Development and Implementation of a Community Based qPCR Monitoring Program for Biological Hazards of Recreational Water

by

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Abstract

Recreational water is an economic and social asset to the public. Its' importance is underscored by the need to protect it, and ensure it is safe to use. There are numerous waterborne pathogens that cause illness each year, including bacteria, human infectious viruses, and parasites, and many of these species are not monitored for due to limited resources. Recreational water monitoring is challenging—to quote Heraclitus, "you cannot step in the same river twice." This statement can be taken literally in the context of water monitoring. Infectious organisms move with the ebb and flow of the tide, and therefore a waterborne pathogen present yesterday, reported on today, is irrelevant to protecting public health. Additionally, there are a huge number of recreational sites (official and unofficial, private and public) which simply cannot all be monitored routinely using the current monitoring scheme in which samples are collected by technicians, shipped to a central laboratory, and analyzed within 48-72 hours of collection.

Taken together, there are an enormous number of waterborne pathogen for which we could monitor, a large number of sites that are routinely used for recreation, and a monitoring scheme that reactively reports waterborne pathogens from days passed—public health is not being protected as effectively as it could be.

Community based monitoring (CBM) has been hailed as a highly effective tool for environmental monitoring. Community based monitoring brings together

partners from communities, industry, academia and government to answer research questions related to monitoring projects. As a methodology it enables data collection and analysis on a large scale (geographically and longitudinally), for less cost. Additionally CBM provides educational value to participants, and engages them in environmental protection. However, managing and implementing these projects can be highly complex, and there is a great deal of uncertainty in the integrity of the data collected.

This thesis seeks to remedy these challenges by implementing a CBM water monitoring system using a molecular technique called quantitative polymerase chain reaction that can detect the DNA of a target organism with incredible specificity and sensitivity. The results herein demonstrate the reproducibility of CBM qPCR as a method of environmental monitoring across a variety of bacterial and avian schistosomes qPCR methods, with interclass correlation coefficients ranging from 0.6, to 0.9 suggesting high reproducibility between experts and CBM partners.

Through the deployment of the CBM qPCR method, I also was able answer scientific and applied research question regarding avian schistosomes. Notably, that the infectious cercariae of these parasites move with wind direction, and have seasonal peaks in concentration which have important implications for managing swimmer's itch risk. Additionally, I demonstrate that copper sulfate applied locally as a molluscicide is ineffective at reducing cercariae concentrations long-term at

recreational beaches, and finally I demonstrate the utility of source-tracking assays to assess changes in parasite composition across four recreational lakes.

The CBM qPCR program also was used to assess fecal indicator bacteria in recreational waters. These results were used to comprehensibly track human-associated *Bacteroides* contamination across an entire lakeshore. Additionally the same approach was used to assess *Enterococcus* spp. concentrations before and after rain events into four recreational lakes. This method was also used to capture increases in *Pseudomonas aeruginosa* concentration in a natural swimming pool environment, and to search for invasive zebra mussel (*Dreissena polymorpha)* veligers in reservoirs in southern Alberta. In summation, this thesis as a whole demonstrates the effectiveness of a community based approach to water monitoring in a wide variety of contexts.

Preface

This thesis is an original work by Sydney Patricia Rudko. This research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project name "Swimmer's itch in Alberta" no. 00048511, May 2012.

The work presented in Chapter 2 of this thesis was part of a collaboration lead by Dr. Patrick Hanington with Ronald Reimink of Freshwater Solutions (LLC), Bradley Peter of the Alberta Lake Management Society, and Jay White of Aquality Environmental Consulting. This work has been submitted for publication to Science of the Total Environment under the title "Democratizing water monitoring: Implementation of a community-based qPCR monitoring program for waterborne pathogens in recreational water. In this manuscript, I designed the study, collected data and performed the statistical analyses, and wrote the paper. My co-authors were also responsible for data collection.

The work presented in Chapter 3 of this thesis has been published in three manuscripts:

Rudko SP, Reimink RL, Froelich K, Gordy MA, Blankespoor CL, Hanington PC. Use of qPCR-Based Cercariometry to Assess Swimmer's itch in Recreational Lakes. Ecohealth. 2018 December 14:4:827-39.

Rudko SP, Turnbull A, Reimink RL, Froelich K, Hanington PC. Species-specific qPCR assays allow for high-resolution population assessment of four species avian schistosome that cause swimmer's itch in recreational lakes. International Journal for Parasitology: Parasites and Wildlife. 2019 Aug 1;9:122-9.

Froelich K, Reimink RL, Rudko SP, VanKempen AP, Hanington PC. Evaluation of targeted copper sulfate ($CuSO_4$) application for controlling swimmer's itch at a

freshwater recreation site in Michigan. Parasitology Research. 2019 May 118:5:1673-7.

All three papers represent an international research collaboration lead by Dr. Patrick Hanington and myaeld. In the first paper, I along with assistance from and Ronald Reimink designed the study. Ronald Reimink, Kelsey Froelich and Curtis Blankespoor collected the water, snail, and bird fecal samples and a small portion of the data. I also collected data and analyzed it. I wrote the manuscript. In the second paper, myself, Ronald Reimink and Patrick Hanington designed the study. I developed the methodologies used to answer the research questions posed. Ronald Reimink and Kelsey Froelich collected samples and some data. Alyssa Turnbull assisted with data collection and validation of the methodologies. I analyzed the data and wrote the paper. In the third paper, Kelsey Froelich and Ronald Reimink designed the study based on a hypothesis I developed in the manuscript published in Ecohealth, they, along with Aaron VanKempen, collected samples and performed some data collection. I also performed data collection and some analysis along with Dr. Patrick Hanington. Kelsey Froelich wrote the first draft of the manuscript and I edited it. I was responsible for revising the second draft of the manuscript (after peer review).

The work presented in Chapter 4 was part of a research collaboration lead by Dr Patrick Hanington, in collaboration with the Alberta Lake Management Society, the Borden Park Natural Swimming Pool, and Ronald L Reimink of Freshwater Solutions in Michigan, USA. These results have not yet been published, but in all cases each respective collaborator collected samples, while I performed data acquisition and analysis. Research questions were developed in collaboration.

"Water does not resist. Water flows. When you plunge your hand into it, all you feel is a caress. Water is not a solid wall, it will not stop you. But water always goes where it wants to go, and nothing in the end can stand against it. Water is patient. Dripping water wears away a stone. Remember that, my child. Remember you are half water. If you can't go through an obstacle go around it. Water does."

-Margaret Atwood, The Penelopiad

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Thank you to my community partners. Ronald Reimink and Kelsey Froelich, our collaboration taught me more than I could have expected about integrity, cooperation, communication, and how much we can accomplish when we work together. Thank you to Bradley Peter, Laura Redmond, and Caitlin Mader of ALMS. It's a rare thing to find a group of brilliant and hilarious people. Working with, and (often) travelling with your team were some of the best parts of my degreeTo my co-supervisor Dr. Norman Neumann, and Dr. Nicholas Ashbolt, thank you for keeping my ideas in check, teaching me patience, and always having my best interests at heart. I have such respect and admiration for both of you and it has truly been such a pleasure working with both of you and your respective labs. This work would not have been possible without my mentor and friend Graham Banting. Thank you for answering hundreds of scientific questions, and I'm so glad I could, that one time, help you figure out why your cloning wasn't working. I'm thankful for the hours spent debating, theorizing, and discussing results. You have always challenged my ideas, and in the process taught me to defend my position—what an invaluable skill you've given me. Thank you to my friend and mentee Alyssa Turnbull, who arrived in the lab just in time to remind me why I started.

I have had the privilege to have so many wonderful teachers during this degree, and even before, each of you, in your own way, made me a better person. I I have also had the privilege of friendship with truly some of the most brilliant

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I could not have picked a better family. I have been so lucky to have your unwavering support throughout this degree. Mom and Dad, your hard work and sacrifice taught me never to quit, thank you for raising me to be the person I am today. Oreste Rudko, while you will never read this, you inspired this journey. To the unbelievably talented Julia Rudko, thank you for all of the evenings spent working at our favorite café, for providing judgment-free spelling and grammar lessons, and for being my best friend and my soul sister. Andrew, thank you for being my partner. Your love, support, and dedication astound me everyday. Thanks for making me lunch, and always coming through with cheeseburgers on my best and my worst days. The best part of the last five years was meeting you. I love you all so much.

