Abundance, Transportation, and Preservation of *Mysis diluviana* eDNA in Freshwater Ecosystems

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Scott Bergson

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Abstract

Environmental DNA (eDNA) has proven to be a valuable tool in detecting rare or invasive species, particularly within the Great Lakes and surrounding aquatic ecosystems. Recent work has shown that sedimentary eDNA (sedDNA) can reveal temporal changes in ecological community composition and can potentially be used to provide restoration guidelines for impacted ecosystems. However, uncertainties currently exist regarding the application of eDNA techniques in both sediment and aquatic freshwater ecosystems. For example, little is known regarding how diel movements affect the short-term persistence and transport of invertebrate eDNA within freshwater lakes and streams. In this study, I examined the effectiveness of both aqueous eDNA and lake sedDNA sensitivity to the presence of *Mysis diluviana*, a keystone macroinvertebrate species. Water and sediment samples were collected from five lakes at the IISD-Experimental Lakes area in Northwestern Ontario. *Mysis* sedDNA was extracted from sections of both freeze and gravity cores and tested using quantitative polymerase chain reactions (qPCR) to evaluate preservation and down-core presence. Day and night eDNA samples were analysed during the fall and compared across the epilimnion, metalimnion, and hypolimnion to assess the short-term persistence within a stratified lake. Water samples were collected between June until October of 2019 from a stream connecting two of the study lakes to examine the effect that seasonal variation within a stream may have on downstream sedDNA distribution. My results demonstrated that the occurrence of *Mysis* eDNA in dated sediment freeze cores closely matched known historical distributions. Further, the absence of significant day/night differences in *Mysis* eDNA between thermal layers in the fall indicates the persistence of eDNA in water over short time intervals. Similarly, high

concentrations of *Mysis* eDNA in streams during fall compared to summer months provided further evidence for persistence as well as transport among lakes during certain time periods. My work demonstrates a valuable method to reconstruct past occupation histories and provide restoration guidelines of impacted ecosystems when applied to sediments by indicating candidate lakes for potential biological reestablishment, as well as indicates spatiotemporal factors that should be considered in the design of eDNA surveys.

Lay Summary

Biodiversity is important, but to measure it you need to know what species are where. New technologies can help with that, and one is the measurement of the DNA that animals shed into their environment. The purpose of my thesis is to better understand how invertebrates are distributed, specifically *Mysis*, which is a small freshwater shrimp, using environmental DNA (eDNA). I looked at how well *Mysis* eDNA was preserved and moved in systems such as lakes, streams and lake sediments. I found that *Mysis* eDNA can be accurately detected in freshwater sediments. Also, *Mysis* eDNA in water can be found in both lakes and their connecting streams during colder times of the year when *Mysis* are most active. *Mysis* do not inhabit streams at the IISD-ELA, and therefore the eDNA found in them must come from upstream lakes that have *Mysis*. Methods from this study can be used to help future research looking at eDNA in sediment that target organisms that do not leave behind anything like hard parts that otherwise allow for their identification and detection. These results suggest that eDNA methods used in this study can help find more organisms in lake and sediments than we have been able to detect before based on existing methods.

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Chapter 1:

General Introduction

The monitoring of biodiversity has become urgent as anthropogenic disturbance increases in scope and frequency. Humans have had a major impact on species loss, with observed extinction rates 8-100 times above background across the globe (Ceballos et al. 2015). As species go extinct faster than we can document them, there is an urgent need for more efficient methods of monitoring and documenting biological diversity (Barnosky et al. 2011). Pereira and Cooper (2006) described multiple challenges with current biodiversity monitoring programs, including poor spatial and taxonomic coverage. While existing biodiversity monitoring strategies can be improved several ways, advances in our technologies to monitor species at low abundances would assist in the detection of species that traditional methods may miss (Lindenmayer et al. 2012). Environmental DNA techniques are among the most promising of these technologies.

The term environmental DNA (eDNA) refers to genetic material that can be collected from water, soil or air samples. (Barnes and Turner 2016, Harper et al., 2019). Environmental DNA is widespread throughout the environment, the dynamics of which are complex due to the multiple ways it can enter, move, and be transformed. Excretion from animals, reproduction, and decomposition are just some examples of the ways eDNA can enter the environment (Barnes and Turner 2016). Environmental DNA can also exist as either extracellular or intracellular DNA (Ogram et al., 1987). Once collected, there are many ways in which eDNA can be collected, extracted, amplified, and identified. One of the initial uses for eDNA was to extract bacterial DNA from soil

(Torsvik 1980). Since then, methods of isolating DNA from environmental materials have expanded greatly. Technological advances have resulted in the use of eDNA as an increasingly popular monitoring tool (Thomsen and Willerslev 2015).

From the perspective of biomonitoring species' distributions in aquatic ecosystems, typical survey methods that are not eDNA-based primarily use physical monitoring techniques, such as traps or nets, which can require extensive sampling to ensure the capture of rare species. Physical monitoring only allows for a snapshot of the target species assemblage because of the limited sampling time. These methods are often costly and labour-intensive. In aquatic ecosystems, however, eDNA allows for a non-invasive and rapid approach to examine lakes and river systems for rare aquatic species (Wilson and Wright 2013; Castañeda et al. 2020). One of the more common current uses of eDNA is in monitoring for early detection of invasive species such as Asian carp (Jerde et al. 2013). In addition, eDNA also has potential to be an important tool in population genetics (Adams et al. 2019).

The investigation of aquatic biological systems using eDNA faces several challenges. Genetic databases of sequenced genomes may not have information on all organisms (Wang et al., 2019). Further, the degradation of eDNA increases in environments with lower pH, higher temperatures and greater exposure to solar radiation (Strickler et al. 2015; Eichmiller et al. 2016a). Despite these challenges, research in aquatic eDNA has produced several intriguing results. There are many recent studies showing the ability to use eDNA to predict biomass or density of organisms using several model based approaches (Lacoursière-Roussel et al. 2016a; Yates et al. 2020; Spear et al. 2021). Recent success in the application of aquatic eDNA methods has been recognized

by the Department of Fisheries of Oceans Canada, as illustrated by their development of a set of standard reporting protocols (Department of Fisheries and Oceans Canada 2020).

Studies assessing eDNA presence within lake sediments (sedDNA) have not shown the same success, interest or activity when compared to aqueous eDNA research. This may be due to challenges in working with sedDNA, including fragmented DNA and potential Polymerase chain reaction (PCR) inhibition due to contamination by humic materials that is commonly present in freshwater sediment (Wilson 1997). There is also a variety of chemicals used during the extraction process that can cause inhibition if not removed before PCR (Hedman and Radstrom 2015). Research to improve extraction techniques that also limit PCR inhibition in sediments is an ongoing area of interest. Examples of efforts to reduce inhibition range from manipulating chemical protocols to remove humic acids (Dong et al., 2006; Torsvik, 1980; You et al., 2017), to manipulating and comparing extraction kits from various popular scientific companies (Eichmiller et al., 2016; Hinlo et al., 2017). However, as knowledge of sedDNA progresses, there have been several promising results. For example, sedDNA has determined historical assemblages of native and non-native fish species in alpine lakes (Nelson-Chorney et al. 2019).

Sediment eDNA provides a promising technique to understand historical changes in aquatic communities but requires validation against ecosystems where background data are extensively documented. The IISD-Experimental Lakes Area (ELA) in northwestern Ontario is an excellent candidate for sedDNA validation with over 50 years of zooplankton community data. From 1976-1983, an acidification experiment was conducted to study the effects of acid rain in a whole ecosystem (Mills et al. 1987,

Schindler et al 1985). Ecosystem responses to this experiment included the extirpation of two vertebrate species, slimy sculpin (*Cottus congnatus*), and fathead minnow (*Pimephales promelas*), as well as two invertebrate species, crayfish (*Orconectes virilis*) and *Mysis* (*Mysis diluviana*). The pH of the lake returned to pre-acidification levels in the 1990's and most of the extirpated species have naturally returned over the past two decades. However, *Mysis* have never re-established.

Mysis diluviana, previously *Mysis relicta* (Audzijonyte & Väinölä, 2005 and henceforth referred to as "*Mysis*"), is a small (<3 mm) freshwater crustacean that occurs throughout the Canadian Shield (Walsh et al., 2012). *Mysis* reach sexual maturity between 1-2 years; mature females begin to develop eggs in their brood pouches during the winter and release them approximately five months later in May, shortly after which the males die (Lasenby and Langford 1972). Ecosystem productivity may affect the life span of *Mysis*, which can live up to four years in environments of low productivity, but only 1-2 years when productivity is high (Morgan 1981, Nero 1981). Productivity is variable among lakes at the Experimental Lakes Area, which suggests differences in life cycles for the *Mysis* which inhabit them. *Mysis* are often found in high abundance; for example, recent estimates of *Mysis* densities in Lake 224 at ELA were estimated to be on average 77/m^2 (Milling, 2020). With high densities of *Mysis* dying off approximately every 2 years at the ELA (Paterson et al., 2010, Nero and Schindler 1983), their carcasses, and genetic material, will likely settle to the lake bottom and in turn introduce eDNA into the sediment.

Understanding persistence and movement of aquatic eDNA can provide important information to eDNA surveys conducted both within lakes where *Mysis* are present and

the streams that connect these *Mysis* lakes to other hydrologically connected lakes. If the eDNA of an organism, like *Mysis,* can traverse the full length of a stream into a neighbouring lake, this could potentially cause false positive detections in downstream systems in which they are absent. In addition, eDNA studies of organisms like *Mysis,* which have certain migration patterns (e.g., diel migrations) or environmental constraints (e.g., thermal constraints), introduces the possibility of false negatives from eDNA surveys due to sampling designs that fail to incorporate these life history traits. Developing a better understanding of *Mysis* eDNA movement and persistence in the water column may provide insights into the accumulation and deposition of their eDNA within lake sediments. For example, seasonal variability in the distribution of *Mysis* within the water column may affect the dispersal of *Mysis* eDNA among lakes and detectability within the water column. In addition, their distribution may also influence the deposition of their eDNA in lake sediments (De Souza et al., 2016).

For freshwater invertebrates, paleoecological research has traditionally focused on organisms that leave behind physical remnants. For example, body parts such as the ephippia or mandibles of *Daphnia* have been used to estimate current day *Daphnia* body size and abundance of planktivorous fish due to these parts preserving in the top layer of the sediment cores (Jeppesen et al. 2002; Korosi et al. 2010). This, however, is not the case for every organism. Many invertebrates do not leave behind any hard parts that can be recovered from sediments. *Mysis* is one example as their mandible and exoskeletons degrade in sediments (Tracy and Vallentyne 1969).

Recent work illustrates the significant potential of eDNA in sediments to reconstruct historical communities for organisms that do not leave behind physical

remains. Studies have shown that fish eDNA was significantly more concentrated within sediments as compared with the water column (Turner et al., 2015, Sakata et al. 2020), and that eDNA decay rates were low in aquatic sediments (Levy-Booth et al. 2007; Pietramellara et al. 2009; Sakata et al. 2020). Additionally, sedDNA has been shown to exhibit slower decay rates in low oxygen environments, which are common in deeper sediments (Pietramellara et al. 2009; Sakata et al. 2020). The binding of humic acids in sediment particles to eDNA contributes to its preservation through protection from biological and chemical decay elements (Greaves and Wilson 1969; Crecchio and Stotzky 1998; Levy-Booth et al. 2007). Humic acids are also one of the main inhibitors in freshwater sediment that make eDNA detection challenging and time consuming (Dong et al., 2006; Santı, 2009; You et al., 2017).

The goal of this thesis was to investigate the detectability of *Mysis* eDNA in boreal aquatic ecosystems. *Mysis* eDNA in water have only been described in one other study (Carim et al. 2016). Recently, a metabarcoding eDNA survey failed to detect *Mysis* at IISD-ELA in lakes known to support *Mysis*; however, the survey was conducted in the summer and during the daytime when *Mysis* would be unlikely to be occupying the water column (Personal communication, Joanne Littlefair). The absence of *Mysis* eDNA detection in this previous survey, combined with known diel migration patterns of *Mysis,* justifies additional research into the persistence of *Mysis* eDNA. In addition, studies have shown that aqueous eDNA can move through systems before settling to sediments (Barnes and Turner 2016). In the first chapter, I examine how *Mysis* eDNA is distributed in the water column and in streams connecting lakes. My second chapter improves on previously published extraction protocols from lake sediments for detecting *Mysis*

sedDNA. I then use these methods to investigate historic and contemporary distributions of *Mysis* within aquatic ecosystems at IISD-ELA. Specifically, I investigated the preservation of *Mysis* sedDNA in lake sediment, diel movement of *Mysis* eDNA within the water column and the transportation downstream from a source.

Chapter 2:

Abundance and Transport of *Mysis diluviana* eDNA

Abstract

The use of environmental DNA (eDNA) for detecting the presence of aquatic organisms in water is a well-studied and proven technique. However, uncertainties regarding diffusion, persistence and transport of invertebrate eDNA from its source in freshwater lakes and streams remain unresolved. I determined quantitatively the spatial distribution of eDNA from *Mysis diluviana,* a small freshwater crustacean that inhabits deep cold-water lakes across North America. An eDNA survey was conducted during fall (when temperatures were optimal for *Mysis* occupancy throughout the water column) in several lakes and outflowing streams in northwestern Ontario. In addition, the transport of *Mysis* eDNA within a single stream was evaluated system across several months. I found significant quantities of *Mysis* eDNA in streams that drained lakes where *Mysis* were present, with an increase in abundance during times of higher flow and cooler water temperatures (< 8°C). Within lakes, I found no significant differences between day and night *Mysis* eDNA concentrations within the same thermal layers in autumn. In addition, significant eDNA transport from upstream lakes during certain times of the year not likely not associated with the movement of organisms themselves suggests the possibility of false positives in downstream lakes. My results indicate that seasonal characteristics should be considered during eDNA surveys to avoid false negatives of organisms of interest.

Introduction

Developing methods for the rapid assessment and monitoring of biodiversity in freshwater ecosystems is a major priority of the Canadian government and environmental monitoring organizations globally (Tognelli et al. 2017). Freshwater environments support hundreds of thousands of freshwater species (Dudgeon et al. 2006), and though they occupy only 2% of the earth's surface, they harbour a disproportionate fraction of global biodiversity. For example, the species diversity of freshwater fishes matches that found in marine environments, which occupy 70% of global area (Carrete Vega and Wiens 2012; Guinot and Cavin 2015). Further, in particular fish taxa, freshwater diversity has shown to be higher when compared to marine species within the same taxa (Manel et al. 2020).

Though most investigations comparing species and genetic diversity between freshwater and marine environments have focused primarily on fishes, invertebrate species make up a large proportion of both the biomass and species diversity in both of these environments (Strayer 2006). Typical monitoring methods used for freshwater invertebrates involve net hauls or sediment grabs, both of which can be physically taxing. These methods can also be limited as they are spatially discrete and can miss species with patchy and/or limited abundance, and have the potential to unintentionally harm rare or endangered species (Lacoursière-Roussel et al. 2016b).

Within the past 15 years, new and previously existing threats to freshwater species have greatly increased from anthropogenic influences (Reid et al. 2019). In general, evaluations of freshwater biodiversity rely heavily on the monitoring of species of significant importance, such as those defined as 'keystone' or having economic value.

This economic focus has generally been directed toward fish species historically; however, several freshwater invertebrate species have been identified as holding keystone status and as a result have been used as freshwater biological indicators (Bond 1994).

The use of environmental DNA (henceforth eDNA) surveys as to tool to assess aquatic biodiversity has increased recently. Compared to traditional methods, it is potentially a more efficient technique by sampling eDNA from numerous species and increasing the sample size and geographical range with a single method (Coble et al. 2019). Environmental DNA refers to genetic material collected from the environment in the form of water, soil, sediment and even air samples (Barnes & Turner, 2016, Harper et al., 2019). As a monitoring tool, eDNA has become an invaluable technique for detecting rare or invasive species (Goldberg et al. 2016). Further, it is less invasive than traditional methods as there is no physical capture of individuals involved (Thomsen and Willerslev 2015) and it has a smaller environmental footprint compared to traditional sampling methods. For example, drawing water is less invasive than setting trap nets, hauling net tows or ponar dredges. Recently, there has been a spike in research attempting to use eDNA to predict biomass and densities of organisms, particularly for fishes. However, these methods often have issues with accuracy and reliability (Chambert et al. 2018). The application of these same techniques to aquatic invertebrates is limited, and many questions remain (Roussel et al. 2015). In particular, research pertaining to invertebrate eDNA distribution on daily time periods (e.g., day versus night) or seasonal variation is scarce. Recent studies have shown that eDNA in fish species such as lake trout (*Salvelinus namaycush*) reflect the seasonal distribution of individuals, based on their thermal requirements (Littlefair et al. 2020). This begs the question as to whether similar

environmental limitations may affect eDNA distribution and detectability of lentic invertebrates.

As the use of eDNA for biological monitoring increases, so does the need to understand factors affecting its persistence and distribution in ecosystems. Persistence of eDNA in laboratory settings has been well studied, indicating a general negative correlation between residence time and detectability of eDNA, which varies among species (Dejean et al. 2011; Pilliod et al. 2014; Spear et al. 2015). However, in the wild, there are many biotic and abiotic factors that can affect the abundance and persistence of eDNA such as pH, light exposure, temperature and bacteria (Strickler et al. 2015). Research has shown that transport of invertebrate eDNA in the wild can be species specific due to shedding rates into the environment and detection limits in primers (Deiner and Altermatt 2014). In stream ecosystems over long distances, eDNA transport is inconsistent with longer streams report a steady decrease in eDNA abundance as it travels from the source (Deiner and Altermatt 2014). Other studies examining streams over shorter distances at high flow have observed constant detections at sample points along the stream during times of high flow (Jane et al. 2015). These differences are believed to be due to resuspension of eDNA from sediments due to fluctuating stream levels and higher rates of flow (Jane et al. 2015; Shogren et al. 2017; Curtis et al. 2020).

Mysis diluviana are recognized as a keystone species as they can cause large shifts or disruptions in freshwater communities if removed (Mills et al. 1987; Milling 2020) and have caused major disruptions when introduced to ecosystems where they are non-native (Lasenby et al. 1986; Spencer et al. 1999). *Mysis* exhibit diel vertical migration (Ahrenstorff et al. 2011), moving from the sediments into the water column to

feed at night. They exhibit a thermal range of approximately 3.5 to 10.2^{o}C (Boscarino et al., 2009), which constrains them to deeper waters in stratified lakes. When surface temperatures are approximately 10^{o}C or less, *Mysis* may migrate throughout the lake and occur closer to shore and in shallower waters (Paterson et al., 2011, Wang et al., 2012). In addition to being more active, they exhibit faster growth in the spring and early summer (Morgan 1981; Nero 1981). *Mysis* also exhibit light sensitivity and favor light ranges between 10^{-7} and 10^{-8} mylux (Boscarino et al,. 2010). These environmental thresholds limit the distribution of *Mysis* in lakes during summer to only cool, well-oxygenated waters (Nero and Davies, 1982), and cause them to exhibit diel vertical migrations with increased occupancy of the water column at night (Ahrenstorff et al. 2011). Thus, these environmental limitations make *Mysis* an excellent candidate for understanding how seasonal and daily variations in distribution might limit their eDNA distribution in the water column. While stream movement of *Mysis* has been documented elsewhere (Ellis et al. 2011), the persistence, preservation and transport of their eDNA in streams is not well understood.

