	0.151	3.404483879	0.53205128	0.10025	33.82505157			
	StDev	0.016687169	45.13788747	1.06969029	0.000618	449.840872			
	%CV	10.9348578	240.3336593	249.25802	0.615147	240.6140741			

Table 12. Tabulation of calculations for HNE concentrations for Plate #2 based on absorbance levels. Absorbance levels are found in Rows A – H and columns 1 – 12. Blanks (A1, B1), Standards (A2-10, B2-10), assayed in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.255	0.136	0.136	0.201	0.166	0.145	0.135	0.151	0.138	0.137	0.134	0.14
B	0.2	0.137	0.123	0.126	0.148	0.144	0.17	0.234	0.176	0.211	0.16	0.144
C	0.153	0.152	0.168	0.203	0.159	0.125	0.169	0.121	0.155	0.135	0.143	0.129
D	0.144	0.142	0.154	0.147	0.166	0.14	0.128	0.188	0.193	0.144	0.137	0.148
E	0.166	0.131	0.137	0.166	0.153	0.133	0.17	0.171	0.15	0.154	0.135	0.155
F	0.158	0.18	0.155	0.126	0.132	0.139	0.149	0.163	0.145	0.14	0.133	0.165
G	0.159	0.178	0.156	0.12	0.118	0.142	0.145	0.159	0.136	0.134	0.15	0.138
H	0.147	0.129	0.133	0.112	0.111	0.122	0.146	0.137	0.15	0.131	0.124	0.146

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass	Conc (ug/g ww)	Stdev	CV%		
									Y Coeff	0.1743

Std1	S1	0.1365	200	2.30103	N/A	N/A	0.000707	0.518027	X Coeff	-0.0162
Std2	S2	0.1295	100	2	N/A	N/A	0.009192	7.098369		
Std3	S3	0.1635	50	1.69897	N/A	N/A	0.053033	32.43609		
Std4	S4	0.157	25	1.39794001	N/A	N/A	0.012728	8.106957		
Std5	S5	0.1445	12.5	1.09691001	N/A	N/A	0.000707	0.489347		
Std6	S6	0.1525	6.25	0.79588002	N/A	N/A	0.024749	16.22868		
Std7	S7	0.1925	3.13	0.49554434	N/A	N/A	0.05869	30.48824		
Std8	S8	0.157	1.56	0.1931246	N/A	N/A	0.02687	17.11469		
Std9	S9	0.174	0	#NUM!	N/A	N/A	0.052326	30.07236		

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass (g)	Conc (ug/g ww)	abs Stdev	abs CV%	Conc (ug protein)
10CL01		0.147	48.4378865	1.68518519	0.1003	482.9300747	0.018385	12.50665	2.421894
10CL08		0.142	98.58870465	1.99382716	0.1002	983.9192081	0.002828	1.99185	4.929435
10CL09		0.1485	39.13745602	1.59259259	0.1	391.3745602	0.006364	4.285496	1.956873
10CL10		0.147	48.4378865	1.68518519	0.0999	484.8637287	0.007071	4.81025	2.421894
10CL11		0.161	6.621976792	0.82098765	0.1002	66.08759273	0.009899	6.148755	0.331099
10CL12		0.175	0.905295004	-0.0432099	0.1004	9.016882506	0.039598	22.62742	0.045265
10CL13		0.1625	5.350508542	0.72839506	0.1007	53.13315335	0.00495	3.045998	0.267525
10CL14		0.1325	380.4056105	2.58024691	0.1006	3781.367897	0.010607	8.004982	19.02028
10CL26		0.1485	39.13745602	1.59259259	0.1	391.3745602	0.028991	19.52281	1.956873
10CL27		0.1545	16.68100537	1.22222222	0.1	166.8100537	0.047376	30.66418	0.83405
10CL28		0.174	1.04356263	0.01851852	0.1002	10.4147967	0.02687	15.44256	0.052178
10CL29		0.1395	140.6527242	2.14814815	0.1027	1369.549408	0.006364	4.561979	7.032636
10CL30		0.14	131.0038173	2.11728395	0.102	1284.35115	0.004243	3.030458	6.550191
06CL01		0.1385	162.1349131	2.20987654	0.1017	1594.246933	0.013435	9.700382	8.106746
06CL02		0.162	5.744592928	0.75925926	0.1005	57.16012864	0.005657	3.491885	0.28723
06CL04		0.1555	14.47084285	1.16049383	0.1006	143.8453563	0.034648	22.28182	0.723542
06CL05		0.146	55.83590765	1.74691358	0.1004	556.1345383	0.012728	8.717755	2.791795
06CL06		0.146	55.83590765	1.74691358	0.1004	556.1345383	0.028284	19.37279	2.791795