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Chapter 1: Introduction

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1.0 Preamble

The goal of this thesis is to broadly consider how to design and implement a community based qPCR water monitoring program for a variety of hazardous organisms. This thesis tested the accuracy of parasitic and bacterial qPCR monitoring methods in a CBM program that I designed. To present this data most logically, this general introduction serves to orient the reader with the principles of community based monitoring, and water quality and assessment in recreational water. Introductions regarding the biology of the organisms of interest will be presented in the introductions of the relevant chapters. This literature review introduces the overarching concept of the thesis and the theories that have informed its conception.

1.1 Water Quality and It's Assessment Through the Ages

Civilizations are built upon fresh water; human settlements were first built in locations where water was available. As settlements expanded, they often outgrew or contaminated their water supplies such that a new supply would need to be found. To overcome these early impediments to expansion, early civilizations dug wells or had water delivered to their homes (Bromehead 1942). As cities grew, many civilizations (most famously the Romans, but also the ancient Mesopotamians, peoples residing in the Indus valley, and the Mayans) built aqueducts to bring water into cities across vast distances

(William Back 1978; Burian et al. 2002). The Roman aqueducts are perhaps the best documented and most renowned. The sophistication of the Romans' irrigation system was a necessity. By 300 BCE, Rome's population had grown to nearly half a million people, an unprecedented number in the ancient world where even large cities' populations rarely exceeded 100,000 people. For the next 500 years, the Romans would make huge advances in engineering, hydrology, and surveying in order to deliver clean water to and remove soiled water from the city of Rome (De Feo et al. 2011; Sedlak 2014).

The Romans surely had some understanding of water quality as demonstrated by their use of turbid waters for agriculture and reservation of clear waters for drinking and bathing (Sedlak 2014). After the fall of the Roman Empire, advances in water technology slowed. Previously, academic opinion was that much of the infrastructure after the collapse of the empire was destroyed, but newer evidence suggests that this was not the case. Evidence from the Middle Ages (5-10 century CE) suggests a patchwork of continuity from the earlier systems, and while some systems were discarded or destroyed, some systems were renewed (Magnusson 2001). Religious organizations of the time possessed much of the knowledge of these water systems, and as monasteries and nunneries expanded, so did their water systems (Bromehead 1942). As towns and cities developed administrations that were able to raise money, hire employees, and had the institutional capacity to develop, finance, administer, and maintain large water projects, they slowly adopted these systems. By the High Middle Ages (11-13 century CE), complex water distribution systems were being adopted and built again throughout Europe (Magnusson 2001).

In 1685, Luc Antonio Porzio published the first illustrated description of a sand filter with multiple filtration steps, including sedimentation and straining (Mays 2013). Of course, sand filtration was not new at the time, and had been reported as early as 2000 BCE (Mays 2013). However, it is around this historical period that society began to make advancements in the area of water treatment and safety. Between 1665 and 1681, Antonie van Leeuwenhoek and Robert Hooke first discovered microscopic life, with Robert Hooke describing the first of the fungi and Anton Von Leuwenhoek describing bacteria, protozoan parasite *Giardia,* and sheep liver fluke *Fasciola hepatica* (Von Leeuwenhoek 1700; Gest 2004; Lane 2015). Two hundred years after Von Leuwenhoek, John Snow documented the first waterborne outbreak associated with a sewage contaminated well in London (Snow 1855). But is was not until Pasteur in 1862 and Koch in 1884 that the development of modern germ theory was established, and all of this knowledge formed the basis for the modern understanding of the importance of faecal bacterial pollution to fresh water and the need for sanitation (Koch 1884; Blevins and Bronze 2010; Berche 2012).

Though we often herald John Snow as the father of epidemiology, it is clear that even in ancient times, people understood that water quality varied, and that some waters could cause illness or death. Ancient Sanskrit writings (circa 2000 BCE) give the first evidence of water treatment. This text, called the Sushruta Samhita, which concerns medical treatment, recommended that "impure water should be purified by being boiled over a fire, or being heated in the Sun, or by dipping a heated iron into it, or it may be

purified by filtration through sand and coarse gravel and then allowed to cool" (Mays 2013).

We also see references to waters of varied quality in folklore and religion. Waters have often been imbued with divine properties, from Hesiod's naiads (nymphs of rivers and lakes), to the various holy wells of Britain and Europe (Hamlin 2000). The powers of these various healing waters varied substantially; some were sites of mystic danger, while others had healing properties. It has been hypothesized that in the Middle Ages, the building of churches near springs or wells was believed to mutually amplify the holiness of these sites (Rattue 1995; Hamlin 2000). Despite the mythos surrounding the wells, this did not preclude them from mundane tasks, and indeed there are legal documents in which monasteries are empowered to divert water from holy wells into towns for domestic use (Rattue 1995; Hamlin 2000; Magnusson 2001). The folklore of water bodies likely also had the effect of differentiating bodies of water in terms of quality and their purpose. An interesting passage from a 12th century guidebook for pilgrims to Compostela in northwest Spain includes a lengthy discussion of safe drinking water sources. In one colorfully written passage, readers are cautioned against trusting local residents regarding safe drinking water. "While we were proceeding towards Santiago, we found two Navarrese seated on its banks and sharpening their knives: they make a habit of skinning the mounts of pilgrims that drink from the water and die. To our questions they answered with a lie saying that the water was indeed safe to drink. Accordingly, we watered our horses in the stream, and had no sooner done so, than two of them died; these men skinned them on the spot." (Melczer 2017) Throughout history, humans have passed down stories to teach

lessons, enforce cultural values, or to caution future generations about hazards. The use of folklore and storytelling to distinguish water of different qualities demonstrates that despite the arbitrary historical events we associate with advances in our scientific understanding of water quality, water quality has been a concern since before the written word, and that communities have passed down these stories to preserve and protect the lives of others.

Nonetheless, as science has advanced, and as water distribution systems have become centralized in large cities, much of the community based knowledge about water quality has too become centralized in the hands of scientists and the regulatory agencies tasked with protecting health.

In the late 19th century, microbiological testing for drinking water was under development (National Research Council 2004). While the primary concerns about drinking water at the time were the presence of *Vibrio cholera* and *Salmonella enterica* serotype Typhi, methods available this time (i.e.: culture based methods) were not capable of detecting these infrequent and low concentration organisms reliably; thus, the concept of the indicator organism was born. Indicator organisms are organisms that may indicate the presence of faecal contamination, and therefore potentially human pathogens could be present (Ashbolt et al. 2001). The indicator organisms used to assess the quality of drinking water at this time were total coliforms (TC) (Smith 1892). Coliforms are in the family of Enterobacteriaceae. The indicator organisms used to assess the quality of drinking water at this time were total coliforms (TC) (Smith 1892). Coliforms are in the family of Enterobacteriaceae, which include the genera *Escherichia, Citrobacter, Klebsiella*,

and *Enterobacter* (Clark and Pagel 1977). The most important member of the coliform group, *Escherichia coli,* is present in high numbers in the feces of humans and other animals (Hofstra and Huis 1988). In the United States, total coliform testing was adopted in 1897, and by 1909, standard methods had been developed to ensure quality of testing for this group of organisms (Wolfe 1972). It was not long after this that total coliform testing became the standard for assessing drinking water quality in the United States; however, complications with the interpretation of this method occurred quickly after its use became widespread (National Research Council 2004). Most troublingly, a number of organisms not of fecal origin can result in a positive total coliform test result (Kabler et al. 1964; National Research Council 2004). Modifications to improve the specificity of total coliform testing resulted in the fecal coliform test, which selects only for fecal coliforms capable of growing at 44.5 °C rather than body temperatures (Kabler et al. 1964; National Research Council 2004). In the last forty years, alternative indicators like *Enterococcus* spp. and *Clostridium perfringens* came into use in drinking and recreational waters, and, in the case of *Enterococcus* testing, have proven useful in assessing the health risks associated with recreational waters (WHO 1984; Wymer et al. 2005; USEPA 2012).