The first objective of this study was to determine the distribution and persistence of *Mysis* eDNA in lentic ecosystems. Previous eDNA metabarcoding studies conducted in northwestern Ontario did not detect *Mysis* in lakes where they are known to be present (unpublished data, Joanne Littlefair). Metabarcoding, however, is used for multi species targeting and is less sensitive when compared to barcoding techniques which is primarily used for single species targeting (Ficetola et al,. 2015). Therefore, I used a barcoding approach to solely target *Mysis* in a subset of these lakes to determine whether their eDNA could be detected. Second, I sought to determine seasonal variation in the

distribution of *Mysis* eDNA in lotic ecosystems connecting lakes where *Mysis* are known to be present. *Mysis* occur throughout the water column in spring and fall when habitat is not limited by high surface water temperatures and low profundal oxygen (Boscarino et al. 2009). With temperatures permitting *Mysis* movement throughout the entire lake in spring and fall, I predicted that there would be better chance of detecting *Mysis* eDNA travelling downstream during these periods. In addition, I hypothesized that within individual strata, eDNA concentrations would shift in favour of more shallow depths during periods of darkness, and in turn exhibit higher concentrations at greater depths during the day. I also hypothesized that when *Mysis* occurred at shallower depths within the water column (e.g, during spring or fall), the concentration of their eDNA in these water layers would also increase. In the fall, there is also entrainment of deeper waters into the epilimnion with the breakdown of the thermocline, which may introduce *Mysis* eDNA into shallower waters (Schindler et at., 1980).

Methods and materials

Study Area

The IISD Experimental Lakes Area (IISD-ELA) is a collection of 58 pristine freshwater lakes in Northwestern Ontario. The lakes at this facility are primarily used for long term monitoring and whole ecosystem experiments (Blanchfield et al. 2009). The lakes are dimictic and surrounded by boreal forest tree species such as jack pine (*Pinus banksiana*), red pine (*Pinus resinosa*) and black spruce (*Picea mariana*). Most lakes at IISD-ELA are connected by streams, making possible the movement of organisms (or their DNA) between lakes. Four lakes and 3 streams from the IISD-ELA were sampled

during the summer and fall of 2019 (Figure 2.1). They were selected based on known presence of an established *Mysis* population, with the exception of Lake 223. Lake 223 (14 m maximum depth) is a relatively small, oligotrophic lake, that was acidified experimentally from 1976 to 1983 (Mills et al., 1987). Lake 223 was included as a pseudo-negative control, as *Mysis* were extirpated from the lake in 1979 due to acidification and is currently the site of a *Mysis* re-establishment experiment. Additions of *Mysis* to Lake 223 from upstream Lake 224 began in the spring of 2018, prior to which and to our best knowledge, the system had been free of *Mysis* for nearly 40 years. Lakes in the study varied in their physical features (Table 2.1). Streams flowing from the lakes had varying depths and pathways, with several having significant barriers to downstream movement such as beaver dams and marsh environments.

Lake sampling

Water samples for eDNA were collected at the deepest part of each lake in the autumn of 2019 (Table 2.2) when surface temperatures in all lakes were <10^{o}C. Prior to sample collection, a temperature probe was used to delimit depths of the epilimnion (Epi), metalimnion (Meta) and hypolimnion (Hypo). The middle of each estimated layer was used as the target depth for eDNA sampling (Table 2.2). A weighted sampling line constructed from 1/4" inner diameter clear vinyl tubing was lowered to the target sampling depth. Using a small peristaltic pump, water was pumped through the tube for a minimum of 2 minutes before filling a 36 oz whirl-pak. Whirl-paks were then placed into a large sealable sandwich bag, which was then immediately placed into a hard plastic cooler with ice packs. A total of 9 replicates were collected per sampling event (3

samples per water layer). Thermometers were also placed in coolers to monitor and ensure temperatures remained close to 4^{o}C during transport to the ELA laboratory.

To aid in minimizing contamination, several extra steps were included during the sampling process. Three sampling lines were kept in separate plastic bags and changed for a clean line before proceeding to sample a new water layer. In addition, bleach water (20%) was run through the pump for a minimum of five minutes, followed by rinsing with 2 L of deionized (DI) water for two minutes. Gloves were replaced when sampling each new layer, between touching new surfaces, or if gloves came into contact with water. To clean the sampling lines for subsequent days, 20% bleach water was pumped through the lines until they were filled and were then left for a minimum of 20 minutes to soak. Lines were then rinsed thoroughly with deionized water and dried using forced air. Epilimnetic water was sampled first, followed by the Metalimnetic and Hypolimnetic waters.

Mysis densities were obtained from lakes to determine correlations between *Mysis* abundance and concentrations of eDNA; *Mysis* densities from lakes 373 and 442 were provided by IISD-ELA staff from routine sampling in 2017. Lake 224 densities for 2017 were reported in Milling (2020). The lakes in this study were not sampled for *Mysis* densities every month at the Experimental Lake Area. The only time estimates aligned within a relatively same timeline across all lakes occurred in August and September of 2017. *Mysis* densities from all lakes were estimated using weighted average densities across all depth intervals to determine densities and abundances of the entire lake (Paterson et al. 2011). *Mysis* densities in 223 were estimated as the number added from Lake 224 prior to sampling in fall of 2019.

Stream sampling

Samples were collected at night from three sites along the stream that connects lake 223 and 224 (~ 250 m) during the last week of each month, from June to October 2019. Two additional streams were sampled during October from the outflow of Lake 373 that connects to Lake 375 (~325 m) and the outflow of Lake 442 to its connecting marsh (~200 m). Depths and temperatures were recorded for each sample (Table 2.3). The first sampling site in a stream was located at the head of the stream as it exited the lake (Figure 2.1). The second site was located at the midpoint of the stream between the inflow and outflow lake, and the third was situated at the inflow of the downstream lake. Three samples per site were collected using 36 oz whirl-paks. Samples were placed in a cooler with ice packs and transported back to the lab following the same procedures as the lake samples.

As an extra measure to prevent eDNA degradation, for both lake and stream samples, I placed ice packs in the cooler with the samples while they were being transferred back to the field lab. Samples placed immediately on ice have shown to exhibit significantly slower degradation rates after 6 hours when compared to samples left in ambient temperature (Yamanaka et al. 2016b).

Filtering procedures

Within 6 hours of collection, water samples were filtered in the laboratory through glass microfibre grade F filter paper, 4.25 cm diameter, with 0.7 μm nominal particle retention (Your Science Hub). Filters were placed in a 35 mL perforated porcelain Buchner funnel (Your Science Hub), which were then inserted into a triple

slotted vacuum manifold. Each sample was filtered separately, in the order they were collected in the field (epi, meta, hypo). One litre of sample water from each replicate was filtered on each separate manifold. Following filtration, filter papers were cut in half and placed in a sterilized 2 mL storage vial (Your Science Hub). To avoid contamination, all equipment was sterilized between filtering samples using an autoclave (if applicable) or 20% bleach water and rinsed thoroughly with DI water.

Extraction

Extraction of DNA for both lake and stream samples was conducted in the eDNA clean lab at McGill University in December 2019. Extraction of DNA from water samples was conducted using the DNeasy blood and tissue kit (Qiagen), with the following modifications: the addition of Qiashredder^{TM} spin columns, overnight incubation and increased amounts of reagents at specified steps (following Appendix S1 of Yates et al. 2020). Extraction blanks were conducted during every extraction session as a negative contamination control. For the final step, DNA was eluted into 130 µL of AE buffer and the final product was stored immediately in a -20^{o}C freezer. Samples were transported overnight on dry ice to Lakehead University and were again stored in a -20 ^{o}C freezer until the amplification process could be completed.

Amplification

A series of quantitative polymerase chain reactions (qPCR) were conducted on the extracted water samples using the ABI 7000 Sequence Detection System for real-time analysis (ThermoFisher Technologies). The species-specific primers and probes used for

this process were developed by Carim et al. (2016) from the Cytochrome oxidase subunit I (COI) region of *Mysis diluviana*. The *Mysis*_A forward primers, 5'-CCAGTGTTAGCAGGGGCTAT – 3' and *Mysis*_A reverse primers, 5' – CCCACCTACAGGGTCAAAGA – 3' (Integrated DNA Technologies) were used to target and amplify a 78 base pair(bp) amplicon (Carim et al. 2016). In addition, I used the *Mysis*_A TaqMan minor groove binding (MGB) probe 5' – TTTAACAGACCGTAATTTAA -3' (Life Technologies INC.), labelled with FAM dye. A quantitative polymerase chain reaction (qPCR) assay was conducted on the extracted samples and analyzed using the ABI 7000 Sequence Detection System for real-time analysis (ThermoFisher Technologies). Assays were conducted using clear 96 well qPCR plates sealed with heat bonding film layer.

Each extraction replicate was amplified in triplicate following a slightly modified qPCR formula (Carim et al., 2016). The qPCR chemistry included the following recipe: 0.75 μL 20x *Mysis* Assay, 7.5 μL Taqman Environmental Mastermix 2.0 (Life Technologies INC), 1.5 μL VetMAX™ Xeno™ VIC positive control DNA, 0.1 μL VetMAX™ Xeno™ VIC positive control assay, 1.15 μL distilled water and 4 μL of Extracted *Mysis* DNA for a final volume of 15 μL. Thermocycling conditions consisted of 95 °C for 10 minutes, and 50 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Using synthetic *Mysis* DNA comprised of gBlocks® Gene fragments (Integrated DNA Technologies), I created a standard series of positive *Mysis* DNA controls. The synthetic DNA was resuspended following the manufacturer's protocol and used to produce a standard dilution curve of 6250, 1250, 250, 50, and 10 copies per 4 μL reaction. Every qPCR assay that was conducted included a standard dilution curve to quantify *Mysis*

eDNA. Any sample that indicated inhibition from the internal positive controls (IPC) that may have affected the results was retested. The standard curve values (R^2) of the qPCR that corresponded with the eDNA abundance data for both lake and stream analyses averaged 0.97.

Statistics

All statistical analyses were conducted using the statistical computer program R (Version 4.0.2, R Core Team, 2019). An initial mixed effects model analysis was conducted to test the differences in day/night and water layer concentrations across all of the lakes. Generalized Linear Mixed effects Models (GLMMs) and General Linear Models (GLM) were run using the glmmTMB package followed by a Wald chi-square test to evaluate significance of model terms. Several distributions were compared such as poisson, gamma, negative binomial, and general poisson, in addition to the inclusion of a random effect. Akiake Information Criterion (AIC) was used to establish the model and distribution that best fit the data. This was determined to be a negative binomial distribution in addition to using lake as a random effect. Using the DHARMa package, statistical assumptions were evaluated by a nonparametric dispersion test of the residuals, diagnostics of the residuals which included a Kolmogorov-Smirnov test of normality and a plot of residual against predicted value to identify outliers or patterns in residuals. To test for differences in eDNA concentrations between day and night within individual lakes, a two-factor ANOVA was conducted to test concentrations of eDNA with the interacting effects of time of sampling (day/night) and water layer (epi, meta, hypo). Assumptions of normality and homogeneity were tested using Anderson-Darling and

Levene's tests, respectively, in addition to a visual evaluation of diagnostic plots of the residuals. Data from Lakes 224 and 373 were log transformed to satisfy both the assumption of normality and homogeneity. Critical alpha values were adjusted using a standard Bonferroni procedure to compensate for comparisons among the 3 lakes. Since only Lake 224 exhibited a significant interaction, I applied for a post-hoc test of pairwise differences among means, which was conducted using a Tukey Honestly Significant Difference (HSD) test.

The stream analysis was conducted in a similar fashion using a GLM and a Wald chi-square test to determine differences in seasonal stream eDNA concentrations for the stream connecting Lakes 224 and 223. The same distributions used in the lake analysis were compared in addition to testing if zero inflation was needed for the abundance of zeros present in the data. Using AIC, I established that a negative binomial distribution with zero inflation best fit the data. Assumptions of the GLM were again evaluated using the DHARMa package. I used a two-factor ANOVA to determine if eDNA concentrations differed among sites across all streams during the fall. With a significant interaction occurring between site and stream, I compared pairwise differences among means with a Tukey HSD test. A combination of diagnostic plots, Anderson-Darling and Levene's tests were again used to test assumptions of the two-factor ANOVA.

Results

Spatial distribution of Mysis eDNA in lakes

No eDNA abundance measures exhibited inhibition and all field extraction and PCR negative controls had no amplification. We detected *Mysis* eDNA through all thermal layers in lakes 224, 373 and 442 in fall samples (Table 2.2, Figure 2.2). Results from the Type II Wald chi-square test of the GLMM indicated no significant interaction between thermal layer and time of day ($\chi^2 = 2.16$; *df = 2; P*= 0.34). *Mysis* eDNA quantities (Copy/L) between day and night did not differ significantly ($\chi^2 = 0.15$; *df = 1; P*= 0.70), but there were significant differences in eDNA abundance among thermal layers ($\chi^2 = 8.91$; *df* = 2*; P*= 0.01). The maximum abundance of *Mysis* eDNA detected was in the metalimnion of lake 373 (77,102 copies/L), whereas the minimum *Mysis* eDNA detected in the metalimnion of lake 442 (516 copies/L; Table 2.2). The DHARMa nonparametric tests indicated all assumptions for the model were met and no outliers were present.

In Lake 224, eDNA abundance was $\log_{10}$-transformed to satisfy assumptions of normality and homogeneity of variance. A two-factor ANOVA revealed a significant interaction between time of day and water layers on *Mysis* eDNA quantities ($F_{2,12} = 6.89$, *P*= 0.01). Tukey post hoc tests indicated *Mysis* eDNA was greatest in the metalimnion, relative to both the epilimnion (*P* = 0.001), and the hypolimnion (*P*= 0.005), which did not significantly differ from each other (*P* = 0.48). Visual inspection would suggest that mean concentrations of *Mysis* eDNA increased at nighttime (Figure 2.2A).

In Lake 373, eDNA counts were also $\log_{10}$-transformed to satisfy assumptions of normality and homogeneity. In this case, the two-factor ANOVA revealed no significant

interaction ($F_{2,12}$ =0.58, P= 0.57), but a significant effect of water layer on *Mysis* eDNA quantity was observed (Figure 2.2B; $F_{2,12}$ =9.31, P= 0.004). We saw no significant difference in *Mysis* eDNA abundance between day and night ($F_{1,12}$ =2.65 , P= 0.13). Similar to Lake 224, Tukey post hoc tests again indicated *Mysis* eDNA was greatest in the metalimnion, in relation to the epilimnion(P = 0.023), and hypolimnion(P= 0.004). There was no significant difference between the epilimnion and hypolimnion(P = 0.56).

Unlike the other two lakes, Lake 442 did not require any transformation to meet assumptions of normality and homogeneity of variance. Similar to lake 373, the two-factor ANOVA revealed no significant interaction between layer and day/night on *Mysis* eDNA quantities ($F_{2,12}$ =0.1.49, P= 0.26), and there was no significant difference in eDNA concentration between day and night in Lake 442 ($F_{1,12}$ =0.64, P= 0.44). Like lakes 224 and 373, there was a significant difference among layers ($F_{2,12}$ =6.88, P= 0.01), but Lake 442 exhibited a different pattern than in Mysis eDNA concentrations among water layers compared with the other two lakes (Figure 2.2C). Tukey post hoc indicted a significant decrease occurred between the epilimnion and the metalimnion (P = 0.008). There was no difference in eDNA concentration in the hypolimnion when compared to the epilimnion (P = 0.4009) and metalimnion(P = 0.09), though nighttime densities of Mysis eDNA in the metalimnion did appear to be higher at night than in the day (Figure 2.2C).

No statistics were conducted on Lake 223 as most of the *Mysis* eDNA concentrations were undetectable (Figure 2.2D) and on average 1-3 orders of magnitude lower than those reported in other lakes (Table 2.2; Figure 2.2). No detections occurred during the night in either the metalimnion or hypolimnion, or during the day in the

epilimnion. The highest average concentration of *Mysis* eDNA of 214 copy/L was found in the metalimnion during the day. Very little eDNA was found in the epilimnion during the night and hypolimnion during the day.

Based on previous surveys, the density of *Mysis* in lake 373 is highest of the 4 lakes with a density of approximately 59 *Mysis*/m^2; which coincided with the highest amount of eDNA observed at a lake average of 31,415 copy/L (Table 2.3). The second highest was lake 224 with a *Mysis* density of 41 *Mysis*/m^2 and an eDNA lake average abundance of 5,404 copy/L. Lake 442 was the next highest with 18 *Mysis*/m^2 and a lake eDNA average of 3,636 copy/L. The lowest abundance of both *Mysis* (0.15 per m^2) and *Mysis* eDNA (18 copy/L) was lake 223. A Pearson correlation test on log-transformed data revealed that there was significant correlation between *Mysis* abundance against volume weighted eDNA concentrations at $\alpha = 0.05$ for both day and night ($r = 0.98$, $T = 3.5$, $P = 0.02$). The pattern indicated on a scatterplot show an association even among these few data points, which is potentially promising, however a larger sample size would be required to evaluate this association more thoroughly (Figure 2.3).

Spatial and temporal distribution of Mysis *eDNA in Streams*

In the stream connecting 224 to 223, results from the Type II Wald chi-square test of the GLM indicated that the abundance of *Mysis* eDNA differed significantly among months of sampling ($\chi^2 = 58.04$; $df = 4$; $P < 0.0001$; Figure 2.4). Concentrations increased in the stream from June to October with October exhibiting the greatest abundance of *Mysis* eDNA overall. Temperatures within the stream remained above the 10°C threshold of *Mysis* from June until September (Table 2.3). During October, stream temperatures

decreased to 8ºC. The depths of the sites also appeared to increase, particularly from August to September (Table 2.3), potentially suggesting increased flow. Minimal detections occurred in June and August with a maximum abundance of 828 and 446 copies/L respectively. We observed no detections in July and Tukey post-hoc analysis indicated no significant difference in *Mysis* eDNA concentrations among June, July and August (Z =+/-0.003, $P_{(\text{June, July})}$ = 0.9999). Between August and September, we observed a nearly 3-fold significant increase in *Mysis* eDNA (Z = 3.79, P = 0.0008). October *Mysis* eDNA densities were nearly 8.5 times higher than in September (Z =4.20, P = 0.0001). The DHARMa nonparametric tests indicated all assumptions for the non-parametric model were met and no outliers were present.

