DNA-Based Environmental Monitoring for the Invasive Myxozoan Parasite, *Myxobolus cerebralis*, in Alberta, Canada

by

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Abstract

Whirling disease is a disease of fish caused by an invasive myxosporean parasite, *Myxobolus cerebralis*. It was first detected in Canada in Johnson Lake in Banff National Park, Alberta, in August 2016, and little is known about the transmission of this parasite in Canada. Current testing focuses on the detection of *M. cerebralis* in fish tissues, requiring lethal testing of both infected and non-infected fish. However, the parasite has a definitive host, the oligochaete worm *Tubifex tubifex* and two environmental stages found in water and sediment that create other avenues for detection. We propose that using the parasite stages found in water and sediment and the alternate worm host, *Tubifex tubifex,* are a reasonable complement to fish sampling and will be especially useful for sampling in areas where fish collection is challenging or prohibitive due to vulnerability of the fish populations. In addition, *T. tubifex* susceptibility to *M. cerebralis* is not consistent across the species, with experiments showing some are refractory. Characterization of these worm populations will help target future monitoring and control programs based on the presence or absence of susceptible *T. tubifex*.

This project utilized ~5000 samples collected over two years. These include sediment samples, invertebrate worm samples and water samples from stocked ponds. DNA was extracted from these samples using different methods tailored to the sample type and tested in a newly developed qPCR assay targeting the *18S* gene of *M. cerebralis*. Additionally, worm samples were barcoded by targeting the *cox1* gene to determine species, since as previous tests for *M. cerebralis* compatibility proved unreliable identification by morphology is unreliable.

I detected *M. cerebralis* with the new qPCR test at 18 different sites including two novel detections in previously negative water sheds, Athabasca and Peace River, where fish testing had all returned negative or was unable to be done. The worm host barcoding showed 37 different

species belonging to 21 genera. The genus *Tubifex* was divided into five different taxonomic groups, with a currently unknown variation in *M. cerebralis* compatibility. This work is an important early step to understanding the biology and providing alternatives for detection of this invasive parasite in Alberta to best inform management decisions in order to protect our natural resources.

Preface

Chapter 3 of this thesis is to be submitted as Barry, Danielle E.; James, Clayton; Veillard, Marie; Brummelhuis, Leah; Turnbull, Alyssa; Oddy-van Oploo, Arnika; Han, Xinneng and Hanington, Patrick C. Comprehensive qPCR-based monitoring for the whirling disease-causing parasite *Myxobolus cerebralis* in Alberta, Canada (**Paper I**)

Chapter 4 of this thesis is to be submitted as Barry, Danielle E.; James, Clayton; Veillard, Marie; Brummelhuis, Leah; Turnbull, Alyssa; Oddy-van Oploo, Arnika and Hanington, Patrick C. Molecular assessment of the invertebrate host, *Tubifex sp*., for the causative agent of whirling disease, *Myxobolus cerebralis,* in a non-endemic area (**Paper II**)

This research was conducted as part of a whirling disease monitoring project with Alberta Environment and Parks, Government of Alberta.

All samples were collected by the Government of Alberta, which was organized by Clayton James and Marie Veillard. I was responsible for data collection from environmental and worm samples along with Leah Brummelhuis, Alyssa Turnbull, Arnika Oddy-van Oploo and Xinneng Han. I was then responsible for analysis and manuscript preparation. Patrick C. Hanington was the supervisory author and was involved in project design, manuscript composition and edits. Fish samples were processed and tested by Government of Alberta employees and results were shared with our research group for comparison to the samples we processed for **Paper I**.

Acknowledgments

I would first like to thank my friends and family for their complete support throughout this process, without their encouragement and feigned interest in parasites, I would not have completed this thesis. I would specifically like to thank Dr. Patrick Hanington for taking a chance on me as an undergraduate and then graduate student, and his guidance throughout this opportunity. I would also like to thank Dr. Simon Otto and Dr. Norm Neumann for their help and support on this project as my supervisory committee.

Thank you to my lab mates, who not only helped me navigate the process of learning, troubleshooting and not giving up on lab experiments, but also helped with the challenges of grad school. Special thanks to Michelle and Brooke for phylogenetic assistance, and Leah for her dedication to the project. Thank you to the larger community of Environmental Health students, who again not only helped with their expertise but also their encouragement. A big thank you to the climbing ladies and ecology students who not only brought perspective but also balance to my grad school experience.

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Finally, I am forever grateful to my Mom, Georgia, and sister, Charlene, for their unwavering support and motivation throughout my post-secondary journey.

"I tell young people: Do not think of yourself, think of others. Think of the future that awaits you, think about what you can do and do not fear anything."

– Rita Levi, 1986 Nobel Prize Winner

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Chapter 1. Background and Introduction

Whirling disease is a disease of fish caused by an invasive myxosporean parasite, *Myxobolus cerebralis*. It was first detected in Canada in Johnson Lake in Banff National Park, Alberta in August 2016, and little is known about the transmission of this parasite in Canada. This thesis work was prompted by an interest to find a more sustainable path for monitoring of this parasite in the province of Alberta, as well as to understand more about the definitive worm host and transmission dynamics in a novel environment.

Myxobolus cerebralis Biology and History

There are around 2200 identified species of myxozoan parasites, many discovered due to their economic and health burden on commercial aquaculture and natural fish populations (Okamura et al., 2015). They are found within the Phylum Cnidaria and are all parasitic with a complex life cycle composed of the myxospore stage, which takes place within a vertebrate host (freshwater, marine or terrestrial), and the actinospore stage, which is found in an invertebrate host (Figure 1). The type of actinospore for *M. cerebralis* is referred to as a triactinomyxon (TAM) due to its shape with three caudal processes.

Numerous species of salmonids can be infected by *M. cerebralis*; however they exhibit varying clinical signs of disease (Gilbert and Granath, 2003). The severity of the disease is defined by several factors in the fish host; size and age at the time of exposure, strain/species of fish, and the dose of TAMs received. TAMs infect fish by attaching to the skin, gills or upper digestive system if swallowed. *M. cerebralis* causes a range of pathologies in the fish host from blackened caudal tail regions, severe skeletal deformities and whirling swimming behaviour, for which the disease is named (Sarker et al., 2015). The parasite targets cartilage in the fish host,

and it is thought that the swimming behaviour is caused by pressure on the spinal cord and lower brain stem from a parasite triggered inflammatory response. It is also thought that this type of damage may cause the caudal tail darkening as the nerves that control pigment deposition become damaged (Gilbert and Granath, 2003). Heavier infections in younger fish result in worse clinical symptoms and are often fatal. In contrast, infections in older fish tend to have less severe symptoms and often older fish are found to be asymptomatic, as the more ossified skeleton of an older fish has less cartilage for the parasite to feed on. Rainbow trout seem to be the species of fish that is most affected by *M. cerebralis* infection, while brown trout appear more tolerant with infections usually being asymptomatic, sometimes showing blackened tail regions but whirling behaviour hasn't been noted (Fetherman et al., 2012; Hedrick et al., 1999).

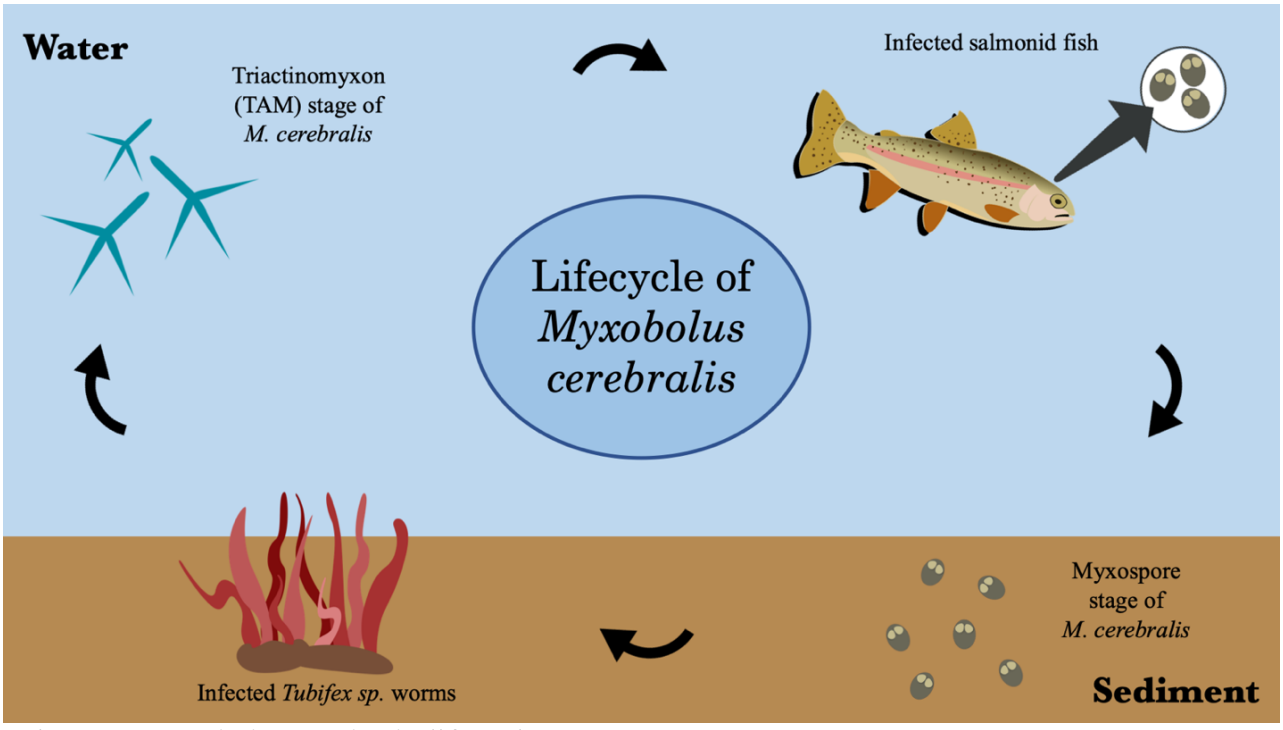

Figure 1. *Myxobolus cerebralis* lifecycle

Tubifex tubifex worms are the definitive host and live in sediment at the bottom of lakes and rivers and feed on detritus and bacteria selectively (Okamura et al., 2015). Currently, they

are the only worm host confirmed to transmit *M. cerebralis*, however it is known that the phylogeny of these worms is likely incomplete (Beauchamp et al., 2002a). Tubifex worms are important in the ecosystem for reworking sediments and being a food source for crustaceans, fishes and insects. They can survive eutrophic conditions and populations are usually kept low by predation and competition. Distribution within a watershed is influenced by substrate composition and organic content. Like other oligochaetes, *T. tubifex* is hermaphroditic, usually reproduces sexually but can reproduce asexually via parthenogenesis.

Originally from Europe, *M. cerebralis* is invasive in North America, having been first confirmed in hatcheries in Pennsylvania in 1958 (Hoffman et al., 1962). This parasite has been responsible for the decline of wild fish populations and stocked trout in North America, most notably in Colorado and Montana (Nehring and Walker, 1996; Vincent, 1996). Due to the presence of numerous salmonid species and favorable environmental conditions, this led to a rapid establishment and expansion of the parasite range. Currently *M. cerebralis* affects fish populations in Colorado, Wyoming, Utah, Montana, Idaho and New Mexico; with some populations being reduced by as much as 90% over the last two decades (Rognlie and Knapp, 1998). Given the impact on important sport fishing species, *M. cerebralis* is considered a serious threat to recreational sport fishing, estimated to be an industry valued over $125 million CAD annually in Alberta alone. In addition to the potential economic impact, salmonid species in Alberta that are known to be susceptible to whirling disease include: Rainbow Trout (*Oncorhynchus mykiss*), Cutthroat Trout (*Oncorhynchus clarkii*), Brook Trout (*Salvelinus fontinalis*), Brown Trout (*Salmo trutta*), and Bull Trout (*Salvelinus confluentus*). Of these species, Westslope Cutthroat Trout (*Oncorhynchus clarkii lewsi*), Athabasca Rainbow Trout, and Bull trout are listed under the *Alberta Wildlife Act* ("Wildlife Regulation, Alberta," 1997) and

Federal *Species at Risk Act* (Government of Canada, 2002). Following Johnson Lake being declared positive in August 2016, the Bow, Oldman, Red Deer and North Saskatchewan River watersheds have also been declared positive for *M. cerebralis* by the CFIA (Canadian Food and Inspection Agency (CFIA).

Other Species of Myxozoans in Canada

No large-scale work has been done to identify myxozoans in Canada or to create a central database on myxozoans and their hosts, despite their economic importance for aquaculture. Therefore, even on a provincial scale, a database of known myxozoans and their hosts is not available. In fact, I have not been able to locate any published information on any myxozoans in Alberta, apart from knowledge of one infecting fathead minnows in southern Alberta via personal communication. In *A Guide to Animal Parasites of Albertan Fish* published in 2000 by the provincial department of Alberta Agriculture, Food and Rural Development, there was no mention of a single myxozoan parasite for any fish species in the province (Government of Alberta, 2000). For diagnostic purposes, the myxozoans we would want to be most aware of are those found in Western Canada (BC-SK), as well as those found in closely related fish species (salmonids) and parasites closely related to *M. cerebralis*.

Currently, nine species of myxozoan parasite have been found in Western Canada: *Myxobolus arcticus* (Urawa et al., 2011) which may be synonymous to *M. neurobius* (Margolis et al., 1995), *Myxobolus neurophilus* (Scott et al., 2015), *Parvicapsula kabatai* (Jones et al., 2006), *Henneguya salminicola* (Fish, 1939), *Myxobolus aureatus* (Margolis et al., 1995), *Myxobolus bibullatus* (Margolis et al., 1995), *Myxobolus commersonii* (Margolis et al., 1995), *Myxobolus sqamalis* (Polley et al., 2013) and *Kudoa thyrsites* (Moran et al., 1999). All species except *M. neurophilus, M. aureatus* and *M. bibullatus* infect at least one salmonid species. Based

on *18S* sequences of Myxozoan parasites, the closest related *Myxobolus* species to *M. cerebralis* are *M. squamalis*, *M. elipsoids*, *M. sandrae*, *M. djragini*, and *M. bramae* (Holzer et al., 2018) and currently only *M. squamalis* has been found in Canada.

Past Monitoring for *Myxobolus cerebralis*

Currently, testing for *M. cerebralis* relies on detecting the parasite in fish tissues, which requires collection and lethal testing of both infected and non-infected fish, as fish are sometimes asymptomatic (Sarker et al., 2015). Both microscopy (spore counts with or without an initial digestion) and molecular methods (PCR and qPCR) for parasite identification have been used for monitoring in the United States (Cavender et al., 2004; Hogge et al., 2004; Kelley et al., 2004). Two acceptable methods exist for microscopy-based tests; the digestion method or the plankton centrifuge method. Both of these rely on the fish being at least 120 days old at time of collection and for the spore stage of the parasite to bebeing directly observed in homogenized fish tissue. Both methods can be affected by the preservation technique used with the sample and the duration of time before the sample is analyzed. Misidentification is possible with microscopy-based techniques indicator?. due to morphological similarities between the myxospore stage of multiple *Myxobolus* species (Cavender et al., 2004; Hogge et al., 2004). To address these myxospore identification concerns, DNA based PCR and qPCR tests have been developed, usually following homogenization or a pepsin-trypsin digest, using pooled fish samples (Cavender et al., 2004; Kelley et al., 2004).

A clear disadvantage of relying on fish samples for testing is that the parasite must have already established in the fish population to be detected at an appropriate level of confidence. When a parasite is establishing in an area, disease prevalence will be low. To gain a 95% level of confidence that an infectious agent has been detected when it is at a low 2% infection

prevalence, 76 out of a population of 100 fish would have to be sampled (Gillespie et al., 1974). This level of sampling would place a large burden on already threatened or endangered fish populations, as well as populations that require continued monitoring over time. Waiting until the parasite has established in the population leaves any preventative measures behind an already established infection. This was noted in many US States where the parasite was only detected once established in fish populations, making control measures more difficult (Chiaramonte et al., 2018). It also leaves a testing gap for locations without fish. It is important in these locations to determine infection status before stocking fish into a pond/aquaculture facility or a watershed in the case of restorative stocking for species at risk. In addition, fish collection and testing, especially when considering many sites and a wide geographic area, is time consuming and costly. Hence, there is an opportunity to use the parasite's lifecycle to our advantage and develop methods for detecting *M. cerebralis* in other stages of its lifecycle.

As the majority of research studies has looked at the infection processes in fish rather than in the worm host, only a few studies have examined the compatibility between various oligochaetes and *M. cerebralis* (Arsan et al., 2007; Beauchamp et al., 2002a; Zendt and Bergersen, 2000a). A *Tubifex* sp. lineage PCR test had been developed to assess different toxicological responses to cadmium in wild *Tubifex* populations, and some research groups have connected these different lineages to the variability in compatibility between specific *T. tubifex* lineages and *M. cerebralis* (Baxa et al., 2007; Beauchamp et al., 2001; Sturmbauer et al., 1999). However, many studies do not use this lineage PCR test and only morphologically identify infected worm hosts based on colour, creating a possibly inaccurate record of infection. This lineage PCR test was developed using worm populations in Europe and the USA, and the accuracy on Albertan worm populations is unknown. These different factors show a lack of well-

designed studies that focus on understanding the worm species responsible for transmitting *M. cerebralis*. Given that worms produce the life stage infectious to fish, it is important to close these knowledge gaps to be able to move toward a more preventative approach of controlling whirling disease.

The Definitive Host- *Tubifex tubifex*

Tubifex tubifex can live for up to three years and can remain infected with *M. cerebralis* throughout their lifespan (Zendt and Bergersen, 2000a). Within this worm host, *M. cerebralis* myxospores extrude polar capsules that attach to the gut epithelium and penetrate the intestinal wall. These cells replicate in the intercellular space of the gut epithelial cells before beginning sexual reproduction via sporogenesis which produces TAMs, and are released with fecal matter through the anus. In experimentally and naturally infected *T. tubifex*, TAMs are released around 75 days post exposure to myxospores, and continued until 132 days post-exposure (pe) (Gilbert and Granath, 2003). Gilbert and Granath (2003) also noted shedding beginning again at 606 days pe supporting that these worms remain infected throughout a normal lifespan and shed viable TAMs at different points in time. Development and shedding of TAMs can also be influenced by water temperature. Lab experiments show that the highest number of TAMs are released between 10-15ºC, which correlates to the finding that the most severe whirling disease cases are found in that temperature range (Rasmussen et al., 2008). The periodicity of TAM release being connected to seasonality would explain peaks in infection rates found in sentinel fish studies. There is a lack of published work on the pathology affecting Tubifex worms when infected by *M. cerebralis*. However, alterations in the coloration and shape of the intestine can be seen in an actively TAM shedding worm (Gilbert and Granath, 2003).