The most pervasive problem in water monitoring is that there are far too many organisms one could monitor for. In recreational water specifically, bacteria represent a single class of biological hazard present in water; however, viruses and parasites are also present and a health risk. Enteric viruses pose a risk in recreational water, and there have been numerous outbreaks associated with noroviruses, adenoviruses, coxsackieviruses, echoviruses, and astroviruses (Sinclair et al. 2009). Additionally, protozoan parasites like

Giardia lamblia and *Cryptosporidium parvum/hominis* cause outbreaks in drinking water as well as recreational water and swimming pools (Betancourt and Rose 2004). We would hope the indicators would be selected in such a way that maximizes the protection of health (whether in the context of drinking or recreational water); more commonly, however, methods are selected based on cost, time requirements, simplicity of testing, and ease of sample processing (National Research Council 2004; Behmel et al. 2016). For these reasons, bacteria methods have dominated monitoring efforts in academia, industry, and government because they are simple to perform, are relatively rapid (only 24-48 hours are required for results), and don't require large volumes of water to process in order to detect the organism of interest (National Research Council 2004). However, as technologies have advanced, molecular techniques like qPCR and metagenomics, which target and detect the DNA or RNA of organisms, have emerged. Such techniques are capable of detecting a variety of organisms (like bacteria, viruses, or protozoa) from a single sample, are rapid, and the cost to do these methods continues to fall. qPCR methods could compliment or replace existing (i.e.: total and fecal coliform testing, microscopic identification of parasites) techniques. Polymerase chain reaction is one such technique, which recently enabled the development of gene targets for specific sewage/animal fecal source identification (with their different human-infectious pathogens) (Li et al. 2019).

Polymerase chain reaction (PCR) is an enzymatic reaction that replicates DNA *in vitro.* This method was discovered in the mid-1980s, and brought together several techniques and discoveries that were emerging simultaneously—notably, the synthesis of

oligonucleotides and the use of these short single stranded DNA fragments to target the production of new DNA copies by employing a thermostable DNA polymerase. What was novel about PCR is the use of a second small oligonucleotide to amplify the region between these two oligonucleotides, and achieving this over concurrent cycles to exponentially amplify the piece of DNA(Saiki et al. 1985; Bartlett and Stirling 2003). Quantitative PCR (qPCR) was developed in the mid-1990s and coupled a fluorescent molecule to the exponential amplification of DNA. The use of a camera to quantify this change in fluorescence allowed for extremely sensitive quantification of the starting amount of DNA within a sample (Higuchi et al. 1993). qPCR methods for the detection of surrogates in water have existed for decades and can be used to detect minute quantities of an organism's DNA in water. This technique is highly sensitive (in theory, capable of detecting a single copy of organismal DNA) as well as very specific for particular regions of DNA(Bartlett and Stirling 2003). Since their inception, PCR and qPCR have been touted as the unifying solution for much diagnostic testing—for water, but also for human diagnostics (Rapley et al. 1992; Bartlett and Stirling 2003; Bustin 2010; Abou Tayoun et al. 2014). However, we have yet to realize this future. While in some fields (notably veterinary and human medicine) PCR testing has become the standard, in the environmental sphere molecular testing operates on the fringes of testing. Nonetheless, qPCR continues to grow in popularity due to its' rapidness, and because qPCR is a platform in so far as testing can be conducted for multiple hazards on the same instrument (or, in some cases, within the same reaction), thus there is an economic argument to be made for incorporating qPCR testing into environmental monitoring

schemes, as the implementation of new tests would cost less once the infrastructure (i.e.: equipment and labor) was established. As more studies utilize the technique trust in the results it generates continues to grow. Research demonstrating the specificity of the technique, and ability to resolve the DNA of a specific species from a complex sample has improved uptake of the technology. Additionally, studies have demonstrated the usefulness of molecular testing in predicting illness.

Notably, the United States Environmental Protection Agency (USEPA) EMPACT study which found that levels of enterococcus as measured by qPCR correlate with risk of human gastrointestinal illness (Wymer et al. 2005). Since then, papers have correlated the amount of human associated *Bacteroides* (i.e. HF183 targets) with human health targets(Boehm et al. 2015; Cao et al. 2018). Screening for toxigenic cyanobacteria species is also moving towards molecular detection method. For example, in Poland, initial screening for microcystin toxin genes in recreational waters is conducted using qPCR, followed by immunochemical analysis to quantify the toxins (Ibelings et al. 2014). In related fields like environmental monitoring, some locales have moved to molecular methods for monitoring for the veliger stage of invasive zebra (*Dreissena polymorpha*) and quagga (*Dreissena rostriformis bugensis*) mussels (De Ventura et al. 2017).

There are numerous advantages to unifying water testing around a single method like qPCR. The most notable is that a variety of targets can be tested for using a single water sample and a single processing method. Thus, fewer samples could be collected and processed, but we could obtain more information from them by performing multiple qPCR tests on the same sample. Currently, qPCR methods function within a traditional

recreational water monitoring program by which samples are collected and transported to a central facility that performs the prescribed testing. Nonetheless there are drawbacks to molecular testing, notably molecular testing is unable to discern viability of an organism, requires trained personnel to perform and understand the methods and their interpretation. Additionally, molecular methods require specialized equipment which can be expensive both to purchase and maintain.

qPCR testing in environmental health could be a particularly powerful methodology if deployed within a community based monitoring framework. Community based monitoring (CBM) is a method of environmental decision making that engages a variety of stakeholders to collaborate to monitor and respond to issues of concern. This methodology could improve efficiency of environmental sampling and qPCR is a technology that could be amendable to CBM. If qPCR testing could be conducted inexpensively and simply in the field by a community partner, industrial partner, or perhaps a local school or camp, more samples could be tested for more targets. It could also reduce costs associated with transporting samples. To my knowledge, qPCR water monitoring has never been deployed in a CBM context. The next section of this literature review will discuss CBM as a method in detail and will discuss the ways in which a qPCR community based monitoring system could function.

1.2 The Evolution of Community Based Monitoring

Amateur observations of biological phenomena have been critical to the progression of science for centuries (Strasser et al. 2018). Neither Anton von Leeuwenhoek

nor Charles Darwin himself were formally trained scientists; however, both left historic contributions to knowledge. In modern times, we refer to these men as "scientists" while simultaneously reserving the word "scientist" for those individuals who have completed a graduate degree (Silvertown 2009).

The divide between professional and amateur scientists is a relatively recent one. Prior to the 19th century, most of the "natural sciences" (i.e.: botany, zoology, geology, ecology) were not performed inside the walls of universities, but instead by amateur scientists. In biology, naturalists (those who, typically for leisure, studied the natural world) had rarely received formal training in the subjects they studied, and mostly earned money from other occupations; biological sciences were a hobby. Science during this Enlightenment period also lacked the divisions it has today; blurred were the lines between work and leisure, research and teaching, or the academy and industry (Road 2001; Vetter 2011).

Many of the Enlightenment naturalists practiced within field clubs and literary and philosophical societies. These societies were formed in the late 18th and early 19th centuries and were populated with men (and some women--though women were rarely given full member status) of diverse socioeconomic and academic backgrounds. The ambition of these field clubs was to promote the sciences to the general population through lecturing and museum collections. These clubs proved to be immensely popular; in 1873, there were over 100 of them in Britain and Ireland alone (Road 2001). Notably, by the time this extensive network of hobbyist naturalists had arisen in Britain, the professionalization of

science was well underway (Golinski 1992). Despite this, these extensive networks of Victorian citizen scientists were considered valuable by the burgeoning academy, which relied upon them for expertise. What's more, partaking in science was considered by society to be a meaningful recreational activity (Strasser et al. 2018). Science and society were inseparable.

Science was a cultural activity during this period, much like visiting a museum or going to a play. This view was due in part to the activities of the Royal Society in London in the early 17th century. During this time, experimentation occurred primarily in secluded laboratories; soon thereafter, though, experiments were converted into public demonstrations. Granted, most of these demonstrations were available only to those of a higher social class, but across Europe, scientific demonstrations also began to take place in the relatively socially accessible spaces of markets or curio shops. It has been argued by historians that the move towards public experimentation by the Royal Society aided in overcoming the suspicion and distrust the public had towards science at this time. As the Royal Society grew, some members took their public experimentation on the road to coffee shops, inns, and across the countryside to burgeoning towns (Golinski 1992; MacLeod 2010).

The 19th century in both Europe and the United States saw the professionalization of science generally, as well as the demarcation of the professional and amateur scientist(Road 2001; Lucier 2010). The rise of experimental sciences (for example, physics, chemistry, and emerging fields like microbiology), was a stark contrast to the natural

sciences, which could be conducted in one's spare time (Road 2001). In short, naturalism was characterized as a less legitimate science. The need for the emerging discipline of biology to legitimize itself prompted its professionalization. Historians have also indicated several institutional factors that prompted this professionalization—most notably, a scarcity of professional biology positions at colleges and universities of the time. Compared to professorships in the physical sciences, biological positions were few and far between. Those with these coveted positions had a vested interest in maintaining their own jobs and creating more positions like theirs. The prolific nature of amateur biologists at the time became a threat (even the vast majority of "professional" biologists at the time got their starts as amateurs working within various societies) because it reinforced the idea that anyone could do biology while decreasing the need for formal professorships. This prompted many "professional" biologists to actively, vehemently discredit and delegitimize the amateur societies they were brought up in (Road 2001). The 20th century saw a shift to the culture of science today—one where the divide between professional and layperson has arguably increased further than ever before (Vetter 2011). Ironically, the scientific community now calls for science promotion, and advocates for the popularization, and increased communication of science to lay audiences. We have come full circle as now, after two centuries of work removing science as a cultural activity, we call for a return to the Victorian spectacle of science and public experimentation, and a reconsideration of the usefulness of the Enlightenment era networks of citizen naturalists (Strasser et al. 2018).