In all streams during autumn, *Mysis* eDNA was detected at all 3 outflow sample locations (Figure 2.5). The greatest amount of *Mysis* eDNA was observed midstream in the Lake 224 outflow during October (approximately 20,00 copies/L, similar to the value reported in Figure 2.4). A two-factor ANOVA revealed a significant interaction between sample site and stream ($F_{4,18}$ =6.85, P= 0.002). Among the streams, Tukey post hoc tests indicated a significant increase from the stream headwater to mid-stream stations (P < 0.0001). Additionally, there was a significant increase comparing the stream headwater and stream outflow into the next body of water (P = 0.013). There was no significant difference in eDNA concentrations between the stream mid-points and outflows combining all of the streams (P = 0.0866). Analyzing the streams individually, Tukey post hoc tests indicated a significant increase in *Mysis* eDNA in the Lake 224 stream from the headwater to midstream (P = 0.0085), followed by a significant decrease from the midstream to the outflow (inflow of Lake 223, P = 0.01). Lake 373 only has a

significant increase from headstream to midstream ($P = 0.002$), with no significant difference between the midstream and the outflow (inflow to Lake 375, $P > 0.05$). There were no significant differences in *Mysis* eDNA among all sites in Lake 442.

Discussion

My results revealed that *Mysis diluviana* eDNA can be found in high quantities in the lakes where they are present and in the streams that drain them. In lakes when conditions were low throughout the water column (i.e., autumn), *Mysis* eDNA was detected in high concentrations in all thermal layers and was particularly well represented within the metalimnion of 3 of the 4 lakes. Overall, abundances of *Mysis* eDNA appeared to reflect the diel movements and seasonal patterns exhibited by *Mysis.* My results also found potential correlation between *Mysis* eDNA abundance and density of *Mysis*.

From my lake results, the differences between day and night *Mysis* eDNA concentrations indicates there is the potential for differences among the individual layers during the fall when temperatures throughout the water column are optimal for *Mysis*. Contrary to what I initially believed, which was clear diel movement of the eDNA within the same layer, concentrations within the epilimnion and hypolimnion of Lake 373 and 442 were essentially the same. Lake 224 differed however and contained a significant interaction between day/night and layer. Even though the post hoc analysis indicated that statistically there were no differences in eDNA within the same layer between day and night, within the epilimnion of Lake 224, the concentrations appear to increase during the night. With Lake 223, I found that even at low densities, concentrations of eDNA increase at within the epilimnion and during the day was primary found within the

metalimnion. This general pattern was exhibited in both Lake 224 and 223 and what was originally hypothesized. Additionally, I found shifts in the overall pattern of mean abundances in *Mysis* eDNA particularly when transitioning from the metalimnion concentrations either decreased (Lake 224 and 373) or increased (Lake 442) to both the epilimnion and the hypolimnion. This was anticipated as *Mysis* are active throughout the water column when temperatures are low (Boscarino et al. 2009). In addition, as the thermocline degrades, it is possible that the entrainment of water from the hypolimnion causes upward movement of eDNA into the water column (Schindler et at., 1980). Based on the seasonal eDNA results in the stream that connects lake 223 and 224, daily patterns in *Mysis* eDNA with the lakes themselves are likely to vary with season as migration patterns are restricted to the bottom of the lake when epilimnetic temperatures are higher (Boscarino et al. 2009). This similar pattern has been illustrated at the ELA by lake trout, which like *Mysis*, exhibit seasonal eDNA thermal restrictions (Littlefair et al., 2020).

My results indicate that *Mysis* eDNA may be influenced by the density of *Mysis* within each lake, a pattern that has been well documented for other aquatic organisms, mainly fish (Chambert et al. 2018; Spear et al. 2021). *Mysis* eDNA counts among the 4 lakes matched their respective overall ranking of the *Mysis* densities. In addition, we found that *Mysis* densities correlated with volume weighted eDNA concentration estimates for both day and night. However, it is important to note only four lakes were used in this study and is a big limitation to my study. Future studies should look to incorporate more lakes. As noted in the methods, the only *Mysis* density estimates available that aligned within the same month occurred in August of 2017. *Mysis* densities may fluctuate considerably from year to year (Audzijonyte and Väinölä 2005, Paterson et

al,. 2011, Rennie et al,. 2019). Lake 224 in particular has reported historical densities of *Mysis* as high as 595/m^2 (Nero and Schindler 1983). More recently, from 2014-2017, monthly *Mysis* estimates decreased from an average of 139 m^2 to 70 m^2 over the four year time span (Unpublished data, Andrew Milling). This illustrates the degree to which *Mysis* densities may vary naturally and that the densities estimated from 2017 may not accurately represent the population in 2019 when the eDNA survey was conducted. In addition, *Mysis* densities vary considerably within years (Paterson et al., 2011). This leaves the possibility that the densities may be considerably larger in lake 373 in 2019 (relative to the 2017 estimates to which they are compared) and explain the larger amount of eDNA observed when compared to *Mysis* abundance estimates in the rest of the lakes.

Seasonal patterns in the stream leaving Lake 224 (which supports *Mysis*) in combination with the eDNA concentrations found throughout the water column in fall suggest lake-wide seasonal changes in eDNA distribution within the water column. Research at the IISD-ELA has already shown that seasonal eDNA distributions for cold water species like lake trout follow their thermal restrictions (Littlefair et al. 2020). During the summer, lake trout eDNA was primarily limited to the deeper, colder parts of the lake, but was observed throughout the water column in fall when water temperatures fell below the thermal optima for this species (Littlefair et al. 2020). Very similar trends can be inferred for *Mysis* eDNA in the current study. Based on knowledge of *Mysis* distribution during the fall seasons, I expected to observe a shift in their eDNA to favour presence in the shallower strata of the water column at night. Overall, Lake 224, 373 and 223 (qualitatively) exhibited a similar overall pattern with the highest concentrations found in the metalimnion. Lake 442 however had lower concentrations in the

metalimnion relative to the epilimnion or hypolimnion. Not including Lake 223, the difference between lakes may be due to lake 442 being much shallower in comparison to 224 and 373. In addition, Lake 442 was much closer to turnover at the time of sampling, with colder temperatures more uniform throughout the water column compared to the other lakes included in this study, causing the thermal stratification among water layers to be less defined in Lake 442. When separation among the thermal layers was more evident, lower concentrations of *Mysis* eDNA were observed in the epilimnion of Lake 224 and 373 when compared to Lake 442. This may indicate greater shifts in eDNA among water layers as lakes become more isothermal. The inverse can also be assumed as temperatures increase during summer months and the stratification becomes more defined, we would predict to see the majority of *Mysis* eDNA shift towards the deeper parts of the lake within the hypolimnion, relative to the epilimnion or metalimnion.

During October, I detected *Mysis* eDNA in the streams sampled at ELA, and at all reaches regardless of distance from source lakes known to support *Mysis.* I expected that eDNA concentrations would be highest at the initial outflow site, then decrease further down the stream to the next two sites. This however was not observed, as I instead saw an increase in downstream concentrations in streams relative to those at the outflow of the upstream lake known to support *Mysis*. Other studies have reported similar findings of consistent high detections throughout every site downstream from the original source during times of high flow (Jane et al. 2015). The trends between the outflowing streams of lake 224 and 373 were nearly identical; the increase in eDNA concentration from the first site at the initial outflow to the second site was essentially the same, followed by a similar downward trend observed from the second site to the last site. Having the eDNA

in two out of the three streams showing the same similarity indicates the potential for downstream movement of eDNA. Based on the morphology of the streams, which include beaver dams and sections cascading over boulders, it is more likely that the eDNA detected is not from movements of *Mysis* through the streams. Lake 442 does not display the same trend in the second site, and this may be due to two reasons. First, whereas the outflows of lake 224 and 373 are unidirectional and flow to a downstream lake, Lake 442 has several branches throughout the stream but appeared to come together at a single point and intersect into a wetland. I believe that the branches may divide or disrupt the eDNA after leaving the headstream due to the varying flows and water levels, then when the branches congregate into one, so does the eDNA. Having the branches of the stream come together at a single point may be why we see the eDNA in the stream of 442 increase to a range similar to Lake 224 and 373 outflows. If Lake 442 had possessed a unidirectional outflowing stream, I believe all three streams would have had exhibited similar patterns of higher counts of an increase of *Mysis* eDNA to the midstream followed by a slight decrease to the outflow.

While there is evidence from other systems of *Mysis* being transported via streams between lakes (Spencer et al. 1999), no evidence of this movement has been documented at ELA, where Lake 223 has been devoid of *Mysis* since their extirpation there in 1979, until they were intentionally reintroduced in 2018. *Mysis* eDNA in streams varied seasonally, and the overall abundance appeared to increase during the times of the year (spring, fall) when higher *Mysis* activity in shallower waters is expected (Boscarino et al. 2009; Paterson et al. 2011). I observed minimal detections in June and August and no detections in July for the Lake 224 to 223 stream, likely in part due to the fact that

samples were collected towards the end of each month as the end of the summer months at the ELA generally have warmer water temperatures. This is most likely due to thermal limitation of *Mysis* that prevented them from occupying the epilimnion (Boscarino et al. 2009). In addition, water levels in the stream were lower in summer months compared to fall. As temperatures began to decrease in September, I saw an increase in eDNA in the stream, and eDNA counts increased significantly in October when thermal conditions were optimal for *Mysis* occupation of the epilimnion in L224. During the fall we also saw higher water levels, indicative of higher flow.

Observations of such large quantities of *Mysis* eDNA in the streams was unexpected. As discussed in the third chapter of this thesis, eDNA is known to readily bind to organic material (Crecchio and Stotzky 1998; Shi et al. 2015; Senapati et al. 2018), which provides protection from degradation during sedimentation. In addition, eDNA is more concentrated in sediment compared to aqueous eDNA (Turner et al. 2015). Studies have shown small organic particles are transported at higher rates during times of high flow (Bilby and Likens 1979; Wipfli et al. 2007). From the depths observed seasonally, the streams appeared to nearly double in depth across all three sites from August to September. During these times of high flow and high *Mysis* eDNA observed in the water column, it is possible that *Mysis* eDNA binds to organic particles, potentially assisting in transport outside of their source lake. As stream flow decreases, organic particles (with eDNA potentially attached) settle into sediments. When flow increases, eDNA bound to organic particles may be re-suspended and carried further downstream (Fisher et al. 1983; Cushing et al. 1993). Given that *Mysis* can be found more frequently in shallow waters closer to the mouth of the headstream in the fall and spring, their

presence in surface waters during this time may facilitate their downstream transport (Wang et al. 2012). Increased occupancy in epilimnetic waters combined with increased stream levels/flow may be the reason for the high concentrations observed in the streams during the fall period under observation. Given that concentrations of eDNA began to increase in September, when stream temperatures were still around 15^{o}C, previously stated is higher than the thermal range of *Mysis* (Boscarino et al. 2009). This is a good indicator that it is the eDNA of *Mysis* moving through the streams as opposed to *Mysis* moving through streams themselves.

There appears to be no correspondence between the epilimnetic concentrations of *Mysis* eDNA and of the concentrations found at the immediate outflow. In Lake 224, concentrations at the first site of the outflow were higher relative to the lake concentrations of in the epilimnion. This difference may represent the near shore migration pattern of *Mysis* during the fall in addition to the distance of center buoy being relatively further from shore and the outflow when compared to the other lakes. Contrary to Lake 224, *Mysis* eDNA concentration in Lake 373 decreased significantly from the epilimnetic concentration to the outflow. The beginning of the outflow in Lake 373 however, is not as pronounced and contains of wall of debris and vegetation in which the water from the lake flows through to enter the stream and in turn acting as a filter and preventing the epilimnetic concentrations of eDNA from entering the stream. The first site of the outflow of Lake 442 did not have any obstructions and was fairly close to center buoy may be why the epilimnetic concentrations are similar.

The eDNA abundances in my study are two to twelve times higher when compared to the results of Carim et al. (2016), who measured *Mysis* eDNA

concentrations across several lakes in Colorado, USA. There are several differences between the two studies that may explain these differences. First, Carim et al. (2016) used a 1.50 μm filter paper compared to the 0.7 μm filter used in this study. By using the smaller pore size paper, it takes longer to filter a 1L sample, which increases the potential for the eDNA to degrade (Li et al. 2018). However, with the risk of the filters clogging more quickly, the smaller pore size used in this study has the potential to capture smaller sized particles or strands of *Mysis* eDNA (Eichmiller et al. 2016c, Prepas and Trimbee 1988). Second, Carim et al. (2016) primarily collected water samples from June to July when larger portions of *Mysis* populations are known to be on the bottom when compared to fall, which can reduce their detectability (Boscarino et al. 2009). On average, the densities of *Mysis* observed in this study were 3 to 10 times higher than previous studies on other lakes (Carim et al., 2016). Additionally, the surface area of the lakes used in my study were 5 – 109 times smaller in terms of surface area when compared to the lakes described in Carim et al, (2016). This may suggest that the eDNA in the lakes used by Carim et al (2016), are more dispersed due to the larger surface area and volume of their lakes where my lakes are more spatially condensed. This may potentially explaining why we observed significantly higher amounts of *Mysis* eDNA. These questions could be answered with future spatial studies comparing eDNA of similar sized lakes.

Great caution was taken to ensure the eDNA during all steps was stable and degradation was minimal. Due to the small pore size of the filter paper used, the filtering process was slow at times with an average filtering time of 1 hour per 1L sample, ranging between 15 minutes to 2.5 hours. The overall time from sample collection to the completion of sample filtering and storage in the freezer ranged from 1 to 9.5 hours with

an average of 4.2 hours. Studies have shown that in the time between sample collection and filtration, eDNA can degrade (Yamanaka et al. 2016a; Hinlo et al. 2017). Under natural conditions, sturgeon (*Acipenser baerii*) eDNA has shown that it was no longer detectable in a pond after as soon as 6 days (Dejean et al. 2011). Hinlo et al, (2017) found a significant degradation of eDNA at room temperature after 48 hours. My sampling times had a maximum time of 9.5 hours, well under the 48 hour limit of degradation reported by Hinlo et al. (2017).

There was little evidence of contamination in my study, with an absence of detections in field and lab blanks. Although there is always a chance of contamination during laboratory processes, I took several steps to avoid this possibility. The used of 96-well plates is standard practice; however, well-to-well contamination is a common occurrence (Walker 2019). In many cases, when contamination occurred, it was only present in 1 or 2 wells of several negative controls, and never consistent in the extraction negative. When a contamination in a negative control was detected, results from that plate were discarded and the entire assay was run again until the contamination was no longer present. This suggests that any instances of contamination that occurred during my analysis was due to droplets from accidental pipetting cross contamination, which was ultimately accounted for. If contamination had occurred during the sampling process, the filtering and extraction blanks would have consistently exhibited detections, which was not observed. Only plates where negative controls exhibited no amplification in either the field blanks or PCR negatives were used in my results.

Due to the unforeseen occurrence of the COVID-19 pandemic, there were several avenues that I was unable to pursue. Lake sampling was planned for the spring and

summer of 2020, to provide a comparison of *Mysis* eDNA distribution in the water column to the fall distributions reported here. I also planned to collect stream samples in the spring to determine if *Mysis* eDNA would have yielded similar results to what I observed in the fall (since temperatures would have also been tolerable throughout the water column for *Mysis*). There were also plans to collect water samples from Lake 302, another lake situated in the IISD-ELA, which would have acted as my negative control for the lake and stream water samples as this lake does not contain (and as far as we know has never contained) *Mysis* (Mike Paterson, personal communication). Therefore, my best and closest option of a negative control was lake 223. Though using 223 as a negative is not a true negative as there was still a faint presence of *Mysis* due to the current addition efforts and inflow from Lake 224. However, the population of *Mysis* in Lake 223 was much lower compared to any of the other lakes surveyed in this study. Only one *Mysis* has been observed during regular sampling at IISD- ELA in the two years the addition efforts have taken place. The quantities that I observed in lake 223 do not indicate anything to suggest that my results from any of the lakes should be over-detecting *Mysis* or amplification of a non-target species. In addition to ensure species specificity and mentioned chapter three, the *Mysis* primers were tested against genomic DNA of *Hemimysis anomala* and crayfish (*Orconectes virilis*) as it is the closest taxonomically related species to *Mysis* at the IISD-ELA

In conclusion, I found significant differences in *Mysis* eDNA abundance between the thermal layers, but no significant differences between fall day and night *Mysis* eDNA concentrations within the same thermal layers, and no significant interactions between thermal layer and time of day in Lakes 373 and 442. This study provides insight into the

persistence of *Mysis* eDNA within the water column over short time periods (e.g., between periods of daily vertical migrations). Overall patterns, however, suggest daily vertical migrations of eDNA may occur and can be detected with eDNA. This can potentially be applied to other organisms that may share the same diel migration patterns, such as lake trout (Sellers et al. 1998; Littlefair et al. 2020). In particular, the findings of this study indicate that the movement of eDNA through streams during the fall may increase the potential for false positives of *Mysis* in downstream lakes where *Mysis* may not be present. The next step is to determine seasonal diel movement of *Mysis* eDNA.

Tables

Table 2.1: Details for the lake survey sites at the Experimental Lakes Area.

Lake	Coordinates (dd.ddddd)	Size (ha)	Max Depth (m)	Volume (m^3)
224	N 49.69000 W 93.71694	25.4	26.7	$3.0x10^6$
373	N 49.74481 W 93.79913	27.3	20.8	$3.0x10^6$
442	N 49.77552 W 93.81754	14	17.8	$1.4x10^6$
223	N 49.698333 W 93.70778	27.3	14.4	$1.9x10^6$

Table 2.2: Field collection summary and average *Mysis diluviana* eDNA estimates (with standard deviation) for four Lakes at the Experimental Lakes Area. Estimates are averages of three sample replicate in addition to three qPCR replicates for each sample. *Mysis* densities of Lake 224 and 373 are from August 2017 (personal communication, A. Milling and M. Paterson) Lake 223 densities are based on the number of *Mysis* added from May 2018 to time of sampling. Depth of strata represents the width of each thermal layer and Depth of sample is the depth in which the eDNA sample was collected.