Regardless of parasite transmission, identification of oligochaete worms based on morphological characteristics is a common hurdle, despite their cosmopolitan distribution (Brinkhurst, 1996). Morphological identification is difficult with most species in the family Tubificinae and the majority can only be identified when mature, which may not be available in a sample (Beauchamp et al., 2001). Many papers have shown the existence of cryptic species within these groups as morphological characteristics are plastic depending on environmental conditions and vary considerably within currently defined taxa (Achurra et al., 2011; Bely and Wray, 2004; Vivien et al., 2015). All of these factors make identification on a large sample prohibitively time consuming.

To understand the *M. cerebralis* transmission dynamics for novel areas, research groups have started to do more in depth assessments of the worm host. Taxonomic groups within *Tubifex* based on genetic tests have shown significant diversity within the genus in the ability to carry a patent *M. cerebralis* infection (Arsan et al., 2007; Beauchamp et al., 2002b; Rasmussen et al., 2008). Previously, 'lineages' based on mitochondrial *16S* ribosomal DNA have been used to group *T. tubifex* based on their ability to transmit *M. cerebralis* (Beauchamp et al., 2002b; Hallett et al., 2009; Sturmbauer et al., 1999). Four separate lineages have been found in the continental United States and Alaska, I, III, V and VI, with others found in Europe (Beauchamp et al., 2001; Sturmbauer et al., 1999). Parasite replication varies among these lineages, ranging from susceptible to infection and producing viable actinospores (I and III), to susceptible to infection and where parasite development is not completed (V), and finally, to no infection at all (VI) (Beauchamp et al., 2002b). Analyzing worm populations in novel environments in the United States based on susceptibility to *M. cerebralis* has become a useful proxy for assessing risk to salmonid populations (Bartholomew et al., 2005; McGinnis and Kerans, 2013).

Considerations for Developing a qPCR Assay

qPCR is a DNA based molecular testing method that utilizes polymerase chain reaction to amplify and quantify a select genetic sequence (amplicon) from a DNA sample. Amplification is accomplished by a pair of primers that bind on each end of the target DNA sequence and allow the DNA to be replicated during the cycling polymerase chain reaction. A probe binds between these primers that gives off a quantifiable fluorescent signal that is detected and recorded. There are a number of considerations for the development of qPCR tests.

First is to select a target gene and amplicon within that gene. Gene selection is obvious when using qPCR to measure gene expression, but when using qPCR for species detection, there are more variables to consider. When the goal is detection of a species in an environmental sample, the organism may be in low abundance (Bohmann et al., 2014), so selecting a gene that is in a high copy within each cell will increase the chance of detection. Next, a gene that has adequate specificity for your desired target is necessary. If one wishes to detect all species in a family or genus, a different gene target may be used than if the assay is to be used for species level resolution. It is also important to select a gene that has adequate coverage for other non-target species' sequences in online databases so an appropriate amplicon and probe region can be selected during *in silico* analysis. The amplicon should be 75-150 bases and be in a location on the gene that allows the primers and probe to bind to areas where the sequence is different between closely related species and/or those that are expected to be found in the sample when dealing with environmental samples (Burd, 2010). G/C rich areas are to be avoided as well as highly repetitive and palindromic areas to facilitate proper primer and probe binding. Ideally the primers will also give specificity to the assay, but if there are no suitable sequence areas, specificity can be achieved through the probe instead. For species detection a hydrolysis probe is

appropriate due to increased specificity, the most common type being TaqMan™. For this probe type, it's melting temperature is usually 7-10ºC higher than the primers to allow for binding to the complimentary DNA strand before extension occurs. The probe contains a fluorescent dye at the 5'end and a quencher on the 3' end which prevents a fluorescent signal. When the polymerase degrades the probe after the primers have bound, the fluorescent dye is released and is no longer being quenched, allowing it to be detected by a camera in the qPCR machine, so quantification can be achieved.

Following the design of the assay, optimization and validation is necessary. Even pre-designed assays need to be validated in-house as different brands of master mix and qPCR machines can affect the test results. It is suggested to check between new sets of primer/probe synthesis or batches of mastermix. A newly designed assay is then used on synthetic genes, such as those made in plasmids, and positive and negative controls. During this testing it is also important to test samples extracted from the same environmental matrix that you plan to be testing samples from as adjustments may need to be made to optimize the reaction for those samples. How well the assay detects and quantifies the desired target can be considered the accuracy of the assay.

Samples are quantified based on a standard curve created from known gene copies used in each run. Ideally, this standard curve spans the range of expected sample concentrations. Often a synthesized form of DNA is made in a known concentration and used to create the standard curve via serial dilutions. With the standard curve, the efficiency of the reaction can be determined first using an equation to calculate the slope of the line of best fit through the points of the standard curve:

$$y = mx + c$$

Then the slope (m) is used to calculate the efficiency which should fall between 90-110%:

$$E = 10^{-1/m} - 1$$

These measurements are often calculated automatically by the software used by the qPCR machine when the known values of the standard curve are inputted. R^2 is also calculated as a measure of goodness of fit of the data points to the line of best fit, this value should be close to 1 which indicates the points are all well fit to the line. This measurement is also referred to as precision, which indicates how close replicates of the same sample are to each other.

Chapter 2. Objectives and Scope

Since *T. tubifex* maintain the infection throughout their lifespans, I believe that these worms can serve as an important and useful target for monitoring efforts. While the prevalence of worms actively shedding TAMs into the water column in natural populations is usually quite low at 0.4 to 1.5% (Beauchamp et al., 2002a), our initial testing by qPCR suggested that the overall prevalence of a detectable infection is in the range of 15-35% at an established site. This means that if worms are tested for *M. cerebralis* DNA instead of looking for shedding worms to confirm parasite presence, the parasite is much more likely to be detected by molecular means. As worms are much easier to collect and at a much higher number in these environments than juvenile fish, monitoring efforts could shift to worm surveillance to assess infection potential at a site.

In addition to utilizing the worm definitive host, the free-living stages can also be used to detect the parasite. Similar qPCR based testing is done for other invasive species such as zebra mussels, and swimmer's itch causing parasites in Alberta (Rudko et al., 2018). Water collected and concentrated using a zooplankton net is a suitable method for targeting free-living water stages, TAMs in our case. We also suggest using sediment samples, which contain myxospores from decomposing fish that were infected, as another, easier environmental target to substitute fish testing. This type of sediment surveillance has also been used to detect chytridiomycosis causing fungus and *Naegleria fowleri* in aquatic environments (Kirshtein et al., 2007; Streby et al., 2015).

I hypothesize that environmental samples, including the worm host, will be suitable and informative alternatives to fish sampling for the tracking of *Myxobolus cerebralis*.

As part of the initial investigation of *M. cerebralis* dynamics in Alberta, worms were tested in the lineage PCR (endpoint) and a qPCR test targeting the *18S* gene of *M. cerebralis* for infection status. Those lineage PCR results did not fit into previously published lineages, with majority of worms producing a band size not consistent with any published band size for this test, or no band at all. These inconsistencies lead us to believe that the lineage test may not be reliable for worm populations in Alberta. Consequently, I decided to assess the worm populations via species barcoding and to have the first large scale molecular study of oligochaete worms in Canada.

I hypothesize that *Tubifex tubifex* will be found to be the primary definitive host of *M. cerebralis* in Alberta, but given the geographical differences between Alberta and locations where whirling disease has been found in the past, there will likely be distinctions between the *T. tubifex* populations.

The overall aims of this thesis are as follows:

1. Validate a qPCR assay to be used to test for the presence of *M. cerebralis* in environmental samples
2. Advance our understanding of the invertebrate definitive host in an Albertan context through species barcoding

Chapter 3. Comprehensive qPCR-based monitoring for the whirling disease-causing parasite *Myxobolus cerebralis* in Alberta, Canada

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Abstract

Myxobolus cerebralis is an invasive aquatic parasite that is the causative agent of whirling disease in salmonid fish. In 2016, this parasite was detected in Canada for the first time, initiating a comprehensive, three-year monitoring program to assess where the parasite had spread. As part of this program, a novel qPCR-based test was developed to facilitate sampling and detection of *M. cerebralis* at the free-living environmental stages of the parasite life cycle and from DNA extracted from the definitive oligochaete host, *Tubifex tubifex*. During this program, ~2800 samples were collected over two years of this three-year project, including sediment samples, oligochaete samples and water samples from stocked ponds. Fish from environmental sampling sites were collected for all three years. Testing for *M. cerebralis* relied on the novel qPCR test based on the *18S* gene of *M. cerebralis* collected in Alberta. The testing showed sites positive for *M. cerebralis* in three fish testing-confirmed positive watersheds and three novel detections where the parasite had not been detected with fish testing. Additionally,

oligochaete samples were tested with the previously reported *T. tubifex* lineage PCR assay, which has been used to assess *T. tubifex* susceptibility to *M. cerebralis* infection in the US. Lineage assessment of 233 *T. tubifex* suggests that there are unique *T. tubifex* populations in Alberta that do not yield expected results in this test. Thus, the lineage PCR may not be appropriate for use in predicting *T. tubifex* compatibility with *M. cerebralis* in Alberta. Based on our results, we propose that using the parasite stages found in water, sediment and the *T. tubifex* oligochaete host is a valuable complementary test to fish sampling and will be especially useful in large scale monitoring programs, particularly for sampling in areas where fish collection is challenging or prohibitive due to vulnerability of the fish populations.

3.1 Introduction

Whirling disease is a disease of fish caused by a myxosporean parasite, *Myxobolus cerebralis*. This parasite has a two-host life cycle, utilizing a salmonid fish host and an aquatic oligochaete worm host (Figure 1) (Markiw and Wolf, 1983). Myxospores develop in the fish host and are released from both live (Nehring et al. 2002) and decaying fish; these spores are infective to the worm host. Actinospores, otherwise known as triactinomyxons (TAMs), are produced by the worm host and are released into the water column where they infect fish by attaching to gills and skin or via ingestion. Originally from Europe, *M. cerebralis* is invasive in North America, having been first confirmed in hatcheries in Pennsylvania in 1958 (Hoffman et al., 1962). This parasite has been responsible for the decline of wild fish populations and stocked trout in North America, most notably in Colorado and Montana (Nehring and Walker, 1996; Vincent, 1996).

M. cerebralis was first detected in Canada in Johnson Lake in Banff National Park, Alberta, in August 2016 (Canadian Food Inspection Agency, 2016), and little is known about the

establishment and transmission of this parasite in Canada. Following the detection in Johnson Lake, four major watersheds have been declared positive for *M. cerebralis* by the Canadian Food Inspection Agency (CFIA), including the Bow River, Oldman River, Red Deer River and North Saskatchewan River watersheds (Figure 2). Salmonid species in Alberta that are known to be susceptible to whirling disease include Rainbow Trout (*Oncorhynchus mykiss*), Cutthroat Trout (*Oncorhynchus clarkii*), Brook Trout (*Salvelinus fontinalis*), Brown Trout (*Salmo trutta*), Bull Trout (*Salvelinus confluentus*), and Mountain Whitefish (*Prosopium williamsoni*). Of these species, Westslope Cutthroat Trout (*Oncorhynchus clarkii lewsi*), Athabasca Rainbow Trout, and Bull trout are listed under the Federal *Species at Risk Act* (Government of Canada, 2002) due to their threatened or endangered status.

Current testing for *M. cerebralis* relies on detecting the parasite in fish tissues, which requires lethal testing of both infected and non-infected fish. Both microscopy (spore counts with or without initial digestion) and molecular methods (PCR and qPCR) for parasite identification have been used for monitoring in the United States (Cavender et al., 2004; Hogge et al., 2004; Kelley et al., 2004). Two acceptable methods are identified for microscopy-based tests: the digestion method or the plankton centrifuge method. Both of these rely on the fish being at least 120 days old at the time of collection, and the spore stage of the parasite is directly observed in homogenized fish tissue. Both techniques can be affected by the preservation technique used with the sample and the duration of time before the sample is analyzed. Misidentification is possible with these microscopy-based techniques as the myxospore stage is not a good diagnostic tool due to morphological similarities between multiple *Myxobolus* species (Cavender et al., 2004; Hogge et al., 2004). To address concerns related to the identification of myxospores, DNA

based PCR and qPCR tests have been developed, usually following homogenization or a pepsin-trypsin digest, usually using pooled fish samples (Cavender et al., 2004; Kelley et al., 2004).

A clear disadvantage of relying on fish samples for testing is that the parasite must have already established in the fish population to be detected at an appropriate level of confidence. When a parasite is establishing in an area and disease prevalence is low in the host population, say 2%, to gain a 95% level of confidence that an infectious agent has been detected, 76 out of a population of 100 fish would have to be sampled (Gillespie et al., 1974). This level of sampling would place a large burden on already threatened or endangered fish populations, as well as populations that require continued monitoring over time. Waiting until the parasite has established in the population leaves preventative measures behind an already established infection. This was noted in many US States where the parasite was only detected once established in fish populations, making control measures more difficult (Chiaramonte et al., 2018). It also leaves a testing gap for locations without fish, such as migratory fish populations or a pond that has not yet been stocked or is between regular stocking intervals. It is important in these locations to determine infection status before stocking fish into a pond/aquaculture facility or a receiving watershed in the case of restorative stocking efforts for species at risk. In addition, fish collection and testing, especially when considering the wide geographic extent of available salmonid habitat, is time consuming and costly. Hence, there is an opportunity to develop methods for detecting *M. cerebralis* in other stages of its lifecycle.

While most whirling disease research has examined the infection and disease processes in fish rather than in the worm host, a few studies have examined the parasite/host compatibility between oligochaetes and *M. cerebralis* (Arsan et al., 2007; Beauchamp et al., 2002a; Zendt and Bergersen, 2000a). Currently, *T. tubifex* is the only worm host species confirmed to transmit *M.*

cerebralis. However, the phylogeny of these worms is likely incomplete and impacted by the presence of cryptic species (Beauchamp et al., 2002a). A *T. tubifex* lineage PCR test, developed to assess different toxicological responses to cadmium in wild *T. tubifex* populations, has been used to demonstrate that different *T. tubifex* lineages display variability in compatibility to *M. cerebralis*, with some being refractory (Baxa et al., 2007; Beauchamp et al., 2002a, 2001; Rasmussen et al., 2008; Sturmbauer et al., 1999). However, many studies only morphologically identify infected worm hosts based on colour and presence of chaetae and do not report the lineage PCR test result. Morphological identification is difficult with most species in the family Tubificinae and the majority can only be identified when mature and morphological characteristics are plastic depending on environmental conditions and vary considerably within currently defined taxa (Achurra et al., 2011; Bely and Wray, 2004; Vivien et al., 2015). As the current lineage PCR test was developed using worm populations in Europe and the USA, and no large-scale oligochaete worm assessments have been done in Alberta, or Canada, the accuracy of the lineage test on Albertan worm populations is unconfirmed. As worms produce the parasite life stage infectious to fish, it is important to fill these knowledge gaps and move toward a more proactive approach of detecting whirling disease by utilizing our knowledge of the parasite lifecycle to our advantage. Characterization of the worm populations within Alberta will also help target future monitoring and mitigation programs based on our understanding of the presence or absence of susceptible *T. tubifex*.

Surveillance for parasites and invasive species using environmental samples and alternate hosts has been utilized in other systems, for both naturally occurring parasites like trematodes that cause swimmers itch (Rudko et al., 2018) using water samples or the snail host and invasive species like zebra mussels by detecting suspended larva in water samples (Ardura et al., 2017).

Using the lifecycle of an organism to our advantage, we can target other stages that may be easier to collect or more cost-effective to monitor.

We propose that using the parasite stages found in water, sediment and the alternate worm host, *Tubifex tubifex,* are a valuable complementary detection tool to fish sampling and will be especially useful in large scale monitoring programs and for sampling in areas where fish collection is challenging or prohibitive due to vulnerability of the fish populations. Targeting parasite lifecycle stages, other than fish, allows for more routine monitoring and can provide data on transmission dynamics, including seasonal peaks. As the parasite must be established in the worm population at a site before stages that infect fish are released, this is an opportunity to detect the parasite before it is established in fish populations.

Therefore, the objectives of this study were to 1) develop and validate a novel qPCR assay with increased specificity to detect all relevant lifecycle stages of *M. cerebralis* in the worm host, sediment and water samples, 2) utilize the newly developed assay to test oligochaete hosts, sediment and water samples from wild sites and stocked ponds throughout the province, and 3) assess the validity of the *T. tubifex* lineage PCR assay on oligochaetes collected from Alberta while aiming to map worm susceptibility across geographic locations. Here, we report on the development, validation and implementation of this qPCR assay to monitor for *M. cerebralis* in environmental and worm samples over a two-year, comprehensive monitoring program that spanned wild sites throughout the eastern slopes of the Alberta Rocky Mountains and stocked ponds in Alberta.

3.2 Methods

3.2.1 *M. cerebralis* qPCR Assay Development and Validation

qPCR Assay Validation

The *18S* gene was selected as the target, based off an assay developed by Cavender et al. (2004), however when the previous assay was aligned with currently available sequences *in silico*, there appeared to be cross-reaction with other known Myxozoan species, and the possibility for nonagreement with the probe sequence for some *M. cerebralis* sequences (Supplemental Table B). As more *18S* sequences for Myxozoan species have become available since 2004, we were able to align and analyze more species for unique regions that could serve as suitable qPCR primer and probe regions to uniquely amplify and detect *M. cerebralis* specifically (Supplemental Table A). Primers were developed for a 120-bp region using the real-time qPCR assay design tool from IDT (www.idtdna.com/PrimerQuest/) to select specific primer and probe sequences, as found in Table 1. The probe used 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and Iowa Black FQ (Integrated DNA Technologies) as a quencher at the 3' end. This assay was confirmed with plasmids containing the assay sequence and from positive control samples from purified and confirmed *M. cerebralis* myxospore samples.

Table 1. qPCR primers and probe for *18S* gene target for *Myxobolus cerebralis*

MC18S_fwd	5'- GCTGATCGAATGGTGCTACTAA-3'
MC18S_rev	5'- TCAACTGCCATCCTTACGC-3'
MC18S_probe	5'-/56-FAM/AGTGTTGGA/ZEN/GTAGTGTGCCGTCTT/3IABkFQ/-3'

Development of a qPCR plasmid standard

A plasmid containing the region of the *18S* gene that is targeted by the qPCR test (Accession Number EF370481.1, nucleotide numbers 645 to 777) was synthesized by GenScript (USA) and inserted into a puc57 vector. Plasmid preparations were transformed into TOP10 cells and plated on 100ug/ml carbenicillin containing LB plates to confirm successful plasmid uptake. Plasmid purification was then done with the GeneJET plasmid miniprep kit (Thermo Scientific) following the manufacturer's specifications.

Standard curve and Limit of Detection

Purified plasmid DNA containing the specific *M. cerebralis 18S* region used to generate our qPCR standard curves was quantified using the Qubit fluorometer (Thermo Scientific). Stocks of 100 000 copies/uL were diluted and frozen at -20 ºC until used. This stock was then diluted to have 50 000, 5000, 500, 50 and 5 copies of plasmid per reaction to create the standard curve used to calculate the *18S* copy number for every qPCR run. Kelley et al. (2004) found 104 copies of the rDNA *18S* gene per cell. TAMs have 70 cells each and spores have six cells each, bringing the total estimated number of *18S* gene copies to ~7000 per TAM and ~600 per spore (Kelley et al., 2004).