In the last 10 years, there has been a new enthusiasm for research to include laypeople. Collectively, academia is working towards popularizing its work, as well as focusing on knowledge translation and communication to the public (Dickinson et al. 2012; Bowen and Graham 2013). In biological fields, this has included a push towards citizen science. Citizen science describes a methodology of conducting large scale research by recruiting volunteers to collect samples or record observations of particular flora and fauna (Conrad and Hilchey 2011b; Dickinson et al. 2012). Notable examples include the North American Breeding Bird Survey (BBS), which recruits thousands of volunteers each year to follow a rigorous protocol to monitor the status of bird populations in North America (Pardieck, K.L., D.J. Ziolkowski Jr. 2018). It is a coordinated effort by the United States Geological Survey, the Canadian Wildlife Survey, researchers, and statisticians. The highly successful Zooniverse program (zooniverse.org), which includes initiatives like PlanetHunter and GalaxyZoo, is a citizen science project in astronomy that brings together participants online to identify a variety of solar objects (Simpson et al. 2014).

In citizen science, volunteers are directly involved in scientific investigation; mostly, they act as sample collectors or provide basic identification of particular phenomena (Silvertown 2009). There has also been an epistemological push in the field to move away from a paradigm of "using citizens to do science" and towards an equal power relationship which views citizens as scientists, thus embracing some of the ideals of participatory action research (Lakshminarayanan 2007; Buyx et al. 2017). Participatory action research (PAR) is a method in public health research that prioritizes the co-

creation of knowledge by both the researcher and participant. It also differs from traditional research paradigms in that it is designed to enable action. Action in a PAR study is achieved through a reflective cycle in which all knowledge creators analyze data, reflect, and then act upon it. Perhaps the most important part of PAR is that it affirms the idea that experience can be a way of knowing, and that experience can lead to legitimate knowledge that can influence practice (Viswanathan et al. 2004; Baum et al. 2006a).

As citizen science has continued to espouse the ideals of PAR, there has been an effort to rename citizen science as *community science*, a more inclusive term (Conrad and Hilchey 2011a). As this field has continued to evolve, different models of implementing these projects have emerged. The examples listed above (the North American Bird Brood Survey and Zooniverse) are two large scale citizen science projects that characterize the type of project people think of when they think of citizen science. This type of project relies on a large number of volunteers interacting largely online to accomplish their projects. Not all community science projects take on this model. Community based monitoring (CBM) is a subset of community science that is a process of collaboration between government, industry, academia, and local community groups to monitor, track, and respond to issues (Conrad and Hilchey 2011a; Serrano et al. 2013; Obama Administration 2015). CBM espouses more of the PAR ideals than larger scale community science projects. It is action oriented, prioritizes partners as co-creators of knowledge, and does not rely on volunteers merely as data collectors, but tries to bring about transformative change by supporting development, implementation, and assessment of projects (Conrad and Hilchey 2011a). For these reasons, it is poised to improve

environmental decision making. CBM improves scientific literacy, builds social capital, improves participation in local issues, and benefits the environment (Trumbull et al. 2000; Whitelaw et al. 2003).

CBM is a necessary methodology for the future of environmental decision making as many of the questions facing society today revolve around complex environmental issues of climate change and water scarcity. Alvin Weinberg (1975) noted that there are questions we can ask of science that cannot be answered by science. He coined a name for such questions, "trans-science", meant to suggest that these questions transcend the questions that can be answered with the scientific method (Weinberg 1972). Carolan (2006) asks the reader to ponder the term "ecosystem health" and "biodiversity". On the surface, he argues, these are scientific facts, but upon further consideration, these are actually value statements. They represent what we think a particular environment should be. Even the term "health" is subjective and defined by personal values. "Health" is not an intrinsic property of an ecosystem; it is a condition we prescribe to an ecosystem that functions the way we judge that it should. Questions of ecosystem health or public safety, while framed scientifically, are indeed value judgments (Carolan 2006). They are questions of policy, not questions of science. Weinberg's contemporaries have elaborated this further, arguing that uncertain and complex trans-scientific problems require democratization because science and scientists alone cannot answer them (Funtowicz and Ravetz 1992; Carolan 2006). This is why the future of environmental decision making must involve some process of collaboration between public stakeholders, industry, government, and academia.

Broadly speaking, environmental sciences are gradually beginning to embrace the ideals of citizen participation. There is the general belief that CBM programs build social capital, educate the public, and are fundamentally useful in enabling data collection and analysis on a large geographical scale (Trumbull et al. 2000; Dickinson et al. 2012). However, some scientists view CBM programs skeptically. Questions of data quality and integrity are the most persistent issue (Dickinson et al. 2010). Some studies have demonstrated issues with data integrity in community science projects. Data may be fragmented, or it may be inaccurate. There has also been a spurious lack of negative data reported by citizen scientists (Conrad and Hilchey 2011a). However, other studies have shown that with proper training, volunteer partners can generate accurate data (Gardiner et al. 2012).

Despite the challenges described above, CBM programs continue to grow and expand. In the United States, as of 2014, there were 1,720 community groups monitoring water alone. Closer to home, in Alberta, the Alberta Lake Management Society's LakeWatch program has been recruiting and empowering volunteers to monitor their lakes since 1996; it has monitored water quality at 116 individual lakes since the program's inception (ALMS 2019; National Water Quality Monitoring Council 2019).

Community based monitoring is a natural fit for recreational water monitoring for several reasons. A single water body likely has a variety of stakeholders, each with different interests in what happens at that site. This likely includes residents of summer villages, private citizens who use the lake for recreation activities like fishing, government (as many lakes have provincial parks on their shores), and local industry. CBM brings

these groups together to work on projects. As described above, community monitoring projects are useful as they can garner large amounts of data across a wide geographic area. The decentralized nature of CBM projects is highly amendable to recreational water monitoring as there can be a large number of sites or lakes that need to be monitored, and often many of these simply cannot be sampled by a centralized regulatory authority due to time and budgetary constraints (Tulloch et al. 2013; Bardar 2019). Similarly, CBM programs can be cost effective; however, it should be noted that volunteers are not *free* labor—financial and human resources are required to train volunteers and maintain these programs (Jacobson et al. 2012). Most CBM programs focused on water monitoring call on volunteers to conduct simple physicochemical measurements on their lakes. Techniques employed include Secchi depths and water chemistry measured by probes (for instance, dissolved oxygen, conductivity, or temperature), but also can include some biological monitoring like zooplankton collection or invasive species monitoring based on a visual identification of organism. While simple methods like these provide much needed information about a lake's health, monitoring methods conducted by governments or researchers have advanced beyond them. Furthermore, these methods are not capable of detecting microscopic organisms that may be hazardous in water.

As the effectiveness of qPCR diagnostic tests continues to be realized, it is apparent that qPCR is an excellent choice for CBM, or, more broadly, a decentralized monitoring system. qPCR is a platform, and with the infrastructure in place, monitoring for additional targets becomes a matter of designing/validating a new test and running it on the established infrastructure (Abou Tayoun et al. 2014; Wood et al. 2019) .

The idea of portable diagnostic technologies that feed information into a surveillance system is attractive for multiple reasons, but the development to implementation gap is often wider than we would expect; this is why we do not see such systems in place. With regards to qPCR, developing simple techniques for a wide range of users and training these users is not within the purview of the research and development divisions of many companies. It may be difficult to engage and maintain partnerships with organizations and individuals. Additionally, these groups may have competing interests or have different expectations, so a program might need to be somewhat tailored for an end user. Additionally, developing sensitive and specific diagnostic tests for these organisms is time consuming, and requires a specialized skillset (Abou Tayoun et al. 2014; Wood et al. 2019). In short, the real world is a messy place, and theorizing about how a system could function doesn't always translate to reality.

This thesis crosses this development to implementation gap by implementing a CBM qPCR monitoring network for recreational water. The first aim of the thesis was to develop qPCR methods to monitor for various microbial hazards that could be conducted by a variety of users outside of the laboratory. The second aim was to deploy these methods amongst a variety of partners and test their sensitivity, accuracy, and feasibility. The last aim of the thesis was to work with community partners using the CBM qPCR approach to answer monitoring questions they had.