Lake	Approximate Density (m^{-2})	Collection Date (time)	Time of Day	Depth of strata (m)	Depth of sample (m)	Temperature (^{o}C)	eDNA quantity (Copy/L)	Standard deviation
224	40.96	Oct.21 2019 (15:30)	Day	Epi (11)	3	9.4	2082	1412
				Meta (1)	12	8.8	9090	400
				Hypo (16)	21	5.2	4960	1216
		Oct.21 2019 (5:30)	Night	Epi (11)	3	9.4	4982	1600
				Meta (1)	12	8.8	8297	2074
				Hypo (16)	21	5.2	3021	925
373	59.48	Oct.20 2019 (15:30)	Day	Epi (11)	3	8.8	26041	3586
				Meta (1)	12	8.3	55276	19381
				Hypo (8)	18	5.3	24136	5363
		Oct.20 2019 (5:30)	Night	Epi (11)	3	8.8	25066	4175
				Meta (1)	12	8.3	38817	22213
				Hypo (8)	18	5.3	19155	4076
442	18.2	Oct.19 2019 (17:00)	Day	Epi (8)	3	8.8	4722	1039
				Meta (2)	8	8.5	1527	880
				Hypo (7)	12	5.5	4035	1552
		Oct. 19 2019 (6:00)	Night	Epi (8)	3	8.8	4676	1108
				Meta (2)	8	8.5	3201	381
				Hypo (7)	12	5.5	3655	1289
223	0.15	Oct. 22 2019 (16:30)	Day	Epi (9)	3	8.9	0	0
				Meta (1)	10	8.6	214	80
				Hypo (3)	12	6.9	18	31
		Oct. 22 2019 (6:00)	Night	Epi (9)	3	8.9	56	96
				Meta (1)	10	8.6	0	0
				Hypo (3)	12	6.9	0	0

Table 2.3: Details for the stream survey sites collected from the stream that connects lake 224 and 223. Samples were collected towards the end of every month from June until October 2019. Temperatures and depth of site were also collected, not including July.

Month	Site	Temperature (C^{o})	Depth (cm)
June	1	17	12
	2	17	5.5
	3	12	3
August	1	13	38
	2	13	10
	3	13	9
September	1	16	48
	2	15	21
	3	14	13
October	1	8	49
	2	8	20
	3	8	16

Figures

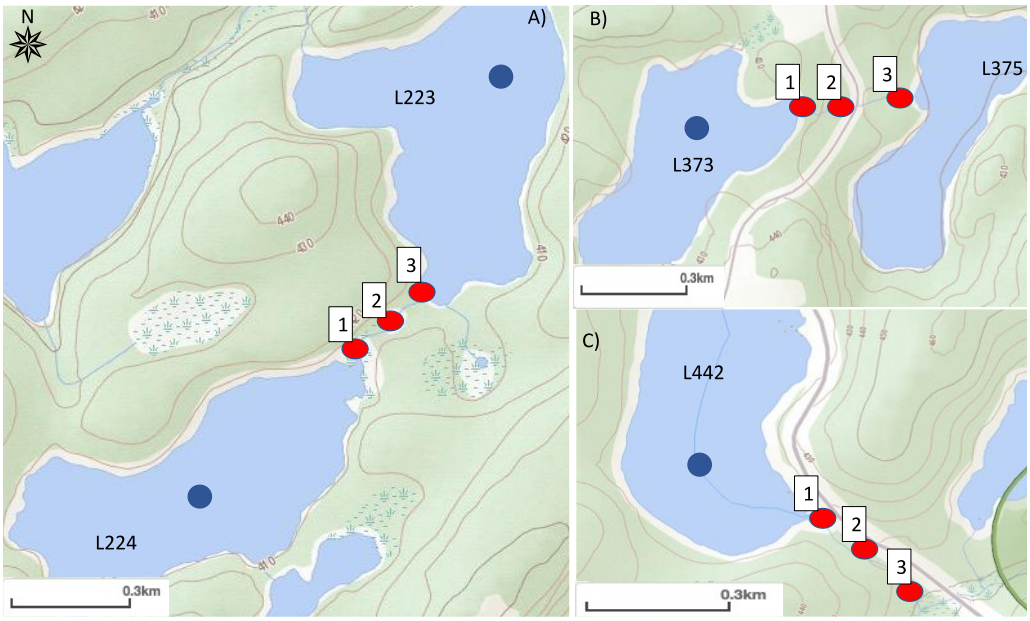

Figure 2.1: Maps of lake and stream survey sites for A) Lake 224, B) 373 and C) Lake 442. Blue dots represent Lake eDNA sampling site (at the deepest part of the lake) and red dots represent stream eDNA sampling sites. Streams flow in the direction of increasing site number. Map and elevation data obtained from ontario.ca.

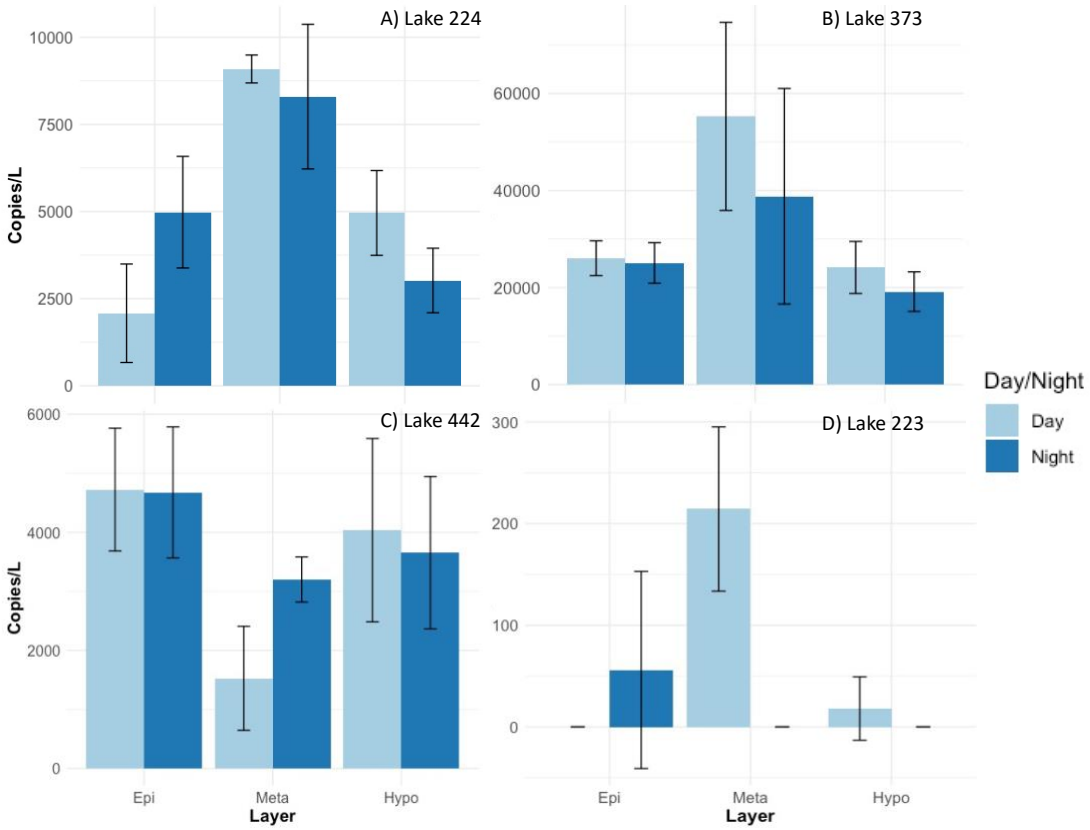

Figure 2.2: Bar plots of *Mysis diluviana* eDNA abundances observed in Lakes 224, 373, 442 and 22 during October 2019, at the IISD - Experimental Lakes Area. Samples were collected from the same depth in the epilimnion, metalimnion and hypolimnion approximately 12 hours apart. Abundances are measured in copies of *Mysis* eDNA per litre with each lake exhibiting a different scale on the y axis. Bars represent the standard deviation around the mean eDNA values.

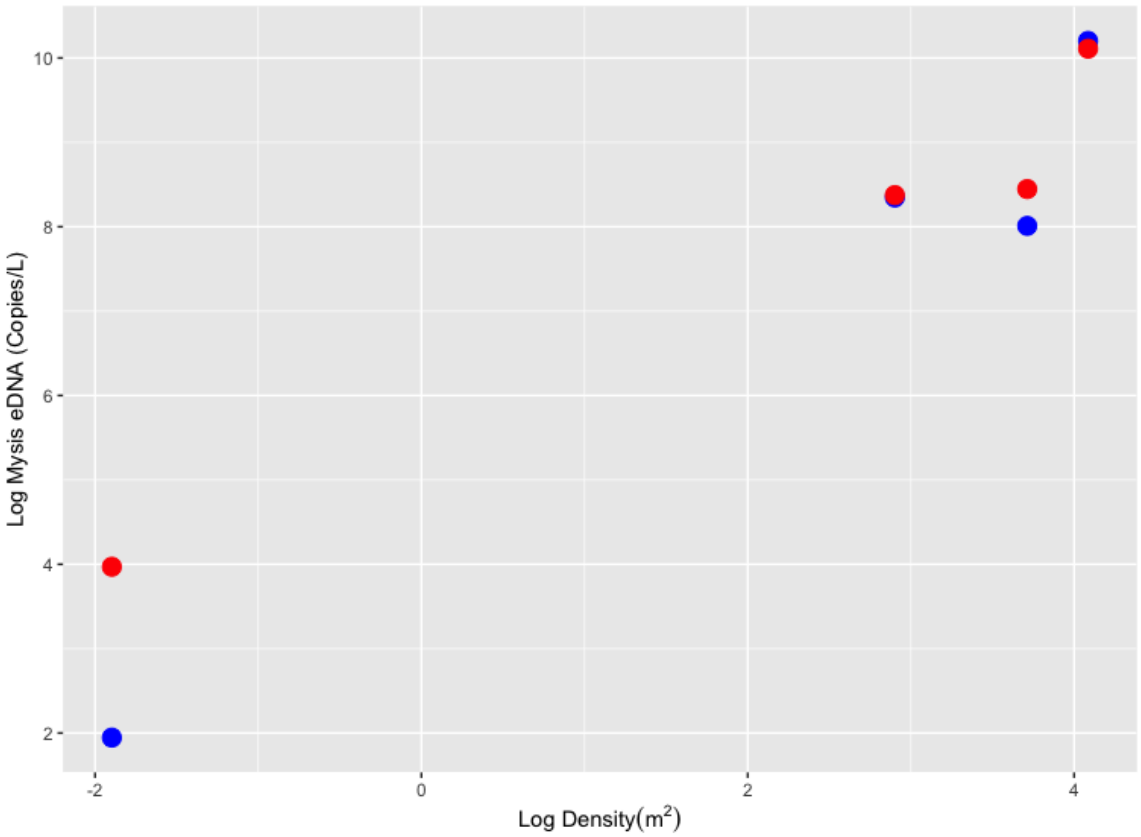

Figure 2.3: Scatterplot of association between day (red) and night (blue) abundance of *Mysis diluviana* eDNA (copies/L) and the approximate density of *Mysis* (m^2). Concentration of *Mysis* eDNA are volume weighted values of each lake. The data for both *Mysis* eDNA abundances and *Mysis* densities were log_{10} transformed.

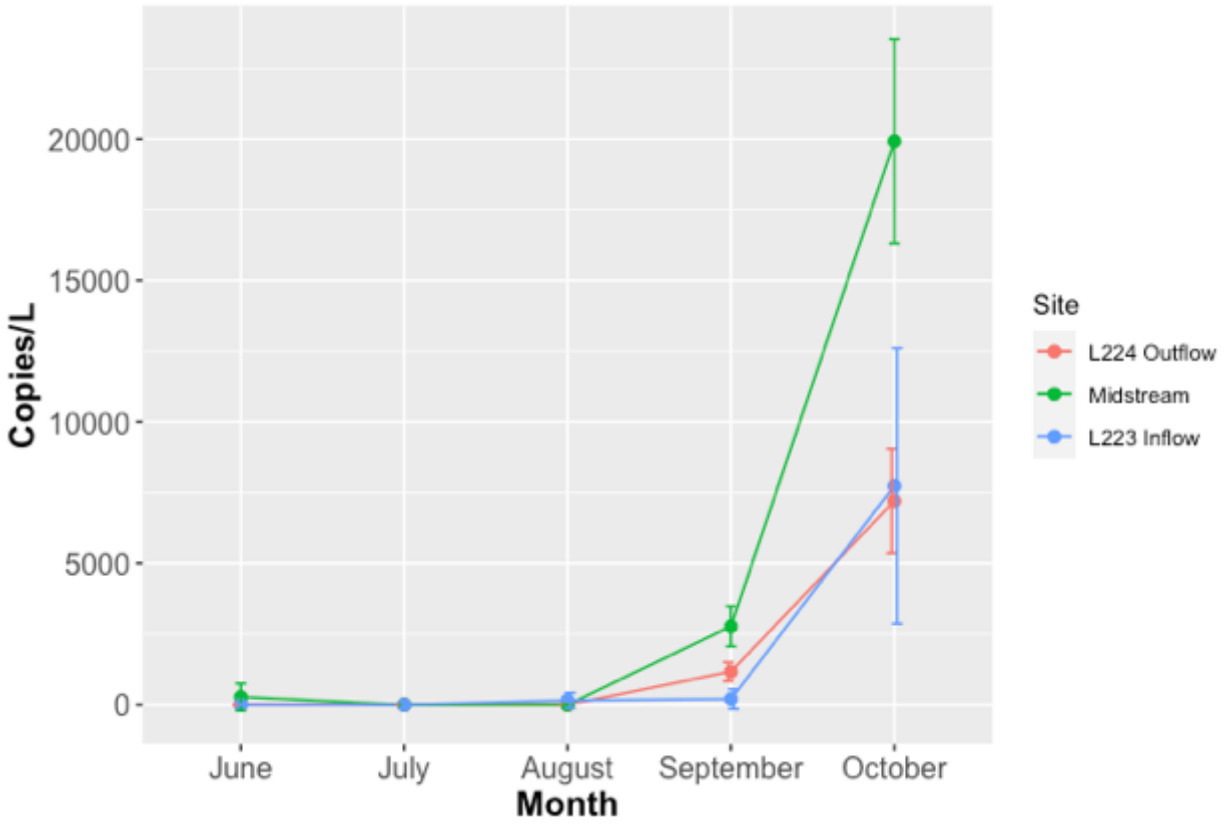

Figure 2.4. Plot of *Mysis diluviana* eDNA abundances (copies/L) observed from June to October at three sites along the stream flowing from Lake 224 to 223.

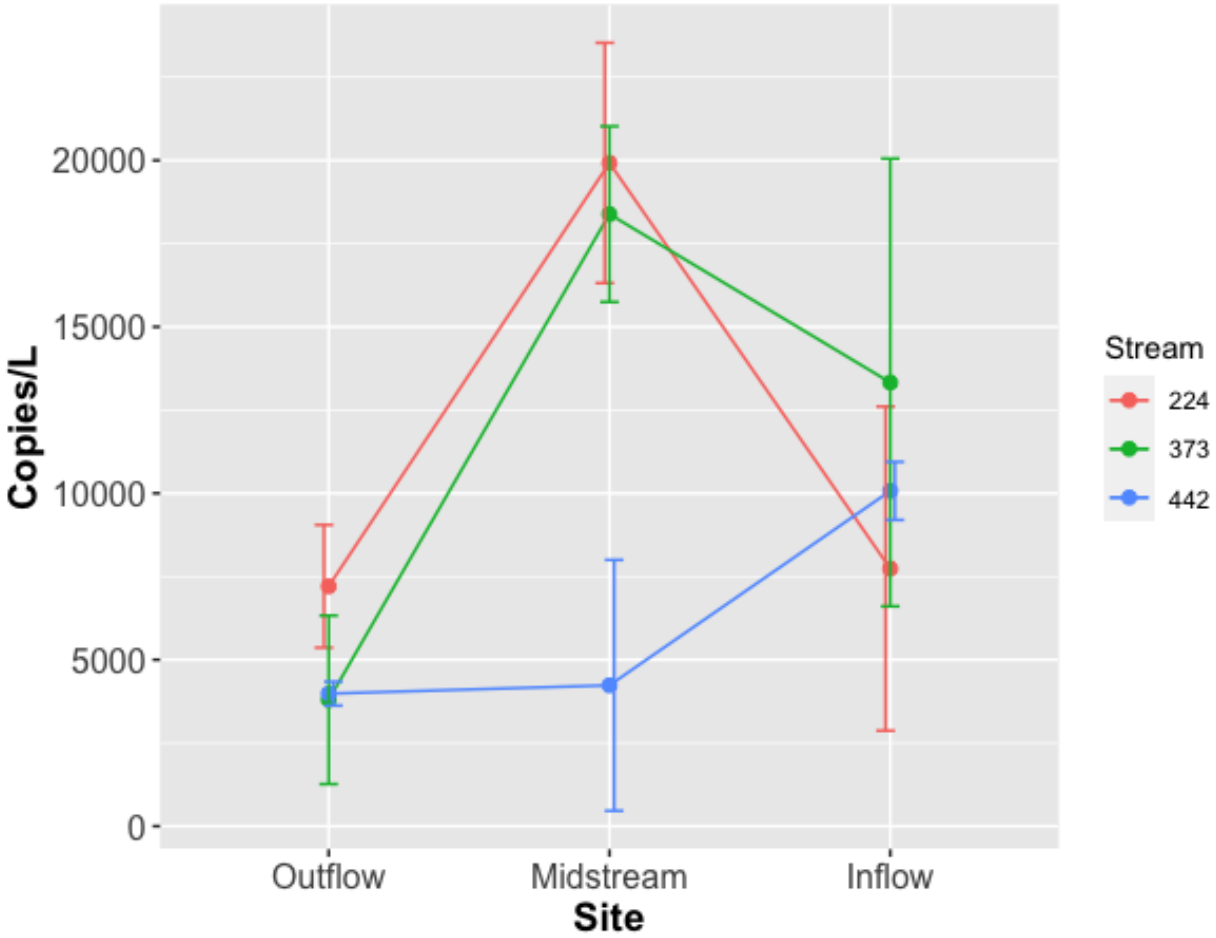

Figure 2.5. Plot of *Mysis* eDNA abundances observed in 3 streams during October 2019, at the Experimental Lakes Area. Abundances are measured in copies of *Mysis* eDNA per litre. Bars represent the standard deviation around the mean eDNA values. When comparing to map, site 1= outflow, site 2= midstream and site 3= inflow.

Chapter 3:

Preservation of *Mysis diluviana* eDNA in Lake Sediment

Abstract

Environmental DNA (eDNA) is a useful tool for the detection of rare species in environments. The use of eDNA in lake sediment (sedDNA) can demonstrate past changes in biological community composition, and potentially be used to provide restoration guidelines for impacted ecosystems. In this study, I describe the preservation of *Mysis diluviana* eDNA in lake sediments. *Mysis* are small freshwater crustaceans that inhabit the Laurentian Great Lakes and many deep, cold-water lakes across North America. *Mysis* eDNA was extracted from gravity cores and from ^{210}Pb-aged freeze cores collected from sediments of three lakes at the IISD-Experimental Lakes Area in Northwestern Ontario, using a *Mysis*-specific primer and quantitative polymerase chain reactions (qPCR) to evaluate eDNA preservation and down-core occurrence. My results revealed that *Mysis* eDNA well preserved in sediment cores and downcore patterns of *Mysis* eDNA closely matched a known extirpation event. My work indicates the power of this method to provide insight into historical aquatic communities, as well as the potential of the method to provide restoration guidelines for impacted ecosystems where biological reestablishment might be attempted.