Each standard was run in triplicate and values for PCR efficiency, slope and correlation coefficient were automatically calculated with the QuantStudio 3 software. The limit of detection with 95% confidence of our assay was determined using the probability of detection-limit of detection (POD-LOD) program with ten replicate standard curves (Wilrich and Wilrich, 2009).

qPCR reaction parameters

All qPCR tests used IDT PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies) and followed manufacturer recommendations. All samples and controls were run

in triplicate. 20uL reactions were run with 5uL of extracted DNA, and 250nM of forward/reverse primer and probe. All reactions were run in 96-well plates in a QuantStudio 3 (Thermo Scientific), using the manufacturer setting for fast cycling: 20sec hold at 95 ºC, followed by 40 cycles of 95 ºC for 1sec, 60 ºC for 20sec. DNA copy numbers were quantified based on a standard curve. Samples were prepared following standard qPCR workflow protocols; master mix is stored and prepared in a pre-PCR clean room, standards and samples are added in a different room with a dedicated dead air box, and the qPCR is run in a post-amplification room where all high copy DNA is handled and processed.

3.2.2 Sampling

Site Selection

Wild site samples were collected from six watersheds in Alberta (Figure 2). Approximately five sites were sampled in each sub-watershed to ensure sample coverage in each area. Sites were selected based on five factors: the abundance of potentially susceptible trout species, high-risk areas for whirling disease (based on stream gradient and temperature), location of parasite vectors (high risk stocked ponds, irrigation canals, and popular fishing locations), geographic breaks related to whirling disease spread (barriers to fish movement such as dams and waterfalls), and accessibility to sites. High-risk areas for whirling disease include slow moving water, which is suitable habitat for the worm host (DuBey and Caldwell, 2004) and parasite release is temperature dependent, with water temperatures above 15ºC being required(Kerans et al., 2005). From 2016 to 2018, 742 unique wild sites were sampled throughout the eastern slopes of the Rocky Mountains.

Stocked ponds were originally sampled based on a CFIA trace out, a study that identified approximately 684 ponds that were stocked from an infected hatchery. In 2017, the ponds identified in this study that were also in close proximity to Calgary AB were sampled. In the 2018 sampling year, ponds were provincially ranked from lower to higher priority based on their hydrogeological connectivity to a watercourse, flood risk and use. Higher connectivity, use and flood risk and proximity to susceptible salmonid populations resulted in a higher priority given to a pond. In total, 36 ponds were sampled in 2017 and 191 in 2018.

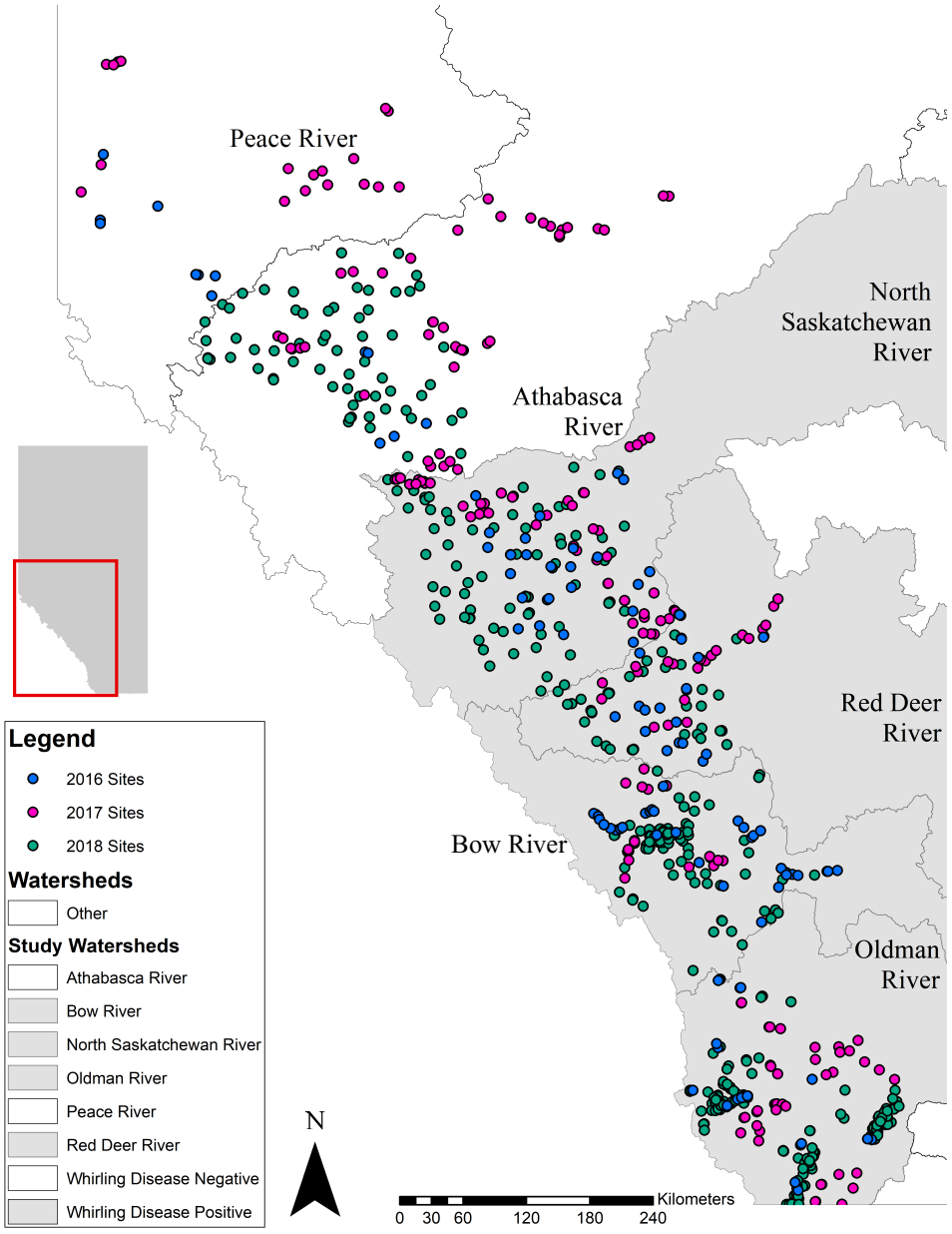

Figure 2. Wild environmental sampling sites for *Myxobolus cerebralis* from 2016-2018 in Alberta, Canada.
These sites were selected by Alberta Environment and Parks to collect sampling for monitoring of whirling disease causing parasites. Watersheds declared positive for *Myxobolus cerebralis* by the Canadian Food Inspection Agency (CFIA), which is based on fish testing, are shaded in.

3.2.3 Fish Sample Collection and DNA Extraction

Wild Fish Collection

Fish were collected in 2016 and 2017 from five to six wild sites within each hydrologic unit code (HUC), with a target of 150-175 juvenile fish (measuring between 40-150mm) per HUC. HUC's are a system of ranked identification numbers based on the hierarchy of hydrologic features from continental drainages at level 1 down to local sub-watersheds at level 12. These numbers indicate the size and location of each water feature, giving a consistent sized area within a watershed to target for collection. Fish were collected via electrofishing with either jet-powered or rowboats depending on what boat launch facilities were present. If boat access was not permissible, backpack electrofishing equipment was used. All fish collected were age classed based on length and were examined for any classic signs of whirling disease (e.g. blackened tail, skeletal/cranial deformities and whirling behaviour) to target testing if possible and monitor for disease impact and use this to compare to DNA-based testing results. Rainbow Trout (*Oncorhynchus mykiss*), Brook Trout (*Salvelinus fontinalis*), Cutthroat Trout (*O. clarkii*), and Mountain Whitefish (*Prosopium williamsoni*) were collected preferentially. Following collection, fish were stored on ice for a maximum of four days and transferred to a -20 ºC freezer as soon as possible for a maximum of two weeks before being stored at -80 ºC long term.

Stocked Pond Fish Collection

Brook trout, brown trout, and rainbow trout were collected from 29 of the 36 stocked ponds that were sampled in 2017. There was no requirement for fish size or age as it was assumed infection would have occurred at the infected aquaculture facility when the fish were young. The number of fish collected at each pond varied based on the perceived risk of the pond containing *M. cerebralis*. Less than 30 fish were collected at a pond that had been stocked from a

positive facility in the last three years. One pond that had not been stocked from a positive facility in three years and also did not have watershed connectivity, had 60-120 fish collected. A high priority pond had fish stocked from a positive facility more than three years ago, has since been stocked with resistant species of fish or has high watershed connectivity with a wild system. All fish were collected using gill nets. Following collection, fish were stored on ice for a maximum of 4 days and transferred to a -20 ºC freezer as soon as possible for a maximum of two weeks before being stored at -80 ºC long term.

Fish Processing and DNA Extraction

Fish were pooled in groups of one to five based on species and age class. The heads were divided sagitally and separated into two samples. One sample was subject to homogenization and the other is processed with a pepsin-trypsin digest (PTD) prior to DNA extraction. The homogenization protocol was based on the protocol from the Fish Health Section of the American Fisheries Society Blue Book (2006). Briefly, fish head sections were homogenized for 60 sec in a stomacher machine in a 1:10 ratio of Dulbeccos medium. The PTD protocol is based on the guidelines outlined in Markiw and Wolf (1974) and briefly, heads are heated for 10-90 minutes and de-fleshed leaving bone and cartilage intact, and then heated for 30-120 min in 20mL of pepsin solution for every gram of fish tissue. The final trypsin digest is in 10mL of trypsin solution for every gram of starting material for 30 min. DNA was extracted from the resulting solution from homogenization and PTD with the Qiagen Blood and Tissue Kit (Qiagen) following the protocols for animal tissue.

3.2.4 Oligochaete Specimen Collection and DNA Extraction

Wild sites where fish were collected in 2016 had corresponding worm samples collected at the same locations in 2017; these will be referred to as 2016 sites. Worm samples were collected in 2017 along with fish samples, at new sites, called 2017 sites. In 2018 collection focused on invertebrate samples, called 2018 sites. Stocked pond sites had worms collected in 2017 and 2018.

Oligochaetes were collected using a 500 μm mesh D-Frame benthic kick net. At wild sites, samplers targeted slow-moving pools with fine sediment substrates or eddies directly behind large boulders where fine sediment deposition occurs. A minimum of five samples were combined from the top 10 cm of sediment at each site. In stocked ponds, oligochaete samples were taken at the same location as sediment samples at the ponds. Samples were refrigerated and transported in 70% ethanol or in stream water. Samples were sorted and all visible oligochaetes were individually isolated and stored in 70% ethanol at -20^{o}C until further processing.

Oligochaete DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). Kit protocols for animal tissues were followed with two modifications: using a minimum of two hours for digestion and 50uL final elution.

3.2.5 Sediment Sample Collection and DNA Extraction

Sediment samples were collected starting in 2017 at the same locations at which fish were collected in 2016, referred to as 2016 sites. Sediment samples were collected in 2017 concurrent to fish sampling at new 2017 sites. In 2018 a small proportion of oligochaete sampling sites had corresponding sediment collected for testing.

Sediment samples were collected from fine sediment habitats associated with slow-moving water (e.g., pool habitat, eddies behind boulders) and collected using a small scoop or shovel within the upper 20-30 cm of substrate. Sediment samples were combined from up to three separate locations (e.g., pools) within each site to ensure adequate coverage of the site. Samples from each location at a site were combined into one ~50 mL composite sediment sample, placed in a 100 mL screw-top container, and topped up with 95% ethanol or stream water.

Sediment was collected from stocked sites in 2017 and 2018, with the addition of composite sediment samples in 2018, where two to nine samples were collected around a stocked pond and combined before testing. Samples were collected from the upper 20-30 cm of substrate at a minimum of the three locations using an Ekman grab sampler or a scoop. Samples were placed into 125 mL bottles, topped up with pond water, and sealed. If sufficient fine sediment (organics, silt, and sand) was not found at any of the initial three locations, additional locations were sampled until three sites containing fine sediments could be collected.

To assess the sensitivity of the qPCR assay when DNA was extracted from a more complex matrix and to test different soil DNA extraction kits, known numbers of spores were spiked into known negative sediment samples and extracted with two commercially available soil extraction kits. We used Soil DNA Isolation Plus Kit (Norgen Biotek Corporation) and Quick-DNA Fecal/Soil Microbe Kit (Zymo Research) and followed all kit protocols for this assessment. This test was done by adding either 100 or 250 spores into 40mL of sediment from a negative pond and mixed thoroughly. Then 1mL of sediment was subsampled from this and was extracted for DNA. With the Soil DNA Isolation Plus Kit (Norgen Biotek Corporation) and the new *18S* qPCR assay, we were able to detect 100 spores in 40mL of sediment. The Norgen

Biotek kit was selected and all kit protocols were followed to extract DNA from all sediment samples for the remainder of the project.

3.2.6 Water Sample Collection and DNA Extraction

Water was collected from 36 stocked ponds in 2017. A composite sample was taken from three locations in each pond. One hundred litres from each location were put through a 20um zooplankton collection net. After the water was passed through, the sides of the net were washed with distilled water to move any material into the collection container at the bottom. Then 250ml of 95% ethanol was added to make the final ethanol concentration ~70%. Due to the turbidity of the samples, further filtering in the lab to remove the ethanol was difficult, so samples were centrifuged at 4200rpm for 5min and supernatant was pipetted off without disturbing the pellet until as much liquid was removed as possible. Five hundred uL was sub-sampled twice from the remaining material and each sample was extracted for DNA.

The water samples were extracted with the Qiagen DNeasy Blood and Tissue Kit (Qiagen). Kit protocols for animal tissues were followed with the exception of an overnight (~14-16hr) digestion.

3.2.7 Molecular Testing

Environmental and Worm

The above qPCR procedure was used to test for *M. cerebralis* in 2800 unique sediment, water and worm samples. Samples from 2017 were stored in 70% ethanol while samples from 2018 were “fresh” in stream or pond water and kept refrigerated (4ºC) and samples were processed as outlined above to extract DNA.

Fish

In 2016 fish testing was run at the Molecular Biology Service Unit at the University of Alberta in Edmonton Alberta. Fish samples from 2017 were processed and tested in a Government of Alberta laboratory. The qPCR test used with the fish samples was an unpublished test from a fish disease testing laboratory in Washington, where the first positive fish samples were tested when *M. cerebralis* was initially detected in the province. When the province took over the testing of fish, they continued to use this test. The test can be found online in a PDF that appears to be an academic poster but was not presented at a conference. None of the author's names on the poster produced any published work in an online database search (Google Scholar and Web of Science) on the qPCR test for *M. cerebralis*. *In silico* analysis appeared to show most of the specificity in the assay was on one primer. At this time, we have no reason to think the assay would cross-react with other myxozoans known to occur in Western Canada. However, continued use of this test should be cautioned as its validation status is unknown, and there has not been a comprehensive assessment of what myxozoan species are present in Alberta. It is, however, potentially favourable that this test was used in fish samples, as myxozoans tend to be tissue/site-specific in their specific hosts. In testing fish heads, we would hopefully be only encountering one myxozoan parasite species at a time. These fish samples were considered positive if either or both of the homogenized or PTD samples came back positive in the unpublished qPCR test.

3.2.8 Tubifex Lineage PCR

At first, all sampled worms that tested positive with the qPCR test and a selection of negative worms were run through the previously published lineage PCR assay to characterize the

worm populations in Alberta based on their ability to transmit *M. cerebralis* and assess whether geographical differences associated with susceptible worm populations. We used a mixture of the four forward primers and universal reverse primer (outlined in Table 2) to produce a band specific in size for each lineage, targeting the *16S* gene. We adapted our protocol from (Beauchamp et al., 2002a) with the following specifications: Cycling parameters: Initial denaturing 95 ºC for 5 min, 35 cycles of 95 ºC for 40s, 44 ºC for 45s, 72 ºC for 1 min, final elongation 72 ºC for 8min; 250 nM primer concentration; 10uL reaction volume and did not include the universal forward primer. The PCR products were then run through a 2.5% agarose gel and imaged using an ImageQuant LAS 4000 (GE Life Sciences). These images were then used to calculate band sizes using Gel-Analyzer (http://www.gelanalyzer.com).

3.3 Results

3.3.1 *Myxobolus cerebralis 18S* qPCR Assay Development

The 95% confidence interval limit of detection was 5.32 gene copies per reaction (lower limit: 3.625, upper limit: 7.83). All triplicate samples had to have amplification within 37 cycles (~5 copies) to be considered a positive sample and the reported copy number is a mean of the three replicates.

Table 2. Sequences of five lineage-specific primers in the *mt16S* rDNA gene of *Tubifex sp.* This test yields one or two lineage-specific amplification products in combination with the *Tubifex* specific reverse primer.

Lineage	Primer Name	Sequence 5'-3'	Band size	Citation
	16sbr- universal reverse primer	CCGGTCTGAACTCAGATCACGT		(Beauchamp et al., 2001)
Lineage I	L1- forward	GGACAAACGAGAATATC	196	(Sturmbauer et al., 1999)
Lineage II	L2- forward	TGTAGGCTAGAATGAAC	400	(Sturmbauer et al., 1999)
Lineage III	L3- forward	TCACCCCCAAACTAAAAGATAT	147	(Sturmbauer et al., 1999)
Lineage IV	L3 and L5		320 and 215	
Lineage V	L5- forward	AAGAAGCTTAAATAAACG	320	(Sturmbauer et al., 1999)

3.3.2 Three Year Monitoring Program Results

In total, 2800 samples from 969 sites were analyzed using our *M. cerebralis* qPCR assay as part of this study. This included 2159 worm samples, 572 sediment samples and 69 water samples. This resulted in 2749 samples being negative overall, 39 positive results and 12 'suspect'- tests where either the triplicates were not the same or the copy number was close to the limit of detection and the sample could not be re-run to confirm the result. These positive results come from 18 different sites, outlined by site type, stocked pond or wild, sample type and watershed in Table 3.

In total, 73% of the positive results from wild sites came from the southern part of the province (Oldman, Bow and Red Deer watersheds). However, some worms were positive in new locations where the parasite had previously not been detected using fish testing, and included

sites 015, 032, and 056. These three sites were in the Athabasca and Peace River watersheds. One site in the Oldman River watershed, where fish were not collected in 2018 but the site had returned positive fish samples in 2017, returned positive worms in 2018 (site 258). (Figure 3 and Table 3).

Based on our testing, 12-23% of the worm samples tested positive for *M. cerebralis* with the qPCR assay at a site where fish tested positive at least a year prior. Site 054 had positive fish tests in 2016, in 2017 a 23% (9/39) infection prevalence was found in the worms collected and a positive sediment sample, the latter two of which were both collected in 2017. Another site, 063, had positive fish samples from 2016 and 12% (7/59) infection prevalence in the worms collected in 2017. qPCR testing returned more positives in the worm population than actively looking for shed TAMs to confirm a positive.