06CL07	0.1425	91.82542836	1.96296296	0.1002	916.4214407	0.014849	10.42052	4.591271
06CL9	0.136	231.311663	2.36419753	0.1	2313.11663	0.004243	3.119589	11.56558
06CL10	0.1595	8.195590435	0.91358025	0.1004	81.6293868	0.014849	9.30987	0.40978
06CL12	0.167	2.822391685	0.45061728	0.1	28.22391685	0.005657	3.387338	0.14112
06CL13	0.1475	45.1150027	1.65432099	0.1008	447.5694712	0.003536	2.396972	2.25575
06CL14	0.147	48.4378865	1.68518519	0.101	479.5830346	0.009899	6.73435	2.421894
06CL15	0.134	307.3649353	2.48765432	0.1007	3052.28337	0.001414	1.055383	15.36825
06CL16	0.16	7.633365353	0.88271605	0.1013	75.35405087	0.007071	4.419417	0.381668
06CL17	0.153	20.64499656	1.31481481	0.1016	203.198785	0.008485	5.545936	1.03225
06CL18	0.1535	19.22873071	1.28395062	0.1009	190.5721576	0.034648	22.57214	0.961437
06CL19	0.1445	69.10447515	1.83950617	0.1	691.0447515	0.016263	11.25499	3.455224
06CL20	0.116	3969.770793	3.59876543	0.0999	39737.44537	0.005657	4.876598	198.4885
10FL04	0.1145	4913.127388	3.69135802	0.1007	48789.74566	0.00495	4.322924	245.6564
10FL06	0.132	408.4238653	2.61111111	0.1003	4072.022585	0.014142	10.71374	20.42119
10FL07	0.1455	59.94842503	1.77777778	0.1004	597.0958669	0.000707	0.485984	2.997421
06FL03	0.148	42.0200718	1.62345679	0.1004	418.5266115	0.015556	10.51105	2.101004
06FL05	0.143	85.52611907	1.93209877	0.0993	861.2902222	0.009899	6.922724	4.276306
06FL14	0.1325	380.4056105	2.58024691	0.1028	3700.443682	0.002121	1.600996	19.02028
06FL17	0.137	200.6638478	2.30246914	0.1043	1923.910334	0.018385	13.41954	10.03319
06FL21	0.142	98.58870465	1.99382716	0.1017	969.4071254	0.005657	3.9837	4.929435

Max	0.175	4913.127388	3.69135802	0.1043	48789.74566
Min	0.1145	0.905295004	-0.0432099	0.0993	9.016882506
Mean	0.146710526	322.6469827	1.70305393	0.100724	3208.72629
Median	0.1465	52.13689707	1.71604938	0.1004	520.4991335
StDev	0.013188668	996.1409642	0.81411532	0.000965	9923.681099
%CV	8.989585458	308.7402076	47.8032611	0.957974	309.2716612

Table 13. Tabulation of calculations for HNE concentrations for Plate #3 based on absorbance levels. Absorbance levels are found in Rows A – H and columns 1 – 12. Blanks (A1, B1), Standards (A2-10, B2-10), assayed in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1	0.196	0.176	0.191	0.201	0.217	0.25	0.274	0.332	0.321	0.186	0.156
B	0.131	0.155	0.166	0.185	0.188	0.198	0.231	0.241	0.278	0.309	0.182	0.165
C	0.262	0.199	0.212	0.164	0.196	0.192	0.175	0.165	0.209	0.187	0.164	0.192
D	0.215	0.19	0.215	0.162	0.185	0.182	0.151	0.138	0.16	0.164	0.183	0.188
E	0.251	0.258	0.22	0.228	0.216	0.247	0.217	0.222	0.278	0.234	0.225	0.203
F	0.229	0.232	0.211	0.204	0.204	0.22	0.206	0.233	0.241	0.259	0.187	0.226
G	0.219	0.221	0.204	0.224	0.204	0.143	0.144	0.275	0.196	0.212	0.212	0.137
H	0.219	0.201	0.199	0.238	0.204	0.158	0.165	0.289	0.194	0.218	0.201	0.137

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass	Conc (ug/g ww)	Stdev	CV%
Std1	S1	0.1755	200	2.30103	N/A	N/A	0.028991	16.5193
Std2	S2	0.171	100	2	N/A	N/A	0.007071	4.135127
Std3	S3	0.188	50	1.69897	N/A	N/A	0.004243	2.256724
Std4	S4	0.1945	25	1.39794001	N/A	N/A	0.009192	4.726164
Std5	S5	0.2075	12.5	1.09691001	N/A	N/A	0.013435	6.474713
Std6	S6	0.2405	6.25	0.79588002	N/A	N/A	0.013435	5.586291
Std7	S7	0.2575	3.13	0.49554434	N/A	N/A	0.023335	9.061951
Std8	S8	0.305	1.56	0.1931246	N/A	N/A	0.038184	12.51927
Std9	S9	0.315	0	#NUM!	N/A	N/A	0.008485	2.69374