Introduction

As more becomes known about the scope and extent of anthropogenic impacts on aquatic ecosystems, management efforts often change focus to the restoration of impacted ecosystems. An example of this progression from impact to restoration has been seen in lakes around Sudbury, Ontario, which suffered from decades of acidification and metal contamination from nearby metal smelters. These lakes have been recovering for nearly 50 years and are still undergoing active monitoring (Keller et al., 2019). In many cases, such impacts pre-date any information on the ecosystems that have been disturbed. Without these historical data, it is often difficult to fully understand the impacts that past anthropogenic events may have had on freshwater ecosystems. As these ecosystems recover chemically, biological recovery often lags behind (Keller 2009) and biological restoration targets are not always obvious.

To establish viable restoration targets, knowledge about past ecosystems is required; however in many cases environmental baselines are unknown. When long term observations are not available, indirect measures of target organisms may be inferred from deposits of organisms left behind in sediments (Smol 1992). For instance, the presence of *Chaoborus* (phantom midge) mandibles can infer presence of fish species (Lamontagne and Schindler 1994). Similarly, *Daphnia* leave behind their ephippia in sediments, and cores and can be used to quantify historic size and abundance of planktivorous fish (Jeppesen et al., 2002). Other organism such as diatoms, other adult cladocerans, and dipteran larvae can be used to infer historical environments (nutrient concentrations, oxygen, etc.) (Frey 1988). The primary reason researchers can take this indirect approach is because these organisms leave physical remnants that preserve in

lake sediments. Even with the presence of physical remains, it can be difficult to make indirect inferences of past presences of closely associated species without historical data (Lamontagne and Schindler 1994). However, not all organisms have structures that preserve within sediments.

Within the past decade, environmental DNA (eDNA) research has increased considerably. Environmental DNA is a broad term that incorporates several areas within eDNA research such as aqueous, air, soil and sediment eDNA (Pawlowski et al. 2020). Sediment eDNA (sedDNA) describes genetic material that has accumulated in sediments of freshwater and marine ecosystems (Barnes and Turner 2016; Cheong et al. 2020). In sediments, eDNA may originate form organisms living within the sediment such as microbes or from eDNA that has settled from animals in the water body above (Ogram et al. 1987; Pietramellara et al. 2009). Studies have implied that eDNA surveys should include both sedDNA and aqueous eDNA as they provide complementary information about target species' distributions (Sakata et al. 2020).

While primary biodiversity surveys focus on aqueous eDNA for conservation purposes and early detection of invasive species (Jerde et al. 2013; Thomsen and Willerslev 2015), there has been a recent increase in studies evaluating sediment eDNA as a means of reconstructing historic species presence. For example, Nelson-Chorney et al., (2019) found evidence of fish species previously considered to be non-native that actually pre-dated known human introductions.

Mysis diluviana ("*Mysis*" henceforth) is a small freshwater crustacean, located widely throughout the boreal ecoregion in Canada (Walsh et al., 2012). Considered a "glacial relict", *Mysis* have inhabited northern continental lakes since the last glaciation

(Audzijonyte et al., 2005). *Mysis* are important food for fish such as lake trout (*Salvelinus namaycush*) that reside in deep cold-water lakes (Ellis et al., 2011), and are frequently considered a keystone species in lakes they inhabit (Boscarino et al. 2007; Stewart and Sprules 2011; Rogers et al. 2014, Spencer et al. 1999). When introduced to non-native lakes, *Mysis* may alter zooplankton communities and directly compete with native fish (Lasenby et al. 1986; Spencer et al. 1999). Community alterations may also occur if *Mysis* are removed from a system. During the late 1970's, a lake in Northwestern Ontario was experimentally acidified, resulting in the extirpation of *Mysis* in the fall of 1979 (Nero and Schindler 1983). Following the extirpation of *Mysis*, changes in both fish and zooplankton biomass occurred (Schindler et al. 1985; Milling 2020). Given the significant role they play in native ecosystems, it makes *Mysis* an ideal biological target for restoration of anthropogenically disrupted ecosystems where they were previously native.

If a goal of habitat restoration is to use historical species assemblages as a target for ecosystem recovery, attempting to determine which species should be included as restoration targets can be challenging without historical data. Until now, this has been particularly problematic for soft-bodied organisms that do not leave behind physical remains in lake sediments, which include *Mysis*. A test of the preservation potential of 20 individuals of *Mysis* in lake sediments after 16 months retained only a single mandible (Tracy & Vallentyne 1969). Given this lack of physical preservation in sediments, eDNA represents a potential means for detecting the historical presence of *Mysis*, and therefore other organisms that do not leave behind physical remains.

Methods for analyzing *Mysis* eDNA in lake sediments have not yet been established. To determine the effectiveness of using sedDNA as a method for evaluating historical presence, I aimed to improve upon previously published extraction protocols applied to lake sediments for other target organisms to determine the reliability of detecting *Mysis* sedDNA. Next, I used those methods to investigate historic distributions of *Mysis* within aquatic ecosystems at IISD-ELA. The first objective of this study was to investigate the use of eDNA for monitoring historic distributions of invertebrates, like *Mysis*, by using a target-specific primer and probe combination to amplify sedDNA using the quantitative Polymerase Chain Reaction (qPCR) process. Due to their abundance found within a lake, I hypothesized that the detection of *Mysis diluviana* eDNA within sediment cores would be achievable. With the optimization of eDNA extraction and amplification methods, I sought to determine the depth to which *Mysis* can be detected within sediment cores. My second objective was to determine if the downcore pattern of *Mysis* eDNA detections matched the known historical patterns of occurrence in (a) lakes known to support *Mysis* and those where they are absent, and (b) a lake from which *Mysis* were extirpated as a result of a whole-lake acidification experiment.

Materials and Methods

Study Area

Three lakes within the IISD - Experimental Lakes Area were sampled for sediments (Table 3.1). Lake 224, the primary positive control lake of the analysis, has an abundant and well-documented population of *Mysis* (Nero and Schindler 1983; Milling 2020). Lake 223 is currently undergoing a *Mysis* reestablishment experiment that began in the spring of 2018. Sediment cores from this lake are ideal for validation of historical detections as *Mysis* were present in the lake prior to known extirpation in 1979 following a whole-lake acidification experiment (Nero & Schindler, 1983; Mills et al. 1987). Lake 302 sediments were used as the negative control since *Mysis* have never been found to occur there in previous surveys (Michael Paterson, Personal Communication).

Sediment sampling

Both freeze core and gravity core sampling methods were utilized, and all sediment samples were collected at the deepest regions of the lakes. Gravity cores were used for method refinement, troubleshooting and preliminary evaluation of downcore patterns, whereas dated freeze cores were used to determine precise patterns in eDNA occupancy and distribution. Using a NLA gravity coring apparatus with a 68mm polycarbonate barrel, samples were collected through the ice from Lakes 223, 224 and 302 in February 2019. From each core, 1 cm slices were collected and placed in labelled whirlpak bags, beginning with the sediment water interface and working down to the bottom of the core. Cores slices were stored at -20^{o}C until eDNA extraction. Freeze cores were sampled from Lakes 223 and 224 through the ice in February 2018 (prior to *Mysis* introduction). A 1.2

x 0.1 x 0.2 meter aluminum box insulated with blue polyethylene foam on three sides was filled with crushed dry ice and methanol; the box was deployed from the surface and left in the sediments for 30 minutes to freeze the sediment in place (Crusius and Anderson 1991). Cores were taken as frozen slabs and sectioned back at the field station using a band saw at 1 cm intervals. Core slices for Lake 223 were collected in 1cm increments up to 22cm, and at 2cm increments thereafter. Sediment that was directly in contact with the corer was removed before analysis. Freeze core samples were divided, with a portion being used for ^{210}Pb radioisotopic analysis to determine sediment age, and another portion for *Mysis* eDNA analysis. Freeze cores were exclusively used for dating to prevent degradation of eDNA, and also because freezer cores are known to have greater accuracy for dating when compared to gravity cores (Crusius and Anderson 1991).

Core dating analysis was conducted by Flett Research Ltd. in Winnipeg, Manitoba using N20110 determination of ^{210}Pb by Alpha spectrometry. Methods used are modified from Eakins and Morrison (1978). Linear regression models were applied to both cores as the average sediment accumulation rates were estimated moderately well. The Constant Rate of Supply (CRS) Model was then used to calculate age estimates. The equation of the CRS model is:

$$A(x) = A(0)e^{\sim kt} \quad (1)$$

Where t is the age of the sediment, x is the depth of the sediment layer used, A(x) is the residual unsupported ^{210}Pb at sediment depth x, and k is the ^{210}Pb radioactive decay constant (Appleby and Oldfield 1978). CRS estimated ages are dependent on the outcomes of the linear regression. There are two important assumptions of the CRS model; first is a constant input of 210Pb and second being that the sediment core is long

enough to incorporate all of the necessary atmospheric ^{210}Pb. If the assumptions cannot be met, CRS can still be calculated by calibrating the required variables against the linear regression. The CRS model in general is preferred over linear regression model because it provides a more accurate age estimations at the bottom of each section, regardless of fluctuating sediment accumulation over time (Appleby et al. 1979).

Extraction procedures

Extractions of eDNA took place in a sanitary room in the PaleoDNA Laboratory at the Lakehead University campus. The DNeasy Powersoil Kit Pro (Qiagen) was used to extract eDNA directly from sediment core samples. Extractions followed the protocol provided by Qiagen with several modifications (Supplementary Information). General modifications included the initial removal of water from samples, overnight incubations with proteinase K and the additional use of 3.25mm ceramic beads to aid in the homogenization process. Concentrations of *Mysis* eDNA in sediment were analyzed in copy of eDNA per gram of sediment (Copy/g). The sediment weights used for the calculations were taken from the sediment after the samples had been spun down from centrifugation and the water in the sample had been removed (see supplementary information). Since all samples were centrifuged in the same manner, any residual water content remaining in the sediments were comparable across all samples. To avoid contamination, several precautionary steps were taken. During extraction, equipment was cleaned between sample analyses with 20% bleach, followed with a rinse of de-ionized water. Blanks were included during every round of extractions. Eluted DNA was stored immediately at -20^{o}C until downstream applications. Freeze core slices were extracted in

triplicate to support occupancy modelling, whereas gravity core slices were extracted only once as they were primarily used for method development and initial analyses.

Amplification

Amplification procedures followed the same protocols as in Chapter 2 and were carried out in the PaleoDNA lab at Lakehead University. Using the same species-specific primers and TaqMan MGB probe (Labelled with FAM) developed by Carim et al.(2016), I targeted and amplified the 78 (bp) amplicon of *Mysis diluviana*. Assays were conducted in 15 µL reactions using a clear 96 well qPCR plates sealed with heat bonding film layer.

Mysis DNA was amplified in triplicate in 15µl reactions following a slightly modified qPCR formula (Carim et al., 2016). The qPCR formula included the following: 0.75µl 20x *Mysis* Assay, 7.5µl TaqMan Environmental Mastermix 2.0 (Life Technologies INC), 1.5µl VetMAX™ Xeno™ VIC positive control DNA, 0.1µl VetMAX™ Xeno™ VIC positive control assay, 1.15µl distilled water and 4µl of extracted *Mysis* DNA for a final volume of 15µl. Thermocycling conditions consisted of 95°C for 10 minutes, and 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. To create positive DNA for a standard series, synthetic *Mysis* DNA of 131bp, 26bp and 27bp on either side of the target amplicon, was created using gBlocks® Gene fragments (Integrated DNA Technologies). Synthetic DNA was resuspended following manufactures protocol and used to produce a standard dilution curve of 6250, 1250, 250, 50, and 10 copies per 4µl reaction. These were included with every qPCR assay that was conducted to quantify *Mysis* sedDNA. To be considered a positive detection of *Mysis*, at

least 1 extraction replicate must have had a minimum 2 out of the 3 qPCR replicates show amplification of *Mysis* eDNA (Ficetola et al. 2015).

Statistical analyses

Mysis eDNA presence in sediment cores was estimated using an occupancy modeling framework with program PRESENCE (Hines, 2006). Repeat extractions for each sediment depth layer in freeze cores (Lakes 223, 224) were used as repeated sampling events, allowing for detection probabilities in each layer of sediment cores (Hines 2006, Schmidt et al. 2013). The primary focus of this analysis was to relate detection probability patterns to known historic distributions, as well as to characterize downcore patterns of detection. The bottom depth of a given slice in the core was used as a covariate in detection probability estimation. Models were applied separately to each core from each lake; the Lake 224 model was considered to be representative of the long-term presence of *Mysis* eDNA in lake sediments, whereas the Lake 223 model described the changes in *Mysis* eDNA occupancy in sediment cores corresponding to a known extirpation event. The occupancy model describes the dependency of the observed state (psi; presence or absence) and the ability to detect *Mysis* sedDNA when it is indeed present (p; Mackenzie et al. 2002). Within each model, parameters were described as either depth-dependent or constant throughout the core and were compared using AIC. To determine if *Mysis* eDNA quantity decreased in older sediments, a Spearman correlation analysis was conducted between sediment depth and eDNA quantity. Spearman correlation was performed individually on freeze core data from Lake 224 and 223, with the top of Lake 223 core (0-14cm) removed due the absence of *Mysis* eDNA in

this part of the core (see results). To determine any patterns of sedDNA concentrations in the freeze core, I plotted the mean concentrations of the three replicates against core slice depth. Spearman's correlation test was conducted using the statistical computer program R (R core team, 2019). Gravity cores were not analyzed in the same manner as freeze cores since they were primarily used for method development and initial results of detection with depth.

Results

Amplification of *Mysis* eDNA in sediment was satisfactory, with R^2 values of the qPCR standard curves averaging 0.964. *Mysis diluviana* eDNA was detected throughout both gravity and freeze cores in two of three lakes examined (Figure 3.1). Variable detections were observed throughout the gravity cores of Lake 224 and 223. *Mysis* eDNA was detected throughout the core from Lake 224, with negative detections appearing occasionally below 15 cm. In Lake 223, there were no detections of *Mysis* eDNA in the top 2cm of the core. No detections of *Mysis* eDNA occurred in Lake 302, a lake where *Mysis* are known to be absent (Figure 3.1A). Freeze cores showed similar consistent detections of *Mysis* eDNA throughout the core from Lake 224. Since freeze cores do not exhibit compression that is observed in gravity cores (Crusius and Anderson 1991), Lake 223 exhibited a considerably larger region of absent *Mysis* eDNA detections in the top of the core.

In Lake 224, AIC indicated equivalence among the top eight models evaluated, which included depth dependence for one or both occupancy and detection probability model parameters (Table 3.2). In Lake 223, the top three models all included depth in one of the model terms. Results for occupancy probabilities and detection probabilities are reported as means percentages weighted arithmetically across the top models with ΔAIC greater than 3. Occupancy modeling of the freeze cores for lakes 223 and 224 showed occupancy probabilities of 10-75% and 97.4% respectively, likely reflecting the absence of *Mysis* from the most recently deposited sediments in Lake 223. Detection probability in Lake 223 increased with depth from 58% at the top of the core to 71% at the bottom. Detection

probability in Lake 224 was consistent, but generally lower than in 223, decreasing with depth from 45% at the top of the core to 35% at the bottom.

Lake 224 exhibited a significant correlation in *Mysis* eDNA concentration (copies/g) in relation to depth of the sediment freeze core (R= -0.39, p = 0.03; Figure 3.2). There was no significant correlation between *Mysis* eDNA concentration and depth for Lake 223 (R= -0.30, p = 0.23) when applied only to the section where *Mysis* were consistently detected (Figure 3.2). For Lake 224, mean *Mysis* eDNA concentrations ranged from 120 to 3112 copies/g. For Lake 223, the eDNA concentrations that were detected ranged from 306 to 3040 copies/g sediment. Visual inspection indicates a possible outlier with a concentration of 8,427 copies/g; however, the outlier does not have an effect on the outcome of the correlation results if removed (Figure 3.2).

The Lake 224 freeze core did not meet the assumption of the CRS aging model, in that it was not long enough to provide a complete ^{210}Pb inventory. Therefore, to use the CRS model, it was calibrated using an atmospheric ^{210}Pb inventory of 55.47 (dpm/cm^2) to predict the same average sediment accumulation rate of 0.0119 g/cm^2/yr as the linear regression model over the core length. The total Pb activity in Lake 224 ranged from 189.35 dpm/g at the top of the core to 3.14 dpm/g at the bottom (Figure 3.3). Based on the determined ages from the calibrated CRS model, the estimated age of the last detection of *Mysis* at the bottom of the Lake 224 freeze core was 169 years old (Figure 3.4). Not every core slice was dated in Lake 224, therefore missing ages were interpolated between slices with determined ages. In Lake 223, both assumptions were not met. Therefore, to use the CRS model, calibration against the linear regression model was again preformed against the 7.5-22.5cm section of the core, using an average

sediment accumulation rate of 0.0098 g/cm^2/yr. In addition, age estimates could only be reliably inferred over the 0-22.5cm interval of the core. Ages were only estimated to 22.5cm due to an exponential decay of ^{210}Pb in cores between 24-26cm and 30-32cm (Figure 3.3a). The ^{210}Pb activity in Lake 223 ranged from 141.4 dpm/g at the top of the core to 10.16 dpm/g at the bottom (Figure 3.3). The last depth that could be dated reliably at 21cm was estimated to have an age of 69 years (Figure 3.5). Core slices that were not aged by Flett LTD between 0-21cm were interpolated and ages below 21 cm were extrapolated using the same average accumulation rate as that between 0-21 cm. The estimated uncertainty for ^{210}Pb in both Lake 224 and 223 was determined to be ± 11% at concentrations between 0.6 and 40 dpm/g at 95% confidence.

Since the recent historical distribution of *Mysis* in Lake 223 (Nero and Schindler 1983) is known, I could validate the precision of the detections. The *Mysis* population was believed to have died off in Lake 223 in the fall of 1979 (Nero and Schindler 1983). This date corresponds almost exactly at a transition in the Lake 223 sediment freeze core at 15 cm depth, where *Mysis* eDNA becomes nearly undetectable from this point to the top of the core (Figure 3.5). I also observed a single positive detection of at the 5cm mark from the sediment core in Lake 223. Based on determined ages of sediments from the Lake 223 core, this detection occurs around 2010. Interestingly, from 2009-2011, Lake 224 exhibited the highest average lake level over 29 years, suggestive of high flow rates from Lake 224 into downstream Lake 223 (Figure 3.6). This was possibly driven by high precipitation over this same three year period, with an average of 802.2 mm annual accumulation over these years (Figure 3.7).