3.3.3 Sample Matrix Comparison

Wild Site Results

2016 Results

There were 110 sites at which fish were collected in 2016 and corresponding sediment and invertebrate samples were taken a year later. Of these 110 sites, 44 sites had positive fish; five had positive environmental or worm samples. Three yielded positive sediment results, three had positive worms and all had fish test positive at the site from the 2016 collection. Only one site produced both positive sediment and worms, 054, in the Crowsnest River in the Oldman River watershed. The number of gene copies per reaction in the positive worm samples (n=17) had an average of 631,726 with a range of 10 to 9,203,676. The average sediment gene copy per reaction was much lower at 955, with a range of 5 to 2,843 gene copies per reaction.

2017 Results

Out of 195 sites at which fish, worms and sediment were collected in 2017, no sites had positive sediment, and two sites had positive worms; both these sites had fish collected but tested negative. These sites were found in the most northern watersheds; Athabasca and Peace River (Figure 3A). The number of gene copies per reaction in the positive worms (n=6) had an average of 72, with a range of 5 to 291. These sites represent an interesting example of where the parasite may be establishing in the worm population as its range expands north in the province but has not reached a detectable level in the fish population.

2018 Results

There was one site with positive sediment in this collection year; however only 45/394 sites had sediment collected. Two sites had positive worms, one in the North Saskatchewan River and one in the Bow River watershed, one of which also had the positive sediment sample (Figure 3B); the other site did not have a sediment sample collected (Table 3). The number of gene copies per reaction in the positive worms (n=3) had an average of 129,811, with a range of 8 to 389,417. The positive sediment sample had a gene copy number of 560. No fish were collected this year for comparison.

Stocked Pond Results

2017 Results

We did not find any positive worms or sediment samples in 36 sites tested. One water sample tested positive (gene copy number 455 per reaction); this site also had fish test positive (Table 3).

2018 Results

No fish samples were collected, but environmental test results from 191 sites showed six sites with positive sediment; only two of these six sites had worms present and both tested negative. Two sites had positive worm samples (gene copy numbers 14 and 98) but the sediment samples were negative. The number of gene copies per reaction in the positive sediment from 2018 (n=6) had an average of 122 with a range of 5 to 453 (Table 3).

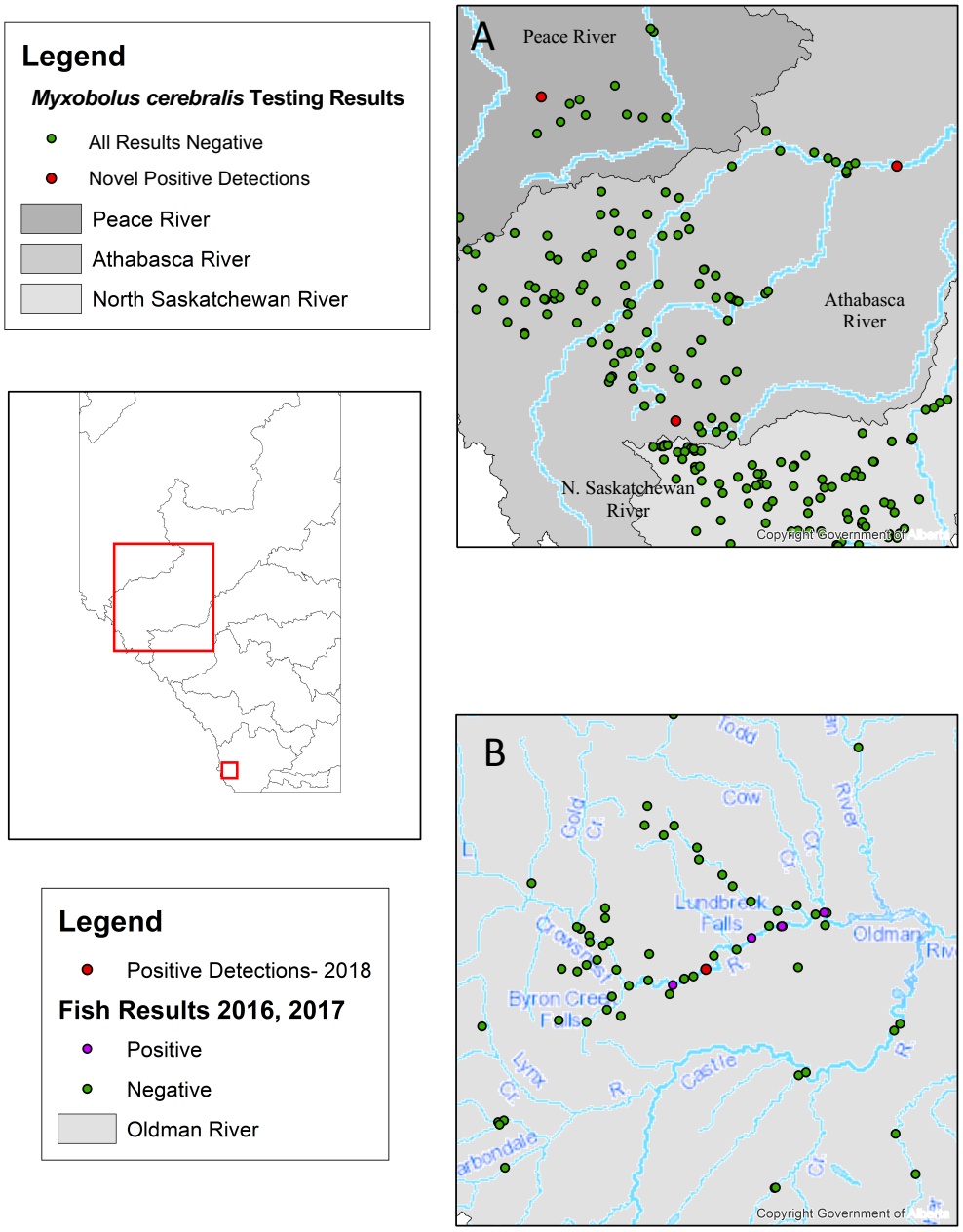

Figure 3. Novel detections of *Myxobolus cerebralis* at select sites in Northern Alberta. (A) Sites in the northern range of our sample area (Peace and Athabasca River watersheds) with novel detections of *M. cerebralis* from positive worms, fish from the same site either tested negative or were not collected. (B) Site in the southernmost watershed (Oldman River) where worms and sediment tested positive for *M. cerebralis*, where fish tested positive in previous years, but no fish were collected in 2018.

Table 3. Environmental positive results from the 18S_MC qPCR test.
Fish results indicated by *-no fish sampled, $^{\theta}$-fish sampled tested negative, $^{\Psi}$-fish sampled tested positive. Coordinates not given for stocked ponds as some are on private land, town/county and watershed is given instead.

Site ID	Year	Type	Gene Copy Count	Location Name	Watershed	Coordinates	
						Latitude	Longitude
075$^{\Psi}$	2017	water	455	Cochrane	Bow River	Stocked Pond	
040*	2018	sediment	22	Calgary	Bow River	Stocked Pond	
097*	2018	worm	14	Calgary	Bow River	Stocked Pond	
083*	2018	sediment	453	Thorsby	North Saskatchewan River	Stocked Pond	
168*	2018	sediment	5	Pincher Creek	Oldman River	Stocked Pond	
135*	2018	sediment	13	Thornhill	Bow River	Stocked Pond	
048*	2018	sediment	198	Chestermere	Bow River	Stocked Pond	
155*	2018	worm	98	Sherwood Park	North Saskatchewan	Stocked Pond	
065*	2018	sediment	43	McKeary	Bow River	Stocked Pond	
054$^{\Psi}$	2016	worm	87	Crowsnest River	Old Man River	49.54976	-114.29543
		worm	48,607				
		worm	15				
		worm	87				
		worm	1,422,774				
		worm	63,837				
		worm	13				
		worm	11				
		worm	31				
		sediment	2,843				
062$^{\Psi}$	2016	sediment	9	Crowsnest River	Old Man River	49.58483	-114.20494
063$^{\Psi}$	2016	worm	28	Crowsnest River	Old Man River	49.59355	-114.17036
		worm	9,203,676				
		worm	23				
		worm	43				
		worm	49				
		worm	17				

		worm	29				
086[ψ]	2016	sediment	12	Dogpound Creek	Red Deer River	51.4161	-114.49941
237[ψ]	2016	worm	10	Fallentimber Creek	Red Deer River	51.62317	-114.72738
015[θ]	2017	worm	291	Moon Creek	Peace River	54.4557	-118.03069
056[θ]	2017	worm	5	Athabasca River	Athabasca River	54.150235	-115.34013
		worm	17				
		worm	105				
		worm	6				
		worm	8				
032*	2018	worm	8	Taylor Creek	Athabasca River	53.004677	-117.01305
		worm	19				
258*	2018	sediment	560	Crowsnest River	Old Man River	49.561532	-114.25748
		worm	389,417				

3.3.4 Lineage PCR

We originally ran worm samples that came back positive in the qPCR test through the lineage PCR. However, we found most worms samples did not come back with any bands or band sizes that matched a published band length for the lineage assay. Published band sizes are found in Table 2, and in our positive worms we found a single band around 240bp or no band at all. In the negative worms, we either had no band, the same band around 240bp and one each of Lineage II and III (Figure 4).

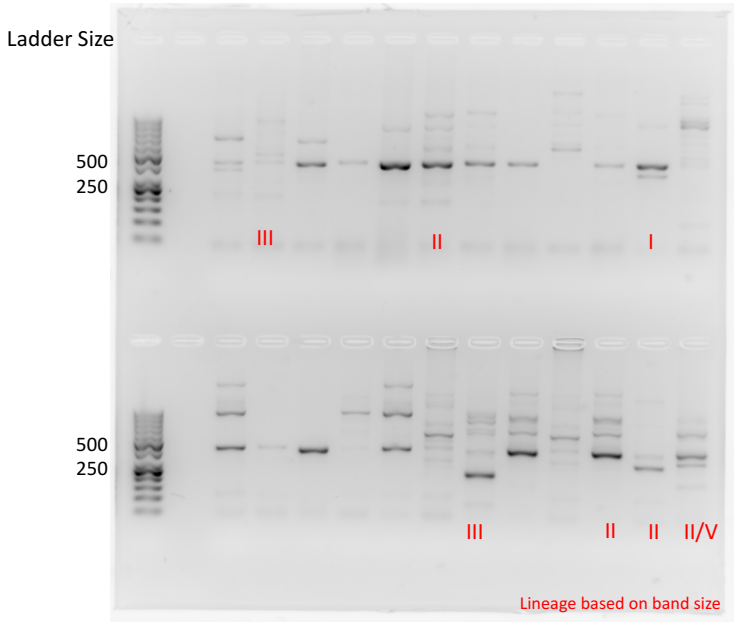

Figure 4. Representative agarose gel electrophoresis for Lineage PCR test results in *Tubifex sp*. worms from Alberta, Canada.
PCR- amplified genomic DNA from oligochaete worms sampled in 2017 using mt *16S* rDNA Lineage-Specific Primers. Band sizes correlate to a Tubifex lineage that informs on *Myxobolus cerebralis* compatibility. A DNA standard ladder (50-bp) is found on the left. 2.5% agarose gel. Lineage results indicated in red. Multiple lineage results were noted in the same sample.

3.4 Discussion

During this two-year-long study, we collected 10519 fish (6320 in 2016, 4199 fish in 2017), 5260 worms, 900 sediment samples and 36 water samples from 969 sites in Alberta. From these samples, 2800 were run through our new qPCR test for *M. cerebralis.* We used these results to assess the distribution of this parasite in Alberta watersheds and select stocked ponds to evaluate the use of all stages of the parasite lifecycle for invasive species detection. Our results from these samples support the findings of traditional *M. cerebralis* fish tests, with more positive detections in the southern part of the province and a few novel detections where fish samples had previously been negative. The latter results suggest an interesting case where it is possible to detect the parasite in the worm population before being at a detectable level in the fish

population, essentially predicting future potential transmission sites. Having this advance knowledge can facilitate necessary management decisions to protect vulnerable fish populations or reduce spread from that area.

In stocked ponds from 2017 to 2018, there was an increase in positive sediment samples, without a corresponding increase in positive worm samples. The increase in positive sediment samples could be due to a change in sediment collection protocol, composite samples in 2018 vs single samples in 2017. In addition, it is possible we have reached the point that the parasite has been present long enough in ponds to be at a detectable level in the sediment as these ponds were stocked from a positive facility as recently as 2016.

qPCR testing of sediment and worms provides an appealing complementary test to lethal fish testing, particularly in waterbodies where the presence of *M. cerebralis* has been confirmed, but the extent of infection is unknown. In these cases, this alternative testing can help identify specific areas of parasite establishment within a waterbody where fish results may be misleading due to fishes ability to move within a waterbody, especially in a stream or river. This qPCR test can also provide insight into the efficacy of management decisions and any control or mitigation efforts by targeting different stages of the parasite lifecycle. For example, if the infection status of a recipient habitat for fish stocking is unknown, worm samples can inform on parasite establishment before assuming the risk of stocking fish into a whirling disease positive system which has been shown to exacerbate the disease in Colorado (Crowl, 2000). In addition, sediment samples can indicate if positive fish have died in that pond or stream system. Water samples can indicate if worms are actively shedding the fish infective TAM stages of the parasite, which has been successfully used to determine infection risk based on parasite prevalence in other myxozoan parasites (Hallett and Bartholomew, 2006). Moreover, fish testing

is difficult to rationalize when targeting species at risk or endangered species and having an alternative approach to fish testing allows for the unification of testing where species at risk are present. Additionally, there may not be enough fish to collect for the desired level of confidence in testing results or pond owners may be reluctant to remove and kill fish they have purchased to stock.

Since *T. tubifex* maintains the infection throughout their lifespans, these worms can serve as an important and useful target for monitoring efforts. While the prevalence of worms actively shedding TAMs in natural populations is usually quite low at 1.2 to 6.8% (Rognlie and Knapp, 1998; Zendt and Bergersen, 2000a), we found positive detections in 12-23% of the worms using our *M. cerebralis* qPCR test. This is likely due to the detection of worms that have consumed myxospores but are not yet actively shedding TAMs. This is an important distinction to make when attempting to assess worm infection prevalence but is also useful when considering monitoring efforts, which are typically focused on positive/negative results rather than confirming completion of the parasite life cycle. As worms are much easier to collect and at a much higher number in these environments than juvenile fish, monitoring efforts could shift more towards worm surveillance to assess transmission potential at a site. In addition, the parasite must be established in the worm population before it is at a detectable level in the fish population, providing an avenue for early detection as a compliment to fish testing.

Given the lack of consistent results from the lineage PCR test where positive worms did not yield expected results in the lineage test, or generated band sizes that did not match currently published lineages, we suggest that this test may not be applicable to all Albertan *T. tubifex* populations. Therefore, we think it is possible to miss susceptible worms in Alberta if the current lineage PCR test is the only test used. Another method may be more appropriate for evaluating

our worm populations' ability to transmit *M. cerebralis*, however until such a test is validated, the lineage assessment and its uncertain connection to parasite susceptibility may not be as valuable as monitoring for infected worms or parasite presence in general at a site.

Molecular methods, including qPCR, have been used for surveillance and early detection monitoring for invasive species (Brown et al., 2016), parasites (Lass et al., 2009) and within waterbodies (Egan et al., 2015). With this study we aimed to demonstrate the utility of such an approach in combination with exploiting environmental lifecycle stages for detecting invasive parasites in water bodies. Molecular assays can answer basic questions about parasite distribution in the environmental pathways used for transmission and help determine intermediate and definitive hosts in the habitat, which is especially important when managing an invasive species. Interrupting the life cycle of a parasite is a way to reduce or even eliminate transmission ono a small scale (Nehring et al., 2018). The efficacy of this type of control measure could be assessed using qPCR assays on the environmental and worm stages of parasites when fish host sampling is restricted. Nehring et al. (2015) found that the myxospore stage of the parasite can only survive in the environment and be infective to worms between six months to one year, contrary to the previous belief that they remain viable for years to decades.

The new molecular test we developed allows us to watch the progress of this parasite in near real-time as it moves through the province. This test is also far more appropriate than previously published tests for confidently amplifying *M. cerebralis* from environmental matrices that likely would contain other myxozoan species, by increasing specificity. We hope that this improved test and an initial survey of parasite presence in the province of Alberta will help to manage the future of this invasive parasite.

Chapter 4. Molecular assessment of the invertebrate host, *Tubifex sp.*, in relation to the causative agent of whirling disease, *Myxobolus cerebralis,* in a non-endemic area

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Abstract

Myxobolus cerebralis is an invasive parasite in North America that causes whirling disease in salmonid fish. The oligochaete, *Tubifex tubifex* serves as the alternate host for *M. cerebralis*. These worms are found throughout fine sediment habitats in freshwater. Within the genus *Tubifex,* variation is observed with respect to susceptibility to *M. cerebralis* infection; however, infection phenotype does not translate into an observable morphological distinction between *M. cerebralis* susceptible and resistant worms. We used DNA sequencing of the *cytochrome oxidase I* (*cox1*) gene for species delimitation of 567 oligochaete specimens collected from six different watersheds in Alberta. Those results were compared with qPCR testing for *M. cerebralis* DNA and a previously used lineage PCR that has been used to determine parasite compatibility. We found 158 unique sequences, belonging to 21 genera and 37 different species. Our results from phylogenetic analysis suggested cryptic speciation in the

Tubifex and *Limnodrilus* genera with five and eight separate taxonomic groups, respectively. Four different taxonomic groups tested positive for *M. cerebralis* DNA via qPCR testing, two belonging to the *Tubifex* genus and two to the *Limnodrilus* genus; it is uncertain of the latter are a suitable host or had simply consumed parasite leading to a positive qPCR test. The lineage PCR did not produce consistent results when compared with the species barcoding results. Our study exposed the possible diversity of worm hosts for *M. cerebralis* in Alberta and was the first large scale analysis of oligochaete worms molecularly in Canada.

4.1 Introduction

When studying an invasive parasite with a multi-host lifecycle, concrete understanding of all hosts, not just those of economic importance, is crucial to ensure the monitoring efforts are time and cost-efficient, and areas of high parasite transmission are identified. Additionally, when parasites are introduced to a new environment, suitable host species may be different from where the parasite is endemic, so hosts should not be assumed to be the same as the parasite's endemic range. These elements are all reflected in the recent introduction of *Myxobolus cerebralis*, the myxozoan parasite that is the causative agent of whirling disease in salmonid fish, in Alberta, Canada. *M. cerebralis* has been responsible for up to 90% die-offs in young of the year salmonid fish in Colorado and Montana (Nehring and Walker, 1996; Vincent, 1996). Given the vulnerable status of some salmonid fish in Alberta (Government of Canada, 2002) and their use for recreational fishing, proper identification of the parasite hosts and lifecycle dynamics in this new environment is important moving forward. Similar research has been done in other areas where whirling disease has spread, but no such work has been done here in Alberta (Anlauf and Moffitt, 2008; Beauchamp et al., 2001; DuBey and Caldwell, 2004; Hallett et al., 2009).