These aims were achieved first by consulting with stakeholders and potential CBM partners in Alberta, Canada, and Michigan, USA, to determine what types of water

monitoring questions these groups had and what kinds of organisms they would be interested in testing for. Next, I developed and vetted a variety of field methods, including how to perform DNA extraction with minimal equipment and a comparison of three field-amendable thermocyclers. qPCR methods were developed or adapted to test for a variety of organisms. Most method development focused on developing sampling methods and qPCR assays for small eukaryotic organisms (including parasitic avian schistosomes and larval invasive mussels in water), a discipline that was wanting for new molecular methods. Existing methods to detect bacterial hazards were also adapted for the field system. CBM partners were trained in these methods and conducted their own monitoring projects. The accuracy and feasibility of the CBM qPCR program was evaluated quantitatively by comparing CBM partner-run samples to expert-run samples, and qualitatively through a long form survey distributed to partners to assess the goodness of fit between the technology and program and their needs. Chapter 2 of this thesis describes the results of the community based monitoring study, and discusses the accuracy of the CBM qPCR method. Chapters 3 and 4 provide case studies into the scientific and applied research questions that we answered using the CBM qPCR method. Chapter 3 focuses on swimmer's itch monitoring in Michigan, USA, while Chapter 4 discusses a variety of bacterial monitoring projects that took place in Alberta utilizing this framework. Appendix 1 contains another, albeit smaller case study utilizing this framework to conduct invasive species monitoring in Alberta. The thesis concludes in Chapter five with an overall discussion of CBM qPCR as a research framework. Methods used in this thesis are located in Chapter 6.

Chapter 2: Democratizing water monitoring: Implementation of a community-based qPCR-monitoring program for recreational water hazards.

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2.1 Introduction

Community based monitoring (formerly referred to as citizen science) is now routinely used for conservation and environmental monitoring. Citizen science describes both a methodology of conducting large-scale research by recruiting volunteers, and refers to the process by which citizens are involved in scientific investigation as researchers. Citizen science can include community based monitoring (CBM) as a process of collaboration between government, industry, academia, and local community groups to monitor, track, and respond to issues (Conrad and Hilchey 2011a; Serrano et al. 2013; Obama Administration 2015).

The earliest incarnations of citizen science and CBM relied on volunteers as data collectors, but the discipline of CBM has grown and evolved. Recent arguments in favor of CBM suggest the field move away from a paradigm of "using citizens to do science" to an equal power relationship which views citizens as scientists, embracing some of the ideals of participatory action research (Lakshminarayanan 2007).

Traditional CBM programs have typically relied on volunteers to conduct biodiversity surveys, do simple tests (i.e. Secchi disk tests for assessing water clarity), or to collect specimens and send them to central facilities for analysis. However, modern monitoring methods conducted in academia, industry, and government have evolved considerably to include large scale spatial assessment methods: for example: algal/cyanobacteria bloom-tracking satellites, and next generation sequencing analysis. CBM programs also must evolve and advance as new technologies become available. In water monitoring especially, quantitative polymerase chain reaction (qPCR) has emerged as the method of choice for conducting routine compliance monitoring of water bodies (Wade et al. 2006). CBM is poised to improve environmental decision making. Its use has been on the rise due to budgetary constraints in both government and academia, but also because CBM can be a powerful methodology for generating large spatial or temporal datasets for monitoring/surveillance purposes. CBM improves scientific literacy, builds social capital, improves participation in local issues and benefits the environment (Trumbull et al. 2000; Whitelaw et al. 2003).

Quantitative PCR methods for the detection of surrogates in water have existed for decades and can be used to detect minute quantities of an organisms' DNA in a complex matrix such as water, soil, or blood. qPCR is highly sensitive (in theory, capable of detecting a single copy of organismal DNA) and is very specific for particular regions of DNA. In the last decade, agencies responsible for monitoring the environment and health have begun to capitalize on the potential of qPCR. Some of the greatest strides have been made in health, especially after the USEPA EMPACT study, which found that levels of enterococcus as measured by qPCR correlate with risk of human gastrointestinal illness (Wymer et al. 2005). Since then, progress has been made in correlating the amount of human associated *Bacteroides* with human health targets (Boehm et al. 2015; Cao et al. 2018). Screening for toxigenic cyanobacteria species is also moving towards molecular detection methods. For example, in Poland, initial screening for toxin genes in recreational waters is conducted using qPCR, followed by immunochemical analysis to quantify the toxins (Ibelings et al. 2014). In related fields like environmental monitoring

some locales have moved to molecular methods for monitoring for the veliger stage of invasive zebra (*Dreissena polymorpha*) and quagga (*Dreissena rostriformis bugensis*) mussels.

As the effectiveness of qPCR diagnostic tests continues to be realized, it is apparent qPCR is an excellent choice for CBM, or more broadly, a decentralized monitoring system. qPCR is a platform, and with the infrastructure in place, monitoring for additional targets becomes a matter of designing/validating a new test and performing it on the established infrastructure. For this reason qPCR and related molecular techniques have been touted as grand solutions for point of care diagnostics in infectious disease monitoring (Abou Tayoun et al. 2014; Wood et al. 2019)

The idea of portable diagnostic technologies which feed information into a surveillance system is attractive for several reasons, but the development to implementation gap is often wider than one would expect. The qPCR CBM described in this chapter narrows this gap by focusing partially on the training, and routine confirmation of their performance of partners to ensure quality data. This chapter also utilizes a participatory action research methodology, which is a methodology that expands the construct of "expertise" beyond the researcher to include the studies participants (Baum et al. 2006b; Bywater 2014). In this way, researchers work in collaboration with partners as co-researchers to investigate problems defined by the partners, which I believe to be central to a successful and accurate CBM program. A portable qPCR method embedded within a strong CBM program that balances its own needs with the needs of volunteer partners could prove to be a strong and beneficial environmental monitoring method. This study is, to my knowledge, the first of its kind to test the rigor of qPCR for detection/quantification of biological hazards and their surrogates in water through a CBM implementation study.

In this chapter I test the feasibility, reproducibility and reliability of implementing a CBM qPCR water monitoring program amongst a variety of groups (government, NGO, and private enterprise). This chapter of my thesis aims to provide evidence of the

robustness and value of a CBM qPCR approach for water monitoring: here, I will present the results of the implementation. Details on any tests developed and validated as part of my thesis, and details about the projects completed during these implementations are located in the chapters that follow this one. The purpose for disconnecting the CBM implementation from specific test development serves to emphasize the universality of this method, and allow for targeted assessment of CBM qPCR independent of the information that has been generated as a result of the monitoring programs. In this chapter I will broadly discuss the reproducibility of the CBM qPCR method, the field equipment and its validated, and how users perceived our program while the details of each individual projects detailed in the proceeding chapters will serve as case studies in CBM qPCR programs.

This CBM project began in 2016 when I partnered with a contractor company in Michigan that was interested in utilizing qPCR to measure swimmer's itch causing cercaria (the larval, environmental stage of trematodes responsible for aberrant penetration of human skin resulting in swimmer's itch) in water samples. In this year our CBM partners didn't run qPCR in the field, but did collect and filter water samples, and then sent them to me for DNA extraction and analysis. In 2017, I recruited additional partners, Alberta Health Serves (AHS) and the Alberta Lake Management Society (ALMS), and these groups in addition to our Michigan based CBM partners performed the qPCR testing method for avian schistosomes. At the request of AHS and ALMS, I also offered testing for the microcystin gene of cyanobacteria using a test developed by Lily Pang's group of the Provincial Laboratory of Public Health (now Alberta Public Labs) (Qiu et al. 2013). Additionally, I began the development and validation of a qPCR diagnostic test for zebra mussels in collaboration with local consulting company, Aquality, and Alberta Environment and Parks (AEP). In 2017, paralleling the CBM implementation I conducted in Michigan, Aquality collected water samples for zebra mussel testing, and I performed DNA extraction and qPCR analysis on these the results of this study are in Appendix I.

In 2018, AHS dropped out of the study, and I added the City of Edmonton as a partner, as a new natural swimming pool had opened at Borden Park, and the facility manager was interested in performing additional testing on top of what Alberta Health Services was conducting weekly. They requested the ability to perform their own testing for P*seudomonas* and human *Bacteroides* and I obliged. Additionally, in 2018 our satellite lab in Michigan expanded, they outfitted themselves with centrifuges and heat blocks and all the equipment required to perform a kit-based DNA extraction. They added sewage marker HF183 monitoring to their program, and ALMS continued with toxic cyanobacteria monitoring targeting the microcystin toxin gene.