Discussion

My study demonstrated for the first time that *Mysis* eDNA is well preserved in lake sediments. To date, assessing historical *Mysis* assemblages has been impossible because they do not leave physical remains; here, I demonstrate an emerging technique to assess historic assemblages of biota that do not leave physical remains. Critically, these results also demonstrated that the pattern of *Mysis* eDNA detections in a sediment core closely matched the timing of *Mysis* extirpation from Lake 223 (Nero and Schindler 1983). The abundance results appear to exhibit slower decay trends to that observed by Sakata et al. (2020); who found decay of fish sedDNA of approximately 0.00033 copy of eDNA per hour. The decay of *Mysis* eDNA appears to be extremely slow as there was only low correlation of eDNA concentration with depth throughout the entirety of the L224 freeze core, with maximum sediment ages >150 years old. Had Lake 223 contained a full core of detections, it may have very well exhibited the same decay pattern as Lake 224. As such, my results emphasize the potential in using eDNA for determining the historical presence of *Mysis* in anthropogenically disturbed lakes to aid in comparing historical to current distributions of *Mysis*. Further, the results indicate the potential for applications of more broadly targeted primers for other invertebrates or vertebrates that do not leave identifiable structures (e.g. copepods or fish) for the reconstruction of historical ecological communities. Examples of such methods have been developed and applied for detection of Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) in sediments of two lakes at Banff National Park (Nelson-Chorney et al. 2019).

In the freeze core from Lake 223, I observed a single positive detection for *Mysis* eDNA, in sediments younger than 1980, which I initially believed to be a false positive detection based on our knowledge of the historical absence of *Mysis* during that time period (Mills et al. 1987). There were no detections in the core slices directly above or below the suspected false positive until 15 cm, which corresponds with the timing of extirpation of the species from the lake. I conducted qPCR of each extracted replicate in triplicate per sample to decrease the probability of false positives. Since 2 out of the 3 extraction replicates exhibited amplification in 2 of the 3 qPCR replicates, this led us to believe that *Mysis* DNA entered the sediment either during or prior to extraction, possibly due to contamination.

An alternative explanation for this 'false positive' may be provided by the hydrological data from the stream flowing into L223 from Lake 224. Effect of variation in water levels and precipitation in a lake can be experienced across several years and as mentioned in the results, there is a degree of uncertainty concerning the ^{210}Pb estimates. In 2010, Lake 224 exhibited the highest lake level recorded over the previous 30 years. Precipitation was also above average during that time, exhibiting an annual total of 955mm, the 3rd highest observation over 27 years of monitoring data. *Mysis* eDNA can be transported through streams (see Chapter 2), and because of higher than normal water levels during seasons when lakes are isothermal, there could have been increased transport of eDNA from Lake 224 to Lake 223. This sum of evidence raises the possibility that the combination of historically high lake levels and high precipitation may have caused an unusually high influx of *Mysis* eDNA into Lake 223 from upstream Lake 224 that was large enough to allow for accumulation within the sediment for that year.

Another explanation may be from redeposition of older sediment as the ^{210}Pb profile is not ideal and contains inflection above 12cm. There also lies the possibility of a brief invasion event of *Mysis* into Lake 223 from Lake 224 that subsequently died out.

When targeting a single organism's eDNA in any sample type, species specificity is crucial. The primers and probes used in this study were previously tested against two of the known closest taxonomically related species present in North America, *Asellus aquaticus* and *Hemimysis anomala,* and were determined to be species-specific for *Mysis diluviana* (Carim et al., 2016). However, Carim et al. (2016) was not able to test their *Mysis*_A primer due the lack of sequences available publicly and could not make a synthetic gene fragment for *Hemimysis anomala* that overlapped with *Mysis diluviana*. Therefore, I tested both primer pairs against extracted genomic DNA of *Hemimysis anomala* and *Orconectes virilis,* a species of crayfish that is believed to be the closest related species to *Mysis* at the IISD-ELA. This ensured species specificity for ELA samples and prevented amplification of non-target DNA.

Environmental factors that are correlated with eDNA degradation in sediment are well documented. Factors such as low pH, increased exposure to sunlight, high temperatures and microbial activity have all shown to increase the degradation of eDNA within a freshwater ecosystem (Pietramellara et al. 2009; Strickler et al. 2015). The conditions within the IISD-ELA vary from lake to lake but in general *Mysis* are found only in deep, and cold lakes (Audzijonyte and Väinölä 2005). In addition, the migration patterns and environmental preferences of *Mysis* keep populations constrained within these conditions (Nero 1981; Boscarino et al. 2009; Paterson et al. 2011). This would suggest that the eDNA released by *Mysis* experience relatively low amounts of exposure

to degrading factors and in turn allow for greater accumulation within the sediment. Lake 224 supported this, as probability of *Mysis* eDNA occupancy was high and detection probability was greatest at the top of the sediment cores. Lake 223 results were unexpected due to the physical and historical conditions of the lake. In comparison to other lakes at ELA that contain *Mysis*, Lake 223 is shallower and had a lower density of *Mysis* historically. I also anticipated leaching of the sulfuric acid into the sediment during the acidification experiment, potentially degrading the eDNA within the sediment. This potentially could have caused a larger absence of detections in the top core of Lake 223. This was not the case as I instead observed *Mysis* eDNA precisely in accordance with known historical presence of *Mysis* and no delay in detections. This may speak to the protective capabilities that substances found in freshwater sediment may possess. According to Greaves and Wilson (1969), lower sediment pH may actually facilitate the binding process of eDNA to sediment particles. During the acidification of Lake 223, the average epilimnetic pH was lowered from a pH of 7 in 1973 to a range of 5.01 – 5.13 in 1981 and held there until 1983 (Mills et al. 1987). Thus, perhaps the lowered pH during the acidification experiment and the naturally low hypolimnetic pH aided in the facilitation and preservation of *Mysis* eDNA into the sediments as they died off and is the reason I observed such high resolution in the results.

In addition to the protection provided to eDNA molecules bound in the sediment, it is also possible for other vectors in the water column to facilitate settling of eDNA. Runoff from woodland watersheds are an important source of dissolved organic carbon (DOC) that is released into oligotrophic systems such as the lakes at ELA (Hall et al. 2019). Studies have shown that approximately 40% of terrestrial DOC accumulates in

lake sediments (Hall et al. 2019). Since eDNA has shown to bind to organic material within sediment (Crecchio and Stotzky 1998; Shi et al. 2015; Senapati et al. 2018), there is a possibility that settling DOC within the water column may bind the eDNA, providing initial protection to degradation prior to accumulation as well as decreasing the time eDNA is in the water column. Depending on the transportability of DOC, it may act as a means of translocating eDNA away from the source, aiding in the accumulation in sediments elsewhere.

Several modifications were made to increase the amount of *Mysis* DNA extracted from the sediment (S1). During the extraction process, I elected to follow the initial steps in the protocols provided by Qiagen. This included removing the water by centrifuge from the sediment sample before carrying out the start of the extraction. Too much water during extraction can dilute the buffer and cause hydrolysis of the DNA (Lindahl 1993). An overnight incubation with proteinase K was added to the beginning Qiagen's protocols. This allowed for additional chemical separation of eDNA bound to sediment molecules (Tsuji et al. 2017). The majority of these modifications took place at beginning of the extraction process and appear to be crucial in the detection of *Mysis* eDNA in sediment.

Avoiding contamination is crucial in any eDNA study. Despite due diligence, however, it is still common for contamination to occur. This may have occurred in my study as samples were only collected from several different lakes in the time span of a week. The gravity coring apparatus was not sterilized in between each sampling event, leaving the possibility of cross contamination between lakes. The negative core (lake 302) was collected after several lakes containing *Mysis* and still exhibited no positive

detections, leading us to assume cross contamination between lakes was minimal to none. However, contamination may still be a possibility within the same core, especially within a gravity corer. As the sediment is extruded, some sediment along the walls of the tubes is left behind, possibly exposing the edges of cores slices at the bottom of the core. The freeze cores in my project may have experienced less exposure to contamination during the sampling process as sediments are extruded and contact between sediment slices are reduced, though some contamination in the slicing procedure is still possible. Once frozen and collected, the edges were removed from the core after which the core was sliced in 1 cm increments using a band saw. During the set up for qPCR analysis, extreme measures are taken to prevent contamination. However, high concentrations of *Mysis* DNA are being handled and even the smallest droplet can lead to a false positive. This could be a problem for my study as during the pipetting process, the tip would sometimes accidentally graze the top of the well on the way out causing the tip to flick as it reached the surface. If there were any liquid remaining on the tip, it may have contaminated adjacent wells. Inversely, to properly remove enough inhibitors present, the sample needs to be exposed to a number of inhibitor removing steps. With each step preformed using an extraction kit, DNA is lost, increasing the probability of a false negative detection (Deiner et al. 2015; Eichmiller et al. 2016b). This illustrates the importance of both extraction and qPCR replicates (Piggott 2016).

My work implies great potential for eDNA methods to provide restoration guidelines of impacted ecosystems by indicating candidate lakes for biological reestablishment. My work can also be important for following invasion trajectories and anthropogenic impacts, which may be of concern especially in North American lakes. For

example, it is currently unknown whether acidification of lakes in eastern North America widely affected *Mysis* in ways similar to what was observed in Lake 223. This is because there have been no surveys and no temporal data that used appropriate methods to examine these impacts, until now. I demonstrated that organisms that do not leave behind physical remnants can still have historical distributions determined using eDNA techniques. The attention of conservation efforts is generally towards fish species or species of economic value. My results show that sedDNA has the ability to incorporate additional species like invertebrates that may be important to the reconstruction of historical food webs. These methods should apply to other organisms; however, further assessment of individual species is required. The hope is that this research will aid current habitat conservations methods and with further research, ultimately incorporate every feasible species known to have been affected during a sedDNA survey. Future tasks will be to apply these sedDNA extraction methods to a recently impacted ecosystem to generate timelines of species occupancy, which will be used in decision making process during the habitat restoration process.

Tables

Table 3.1: Characteristics of lakes 224, 223 and 302 from the Experimental Lakes Area used in the sediment analysis.

Lake	Coordinates (latitude, longitude)	Size (ha)	Max Depth (m)	*Mysis*	Core type	Core Depth (cm)
224	N 49.69000 W 093.71694	25.9	27.4	Present	Freeze	30
					Gravity	48
223	N 49.698333 W 093.70778	27.3	14.4	Present	Freeze	40
					Gravity	40
302	N 49.672830 W 93.727912	12.8	13.8	Absent	Gravity	32

Table 3.2. Model selection results for detection probability of *Mysis diluviana* in two lakes at the IISD- Experimental Lakes Area. Models were run using the program PRESENCE. Models consist of occupancy probability (psi) and detection probability (p). Periods represent constants and "depth" represents depth dependence in the parameter of interest. Depth was modeled as both a continuous (depth), and a categorical (depth 2) variable. Differences between AIC values of the models and strength of the model are indicated by ∆AIC and w respectively

Lake 224			Lake 223		
Model	∆AIC	w	Model	∆AIC	w
psi(.),p(depth)	0	0.208	psi(depth2),p(.)	0	0.399
psi(depth),p(depth)	0	0.208	psi(depth2),p(depth)	0.55	0.303
psi(.),p(.)	0.82	0.138	psi(depth2),p(depth2)	1.86	0.158
psi(depth),p(.)	0.82	0.138	psi(depth),p(depth)	4.94	0.034
psi(.),p(depth2)	2	0.077	psi(.),p(depth)	5.15	0.030
psi(depth),p(depth2)	2	0.077	psi(.),p(.)	5.43	0.026
psi(depth2),p(depth)	2	0.077	psi(depth),p(.)	5.68	0.023
psi(depth2),p(.)	2.82	0.051	psi(depth),p(depth2)	6.70	0.014
psi(depth2),p(depth2)	4	0.028	psi(.),p(depth2)	7.07	0.012

Figures

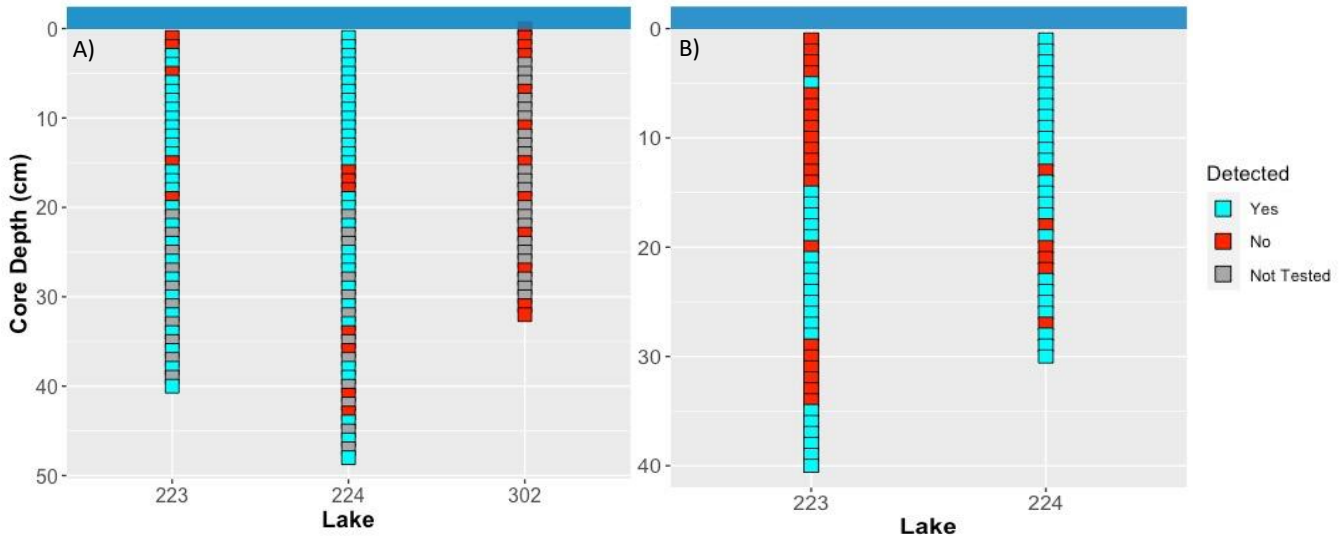

Figure 3.1: Plot of *Mysis diluviana* eDNA detections in lake sediment from gravity cores (A) and freeze cores (B). Lakes sampled include Lake 223, Lake 224 (positive control) and lake 302 (negative control). No detections occurred in Lake 302.

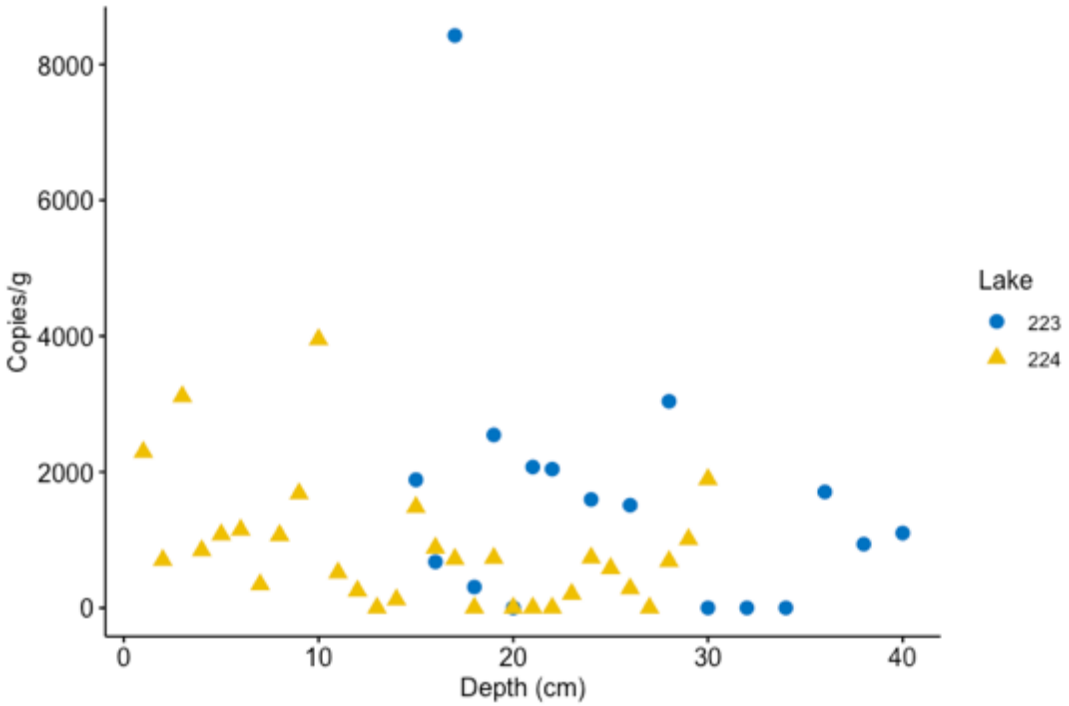

Figure 3.2: Plot of *Mysis diluviana* eDNA concentration(copy/g) from freeze core samples of Lake 224 and 223. Lake 224 exhibited significant correlation (R= -0.39, p = 0.03), Lake 223 did not show correlation (R= -0.3, p = 0.23). Top 15 cm from Lake 223 was omitted due to the influence of the acidification event.

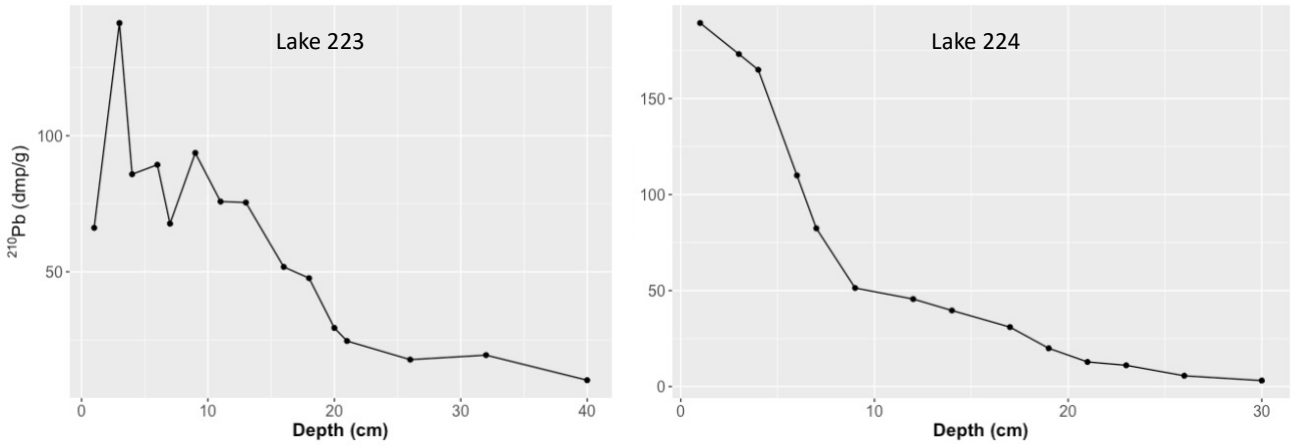

Figure 3.3. Plot of total ^{210}Pb activity in decays per minute per gram (dmp/g) vs depth in Lake 223 and Lake 224.