Myxozoan parasites typically rely on a definitive aquatic invertebrate host and an intermediate vertebrate host. There are two distinct waterborne stages between hosts, myxospores from the vertebrate host and actinospores from the invertebrate host (Figure 1). For *M. cerebralis,* oligochaetes of the genus *Tubifex* are known to be susceptible to infection and specific 'lineages' of *Tubifex tubifex* have been characterized as differing in their susceptibility to *M. cerebralis* (Arsan et al., 2007; Beauchamp et al., 2002b; Rasmussen et al., 2008). These lineages are based on mitochondrial *16S* ribosomal DNA, first based on *Tubifex sp*. resistance to environmental cadmium (Sturmbauer et al., 1999), then applied to their ability to transmit *M. cerebralis* (Beauchamp et al., 2002b; Hallett et al., 2009). Four separate lineages have been found in the continental United States and Alaska, I, III, V and VI (Beauchamp et al., 2001), with II and IV only found in Europe (Sturmbauer et al., 1999). Parasite replication varies from susceptible to infection and produce viable actinospores (I and III), to susceptible to infection and parasite development is not completed (V), to no infection at all (VI) (Beauchamp et al., 2002b). Lineages II and IV have not been analyzed for *M. cerebralis* susceptibility as the parasite is endemic in Europe and generally not of high research concern. What underpins this spectrum in compatibility with *M. cerebralis* remains unknown; however, being able to identify susceptible and resistant *Tubifex* populations is important for understanding invasion dynamics and attempting interventions. Moreover, developing a fast and reliable test for assessing where there may be areas of higher parasite transmission from the worm host can indicate where the most fish host impact may occur and target management decisions (Bartholomew et al., 2005; Zendt and Bergersen, 2000b). Analyzing worm populations in novel environments for *M. cerebralis* has been a useful proxy for assessing risk to salmonid populations in locations in the USA (Bartholomew et al., 2005; McGinnis and Kerans, 2013).

Regardless of parasite transmission, identification of oligochaetes based on morphological characteristics is a common hurdle, despite their worldwide distribution (Brinkhurst, 1996). Morphological identification is difficult with most species in the family Tubificinae and the majority can only be identified when mature, which may not be possible depending on the timing of sampling (Beauchamp et al., 2001). Morphological characteristics are plastic depending on environmental conditions and vary considerably within currently defined taxa (Achurra et al., 2011; Bely and Wray, 2004; Vivien et al., 2015). When morphometrics are unreliable to identify species, physiological differences can be used to distinguish species, such as preferred food sources, reaction to environmental stressors or parasite compatibility (Vellend et al., 2011). However, using these techniques to survey multiple populations and species over a landscape is time-consuming and is dependent on collecting live specimens. An alternative is to use DNA based barcoding. This approach is appealing for the identification of species that are difficult to distinguish morphologically. When these DNA sequences are analyzed for the same gene from multiple individuals, a phylogeny can be created to analyze the relatedness between those individuals, and acts as a proxy for the magnitude for the phenotypic difference (Vellend et al., 2011). Parasite compatibility can be connected to each sequence group, allowing for an assessment of compatibility across a landscape.

Variation within each lineage of *Tubifex* with respect to their ability to produce parasites has been described (Rasmussen et al., 2008). As well, lineage I *T. tubifex* from an Alaskan population were found to be resistant to infection (Arsan et al., 2007) while individuals mapping to the same lineage are able to produce actinospores in the continental United States (Beauchamp et al., 2002b). These inconsistencies put the status of *T. tubifex* in Canada in question, a status that is amplified by the fact that few comprehensive studies of freshwater oligochaetes have been

undertaken using a DNA-barcoding approach, often without specimens from western Canada. As part of the initial investigation of *M. cerebralis* in Alberta, oligochaetes were tested using the lineage PCR and a qPCR test targeting the *18S* gene of *M. cerebralis* for infection status (Chapter 3). Many lineage PCR results did not fit into expectations based on previously published lineages, with the majority of oligochaetes we analyzed producing a band size pattern inconsistent with any published band sizes for this test, or no band at all. Moreover, worms that tested positive for *M. cerebralis* based on the qPCR test did not consistently show up as a previously determined susceptible lineage (I or III). These inconsistencies lead us to believe that the lineage test may not be reliable for assessing *M. cerebralis* susceptibility in the *T. tubifex* populations in Alberta. To take a more unbiased approach to oligochaete identification, we switched to molecular barcoding to assess the worm hosts for *M. cerebralis* in Alberta and in parallel, completed the first large scale assessment of these worms in Canada.

4.2 Methods

4.2.1 Worm Collection and Sorting

Working with Alberta Environment and Parks, invertebrate samples were collected from 348 wild sites and 36 stocked ponds in Alberta from June to November in 2017. This was done using a D-framed benthic kick net to collect an assorted invertebrate sample. These samples were preserved in 70% ethanol and transported to our facility on campus at the University of Alberta in Edmonton, Alberta. Once there, the samples were manually sorted to remove any oligochaete-like worms and store them individually in 70% ethanol at -20ºC. Morphological identification to genus or species was not plausible as the ethanol caused the identifying features to become brittle and break during storage and sorting. The original plan was to barcode ten worms from

every site; however many sites did not have suitable worm samples, so up to ten were barcoded from every site that had oligochaete worms with more being done at sites where they tested positive for *M. cerebralis* or had other special interest, such as a positive fish or sediment test result, or a pond stocked previously from a positive aquaculture facility.

4.2.2 DNA Extraction

DNA from 1815 worms were extracted using the DNeasy Blood and Tissue extraction kit from Qiagen, following the manufacturer's specifications. Ethanol was pipetted off prior to digestion for a minimum of two hours, up to overnight (~16 hours). The kit protocol was followed except for an elution volume of 50uL.

4.2.3 Lineage PCR Test

Select worms were run in the previously published Lineage PCR test; methods are described in Chapter 3, briefly: a mixture of four forward primers and one universal reverse primer is used in a PCR reaction to produce a certain band size when the PCR product is run in a 2.5% agarose gel. These band sizes correlate with which lineage a Tubifex worm belongs to, which should have implications for the ability of these worms to successfully transmit *M. cerebralis*. We will combine these results with the species barcoding, explained below, to confirm if this lineage test is accurate or useful for the oligochaete worm population in Alberta.

4.2.4 Sequencing

Of the extracted worms, 609 were prepared for Sanger sequencing. Partial *cox1* sequences in the Folmer region were amplified by PCR in 10uL reaction volumes in IDT

PrimeTime master mix with 250nM concentration of LCO and HCO primers and 4uL of extracted DNA. The thermocycler protocol was: initial denaturing 95ºC for 5 min, 35 cycles of 95 ºC for 40s, 44 ºC for 45s, 72 ºC for 1 min, final elongation 72 ºC for 8min.

PCR products were run in a 1% agarose gel and target amplicons extracted using the GeneJet Gel Extraction kit. Purified amplicons were sent to Macrogen Inc. (Korea) for Sanger sequencing. The same primers for the PCR reaction were used for sequencing both forward and reverse sequences. Samples with poor PCR results or too low DNA concentration were not sent for sequencing.

4.2.5 Alignments

All sequences were checked for quality and primers regions trimmed using the 4peaks (Nucleobytes) software and were transferred to Geneious Prime2019 ("https://www.geneious.com," 2019) to align the forward and reverse sequences. Twenty-seven had a poor-quality forward or reverse sequence, so a single sequence was used instead of an alignment. Each resulting sequence was then searched in the NCBI GenBank BLASTn database. A representative sequence from every species with an over 80% match was used to align to each consensus sequence and produce a percent identity matrix. 5% was used as the match cut off value to make an initial species call as literature values vary (Achurra et al., 2011; Bely and Wray, 2004). If no match was found within this cut off, the next highest match was used. After accounting for poor PCR or sequence quality, 567 sequences were included in the final assessment.

4.2.6 Phylogenetic Reconstruction

Alignments were trimmed to the shortest sequence length prior to any analysis. MegaX was used for model testing using nucleotide substitution for each group of sequences. Bayesian inference (BI) reconstructions were made using the Mr. Bayes plug-in (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) in Geneious Prime2019 with a burn-in of 100 000, a chain length of 1 000 000, and sub-sampling frequency of 200. Maximum-likelihood (ML) analyses were run in the PhyML plug-in (Guindon et al., 2010) for separate genus-level analysis. The following settings used were: 200 bootstraps, proportion of invariable sites was fixed at 0, the number of substitution rate categories was 4, the gamma distribution parameter was set to estimated and "topology/length/rate" was selected to be optimized. Substitution model selection was the same for both BI and ML analyses and is described in each section below.

4.2.7 Species Determination

4.2.7.1 Phylogenetic Tree Analysis

All Unique Sequences

A total of 158 unique sequences found in our study were aligned with a sequence of the outgroup *Hirudo medicinalis* (HQ333519.1) and the alignment was 586bp long. GTR + invgamma was the best-supported nucleotide substitution model available in the MrBayes plug-in in Geneious for BI analyses.

Next, 11 unique sequences belonging to the genus *Tubifex* were assessed together in BI and ML analyses, via Automatic Barcode Gap Discovery and using p-distance along with representatives from GenBank to look for any cryptic speciation at a finer scale. The same assessments were done with 49 unique sequences from the genus *Limnodrilus*.

Tubifex sp.

Based on their percent match to sequences in GenBank and placement in the above tree, 25 sequences were selected within the genus *Tubifex*, 18 from this study and eight from GenBank. The alignment was 555 bps long, and *H. medicinalis* (HQ333519.1) was used as the outgroup. The best fit model was HKY85 + invgamma was the best-supported nucleotide substitution model available in the MrBayes and PhyML plug-in's in Geneious.

Limnodrilus sp.

Based on their percent match to sequences in GenBank and placement in the General tree, 55 sequences were selected within the genus *Limnodrilus*, 49 from this study and six from GenBank. The alignment was 552 bps long, and *H. medicinalis* (HQ333519.1) was used as the outgroup. The best fit model was HKY85 + invgamma was the best-supported nucleotide substitution model available in the MrBayes and PhyML plug-in's in Geneious.

4.2.7.2 Automatic Barcode Gap Discovery

Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) was used to confirm the natural breaks in the phylogenies and assess the previous species percent cut-off of 5%. It was run online and all default values were used (Pmin: 0.001, Pmax: 0.01, Steps:10, X(relative gap width):1.5, Nb bins:20, distance measurement: Jukes-Cantor(JC69).

4.2.7.3 P-Distances

In addition to ABGD, we used p-distances to confirm the separation of taxa in the genera of *Tubifex* and *Limnodrilus*, as many of our sequences within each of these genera had great enough diversity to suggest cryptic speciation, so we used this more in-depth analysis to confirm

these species divisions. p-distances were calculated in MegaX, calculating within and between-group distances, using all preset functions.

4.2.8 *Myxobolus cerebralis* Infection Prevalence

Worm samples were tested for *M. cerebralis* infection via qPCR testing, as described in Chapter 3.

4.3 Results

4.3.1 Oligochaete Species in Alberta

4.3.1.1 All Sequence Results

Unbiased barcoding of 567 oligochaetes samples throughout Alberta between 2017 and 2018 led to the identification of 158 unique sequences (Table 4). These sequences are numbered into Operational Taxonomic Units (OTU) which are groups of closely related individuals. Based on their *cox1* classification, we would expect these individuals to have the same phenotypes in regards to parasite compatibility and other genetic test results. These numbered groupings also allow for easier reference in the rest of the paper. BLAST analysis of the *cox1* sequences led to the assignment of 37 unique species belonging to 21 different genera (Table 4). 42.3% (240/567) belonged to a *Tubifex sp.* complex and 41.8% (237/567) belonged to a *Limnodrilus sp.* complex, which are covered in more detail below. The species results were separated based on the watersheds from which they were sampled from (Table 7) and were assessed visually for differences in the proportion of each species by watershed (Figure 7). Species results were relatively consistent across watersheds (Table 7) without any obvious differences in population structure. The most numerous species were found in every watershed and included *Tubifex sp.*

T3, *Limnodrilus sp*. L1, L5 and L7, with the exception of Peace River, likely due to its small sample size. Two *Tubifex sp*. were found in higher numbers in all but one watershed; *Tubifex sp*. T1 not found in the Red Deer River and *Tubifex sp*. T2 was not found in Old Man River.

Table 4. Breakdown of the 158 unique operational taxonomic units (OTUs) produced by *cox1* barcoding on oligochaete specimens from Alberta.
The number of positive qPCR results in each OTU from *M. cerebralis* testing, Lineage PCR result and highest species/sequence match from GenBank.

OTU Group Number	Number of Worms in OTU	Species Complex Number	(+) qPCR Test Results	Lineage if applicable	Highest GenBank Species Match	% Match	Top Match Accession #
1	48	T1	2	nb	*Tubifex tubifex*	100	EF089365.1
2	11	T1		II, nb	*Tubifex tubifex*	100	EU311376.1
3	2	T1			*Tubifex tubifex*	99.83	EF089365.1
4	2	T1	1		*Tubifex tubifex*	100	EF089365.1
5	1	T1			*Tubifex tubifex*	99.62	LN810379.1
6	1	T1			*Tubifex tubifex*	99.49	EF089365.1
7	1	T1			*Tubifex tubifex*	100	EF089365.1
8	1	T1			*Tubifex tubifex*	99.81	EF089365.1
9	1	T1			*Tubifex tubifex*	99.68	EU311341.1
10	1	T1			*Tubifex tubifex*	100	EF089365.1
11	4	T1		nb	*Tubifex sp.*	98.772	AF534867.1
12	1	T1			*Tubifex sp.*	98.694	AF534867.1
13	34	T3	1	III, V, 240bp, nb	*Tubifex tubifex*	100	EF089374.1
14	4	T3			*Tubifex tubifex*	99.813	LN999275.1
15	4	T3			*Tubifex tubifex*	100	EF089374.1
16	1	T3		nb	*Tubifex tubifex*	100	EF089371.1
17	1	T3			*Tubifex tubifex*	100	EF089374.1
18	25	T3	6	nb	*Tubifex tubifex*	100	LN999203.1
19	2	T3	1		*Tubifex tubifex*	100	LN999203.1
20	1	T3			*Tubifex tubifex*	100	LN999203.1
21	1	T3			*Tubifex tubifex*	100	LN999203.1
22	23	T3		III, nb	*Tubifex tubifex*	100	EF089379.1
23	1	T3			*Tubifex tubifex*		HQ920531.1
24	1	T3		V	*Lumbriculda sp*	92.5	EF089379.1
25	1	T3			*Tubifex tubifex*	100	EF089377.1
26	1	T3			*Tubifex tubifex*	100	EF089379.1

27	1	T3		V	*Tubifex tubifex*	100	EF089377.1
28	6	T4		I	*Tubifex tubifex*	100	EU311371.1
29	1	T4		I	*Tubifex tubifex*	100	EU311371.1
30	1	T4		III, 240bp	*Tubifex tubifex*	99.29	EF089386.1
31	4	T5		nb	*Tubifex tubifex*	99.681	LN810423.1
32	1	T5			*Tubifex tubifex*	100	LN810423.1
33	27	T2		240bp, nb	*Tubifex tubifex*	100	LN810418.1
34	6	T2			*Tubifex tubifex*	100	EF179543.1
35	16	T2		nb	*Tubifex tubifex*	99.65	LT903789.1
36	1	T2			*Tubifex tubifex*	99.65	LT903789.1
37	1	T2		nb	*Tubifex tubifex*	99.475	LT903789.1
38	1	T2			*Tubifex tubifex*	99.65	LT903789.1
39	29	L7		nb	*Limnodrilus hoffmeisteri*	99.5	EF089358.1
40	1	L7			*Limnodrilus hoffmeisteri*	99.35	EF089358.1
41	1	L7			*Limnodrilus hoffmeisteri*	99.65	EF089358.1
42	1	L7			*Limnodrilus hoffmeisteri*	99.51	EF089358.1
43	4	L7	1	nb	*Limnodrilus hoffmeisteri*	99.537	EF089358.1
44	3	L7			*Limnodrilus hoffmeisteri*	98.95	EF089358.1
45	1	L7		nb	*Limnodrilus hoffmeisteri*	98.9	EF089358.1
46	2	L7			*Limnodrilus hoffmeisteri*	98.51	EF089358.1
47	23	L7	1	nb	*Limnodrilus hoffmeisteri*	90.988	EF089358.1
48	2	L7			*Limnodrilus hoffmeisteri*	91.55	EF089358.1
49	8	L7	1		*Limnodrilus hoffmeisteri*	91.071	EF089358.1
50	5	L7	1		*Limnodrilus hoffmeisteri*	91.07	EF089358.1
51	1	L7			*Limnodrilus hoffmeisteri*	91.071	EF089358.1
52	1	L7			*Limnodrilus hoffmeisteri*	91.27	EF089358.1
53	4	L7			*Tubifex tubifex*	78.3	EF089379.1
54	1	L7			*Limnodrilus hoffmeisteri*	90.31	KY369529.1
55	1	L7			*Limnodrilus hoffmeisteri*	89.97	EF089358.1
56	1	L7			*Limnodrilus hoffmeisteri*	89.94	EF089358.1
57	12	L7	2	nb	*Limnodrilus hoffmeisteri*	91.52	EF089358.1
58	16	L7	1	nb	*Limnodrilus hoffmeisteri*	89.279	KY369576.1
59	1	L7			*Limnodrilus hoffmeisteri*	90	EF089358.1
60	5	L8		nb	*Limnodrilus hoffmeisteri*	99.543	KY369538.1
61	1	L8			*Limnodrilus hoffmeisteri*	99.54	KY369537.1
62	2	L8		nb	*Limnodrilus hoffmeisteri*	98.78	KY369538.1
63	1	L8			*Limnodrilus hoffmeisteri*	96.68	LN999131.1
64	1	L8		nb	*Limnodrilus hoffmeisteri*	100	LN999131.1
65	16	L1	1	240bp, nb	*Limnodrilus claparedeanus*	98.403	LN999079.1
66	5	L1		nb	*Limnodrilus claparedeanus*	98.5	LN999079.1
67	3	L1			*Limnodrilus claparedeanus*	98.27	LN999079.1