In 2018 Aquality performed their own qPCR testing on water samples from reservoirs in southern Alberta. In 2019, the Michigan group began *Enterococcus* testing via USEPA Method 1611, and ALMS began using a qPCR test for total cyanobacteria (targeting the 16S gene), in addition to their microcystin testing, and the City of Edmonton all increased the number of samples they collected and analyzed.

2.2 Results

2.2.1 Determination of field-appropriate qPCR thermocyclers and master mix

The details of the field-testing method are found in section 6.1.1 of the Methods chapter; however, briefly I chose the ChaiBio Open qPCR thermocycler because of its capability to perform 16 samples at a time, which enables the use of a short standard curve and negative controls, with the ability to run multiple samples. It is less portable, but CBM partners did not express a desire to perform qPCR in the field, and preferred to take samples back to an office or central location for testing. The Biomeme™ field DNA extraction kit was selected for DNA extraction because it was fast, user friendly, and required no centrifugation or heating steps. The IDT DNA qPCR master mix was chosen for thermocycling as it is light and temperature stable.

2.2.2 Implementation groups

The CBM partners recruited included a consulting firm in Michigan USA comprised of a retired high school science teacher, his daughter, a high school biology teacher, and her husband, a high school gym teacher. In my first study year I recruited two members of the Alberta Heath Services recreational water monitoring program, the Alberta Lake Management Society, and Aquality, a consulting firm specializing mostly in environmental restoration. The Facility Manager of the Borden Park Natural Swimming Pool also participated in 2018 and 2019 (Table 1)

2.2.3 Organisms selected for testing

I employed a participatory research methodology for this project whereby I worked with stakeholders (CBM partners) to discuss their needs and goals and worked with them to develop either monitoring or research questions. This resulted in CBM qPCR deployment of assays to detect avian schistosome cercariae, invasive zebra mussel larvae (*Dreissena polymorpha*), human *Bacteroides* (HF183 marker), enterococcus testing, *Pseudomonas aeruginosa,* microcystin E gene testing for toxic cyanobacteria species, and total cyanobacteria species.

2.2.4 Detection limits of the Open qPCR thermocyclers

The limit of detection 95 (LOD_{95}) of the Open qPCR thermocyclers is 63.4 gene copies (GC)/5uL (lower limit 43.7 GC/5uL, upper limit 89.2 GC/5uL, n= 40, based on all qPCR tests). This is approximately 1-log higher than the same assays (Avian schistosomes $LOD_{95:}$ 3.4 GC/5uL; Toxic cyanobacteria LOD_{95}: 6.25 GC/5uL ; HF183 LOD_{95}: 7.2 GC/5uL; *D. polymorpha* LOD95: 4 GC/5uL; *P. aeruginosa* LOD95: 6 GC/5uL ; Enterrococcus spp. LOD95: 3 GC/5uL, Total cyanobacteria LOD95: 4 GC/5uL (Table 5.2). performed using our laboratory ABI 7500/QuantStudio 3 thermocycler.

2.2.5 Comparison of Control Standards

A comparison of 10 independent standard curves performed in the Open qPCR and the Core thermocyclers (ABI 7500, or the QuantStudio 3) was undertaken. The average cycle thresholds for each assay were calculated with their standard deviations and from these values the correlation coefficient, slope of the standard curve, and efficiency of the reaction were calculated. Ideal standard curves have an efficiency of between 0.98 and 0.99, a slope of -3.32, and an efficiency of 100%, which can also be represented as an amplification factor of 2, suggesting product has doubled every cycle. (Table 1)

2.2.6 Comparison between machines

Interclass correlation coefficients (ICC) were calculated to compare CBM partner DNA extracts run on the Chaibio Open qPCR machine, and our laboratory ABI 7500/QuantStudio 3. High levels of agreement would suggest that the different thermocyclers are capable of collecting similar results. Results are shown in Table 2. The avian schistosomes assay consistently has the highest ICC coefficient, suggesting high levels of agreement. Toxic and total cyanobacteria tests have similarly high ICC coefficients. In 2019 the total pseudomonas qPCR assay showed lower ICC's than expected (Table 3).

2.2.7 Semi-quantitative analysis using Bland-Altman plots

Reproducibility was assessed using the semi-quantitative Bland-Altman plot. Bland-Altman plots graph the average of two measurements on the x-axis and the difference between these measurements on the y-axis. The Bland-Altman plot for avian schistosomes monitoring for 2017 and 2018 show a linear pattern at lower copy numbers, but at higher copy numbers show uniform variability (Figure 2.2). Bland-Altman analysis of the toxic cyanobacteria test shows uniform variability within the limits of agreement (2 times the standard deviation).

2.2.8 Interclass Correlation Analysis

ICC analysis was also performed to compare user and lab samples; this information is also shown in Table 1 The ICC for the avian schistosome assay in 2017 and 2018 was

similarly high, as was the level of agreement between the toxic cyanobacteria test. Interestingly, in 2019 when the total cyanobacteria test was deployed alongside the toxic cyanobacteria test, the ICC for total cyanobacteria was low, despite the fact that these tests were conducted by the same users performed on the same sample extracts. In 2019 I also deployed *Enterococcus* testing via USEPA Method 1611 DNA extraction. Agreement on this test was low (Table 2).

2.2.9 Inhibition controls

PCR Inhibition was tested on partners DNA extractions and on DNA extractions performed in house. Between 5-8% of samples were slightly inhibited in both partner and in house extractions. Cyanobacteria samples were most likely to be inhibited. Inhibited samples were excluded from analyses.

2.2.10 User Perceptions

User perceptions of the program were captured through a written survey that was administered to participants. A total of 6 respondents filled out the survey. The questions are available in Table 4. Thirty-three percent (33%) of respondents stated that they had some prior knowledge of molecular biology, PCR (polymerase chain reaction), eDNA, or DNA based detection in general prior to the use of the qPCR field method. Fifty percent (50%) reported having low prior knowledge and one participant had no prior knowledge. The same 33% of respondents who reported some knowledge with molecular biology and methods also reported having performed some form of PCR in the past. The rest of respondents reported not having performed PCR (50%) and one participant did not remember.

2.2.11 Thematic analysis

User surveys underwent deductive thematic analysis whereby surveys were coded, and then codes were organized into themes (Braun and Clarke 2006). The codes were identified and relevant excerpts from the surveys are presented in Table 2.6. The first theme identified is “Rapidly responding to hazards”. This theme captured the CBM

partners' perceptions on the speed of the qPCR method and their perceived ability to respond to issues quickly. The second theme identified was the question of who controls the CBM monitoring system. This theme emerged from CBM partners expressing a desire for independence and control over the interpretation of results. The third theme identified was that the triangulation of training was valuable in that most CBM partners suggested that the written and video protocols (complemented with a few in person training sessions) were important to them and enhanced their learning. A subtheme that emerged from this theme was "learning and communication".

2.3 Discussion

2.3.1 Framework for the CBM program

In this study, the accuracy of a community based qPCR-monitoring system was assessed. I assessed the accuracy and reproducibility of the portable qPCR machines relative to a "core" machine and the ability for CBM partners to execute the method. Our analyses have demonstrated that a CBM qPCR monitoring program can yield accurate results for different targets (i.e.: eukaryotic versus prokaryotic); however, if the method itself is too time consuming or challenging to be completed by relatively novice CBM partners, a larger scale implementation of the CBM monitoring program could be less reliable.

My intention was to implement a CBM qPCR system in a real world context. As Figure 2.1 details, I began the development of this project by consulting with local stakeholder groups and assessing their interest in the project and what types of biological hazards and surrogates they might be interested in monitoring for. The goal was to have partners run a sufficient number of tests, not to prescribe a particular test for CBM partners to run. Therefore, I adapted to the needs of our CBM partners and adapted a variety of existing qPCR tests to the field equipment and testing protocol. Additionally, some of the groups I worked with had their own scientific questions they wanted to answer, so I facilitated this.

The selection of appropriate CBM qPCR materials was informed in part by what was available and what equipment seemed most appropriate. The Open qPCR system was selected as it performed consistently, and as none of our groups had a desire to perform qPCR at the site of collection and all opted to transport samples back to their respective locations to conduct the testing, it fit the function they were going to use it for. Additionally the 16 well system left room for controls and a reasonable number of samples.