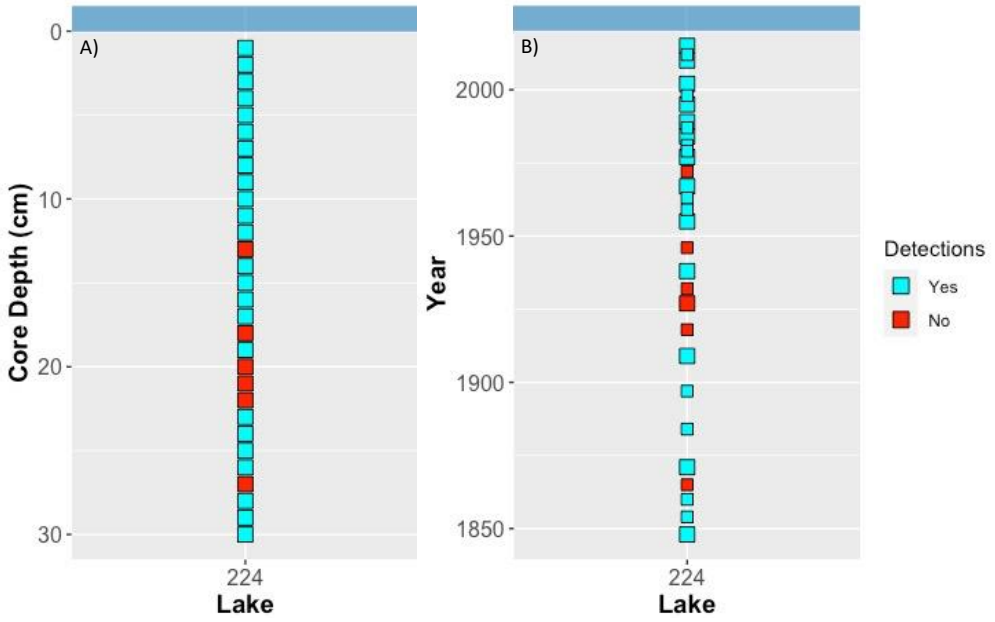

Figure 3.4: Plot of *Mysis diluviana* detections in Lake 224. A) represents detections vs sediment core depth in 1 cm increments. B) represents detections vs date of sediment as determined by Pb^{210} aging analysis. Smaller points represent missing ages between estimated ages that were interpolated.

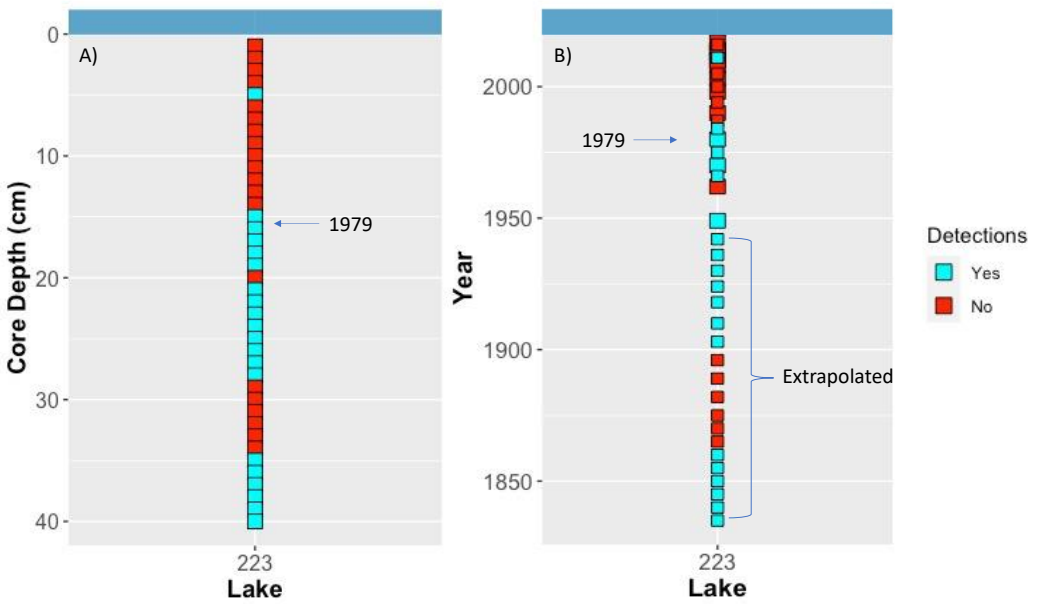

Figure 3.5: Plot of *Mysis diluviana* detections in Lake 223. A) represents detections vs sediment core depth in 1 cm increments. B) represents detections vs date of sediment as determined by ^{210}Pb aging analysis using the CRS model. Smaller points represent sections that were not analyzed for ^{210}Pb and where estimated ages that were interpolated or extrapolated.

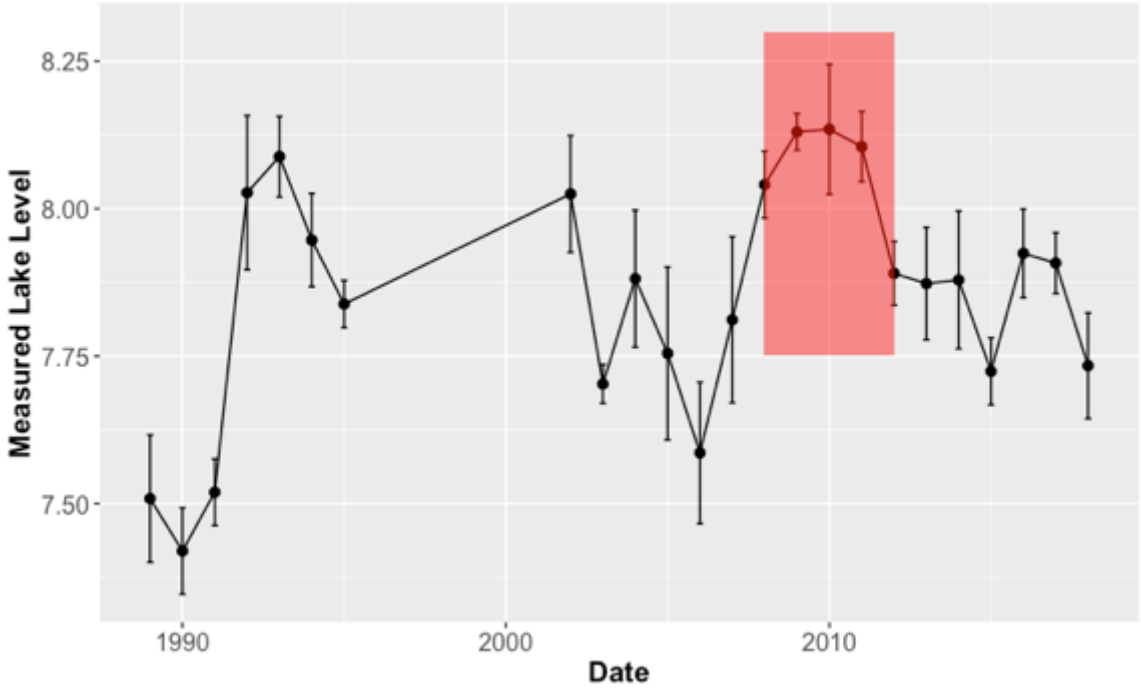

Figure 3.6: Plot of lake level measurements of Lake 224 from 1989 to 2018. Lake levels values are shown as mean +/- standard deviation. Highlighted area represents the approximate timeline in which the suspected false positive occurred.

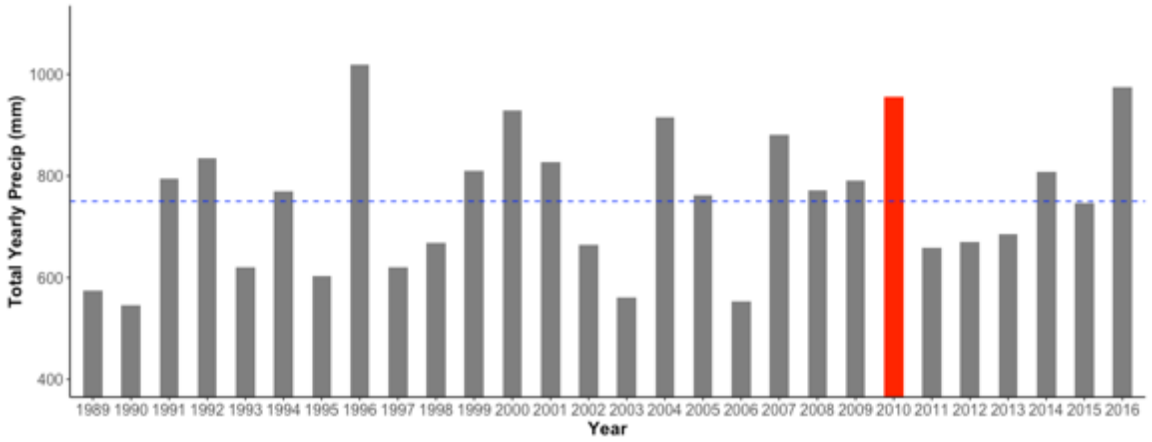

Figure 3.7: Plot of total yearly precipitation (mm) collected from the ELA meteorological site. The red highlighted area represents the approximate timeline in which the suspected false positive occurred. Dashed blue line represent the mean precipitation from 1989 to 2016.

Chapter 4:

Conclusion

The purpose of this thesis was to explore the extent to which eDNA techniques can be used to detect *Mysis diluiana* eDNA in freshwater ecosystems. This was accomplished by fine tuning eDNA extraction methods to target *Mysis* eDNA in both sediment and water samples. The IISD-ELA provided an ideal choice of study site as the biological record for over 50 years is unmatched, particularly in the face of known disturbances. This study offers clear evidence that historical distribution of invertebrates that do not leave detectable physical remains can be determined in sediment cores through the use of eDNA techniques. In addition, short term persistence and seasonal movement within lakes and stream can be monitored through the use of eDNA. The acidification project that took place in Lake 223 provided a unique scenario that allowed for the verification of my methods. The results of this study indicate that the usefulness of eDNA methods is greater than previously recognized.

In chapter two, I focused on persistence of *Mysis* eDNA in lake and streams at several lake at the IISD-ELA. Overall, *Mysis* eDNA was found in abundance in all three of the lakes with established populations of *Mysis* as well and in their corresponding stream outflows. When investigating daily persistence within the water column I observed no differences within the same depth strata between day and night. These samples however were only collected during autumn when *Mysis* are known to be more active due to colder temperatures (Sellers 1995). I believe future studies may find that these results differ temporally. During warmer seasons differences between strata may increase and before the COVID-19 pandemic my intentions were to determine this.

Regardless, results from my stream data are suggestive. Within the streams I observed a significant increase in *Mysis* eDNA during the fall when compared to the summer. In the summer months (June, July and August), there was little to no *Mysis* eDNA observed. With no *Mysis* eDNA occurring at these times the assumption can be made that the *Mysis* eDNA within the lake was concentrated towards the bottom of the lake along with the physical population.

Both the lake and stream results have implications for aquatic eDNA survey design. While my results show large quantities of *Mysis* eDNA throughout the lake, a recent metabarcoding survey did not detect any *Mysis* eDNA (personal communication, Joanne Littlefair). There are two possible reasons why these false negatives may have occurred. The first is that the survey sampling took place during the summer and eDNA may not have been present within the sampled strata. Secondly, metabarcoding techniques are less sensitive than single target barcoding and the primers used appear not to have amplified *Mysis* eDNA. Barcoding surveys have becomes significantly more reliable and because of this government organization like Department of Fisheries and Oceans Canada have begun to implement new management standardized reporting protocols for eDNA surveys targeting single species (Department of Fisheries and Oceans Canada 2020).

The third chapter of this thesis primarily focused on the presence of *Mysis* eDNA within freshwater lake sediment. When targeting the eDNA of a specific species in lake sediment, it is imperative to use methods tailored to that organism. Using qPCR or comparable approaches are essential as the process is considerably more sensitive than other means such as metabarcoding (Wood et al. 2019). There was an abundance of

Mysis sedDNA found in the sediment cores of lakes where *Mysis* are known to have been historically present. In Lake 223, I was able to discern a known extirpation event based on detection patterns of *Mysis* eDNA within a sediment core. When combined with ^{210}Pb dating techniques, the historically recorded timeline of matched well with the estimated age of the sediment in which *Mysis* eDNA detections no longer occurred. In addition, *Mysis* eDNA was detected as far back as 1850 in both Lake 223 and 224. Future studies may include methods that can date beyond 150 years as this is the extent of ^{210}Pb aging techniques.

Future studies may also want to incorporate a spatial aspect of the site in which cores are collected. In the third chapter, I argued for the reason behind the occurrence of a false positive in the top of the core in Lake 223. Since the core was collected at the deepest point of the lake, the distance was relatively far from the inflow. In addition, the stream itself is comparatively smaller as it varies in size throughout the entire length of the stream and in general has lower flow for the majority of the year. This is why I speculated it took extremes of both lake level and precipitation to provide enough flow in the stream to carry the *Mysis* eDNA far enough in the lake to cause the alleged false positive. Had the cores been collected closer to the inflow the results may have been different. Future studies in lakes with larger inflows may want to collect cores in a gradient to determine the extent to which eDNA contamination from upstream can travel and settle within a lake. This will help determine optimal sediment sampling locations and provided more accurate occupancy results.

In conclusion, I have indicated that eDNA techniques can and should be used during habitat restoration processes and other paleolimnological projects that monitor

historical anthropological impacts. My work also implies that aqueous and sediment eDNA should be used in tandem during eDNA surveys. Depending on the proposed research questions, surveys occurring during certain times of the year when flow is high should be avoided to minimize the risk of false positive detections. Concurrently, factors such as seasonal and daily migration patterns need to be considered to avoid missing important organisms. These issues may vary from species to species; however, and eDNA surveys should be approached holistically to provide trustworthy results. This research suggests that eDNA sampling is suitable determining restoration targets and that *Mysis* themselves can be used as a biological target. Furthermore, this study opens up the possibilities for many more biological targets. In particular, by using eDNA techniques we can now use softer bodied organisms that previously were unable to determine historical presence within lake sediments. This will allow for more complete assessments of biological communities during habitat restoration processes.

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Supplementary Information

Mysis diluviana eDNA Extraction Protocol using DNeasy Powersoil Kit Pro

Legend:
Black = Qiagen protocol
Red = Modifications made as part of this study
Blue = Additional notes

Important notes before starting:

- Ensure that the PowerBead Pro Tubes rotate freely in the centrifuge without rubbing.
- If Solution CD3 has precipitated, heat at 60°C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15–25°C).
- Include an extraction blank with every set of extractions

Procedure:

1.. Remove contents from the PowerBead Pro Tube (beads) and transfer into another sterile microcentrifuge tube (not provided). **Weigh the empty tube. Add between 500-600 mg** of soil sample to PowerBead Pro Tube **(weigh tube again)** and centrifuge at room temperature for 30 s at 10,000 x g. Remove as much liquid as possible with a pipette tip. **Weigh tube again and subtract from previous tube weight to determine weight of sediment without water content to be used for concentration calculations. Add 800 µl of Solution CD1 and 40 µl of Proteinase K, vortex for 10 seconds to mix. Place tubes in an incubator at 56ºC and incubate overnight.**

Note: Make sure to weight tube at every step. Qiagen protocol asks for 250mg of soil however, the purpose of initially adding 500-600mg of sediment is due to the high water content. After centrifuging and removing the liquid the amount of sediment left should be very close to 250mg (+/- 50mg).

2. **After incubation add the tubes back to the Powerbead Pro Tube and add 3 additional 3.25mm ceramic beads (QIAGEN) to each tube. Place tubes in a vertical homogenizer for 5min at 25 hertz, rotate tubes 180 degrees and run again for another 5min.**

Note: Qiagen's protocols say to secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min. In the "Protocol: Detailed" section they describe alternative ways to homogenize samples including vertical homogenizers which is what I used instead.

3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.

4. Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided). Note: Expect 500–600 µl. The supernatant may still contain some soil particles.

Note: Expect more than 500-600 µl due to sediment.

5. Add 200 µl of Solution CD2 and vortex for 5 s.

6. Centrifuge at 15,000 x g for 1 min at room temperature. Avoiding the pellet, transfer **all of the** supernatant to a clean 2 ml Microcentrifuge Tube (provided).

Note: Qiagen protocol says to transfer up to 700 µl however, there will be more due to the amount of liquid in the sediment. To avoid missing any Mysis eDNA, transfer all of the supernatant.

7. Add 600 µl of Solution CD3 and vortex for 5 s.

8. Load 650 µl of lysate to an MB Spin Column. Centrifuge at 15,000 x g for 1 min.

9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.

10. Carefully place the MB Spin Column into a clean 2 ml Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.

11. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

12. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.

13. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.

14. Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube (provided).

15. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided).

16. Add 50–100 µl of Solution C6 to the center of the white filter membrane. **Place MB spin column and elution tube in an incubator at 56 ºC for 5 minutes.**

17. Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications. Note: We recommend storing the DNA frozen (–30 to –15°C to °C or –90 to –65°C) as Solution C6 does not contain EDTA.

Appendix

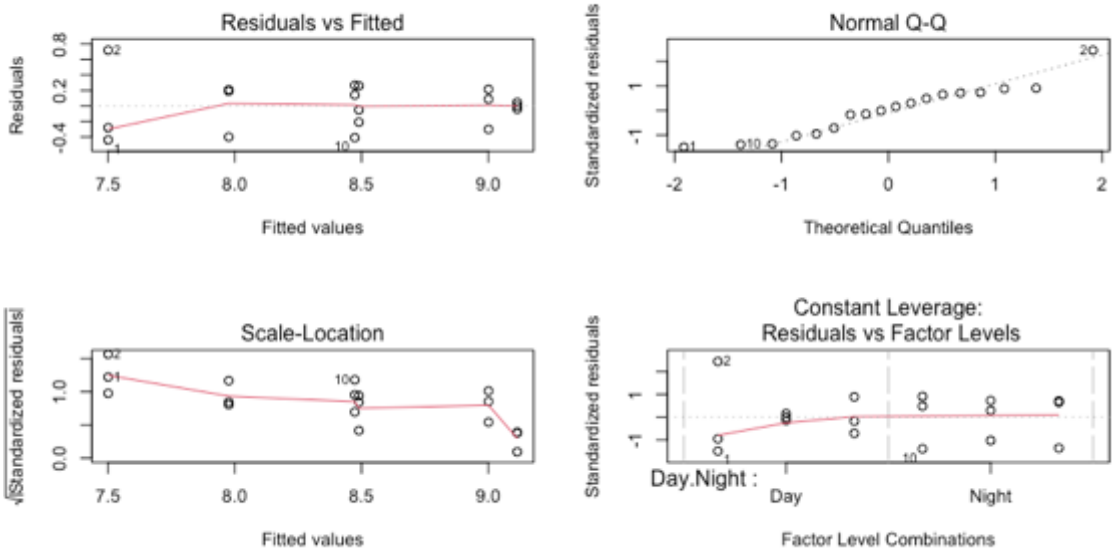

Figure A2.1. Diagnostic plots of the linear model for *Mysis* eDNA as a function of time of day and layer for Lake 224.