68	2	L1				*Haplotaxida sp*	99.83	HQ961579.1
69	1	L1				*Limnodrilus claparedeanus*	98	LN999079.1
70	1	L1				*Limnodrilus claparedeanus*	98.167	LN999079.1
71	1	L1				*Limnodrilus claparedeanus*	97.93	LN999079.1
72	1	L1				*Limnodrilus claparedeanus*	98.53	LN999079.1
73	1	L1	1	nb		*Limnodrilus claparedeanus*	98.421	LN999079.1
74	1	L1				*Limnodrilus claparedeanus*	98.47	LN999079.1
75	1	L1				*Haplotaxida sp*	99.82	HQ961579.1
76	1	L1				*Haplotaxida sp*	99.83	HQ961579.1
77	1	L1				*Limnodrilus claparedeanus*	98.44	LN999079.1
78	1	L1	1	nb		*Limnodrilus claparedeanus*	98.415	LN999079.1
79	1	L1		nb		*Limnodrilus claparedeanus*	100	LN999079.1
80	4	L1				*Limnodrilus claparedeanus*	99.3	KY369628.1
81	2	L1				*Limnodrilus claparedeanus*	98.68	KY369564.1
82	1	L1				*Limnodrilus claparedeanus*	98.81	KY368564.1
83	1	L1				*Limnodrilus claparedeanus*	99.45	KY369706.1
84	1	L1				*Limnodrilus claparedeanus*	99.13	KY369564.1
85	1	L1				*Limnodrilus claparedeanus*	98.79	KY369567.1
86	1	L1				*Limnodrilus claparedeanus*	98.4	KY369564.1
87	1	L1				*Limnodrilus claparedeanus*	98.243	KY369564.1
88	2	L6		nb		*Limnodrilus profundicola*	94.338	KY636925.1
89	2	L6				*Haplotaxida sp*	99.18	HQ961524.1
90	1	L6				*Limnodrilus udekemianus*	98.26	HQ961524.1
91	27	L5		nb		*Limnodrilus hoffmeisteri*	100	KY369635.1
92	3	L5				*Limnodrilus hoffmeisteri*	99.84	KY369635.1
93	1	L5				*Limnodrilus hoffmeisteri*	99.699	LT905374.1
94	1	L5		nb		*Limnodrilus hoffmeisteri*	100	KY369666.1
95	1	L5		nb		*Limnodrilus hoffmeisteri*	100	KY369663.1
96	1	L4				*Tubifex tubifex*	93.22	HM138066.1
97	9	L2		nb		*Limnodrilus hoffmeisteri*	100	KY369557.1
98	1	L2				*Limnodrilus hoffmeisteri*	99.208	KY369579.1
99	1	L2		nb		*Limnodrilus hoffmeisteri*	100	KY369496.1
100	1	L2				*Limnodrilus hoffmeisteri*	100	KY369471.1
101	7	L3				*Limnodrilus hoffmeisteri*	96.423	EF089357.1
102	1	L3				*Tubificinae sp.*	99.843	LN999273.1
103	6					*Ilyodrilus templetoni*	98.19	EF089359.1
104	2					*Ilyodrilus templetoni*	97.83	EF089359.1
105	1					*Ilyodrilus templetoni*	98.01	EF089359.1
106	1					*Ilyodrilus templetoni*	98.01	EF089359.1
107	1					*Ilyodrilus templetoni*	97.83	EF089359.1
108	1					*Ilyodrilus templetoni*	97.83	EF089359.1

109	1		*Ilyodrilus templetoni*	98.15	EF089359.1
110	1		*Ilyodrilus templetoni*	99.1	EF089359.1
111	2	240bp	*Aktedrilus sp.*	82.43	AF064042.1
112	1	nb	*Alexandrovia sp.*	81.7	KY636933.1
113	1		*Alexandrovia sp.*	82.18	KY636933.1
114	3	nb	*Limnodrilus sp.*	93.11	LT598633.1
115	1		*Limnodrilus sp.*	92.95	LT598633.1
116	2		*Limnodrilus udekemianus*	98.26	LT598633.1
117	1		*Limnodrilus sp.*	92.632	LT598638.1
118	1		*Potamothrix bavaricus*	99.84	KY636942.1
119	1	nb	*Aulodrilus pluriseta*	99.836	LT905384.1
120	3	nb	*Ophidonais serpentina*	100	LT903842.1
121	1		*Nais christinae*	98.95	JQ519824.1
122	1		*Nais sp.*	99	MF544513.1
123	2		*Naididae sp.*	99.647	KF000315.1
124	1		*Naididae sp.*	99.463	KF000315.1
125	3	II	*Lumbriculda sp.*	92.93	KM612088.1
126	2	nb	*Lumbriculda sp.*	92.5	KM612088.1
127	1	nb	*Lumbriculda sp.*	92.76	KM612088.1
128	2	nb	*Lumbriculda sp.*	92.58	KM612088.1
129	1	nb	*Lumbriculda sp.*	92.79	KM612088.1
130	1		*Lumbriculda sp.*	92.79	KM612088.1
131	1	nb	*Rhynchelmis elrodi*	99.097	GU592306.1
132	4		*Eiseniella tetraedra*	100	LN810249.1
133	1	II/V*	*Octlasion cyaneum*	98.769	KM611829.1
134	5	nb	*Fridericia sp.*	84.01	GU902074.1
135	3	nb	*Fridericia sp.*	84.36	GU902074.1
136	1		*Fridericia sp.*	84.28	GU902074.1
137	1		*Haplotaxida sp.*	85.17	HQ945774.1
138	5		*Enchytraeidae sp.*	84.24	KM206488.1
139	2		*Amynathas sp.*	84.05	KF205464.1
140	1		*Fridericia sp.*	85.156	MF547670.1
141	4		*Henlea sp.*	90.861	KR872332.1
142	1		*Henlea sp.*	90.5	KR872332.1
143	1		*Henlea sp.*	90.56	KR872332.1
144	1	nb	*Henlea perpusilla*	96.709	GU902084.1
145	1		*Henlea perpusilla*	97.065	LT903838.1
146	1		*Henlea perpusilla*	96.636	LT903838.1
147	1		*Henlea perpusilla*	99.161	GU902084.1
148	1		*Henlea perpusilla*	99.091	GU902084.1
149	1	nb	*Henlea perpusilla*	99.138	LT903838.1

150	2	nb	*Enchytraeidae sp.*	99.581	KT706560.1
151	1		*Enchytraeidae sp.*	99.694	KT706560.1
152	1		*Enchytraeidae sp.*	99.88	KT706560.1
153	1		*Enchytraeidae sp.*	99.87	KT706560.1
154	1		*Fridericia ratzeli*	100	MF544143.1
155	1		*Henlea sp.*	88.235	GU902083.1
156	1	II	*Marionina riparia*	95.853	GU902096.1
157	1		*Marionina riparia*	98.24	GU902096.1
158	1		*Mesenchytraeus sp.*	91.8	KF672423.1

* = this sample tested as both lineages

4.3.1.2 *Tubifex* sp.

Bayseian inference (BI) and Maximum-likelihood (ML) analyses agreed on species separation into five groups (numbered T1-T5) and tree topology with good statistical support (Figure 5). Results also showed five distinct groupings for taxonomic group cut off based on the ABGD results (JC p_{max}= 0.0001) with an interspecific divergence of 6-13%. P-distances confirmed these five groups with no groups having intraspecific diversity high enough to suggest any further speciation (0-3%) (Table 5).

4.3.1.3 *Limnodrilus* sp.

BI and ML analyses agreed on species separation into eight groups (numbered L1-L8) and tree topology with good statistical support (Figure 6). Results showed eight distinct groups for taxonomic group cut off based on the ABGD results (JC p_{max}= 0.0001) with interspecific divergence of 11-13%. P-distances confirmed the eight groups with no groups having high enough intraspecific diversity to suggest further speciation (0-7%) (Table 6).

Some sequences that matched to *Limnodrilus udekemianus* via GenBank did not group with the other *Limnodrilus sp.* complex found in this study (Figure 8). The *L. udekemianus* sequence is a specimen from Europe, so it is unclear if this is due to a difference in oligochaete

populations between the continents, or misidentification of the specimen in GenBank, given the challenges with oligochaete identification.

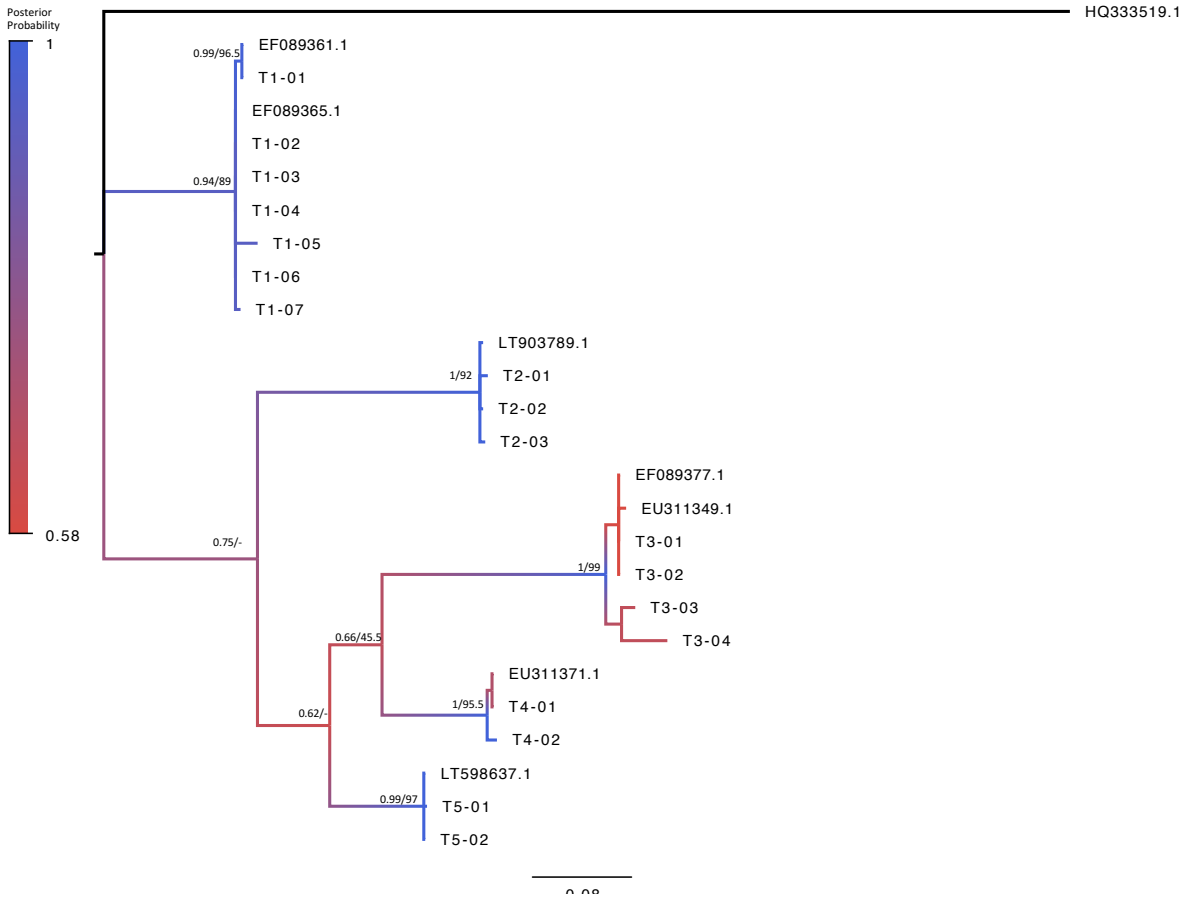


Figure 5. Maximum-likelihood phylogeny of *Tubifex tubifex* based on *cox1*. Posterior probabilities >50 (also reflected in branch colour with blue being highest) and bootstrap values >50 are reported at the nodes, respectively. GenBank accession numbers are given for one sequence in each clade not from this study, identified as *Tubifex tubifex*. This phylogeny shows five well-supported taxonomic groups within this species.

Table 5. p-Distances between five *Tubifex sp.* taxonomic groups in Alberta.
The number of base substitutions per site from averaging over all sequence pairs between groups are shown below the diagonal. Standard error estimate(s) are shown above the diagonal. Average within-group divergence is given on the diagonal. Analyses were conducted using the Maximum Composite Likelihood model. This analysis involved 25 nucleotide sequences. There were a total of 557 positions in the final dataset.

	***Tubifex* sp. G1**	***Tubifex* sp. G2**	***Tubifex* sp. G3**	***Tubifex* sp. G4**	***Tubifex* sp. G5**
***Tubifex* sp. Group1**	0.01	0.177	0.16	0.153	0.162
***Tubifex* sp. Group2**	0.289	0.03	0.139	0.162	0.184
***Tubifex* sp. Group3**	0.26	0.212	0.01	0.108	0.168
***Tubifex* sp. Group4**	0.248	0.239	0.16	0	0.155
***Tubifex* sp. Group5**	0.28	0.297	0.254	0.239	0.01

Table 6. p-Distances between eight *Limnodrilus sp* taxonomic groups in Alberta.
The number of base substitutions per site from averaging over all sequence pairs between groups are shown below the diagonal. Standard error estimate(s) are shown above the diagonal. Average within-group divergence is given on the diagonal. Analyses were conducted using the Maximum Composite Likelihood model. This analysis involved 54 nucleotide sequences. There were a total of 552 positions in the final dataset.

	Lim. sp. G1	**Lim. sp. G2**	**Lim. sp. G3**	**Lim. sp. G4**	**Lim. sp. G5**	**Lim. sp. G6**	**Lim. sp. G7**	**Lim. sp. G8**
***Limnodrilus* sp. Group1**	0.02	0.036	0.043	0.029	0.034	0.039	0.027	0.037
***Limnodrilus* sp. Group2**	0.237	0.06	0.036	0.042	0.045	0.031	0.041	0.028
***Limnodrilus* sp. Group3**	0.286	0.241	0	0.036	0.043	0.037	0.045	0.036
***Limnodrilus* sp. Group4**	0.205	0.268	0.234	n/c	0.021	0.038	0.034	0.035
***Limnodrilus* sp. Group5**	0.24	0.3	0.273	0.151	0.06	0.044	0.036	0.038
***Limnodrilus* sp. Group6**	0.265	0.217	0.247	0.254	0.293	0.02	0.04	0.026
***Limnodrilus* sp. Group7**	0.189	0.255	0.285	0.225	0.246	0.267	0.07	0.038
***Limnodrilus* sp. Group8**	0.25	0.198	0.239	0.233	0.263	0.185	0.25	0.05

*n/c= not compared, not enough samples in this taxonomic group to calculate within-group divergence

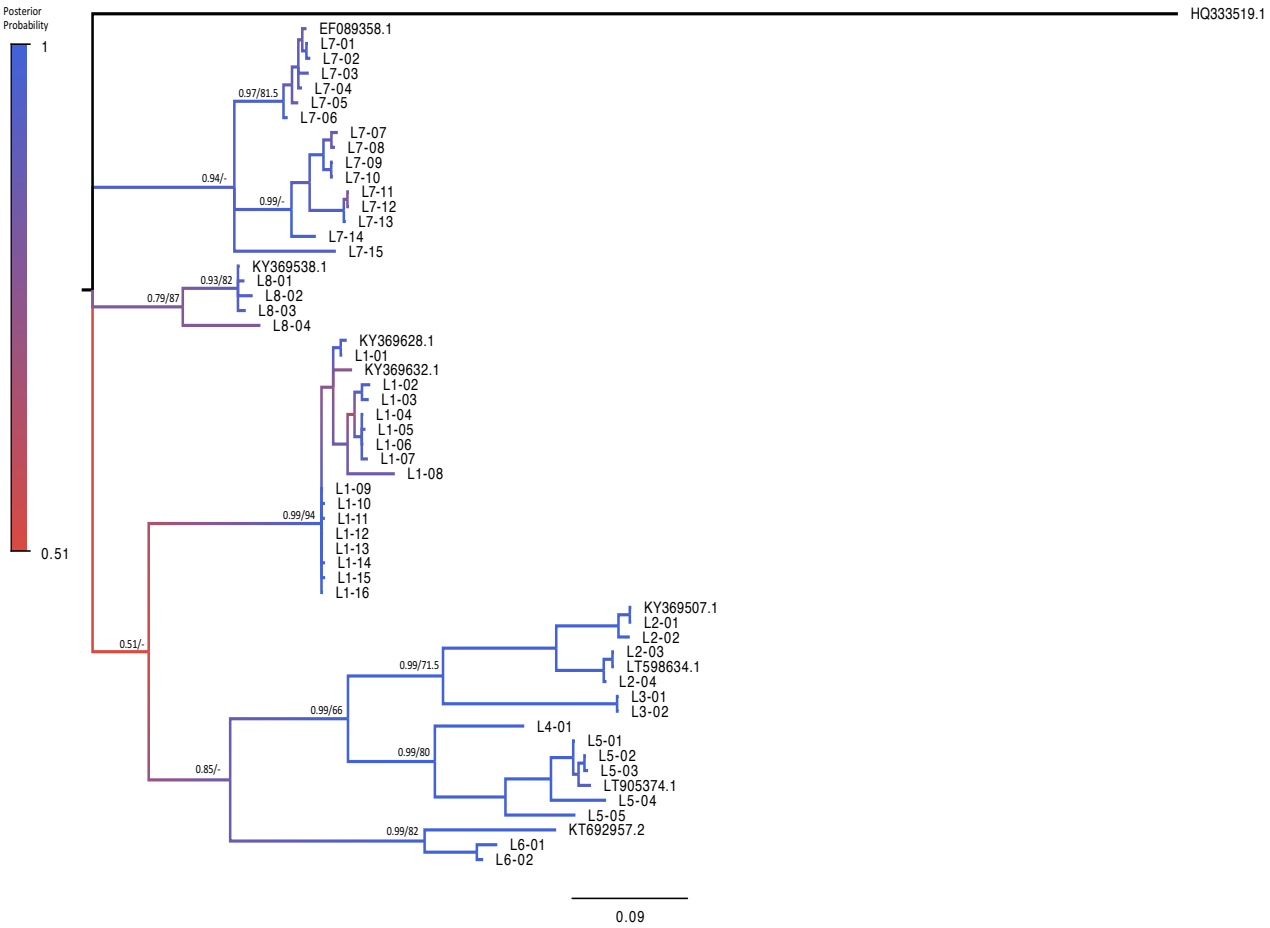


Figure 6. Maximum-likelihood phylogeny of *Limnodrilus* taxonomic groups based on *cox1*. Posterior probabilities >50 (also reflected in branch colour with blue being highest) and bootstrap values >50 are reported at the nodes, respectively. GenBank accession numbers are given for one sequence not from this study in each clade, identified as *Limnodrilus sp*. This phylogeny shows eight well-supported groups within this species.

Table 7. Oligochaete species breakdown by the six Albertan watersheds included in our study. The species determinations were made based on *cox1* barcoding and phylogenetic analysis. Species totals are included on the right-hand side, and watershed totals are located at the bottom of the table.