I distributed all materials required to complete testing to users, and I prepared all qPCR master mix components (enzyme mix, primers and probes), and aliquoted these into individual reaction tubes for users. The purpose of this was twofold: to prevent contamination of CBM partners' qPCR reactions, and for simplicity for the partners. Our laboratory facilities are equipped with a PCR clean room, and we have separate pre and post amplification rooms. By preparing reaction tubes and controls, I could prevent CBM partners handling high copy number controls (a likely source of contamination). Additionally, CBM partners were instructed not to open tubes that had undergone qPCR. The Biomeme™ DNA extraction does not utilize pipettes, but all users were supplied with filter tipped 20uL pipettes to add their purified DNA into their reaction tubes. Preparing reaction tubes made running qPCR as simple as adding the DNA and selecting "start" on the Open qPCR machine.

Analysis of the qPCR data was also performed by myself. CBM partners would download their spreadsheets from the Open qPCR and send them (either via email or Google Drive) to the lab, where I would analyze control data, and calculate copy numbers and, where possible, organismal numbers for partners. Again, this was done in attempt to preserve the simplicity of the method, and because analysis of qPCR data is complex and requires an expert eye.

2.3.2 Assessing the reproducibility of the CBM qPCR program

The LOD_{95} is the lowest concentration of DNA that can be reliably detected in 95% of samples. The Open qPCR thermocycler has a higher limit of detection when using a Taqman fluorescein probe than our ABI core thermocyclers (63.4 DNA copies/5uL versus >10 DNA copies/5uL across all methods). The field thermocyclers are less sensitive than the core laboratory machine. Understanding this change in detection limit is important to determining if the CBM qPCR system would be effective for a particular test. For instance, if the concentration of the target that might constitute a risk is below the LOD_{95} for the Open qPCR thermocyclers, "risky" samples will appear negative as the thermocycler is not capable of detecting them.

For example, in 2018 I deployed the human associated *Bacteroides* HF183 CBM testing for recreational shoreline source-tracking in Michigan, USA; the CBM partners reported only a single positive sample. However, when these DNA extracts were analyzed, 22.7% (54/237), were found to be positive for between 15-35 copies DNA/5ul. Seven (0.07%) of these samples approached the LOD of the Open qPCR thermocycler, and CBM partners detected one of these samples. A recent study found that a HF183 gene copy number of 4200 HF183/100ml exceeds the USEPA benchmark risk of GI illness (Boehm et al. 2015). This level is equivalent to a gene copy number of 210 HF183 GC/5ul—well above the detection limit of the Open qPCR thermocycler. Thus, outbreak scenarios would be clearly discernable. However, this also illustrates an example of how the monitoring project must be clearly rooted in a management outcome. If the intention of the monitoring program is to detect potential outbreak scenarios and initiate action, the increased detection limit is acceptable; yet, if the management context is detection of leaking septic areas or source-tracking fecal markers on a beach, this detection limit may be inappropriate to answer such questions. This example highlights the importance of working closely with CBM partners to understand their specific monitoring questions, as well as critically appraising and assessing if CBM qPCR is capable and appropriate to answer these questions.

I prepared users' standard curves. Having users prepare these would be both time consuming and a potential source of contamination of their reactions. CBM partners were instructed never to open the standards, which contained a 5000 copy standard, a 500 copy standard, and a 50 copy standard and a no template control. Standard curves performed by partners and in my laboratory agreed closely, suggesting that users were capable of placing reaction tubes in the thermocycler and operating the thermocyclers (Table 2.1)

Interclass correlation coefficients (ICC) were calculated to assess agreement between partner and expert samples. ICC is a dimensionless quantity that ranges from zero to one; it is the proportion of real information captured by the measurement. A value of zero means that all of the variability in measurements is due to random variation, while a value of one means that the method captures 100% of the variability of the construct being measured.(Bartlett and Frost 2008) ICC analysis for the avian trematode assays showed high level of agreement, with ICCs between 0.57 and 0.9, between the Open qPCR thermocycler and the core thermocyclers (Table 2.1), and maximum log differences between 0.44 and 1.5. I can expect highly reproducible results between the core machines and the field units. This also suggests that partners can successfully dispense purified DNA into the reaction tubes and operate the thermocycler (Table 2.2).

In 2018 the toxic cyanobacteria test showed lower levels of agreement between the field thermocycler and the lab thermocycler. and the confidence interval surrounding the ICC widened. I discovered through analyzing the control standards that the heated lid on the field thermocycler was loose, and therefore was failing to engage properly with the tops of the reaction tubes (i.e. machine failure). However, from a quality control perspective, the fact that I was able to detect a probable machine failure with a sample size comparison of merely 12 is extremely promising for future larger scale CBM qPCR systems. It suggests that it would be possible with a relative low number of samples being confirmed by a core facility or quality control partner to detect user or machine error once a baseline level of agreement for a single test had been established.

The comparison between CBM partners performing DNA extraction and myself

performing the DNA extraction was first assessed semi-quantitatively using the Bland-Altman plot. The results of this analysis for the almost all targets show a linear and negative linear pattern at lower gene copy numbers. This can be due to bias between methods, but can also be caused by a difference in the within-subject standard deviation (Bartlett and Frost 2008). This seems plausible as users with potentially very different skill levels are performing the two methods. A paired t-test using the log-transformed data was used to compare the within-subject standard deviations. They were significantly different based on an F-test and Welch's t-test ($p < 0.0001$, $F=6288$, mean difference ± SEM: 20326 ± 9843), suggesting that the linear pattern observed is due to an increased variability in CBM partner data.

Partner extracted samples are typically lower in copy number than expert extracted samples. This is likely due to differences in DNA extraction efficiency between the CBM partners and myself. However, it seems more experienced users become better at DNA extraction over time as both the avian schistosomes monitoring group and the toxic cyanobacteria monitoring groups seem to improve over time. This has important implications for the CBM program. Notably, an experienced CBM partner may require less oversight from the QC program, allowing the manager of the program to focus more efforts on new CBM partners (Figure 2.2).

I hypothesized that the ICCs and maximum log differences would be higher when comparing partner and expert extracted DNA samples due to the highly variable nature of DNA extraction, and because the duplicate samples I ran could never be expected to contain exactly the same amount of organism. The ICCs of the DNA extraction comparison ranged from 0.54 to 0.67, with maximum log differences ranging from 1.3 to 1.4 (Table 2.2). It is important to note that for the avian schistosomes monitoring program, a change was made in 2018 to establish a full functional remote laboratory, and move these partners onto using the Qiagen DNAeasy™ DNA extraction kit. This change was made at the request of the CBM partners, who would typically collect and analyze

hundreds of samples each field season. Details about the equipment in this satellite laboratory can be found in Table 2.5.

Ebentier et al. (2013) conducted a reproducibility analysis of five core laboratories on a panel of microbial source-tracking qPCR markers. They calculated reproducibility as the maximum expected log difference (within 95% confidence) between the different laboratories. Their analysis demonstrated reproducibility coefficients for different qPCR assays were highly variable—between 0.09-0.66 logs. The methods that were likely to produce higher copy numbers, like *Enterococcus* qPCR testing via USEPA Method 1611, showed higher reproducibility coefficients than methods that were likely to produce lower copy numbers, like human associated *Bacteroides* marker HF183. They also analyzed the contribution to variability of a variety of factors (the sample itself, equipment, procedures) to the measurement. Their paper concluded that when protocols and reagents were not standardized, agreement between methods decreased. They highlighted the need for standardization of protocols and consumables before implementation of studies involving multilaboratory experiments (Shanks et al. 2012; Ebentier et al. 2013).

The maximum log difference of the CBM qPCR monitoring program higher than the values reported in the Ebentier et al. (2013) paper. Reproducibility between the same extract performed by myself and the CBM partners ranged from 0.44 to 1.5 log, and reproducibility coefficients of between partner and expert extracted split samples ranged from 1.3 to 1.4 log. It should be noted that the majority of the qPCR methods deployed routinely detected copy numbers in excess of 1 log, thus we might expect higher variability between replicates at these larger copy numbers (Figure 2.2). CBM qPCR monitoring programs will likely generate data that does have higher variability. It's important to weigh the pros of a CBM qPCR approach, notably that a CBM qPCR approach may result in increased numbers of samples from across a larger geographic area, while building relationships and partnerships across sectors.

2.3.3. Troubleshooting and improving the CBM qPCR program

In 2019 at the request of CBM partners, I validated two more tests: a total (16S rDNA targeting) cyanobacteria qPCR test, and a total (16s rDNA targeting) *Pseudomonas* qPCR test. The validation of these tests is detailed in chapter 4. However, pertinent to this chapter, these tests have notably lower ICCs for differing DNA extracts (Table 2.3).