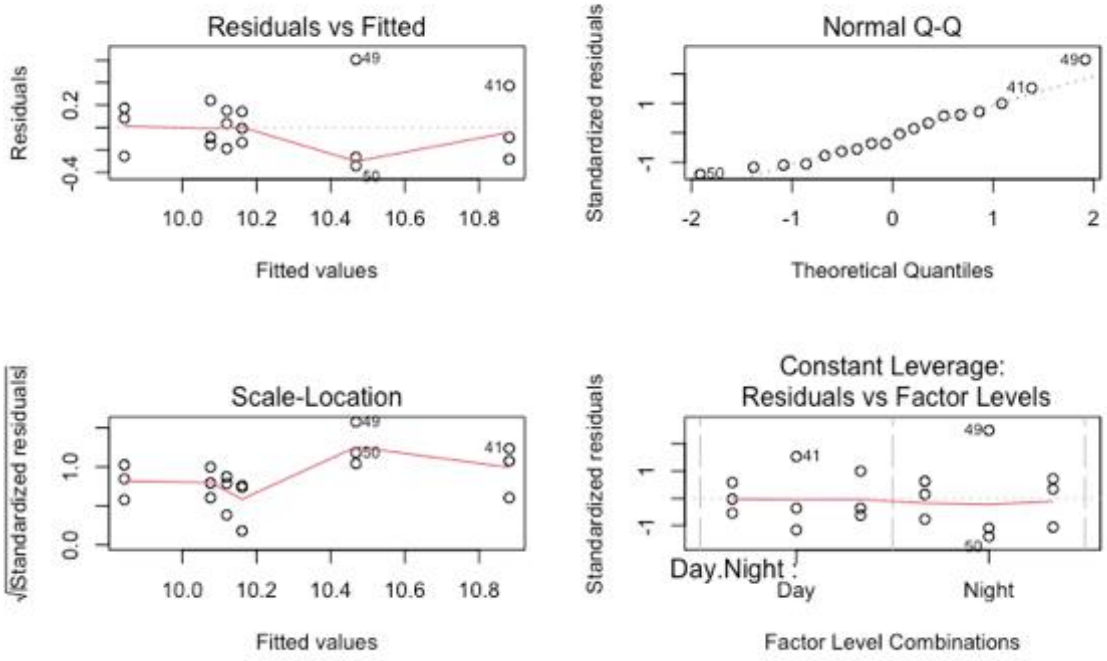

Figure A2.2. Diagnostic plots of the linear model for *Mysis* eDNA as a function of time of day and layer for Lake 373.

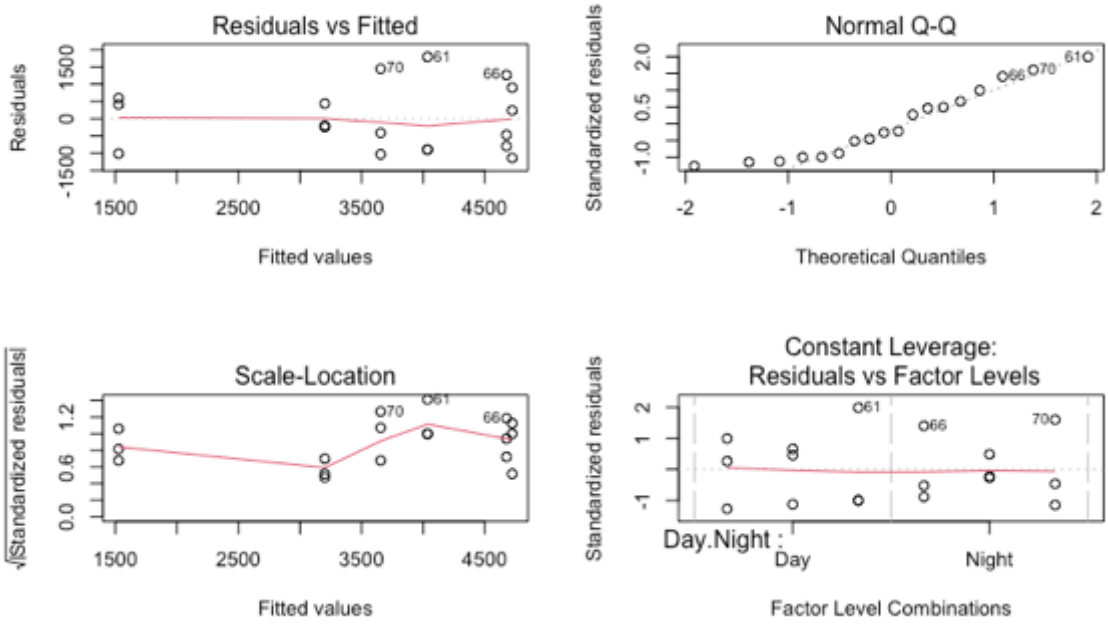

Figure A2.3. Diagnostic plots of the linear model for *Mysis* eDNA as a function of time of day and layer for Lake 442.

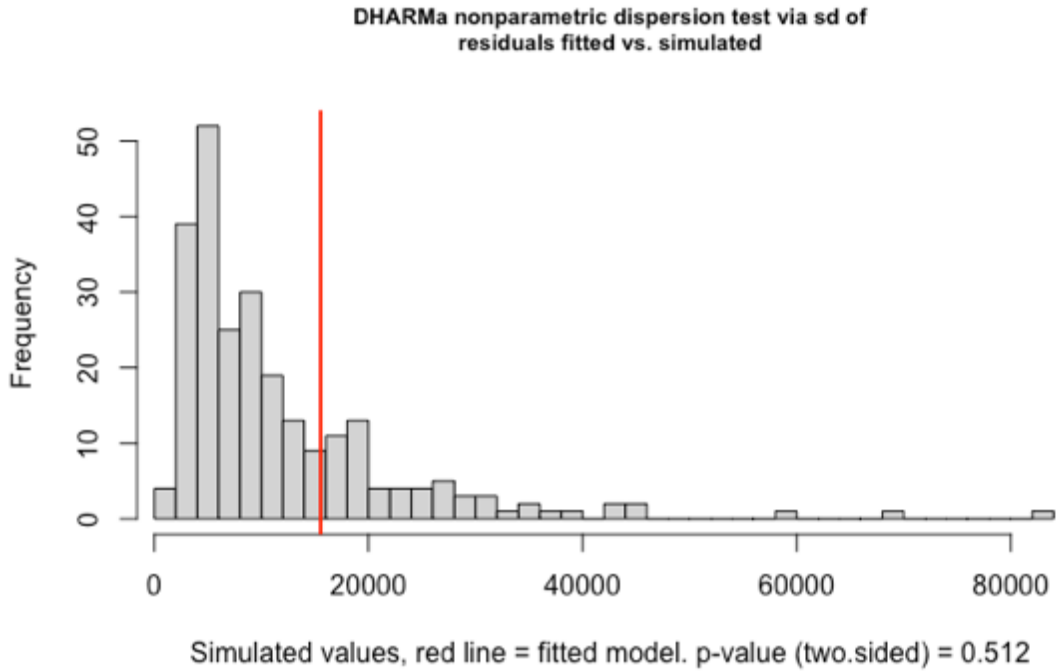

Figure A2.4. Dispersion test of the general linear mixed model for *Mysis* eDNA as a function of time of day and layer fit to a negative binomial distribution.

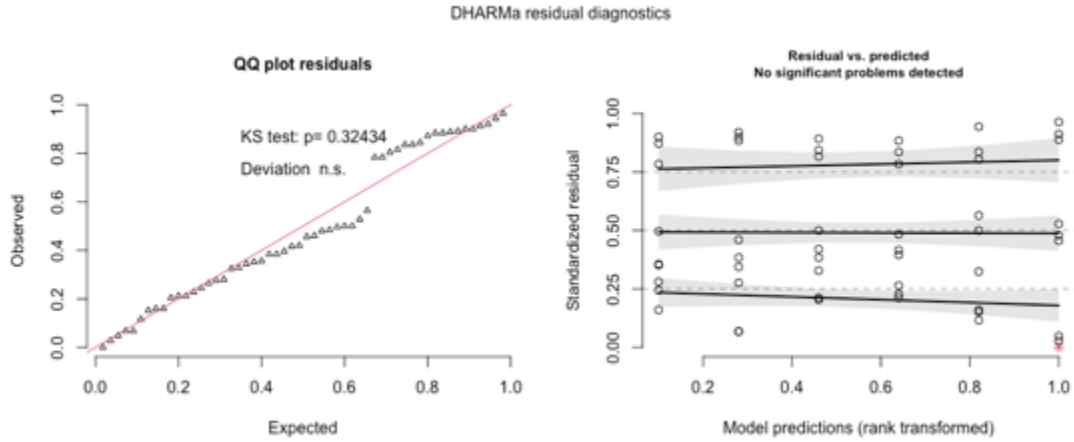

Figure A2.5. Diagnostic plot of the general linear mixed model for *Mysis* eDNA as a function of time of day and layer fit to a negative binomial distribution.

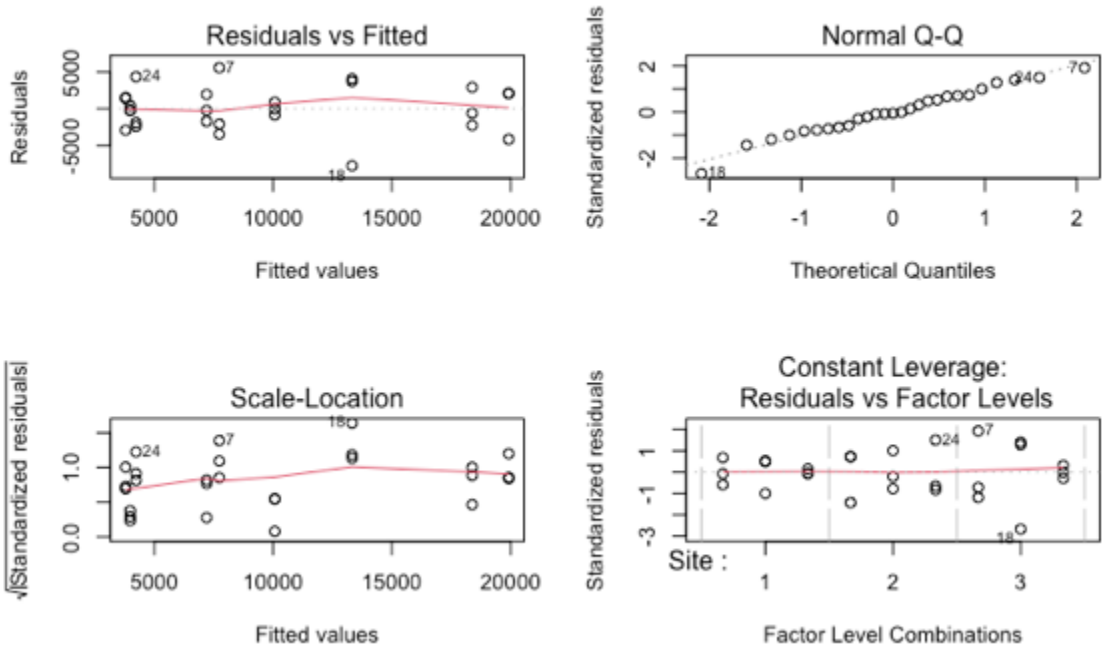

Figure A2.6. Diagnostic plots of the linear model for *Mysis* eDNA as a function of sample site and stream for all streams in the fall.

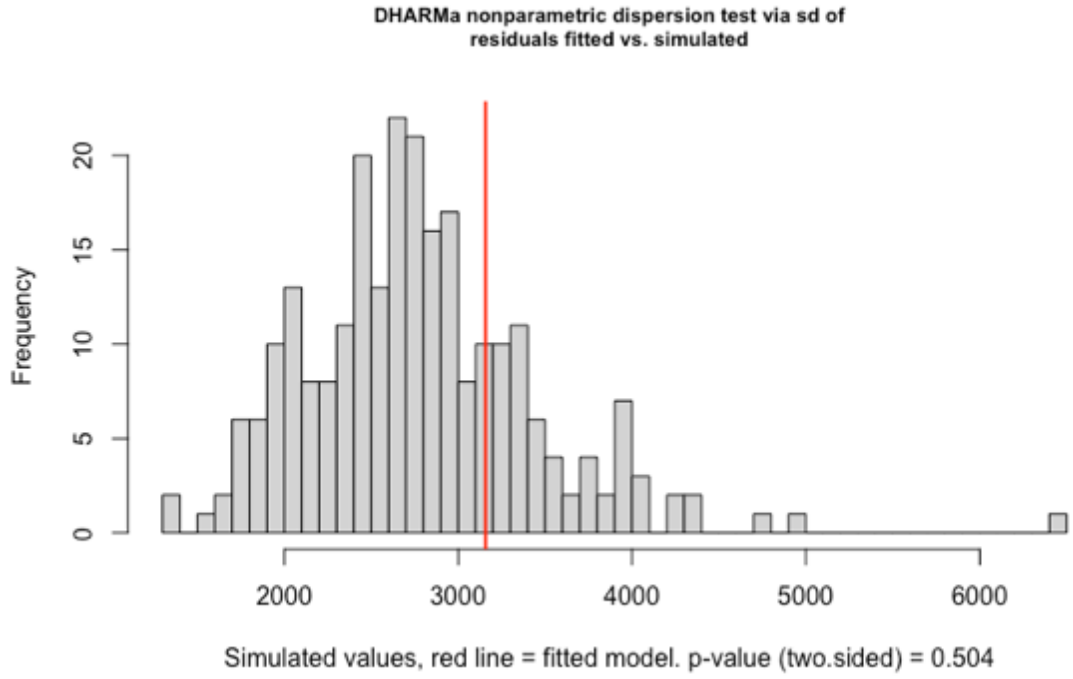

Figure A2.7. Dispersion test of the general linear mixed model for seasonal stream *Mysis* eDNA as a function month fit to a negative binomial distribution.

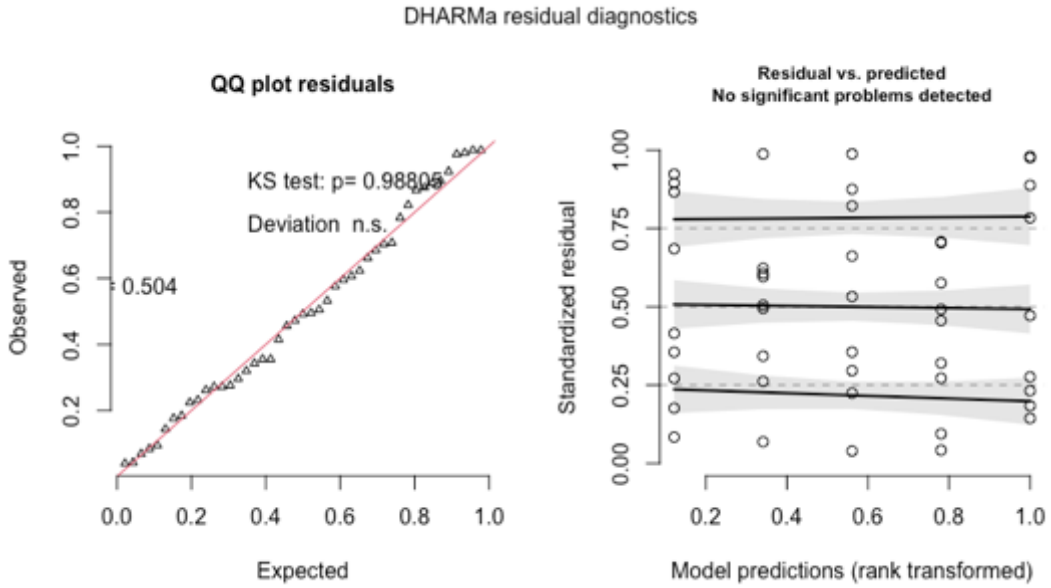

Figure A2.8. Diagnostic plot of the general linear mixed model for seasonal stream *Mysis* eDNA as a function of month fit to a negative binomial distribution.

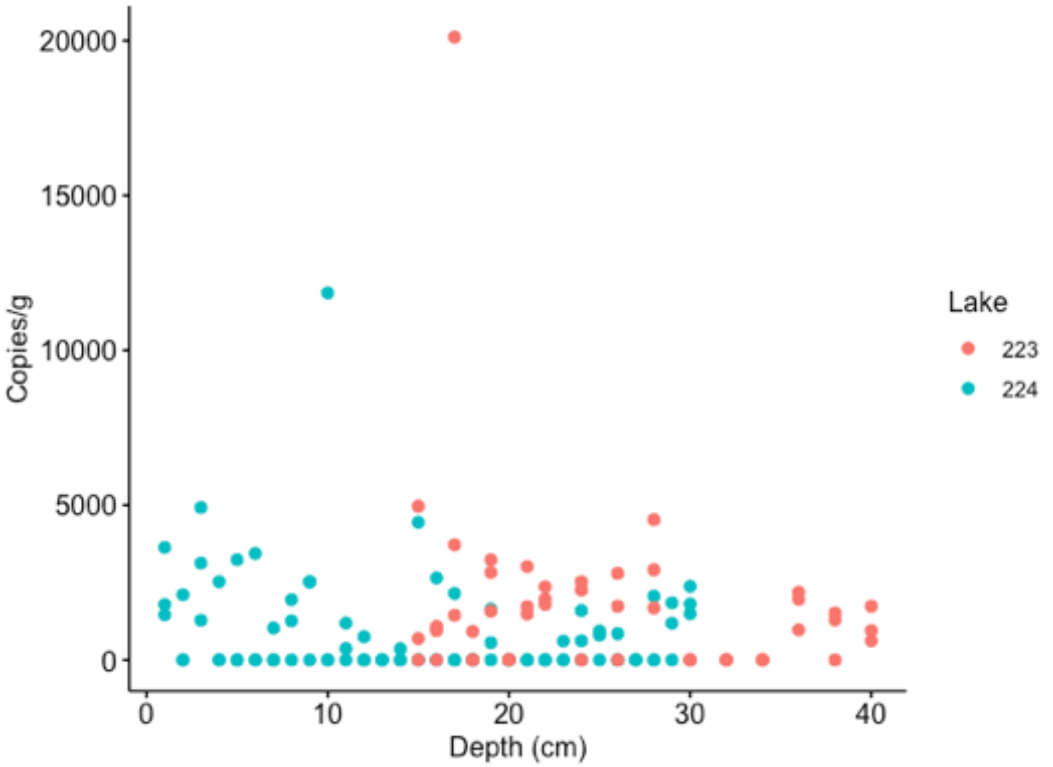

Figure A3.1: Scatterplot of *Mysis* eDNA concentrations for each replicate collected to check for outliers in lake 224 (blue) and Lake 223 (red).