Watershed	Peace River	Athabasca River	North Saskatchewan River	Red Deer River	Bow River	Old Man River	Watershed Total*	Complete Total
Species							Total	
Tubifex T1	0	17	5	0	29	23	74	74
Tubifex T2	0	11	9	5	20	0	45	51
Tubifex T3	5	10	25	15	16	30	101	101
Tubifex T4	0	2	4	1	1	0	8	8
Tubifex T5	0	0	0	0	5	0	5	5
Limnodrilus L1	1	26	4	1	3	11	46	49
Limnodrilus L2	0	2	0	0	8	2	12	12
Limnodrilus L3	0	0	1	0	4	3	8	8
Limnodrilus L4	0	1	0	0	0	0	1	1
Limnodrilus L5	0	1	17	3	11	2	34	34
Limnodrilus L6	0	1	2	0	2	0	5	5
Limnodrilus L7	0	3	12	11	46	35	107	118
Limnodrilus L8	0	0	0	3	0	7	10	10
Aktedrilus sp.	0	0	1	1	0	0	2	2
Alexandrovia sp.	0	0	2	0	0	0	2	2
Amynathas sp.	0	0	0	0	2	0	2	2
Aulodrilus pluriseta	0	0	0	0	1	0	1	1
Eiseniella tetraedra	0	0	0	0	4	0	4	4
Enchytraeidae sp.	0	0	3	6	1	0	10	10
Fridericia ratzeli	0	0	1	0	0	0	1	1
Fridericia raxiensis	0	0	0	0	1	0	1	1
Fridericia sp.	0	0	9	0	0	0	9	9
Haplotaxida sp.	0	0	1	0	0	0	1	1
Henlea sp.	0	0	0	0	1	0	1	1
Henlea perpusilla	0	0	3	0	8	1	12	12
Ilyodrilus templetoni	0	0	6	4	0	3	13	14
Limnodrilus udekemianus	0	3	0	1	0	0	4	6
Lumbriculda sp.	0	0	0	0	10	0	10	11
Marionina riparia	1	0	0	1	0	0	2	2
Mesenchytraeus armatus	0	0	1	0	0	0	1	1
Naididae sp.	0	2	1	0	0	0	3	3
Nais christinae	0	1	0	0	0	0	1	1
Nais sp.	0	0	0	1	0	0	1	1
Octlasion cyaneum	1	0	0	0	0	0	1	1
Ophidonais serpentina	0	0	1	0	2	0	3	3
Potamothrix bavaricus	0	0	1	0	0	0	1	1
Rhynchelmis elrodi	0	0	0	0	1	0	1	1
Total	8	80	109	53	176	117	543*	567

**some stocked pond locations are missing as they were sampled outside our six main study watersheds, so the complete species total is greater than when separated by watershed*

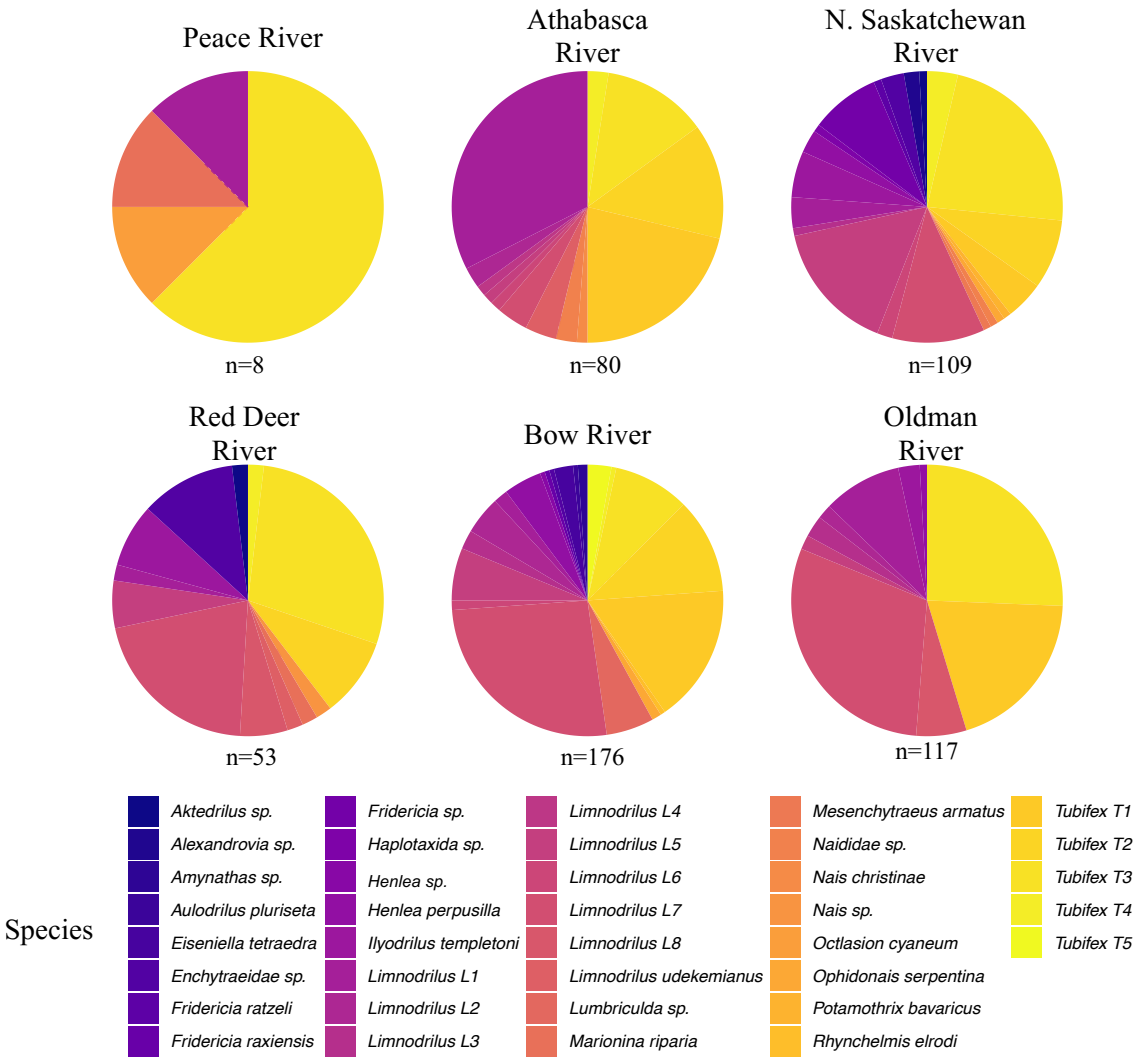


Figure 7. Visual representation of the oligochaete species proportions found in each of our six study watersheds. The sample number is included under each pie chart. Species were determined via barcoding and phylogenetic analysis.

4.3.2 Infection Prevalence and Oligochaete/*Myxobolus cerebralis* Combinations

Only worms from the genera *Tubifex* and *Limnodrilus* tested positive for *M. cerebralis* DNA. Infection prevalence in all the oligochaete specimens included in this study, based on a positive qPCR result, was 3.7% (21/567) while in the *Tubifex* genus was 4.6% (11/240) and

Limnodrilus was 4.2% (10/237). We cannot be sure these are all patent infections of worms actively shedding actinospores.

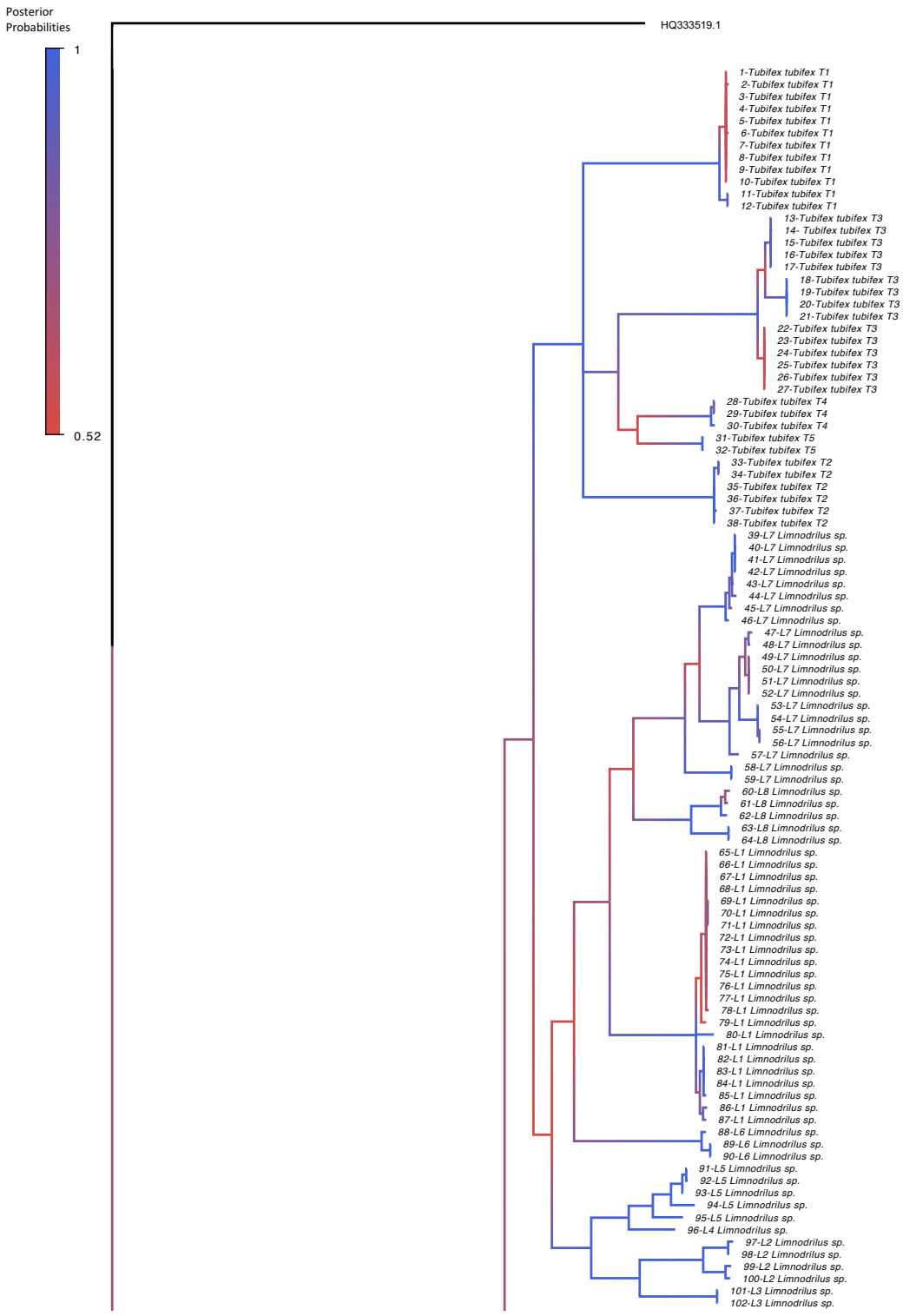
Limnodrilus sp. L1 and L7 both had positive results for *M. cerebralis*. *Tubifex sp*. T1 and T3 had positive results (Table 4). The DNA copy numbers from the positive qPCR tests ranged from 11 to 87 for *Tubifex sp.* T1, 15 to 9 203 676 for *Tubifex sp.* T3. *Limnodrilus sp.* L1 had a range of 6 to 105 and *Limnodrilus sp*. L7 had a range of 5 to 29. As noted in Chapter 3, a myxospore has ~600 gene copies while an actinospore has ~7000 (Kelley et al., 2004). The lower overall copy number in *Limnodrilus sp.* and *Tubifex sp.* T1 indicates that these groups can consume spores and then test positive for *M. cerebralis*, but we do not know at this time if they can carry a patent infection and produce viable actinospores. The very high gene copy number in *Tubifex sp.* T3 is highly suggestive of a patent infection.

4.3.3 Lineage PCR and Barcoded Species Comparisons

Tubifex lineage I and III have been previously identified as being able to transmit *M. cerebralis* actinospores. Lineage V and VI are described as being not susceptible to *M. cerebralis* infection and lineage II and IV are considered to be only endemic in Europe.

In our assessments, oligochaetes identified as *Tubifex* via *cox1* barcoding produced lineage assay results representative of lineages I, II, III, and V, as well as a single band around 240bp, which does not match with any published lineage, or no bands at all. *Tubifex sp.* worms that tested positive for *M. cerebralis* produced results consistent with lineages III, V, the 240bp band or no band at all. Additionally, worms belonging to the same OTU or even the same species, based on *cox1* barcoding, produced multiple lineage PCR results (Table 4). We would have expected that these closely related individuals would produce consistent results in another

genetic test. The individual *Limnodrilus sp.* worms that tested positive for *M. cerebralis* did not produce any bands in the lineage PCR. However other closely related specimens, from the same OTU that were negative for *M. cerebralis,* yielded the 240bp band. One sample, which was identified as *Octolasion cyaneum* based on *cox1* barcoding, yielded a banding pattern that matched both Lineage II and V. Two worms produced a banding pattern that matched to Lineage II; OTU 125 identified as *Lumbriculda sp* and OTU 156 identified as *Marionina riparia*. A worm in OTU 111 produced a 240bp band and was identified as *Aktedrilus sp*.


Posterior Probabilities
1
0.52
HQ333519.1
1-Tubifex tubifex T1
2-Tubifex tubifex T1
3-Tubifex tubifex T1
4-Tubifex tubifex T1
5-Tubifex tubifex T1
6-Tubifex tubifex T1
7-Tubifex tubifex T1
8-Tubifex tubifex T1
9-Tubifex tubifex T1
10-Tubifex tubifex T1
11-Tubifex tubifex T1
12-Tubifex tubifex T1
13-Tubifex tubifex T3
14- Tubifex tubifex T3
15-Tubifex tubifex T3
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17-Tubifex tubifex T3
18-Tubifex tubifex T3
19-Tubifex tubifex T3
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24-Tubifex tubifex T3
25-Tubifex tubifex T3
26-Tubifex tubifex T3
27-Tubifex tubifex T3
28-Tubifex tubifex T4
29-Tubifex tubifex T4
30-Tubifex tubifex T4
31-Tubifex tubifex T5
32-Tubifex tubifex T5
33-Tubifex tubifex T2
34-Tubifex tubifex T2
35-Tubifex tubifex T2
36-Tubifex tubifex T2
37-Tubifex tubifex T2
38-Tubifex tubifex T2
39-L7 Limnodrilus sp.
40-L7 Limnodrilus sp.
41-L7 Limnodrilus sp.
42-L7 Limnodrilus sp.
43-L7 Limnodrilus sp.
44-L7 Limnodrilus sp.
45-L7 Limnodrilus sp.
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63-L8 Limnodrilus sp.
64-L8 Limnodrilus sp.
65-L1 Limnodrilus sp.
66-L1 Limnodrilus sp.
67-L1 Limnodrilus sp.
68-L1 Limnodrilus sp.
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77-L1 Limnodrilus sp.
78-L1 Limnodrilus sp.
79-L1 Limnodrilus sp.
80-L1 Limnodrilus sp.
81-L1 Limnodrilus sp.
82-L1 Limnodrilus sp.
83-L1 Limnodrilus sp.
84-L1 Limnodrilus sp.
85-L1 Limnodrilus sp.
86-L1 Limnodrilus sp.
87-L1 Limnodrilus sp.
88-L6 Limnodrilus sp.
89-L6 Limnodrilus sp.
90-L6 Limnodrilus sp.
91-L5 Limnodrilus sp.
92-L5 Limnodrilus sp.
93-L5 Limnodrilus sp.
94-L5 Limnodrilus sp.
95-L5 Limnodrilus sp.
96-L4 Limnodrilus sp.
97-L2 Limnodrilus sp.
98-L2 Limnodrilus sp.
99-L2 Limnodrilus sp.
100-L2 Limnodrilus sp.
101-L3 Limnodrilus sp.
102-L3 Limnodrilus sp.

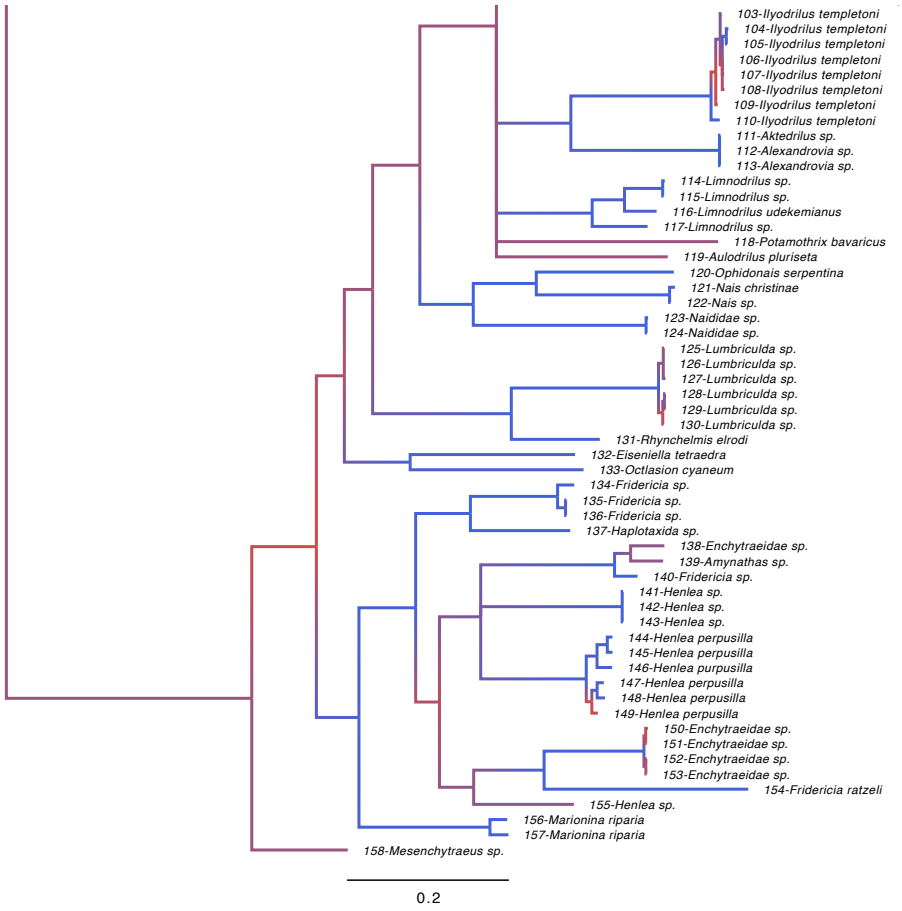

Figure 8. Bayesian inference phylogeny of all 158 unique sequences found in our study based on *cox1*.
OTU number is given prior to the species name. Posterior probabilities are reflected in branch colour with blue being the highest and red the lowest.

4.4 Discussion

This project started as an assessment of the worm host for *M. cerebralis*, to aid in making management decisions by understanding which oligochaete worm species we have in Alberta, and looking for any geographic differences. We thought we would see some differences in the oligochaete populations that would be defined by watershed, perhaps creating a sort of ecological barrier to the spread of *M. cerebralis*. However, our findings did not confirm this, with potentially susceptible worm hosts found in every watershed. We also found that an oligochaete worm testing positive for *M. cerebralis* does not tell the whole story, as *Limnodrilus sp.* (not a

known *M. cerebralis* host) tested positive for *M. cerebralis* in the qPCR assay, but at an overall lower gene copy number than some *Tubifex sp.*, suggesting that they may have consumed myxospores but are not carrying a patent infection. This is an important distinction because to successfully manage the parasite and protect fish populations, the oligochaete host has to be targeted as it produces the parasite stage infective to fish. A worm testing positive for *M. cerebralis* that is not able to carry a patent parasite infection is of less concern for management.

When identifying species, ideally, more than one technique should be used in designating a species. This can be morphometrics and a gene phylogeny or often multiple phylogenies using different genes to compare species separation results. As past studies in Canada have mainly focused on morphology, we are adding a new dimension to oligochaete taxonomy by recording 567 new *cox1* sequences. Other groups have shown that *cox1*, *ITS2* and morphology-based phylogenies tend to align well for oligochaete worms, giving support to our findings from a single gene phylogeny (Achurra et al., 2011; Vivien et al., 2017). We encountered a few concerns: the coverage of oligochaete species available in the sequence databases and the accuracy of the identification of these other species. For the former, we had a number of OTUs that did not have any sequence matches over 90% nucleotide identity and some that were below 80% (Table 4). Additionally, as indicated in previous work, often any small pink worm found in an aquatic environment is called a "*Tubifex*" worm without further proper identification (Vivien et al., 2017). As well, features used for morphological identification, such as chaete, often become fragile and break in a sample that has been preserved, limiting accurate identification. We think these difficulties may lead to similar small pink worms falling under the umbrella of "*Tubifex*," which we believe might be reflected in OTUs 53 and 96.