Y Coeff	0.2999
X Coeff	-0.0646

Sample	Well Code	Mean abs	Conc (ug/mL)	Log Conc	Mass (g)	Conc (ug/g ww)	abs Stdev	abs CV%	Conc (ug protein)
10CL01		0.184	62.24688844	1.79411765	0.1003	620.6070632	0.002828	1.537189	3.112344
10FL08		0.1605	143.8449888	2.15789474	0.1002	1435.578731	0.006364	3.965085	7.192249
06FL01		0.2385	8.922044731	0.9504644	0.1006	88.68831741	0.033234	13.9346	0.446102
06FL02		0.1945	42.81332399	1.63157895	0.101	423.8942969	0.006364	3.271959	2.140666

06FL06	0.2135	21.75010212	1.3374613	0.1014	214.4980485	0.002121	0.993593	1.087505	
06FL22	0.163	131.5815647	2.11919505	0.1007	1306.668964	0.001414	0.867616	6.579078	
06FL23	0.1905	49.37401584	1.69349845	0.1027	480.7596479	0.007778	4.083031	2.468701	
06FL24	0.187	55.93427568	1.74767802	0.1025	545.7002506	0.007071	3.78132	2.796714	
06FL25	0.163	131.5815647	2.11919505	0.1023	1286.232304	0.016971	10.41139	6.579078	
06FL26	0.1515	198.2501946	2.29721362	0.1005	1972.638752	0.019092	12.6019	9.91251	
06FL31	0.1845	61.14735966	1.78637771	0.1023	597.7259009	0.034648	18.77953	3.057368	
06FL33	0.1755	84.27457954	1.92569659	0.1011	833.5764544	0.016263	9.266926	4.213729	
06FL34	0.1735	90.50161865	1.95665635	0.1001	904.1120744	0.013435	7.743532	4.525081	
06FL35	0.19	50.26184078	1.70123839	0.1027	489.4044867	0.002828	1.488646	2.513092	
06ML01	0.24	8.457550157	0.92724458	0.1008	83.90426743	0.015556	6.481812	0.422878	
06ML03	0.245	7.076934911	0.8498452	0.1019	69.44980285	0.018385	7.50399	0.353847	
06ML05	0.2155	20.25356826	1.30650155	0.1008	200.9282565	0.006364	2.953114	1.012678	
06ML06	0.216	19.89580931	1.29876161	0.1003	198.3630041	0.016971	7.856742	0.99479	
06ML07	0.21	24.64000927	1.39164087	0.1007	244.6872817	0.008485	4.04061	1.232	
06ML12	0.2335	10.6626162	1.02786378	0.1008	105.7799226	0.019092	8.176395	0.533131	
06ML14	0.2115	23.35721469	1.36842105	0.1004	232.6415806	0.007778	3.677624	1.167861	
06ML15	0.2275	13.20514073	1.12074303	0.1004	131.5253061	0.007778	3.418978	0.660257	
06ML16	0.2595	4.220724418	0.625387	0.1007	41.91384725	0.026163	10.08206	0.211036	
06ML17	0.2465	6.708499427	0.82662539	0.1008	66.55257368	0.017678	7.171468	0.335425	
06ML18	0.206	28.41583168	1.45356037	0.1005	282.7445938	0.02687	13.04372	1.420792	
06ML19	0.2145	20.98850109	1.32198142	0.1004	209.0488156	0.016263	7.582031	1.049425	
06ML21	0.219	17.87812549	1.25232198	0.1008	177.3623561	0	0	0.893906	
10CL12	0.211	23.77721532	1.37616099	0.1004	236.8248538	0.014142	6.702434	1.188861	REDO
10CL15	0.2015	33.35952163	1.52321981	0.1001	333.2619543	0.003536	1.754607	1.667976	
10CL27	0.231	11.65637384	1.06656347	0.1	116.5637384	0.009899	4.285496	0.582819	
10CL28	0.204	30.51547425	1.48452012	0.1002	304.5456512	0	0	1.525774	
10CL32	0.1505	205.4440172	2.3126935	0.1002	2050.339493	0.010607	7.047576	10.2722	
10FL11	0.1545	178.1451462	2.25077399	0.1002	1777.89567	0.014849	9.61116	8.907257	

10ML01		0.282	1.89273487	0.27708978	0.1003	18.87073649	0.009899	3.510459	0.094637
10ML04		0.195	42.05706961	1.62383901	0.1	420.5706961	0.001414	0.725238	2.102853
06CL04		0.215	20.61776029	1.31424149	0.1006	204.9479154	0.004243	1.973321	1.030888
06CL18		0.2065	27.91389454	1.44582043	0.1009	276.6491035	0.007778	3.766671	1.395695
	Max	0.282	205.4440172	2.3126935	0.1027	2050.339493			
	Min	0.1505	1.89273487	0.27708978	0.1	18.87073649			
	Mean	0.204459459	51.71957015	1.47740775	0.100827	513.1204517			
	Median	0.2065	27.91389454	1.44582043	0.1006	276.6491035			
	StDev	0.0311565	55.95102006	0.48229876	0.000776	556.7328906			
	%CV	15.23847312	108.1815257	32.6449322	0.76954	108.4994544			