Overall, these results show much greater species diversity than previously thought within the genera of *Tubifex* and *Limnodrilus*, with five and eight taxonomic groups respectively, suggesting cryptic speciation. The tests used previously, lineage PCR, to assess *Tubifex* populations in the context of whirling disease management appear to be inaccurate for worm populations in Alberta. There did not appear to be consistent results from the lineage PCR matching the OTUs or group separation found in this study. As well, we found worms in Alberta that were not identified as *T. tubifex* based on *cox1* barcoding (OTUs 2, 125 and 133) that gave lineage PCR test results identifying them as lineages of *T. tubifex* that should not be found in North America (II or IV) (Table 4). As the parasite was first found in southern Alberta and the biggest impacts have been in Bow and Oldman River watersheds (Chapter 3), we were curious if this difference in infection prevalence had to do with variation in the worm host population or is simply a matter of parasite invasion delay. The worm species across watersheds in abundance and proportion were quite consistent, with most areas having approximately 50% *Tubifex* sp. complex worms. Worm distribution seems to be more affected by local water conditions than any largescale differences across our study area (Zendt and Bergersen, 2000b). Unfortunately, this suggests that there are likely susceptible worm hosts across the province and no ecological barrier, being an area without appropriate hosts to maintain the parasite lifecycle, to prevent spread.

All four of the taxonomic groups found positive for *M. cerebralis* were also the most numerous worm species found. This may influence the prevalence estimate because they were the only species found positive given the low overall infection prevalence. While positive qPCR results do not guarantee the worms are able to shed viable infective parasite stages, potentially susceptible intermediate hosts could be missed if we only rely on lineage testing or focus solely

on *Tubifex* sp. for surveillance. For example, a worm in OTU 65 (*Limnodrilus sp.*), which matched at 98.403% to *Limnodrilus clapardeanus* from GenBank, reported 105 gene copies in the qPCR test for *M. cerebralis*. While unconfirmed if this worm was able to actively transmit the parasite, using qPCR testing for early detection of the parasite is still useful when dealing with an invasive species. For general monitoring, it is helpful to know the parasite is present at a location, especially along the edge of the known parasite distribution, and worms can act as a concentrator of *M. cerebralis* as it moves through the environment and consume spores.

The next steps to confirm actual patent infection in separate *Tubifex* groups and in non-Tubifex worms would be an infection challenge in the lab and then monitoring for parasite development. As detection of *M. cerebralis* DNA does not confirm an infection that is able to produce actinospores. Once the target worm species have been identified, high throughput sequencing has been suggested as a good technique to identify multiple species in an environmental sample at once (Bohmann et al., 2014). Alternatively, a multiplex-qPCR could be developed targeting *M. cerebralis* and the oligochaete species able to produce actinospores and could be used to confirm parasite and suitable host presence in one qPCR run with a single sample.

In all, this study added 567 barcoded oligochaete sequences from six different watersheds in Alberta, uncovering greater diversity than was previously thought, increasing our understanding of freshwater invertebrate diversity in Canada. This has also increased our understanding of the oligochaete worm host for *M. cerebralis* and has opened new research questions in order to best manage this invasive parasite.

Chapter 5. Discussion and Conclusions

Overview

Parasites are everywhere and are known to influence ecological interactions (Lafferty et al., 2008). They can have effects on population size, either by influencing host fecundity (Dobson and Hudson, 1992) or by affecting host fitness (Johnson and Hoverman, 2012). Parasites can impact host behaviour, such as control of ants to facilitate the transmission of *Dicrocoelium dendriticum* (Martín-Vega et al., 2018), and host behaviour can also impact parasite success, such as the grooming for removal of external parasites like lice (Ezenwa et al., 2016). Biodiversity can be impacted by disproportionate effects on host species, such as the maintenance of a parasite population by host species that are asymptomatic, leading to continual reinfection of other host species that are negatively impacted, reducing their numbers (Price et al., 1986). The effects of parasites can be more pronounced when they act as an invasive species having unknown influence in a novel landscape. New host species, when a non-endemic parasite is encountered, lack the benefit of thousands to millions of years of co-evolution, and this can have harmful effects on the population. For myxozoan parasites, these impacts are reflected in instances of disease where the parasites infect an atypical host, as generally, myxozoans are of low ecological concern when in their native ranges (Gilbert and Granath, 2003). Of course, the introduction of *Myxobolus cerebralis* to North America is a strong example, resulting in steep declines of rainbow trout populations in the USA (Vincent, 1996). Some fish species, such a brown trout, are also susceptible to this parasite but are not negatively impacted as they are also an introduced species from the same native range as *M. cerebralis* in Europe. This means the asymptotic brown trout can act as parasite reservoirs and maintain the parasite population in an

ecosystem. Complex parasite lifecycles, where more than one host is used, like those of myxozoans, can make it more difficult to study and understand parasite biology, but they also give more opportunity for alternate ways for detection and control of important parasites. This benefit is most apparent when looking at wild systems not under human control, such as a river system compared to an aquaculture facility. Due to the increase in research during the impacts of whirling disease in the USA, we understand that the alternative host to fish, an oligochaete worm, is also an important factor to consider when monitoring and managing this parasite (Rognlie and Knapp, 1998).

Since monitoring for the presence of an invasive parasite is important to protect vulnerable host species, introduce any mitigation efforts and prevent negative outcomes, suitable techniques for the invasive species being monitored are necessary. Previously, monitoring for *M. cerebralis* has focused on the fish host, whether by collecting fish from the waterbody or using sentinel testing, placing contained fish into the waterbody and monitoring for parasite infection. There are constraints with fish testing, such as waterbody access, permits, and protecting vulnerable fish populations, so having an alternative test for detection and monitoring is an important management tool. We can use the complex lifecycle of *M. cerebralis* to our advantage and utilize the multiple parasite stages for other monitoring opportunities.

Sampling can target alternative hosts that are more numerous, easier to collect or not threatened/endangered. Sampling can also focus on environmental stages, whether waterborne or terrestrial. Approaching detection with more than one test using different parasite stages could be helpful where host sampling is limited: a host is inaccessible, sampling requires lethal host testing or the host is already experiencing population pressure. These aims can all be achieved by developing detection tests with adequate sensitivity and specificity to use them in more complex

media like an environmental sample, and also having a solid understanding of the parasite dynamics and biology to reduce sampling effort and increase useful data. This thesis used the invasion of *M. cerebralis* in Alberta, Canada, to test all these ideas and increase our knowledge of its ecology in this new environment to facilitate the best management decisions. This work included the development of a more specific qPCR assay, its use in ~2800 samples and genetic analysis of the oligochaete worm host.

We hypothesized that environmental samples, including the worm host, will be suitable alternatives to fish sampling for the tracking of whirling disease-causing parasites in water bodies. We also hypothesized that the definitive worm host-parasite relationship would be dominated by *Tubifex tubifex,* but given the geographical isolation of Alberta in comparison with where whirling disease has been studied in the past, may lead to other species being used as a definitive host.

Key Findings

The results of this thesis work indicate that *M. cerebralis* can be detected in sediment and worm samples with a qPCR assay, with the latter able to produce positive results before the parasite infection is at a detectable level in fish. Focusing on only finding infected fish is not giving the full parasite story. The testing using sediment samples or the worm host produced less positive results overall than with fish testing. It is possible that there are localized areas of transmission with infected worms, releasing TAMs into the waterbody, producing infected fish that are more spread out than infected worms. Only one TAM in 500L of water is required to maintain the infection in a fish population (pers. comm.), so a smaller number of worms being responsible for more widespread fish infection is possible. Also given the dynamics of a water

system where TAMs could easily be dispersed or carried downstream, an infected worm hotspot up a river could be responsible for fish infections in multiple locations. Additionally, fish move and may not be found in the location where they were infected.

Our results for the oligochaete species found in Alberta and the poor performance of the widely used lineage PCR suggests that when dealing with a parasite in a novel area, it is inappropriate to assume the same hosts previously determined are being used in the local life cycle. The lack of consistent results in the lineage PCR also suggests that oligochaete populations can have a lot of variation on the same continent, and it cannot be assumed the species are consistent. On the other hand, these results may be due to a flaw in the lineage PCR test. The selected region of the *16S* gene may not be a suitable determinant of susceptibility to *M. cerebralis* or different primers or a different region may give more consistent and accurate results than what our study and other groups have seen (Rasmussen et al., 2008).

Strengths and Limitations

This thesis utilized a large dataset of samples collected over a large geographical area. This was possible due to the collaboration with Alberta Environment and Parks, which facilitated the collection of these samples. The geographical range allowed us to work with samples from known *M. cerebralis* positive watersheds (Old Man, Bow, Red Deer River), watersheds in a transition zone (North Saskatchewan) and watersheds presumed negative (Athabasca and Peace). We also had good coverage to collect a variety of oligochaete worms increasing our identification of those populations in Alberta.

Assessing sensitivity and specificity for our new qPCR test for *M. cerebralis* is challenging as those calculations are done by comparing the new test results to a gold standard.

The most recent qPCR assay developed for *M. cerebralis* before the one in this study was designed to be used in fish samples and shown to not be specific in our tests, due to positive results in non-salmonid fish. Calculation of sensitivity or specificity of our test for worm and sediment samples in our study would require comparison to fish testing results. The latter not only used an unpublished test but also in an entirely different media and different life stage of the parasite. Using that comparison would actually calculate the sensitivity and specificity of the physical worm or sediment sampling in combination with our qPCR test, which we fully expect to be different from fish sampling. However, sensitivity and specificity can also be used to measure the performance of the qPCR assay on a sample. We developed the new qPCR assay because the currently available tests showed the possibility of cross-reaction *in silico*, gave a questionably high number of positives when first run and also have positive results when used with a fish sample that was not a host species for *M. cerebralis* (3-spine stickleback). We designed the new test to be specific for *M. cerebralis* compared to all currently available myxozoan species *18S* sequences. Sensitivity can also be measured by the lowest gene copy number in a sample that can be detected as a positive result. Using a plasmid with our gene sequences inserted, we were able to use a standard curve of serially diluted concentrations to determine the lower limit of detection of five gene copies in a sample, which is much less than one myxospore (~600 copies).

Samples from each site were not always collected at the same time, as explained in Chapter 3. This limits some conclusions we can make about the correlation between samples. Worm sample collection was not always successful, and sediment was not always collected, also limiting our power to make conclusions.

Future Directions

Going forward, in addition to sediment and worm samples, water samples, which would target the suspended actinospore stage, should also be considered. Water samples would be the last step in completing the picture of what the parasite is doing in a waterbody and would confirm the presence of the parasite stage responsible for the fish infection. Water sampling techniques have been developed for monitoring for many other organisms and could be adjusted for *M. cerebralis* monitoring (Ardura et al., 2017; Rudko et al., 2018).

After determining the Albertan oligochaete worm host *M. cerebralis* susceptibility profiles, a new assay can be developed once we understand which worms to target based on that parasite compatibility. If an improved PCR test can be developed, it would be less expensive and faster to run than using barcoding for each individual worm. High throughput sequencing has also been proposed as an option when looking for multiple sequences at once in an environmental or composite worm DNA sample (Bohmann et al., 2014).

Using these non-fish stages lends itself well to being used in aquaculture facilities, where fish have been removed after being found to be *M. cerebralis* positive. These sites would need to be confirmed cleared of the infection before fish could be added, which could potentially be done by the testing proposed in this thesis. Overall, this work is an important step forward in managing this invasive parasite in Canada.

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Appendix A. Additional Tables

Supplemental Table A. Alignment of *18S* gene of *Myxobolus cerebralis*, other myxozoan species and the *18S* qPCR assay developed in this study.
Base differences to the primers or probe are highlighted. GenBank accession numbers are indicated for each sequence.

Species /Primer /Probe		Accession Number
MC_18S Fwd	---GCT-GATCGAAT-------GGTGCTACTAA----------	
MC_18S Probe	----------AGTGTTGGAGTAGTG-TGCCCGTCTT----------	
MC_18S Rev	----------GCGTAAGGATGGCAGTTGA-----	
M. cerebralis	TAGGCT-GATCGAAT-------GGTGCTACTAACTGCTCCAGCGTTGAATTTCAAATTCAGTGTTGGAGTAGTG-TGCCGTCTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	EF370481
M. lepomis	TGGGCGTGATATTGTTCGCTATTGACCTTGTAACTGGGCTGGCGTGTCCTCACGTCAGTCTCGGTACGAGGGAGCAGCAGTGTGC-GTCAAAAAGCTGGCGTGCAGTAATTTCACGCGAGGATGGCTTTGACCTTGA	KY203391
M. bramae	TAAGCT-GATTGAAT-------GGTGCT-CCAACTGTTTTGGTGATAAATTTCTA-TTTTATTACTAAACAGTG-TGCT-TCTTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	AF085177
M. ellipsoides	TAAGCT-GATTGAAT-------GGTGCT-CCAACTGTTTTGGTGATAAATTTCTA-TTTTATTACTAAACAGTG-TGCT-TCTTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	AF085178
M. djiragini	TAAGCT-GATTGAAT-------GGTGCT-CCAACTGTTTTGGTGATAAATTTCTA-TTTTATTACTAAACAGTG-TGCT-TCTTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	AF085179
M. sandrae	TAAGCT-GATTGAAT-------GGTGCT-CCAACTGTTTTGGTGATAAATTTCTA-TTTTATTACTAAACAGTG-TGCT-TCTTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	AF085181
M. hungaricus	TAATCTAGGCAATGT-------ATGCTGACGATTGCCTCAGGGCGTA-TCGGATCTTCATTGGCTCAGATGTT----------TAT--GCTGAATGCTACTATTTGCACACAAGTGTGGTATTTGGCCTTAA	AF448444
M. squamalis	TAAGCT-GATTGAAC-------G--GTACTAC-ATCTGTTTCAACGTTTAAATTTCTATTTGA-TGTTGAACAGTG-TGCCCTCTTTCAGTTATTCGCCAATTTACACTACTTACGCGTAAGGATGGCAGTTGACCTTTA	JX910363
M. kingchowensis	TAGTCTAATAGCGAA----------CATTTGGCTGATTCA--GCTGATGTTCGCAATAGATAACAA----------CGCAAACTGTTACTATTTAATCGCAAGTATGTTGATTCACCTTTA	KP400625
M. sheyangensis	TTACATAGATT----------GGTTCTTTTGAC-------GATTTTCTTTTACTAGATTATTGAATCTTGGCCC--GTCTTGTGTTA-CCGCTGCATGTTACTATTTGCACACAAGTATGGTATTTGGCCTTTA	KU313685
M. parvus	TGGGTTATGAAGTCTAC-----GTCAAAAACCAGAAAATCCCCTGTGTGTCATCGCATGGCGAAGGATTTGGCGAG-CTTCGTGCCGTAAGCCGGTGGATAGTAATTGCACGTCAGGATAGCACTGACCTTGA	KX242161

Supplemental Table B. Alignment of *18S* gene of *Myxobolus cerebralis*, other closely related myxozoan species and the *18S* assay from Cavender *et al.* 2004. Base differences to the primers or probe are highlighted. GenBank accession numbers and year published are indicated for each sequence.

Species/Primer/Probe	SEQUENCE	Accession Number	Date
Forward: 811F	-TGAATAAATCAGAGTGCTCAAAGC----------	n/a	n/a
Probe: 888P	----------TGTGACAAATAGCG----------	n/a	n/a
Reverse: 937R	----------CAGTTA-CAGCATAC--AGCACCAA--	n/a	n/a
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	AF115254	1999
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	EF370478	2007
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	EF370479	2007
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	EF370480	2007
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	EF370481	2007
Myxobolus cerebralis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACAAATAGCGATCG-GTCTTTGACTGAA--TGTTATT-CAGTTA-CAGCATAC--AGCACCAACC	U96492.1	2001
Myxobolus hungaricus	TTGAATAAACAGAGTGCTCAAAGCAGGCGA-AAGCTTGAATGTT-ATAGCATGGAACGAACAAACGTGTATTTGCGTATTGTT-------TAGTGATGA----TTGGGGGCAACTCTGATTGTTGGTG--GCATAGCGCAGCACCTACC	AF448444.1	2002
Myxobolus kingchownensis	TTGAATAAACAGAGTGCTCAAAGCAGGCGAA-CGCTTGAATGTT-GTAGCATGGAACGAACAAACGTGTATTCGCGTGCATTGTCGA--AGTGAGTGTG-CTTCTGGTGCGCTTGCC-TTGTTGCTTTTMGCAT-----CACCGCGCC	KP400625.1	2016
Myxobolus sheyangensis	TTGAATAAACAGAGTGCTCAAAGCAGGCGA-ACGCTTGAATGTT-ATAGCATGGAACGAACAAACGTTTATTTG----------CGGATAGC---CGGATAGTTGGGGGCA---ACTTTGACGTTT-GGCATAGCGTAGCACCTGCC	KU313685.1	2016
Myxobolus parvus	TTGAGTAAATCAGAGTGCTTAAAGCAGGCTTTTAGCGCTTGAATGTT-ATAGCATGGAACGAACAATCGTGTA-TGGTGTA--TGCTGGCAATGA----------TTTTGT-TAAACGTAGTT-TTGAGCGTCATCAGCATAC--GACACCGACC	KX242161.1	2016
Myxobolus lepomis	TTGAGTAAATCAGAGTGCTTAAAGCAGGCGA-AAGCTTGAATGTTAATAGCATGGAACGAACAATCGTGTA-TGGTGTG--TAGTGA-----ACCGAGCGTGACCTTCTCTCGAGTTGGC---GC-GTTTGCAGTTGAGC--GACACCGACC	KY203391.1	2017
Myxobolus arcticus	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GNCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085176.1	2001
Myxobolus bramae	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085177.1	2001
Myxobolus djiragini	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085179.1	2001
Myxobolus elipsoides	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGTTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085178.1	2001
Myxobolus neurobius	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATATTAATAGCATGGAACGAGCAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085180.1	2001
Myxobolus sandrae	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	AF085181.1	2001
Myxobolus insidiosus	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTATG--TTGTGACAATAGCGATCG-GTCTTTGACTGAA--TGCTATTGCTGTTA-CAGCATAC--AGCACCAACC	U96494.1	2001
Myxobolus squamalis	TTGAATAAATCAGAGTGCTCAAAGCAGGCTT-TTGCTTGAATGTTAATAGCATGGAACGAACAATTGTGTAGTAGTGTG--TTGTGACGAATAGCGATCG-GTCTTTGACTGAA--TGTTGTTGCTGTTA-CAGCATAC--AGCACCAACC	JX910362.1	2013