In 2019, ICC between same sample extracts of the total *Pseudomonas* test was very low. After working with our CBM partner on this test and doing an in-person re-training session I ascertained that the partner wasn't dispensing the purified DNA into the enzyme mixture and instead was dispensing the 5uL of DNA extract onto the side of the reaction tube and not ensuring the DNA extract made it into the enzyme mixture prior to thermocycling. This issue is also reflected in the DNA extract comparison; however, it is important to note the high confidence interval surrounding this ICC. Once the problem was realized, our CBM partner took steps to improve her ability to do the method (Figure 2.2).

The data surrounding the total cyanobacteria assay is somewhat more complex. In 2019 the group performing the total cyanobacteria assay, as well as the microcystin assay had hired two interns to conduct the qPCR monitoring program. While I conducted training with the interns, it was difficult to engage them properly as they worked unpredictable hours. While I make a point to stop by and visit our CBM partners regularly during the summer, checking in, picking and dropping off samples and supplies, and generally staying apprised of what's happening with the various projects and I often found myself communicating with the interns through the ALMS staff indirectly. There was also a large gap between when samples were collected and when the partners ran them. I received samples for testing from July and August at the beginning of September, while the partners didn't perform analysis on these samples until October.

The ongoing challenge of a CBM program, especially one that offers a multitude of testing options is continuously engaging, training, and ensuring compliance with methods

across a wide variety of users. The data suggest that CBM partners do improve over time as exemplified by the avian schistosomes monitoring program. However, this success stems from establishing strong relationships with partners, training and retraining as required to ensure data quality is high, and perhaps most importantly having passionate partners who care about the data and always want to learn more. Thus, CBM programs may require more hand-on mentorship and quality control initially, with less oversight being required as partners become more experienced

That isn't to say that there is not a continued challenge to embarking on a new method even with experienced CBM partners. In 2019 we also rolled out *Enterococcus* USEPA method 1611 testing with our experience Michigan CBM partners. This method involved learning a new (but arguably simpler) DNA extraction protocol. ICC of qPCR analysis performed by partners and by me showed reasonable levels of agreement; however, these levels were lower than for our other qPCR tests (Table 2.2). I had anticipated a slight decrease in ICC and a high maximum log difference between these samples because DNA extracts processed via Method 1611 don't undergo steps to remove proteases or nucleases, this method of DNA extraction relies on bead beating to lyse bacterial cells. When I extracted frozen filters from partners I was very surprised by the dramatic drop in ICC and increase in maximum log difference between the data sets. I suspect this extreme difference is likely due to changes in extraction efficiency between the field users bead beating apparatus and our lab bead beating apparatus, and the focus of future work will be assessing DNA extraction efficiencies between field and core laboratories.

However, none of these projects were totally in vain. As CBM partners collect duplicate samples that I am able to analyze separately, even when a method breaks down in the field, the information from the samples collected and filtered is not lost and these samples can always be assessed in the core laboratory environment. The results of the *Pseudomonas*, cyanobacteria, and *Enterococcus* monitoring projects are described in Chapter 3. Even though I performed sample analysis in our core lab, the knowledge

generated for partners by these projects is extremely valuable, moreover the samples themselves would not exist without a CBM program having guided their collection in the first place.

2.3.4 Assessing partner buy-in

Improving a CBM program is an iterative process that requires by-in from CBM partners. Establishing long term partnerships with organizations that are willing to constantly work to improve their performance is essential. As the partnership between core laboratory "experts" and community based partners is just that: a partnership. I wanted to assess the CBM partners' thoughts on the program. Specifically, I wanted to understand if partners felt the program was valuable to their organization, and if the method was too difficult or time consuming for them, and I wanted more information about what drove them to participate. My goal in assessing this qualitative component of the program is that if this CBM program or something similar could ever be scaled up, it would require some understanding of what makes individuals buy into the program and what they need to continue to buy in. The CBM partners participating in this study performed qPCR analysis on over 2000 total samples over the three years of this program.

Thematic analysis (TA) was performed to analyze CBM partner surveys, which is a method of analysis by which codes and theme development were directed by our existing research questions. Thematic analysis is a qualitative research method for discovering, analyzing, and reporting patterns in data. The goal of thematic analysis is for a researched to find and make sense of the experiences of a collective of people. By developing a codebook to describe common ideas expressed by participants in a TA study, the research then organizes these codes into detail rich themes, which are important to answering the specific question the researcher has. Numerous patterns of meaning could be identified in any one dataset, but the goal of the analysis is to answer a specific research question (Braun and Clarke 2006; Clarke et al. 2013; Braun et al. 2019).

Three primary themes emerged from this analysis (Table 2.5). The first theme identified was “Rapidly responding to hazards”. Our CBM partners liked that the “time requirement from the qPCR testing method was less than the traditional operational time frame…" However, when asked about the time it took for the method to be completed and if this time was appropriate, all of our CBM partners equated rapidness of the method to a rapid policy response to these hazards. This was likely not the reality as any hazardous microorganism was dealt with formally through our existing regulatory system which is currently still adapting to the implementation of qPCR methodologies for water monitoring at their core facilities, and for which clear policy frameworks and courses of action do not yet exist for qPCR test results. The only exception to this was the avian schistosomes monitoring group, who liked “that [they] could use the next day to change [their] field procedures and experimental designs".

The second theme was “Independence and verification of a CBM monitoring system”. Two codes that emerged during analysis were CBM partners expressing a desire for more independence and more control over the interpretation of results. Our study was designed to remove data interpretation from participants’ hands, and instead place it in our own hands (with the vision that in a CBM monitoring system, data analysis would be accomplished by a central data processor or the enforcement agency). I thought this would be beneficial because the interpretation of qPCR data is not trivial (especially for quantitative tests that can be correlated to organismal or health outcome levels), and to prevent panic if CBM partners saw positive samples that, while meaningful, might not constitute a real concern. Nonetheless, CBM partners said “the only way these results would be more valuable would be to have a quantitative number which would correlate to specific standard or relative unit conversation chart.” In reality, this was what I was doing for CBM partners, but this group expressed a desire to conduct this independent of our assistance. Additionally, I had a group suggest that they wished the data were published online, "If the data was available or if there was a way to input the data online into a database. Then I could use the results more easily,” they said. Our CBM partners also expressed a desire to validate their results and have access to quality control data. One

user suggested “a visual that compared our results to yours so I have some idea of if I were capturing the results accurately." Another specifically suggested that "…third party verification can be one method to enhance validity of the results," suggesting a desire for some oversight to ensure data quality, but also a desire for CBM partners to know that they are contributing meaningful and accurate results.

One of the biggest challenges for CBM programs is data validation, storage, and visualization. Many communities lack the ability to share CBM data online. However, tools are emerging to address this challenge, including the Lake Observer mobile app through the Global Lake Ecological Observatory Network, the DataStream through the Gordon Foundation, or the ABMI’s NatureLynx. Allowing community partners to upload and visualize their results may help to create a sense that partners are part of something bigger than just their lake. It might allow them to contextualize their results relative to other water bodies, log additional environmental observations, or upload photographs of recreational waters. These apps can also be helpful to track long term results, or to have the data incorporated into reporting by other agencies.

The third theme I identified was that the triangulation of training was valuable. CBM partners appreciated the three forms of training. Most CBM partners found "the training videos were really useful." CBM partners found the written protocol useful as a reference, but suggested that after "around 2-3 runs of the machine this resource was no longer needed." Most CBM partners stressed the importance of the in-person training and one user stated that "the in-person training went a long way in creating and (sic) increased comfort and confidence in the machine." Studies conducted assessing training in citizen science or CBM projects have found that multiple training sessions can improve data accuracy (Ratnieks et al. 2016).

A subtheme that emerged during analysis was that CBM partners appreciated the learning process. One user stated that they were "…always up for learning new methodologies to answering scientific questions." CBM programs are often touted for the positive learning experiences they create, and it is nice to see that ours also had positive learning outcomes for participants (Trumbull et al. 2000). A number of CBM partners also

suggested that they appreciated the ability to communicate results quickly to their volunteers or to residents on the lakes they worked on. This type of a CBM project could greatly improve science literacy and communication.

2.3.5. A Vision For The Future

Rapid monitoring approaches, including CBM qPCR, should be deployed within the context of a policy framework and management response plan that can support acting upon the results generated. The response plan for samples that might constitute a hazard should be clear to CBM partners. If response plans lack transparency, a CBM partner who encounters a sample that contains a high level of an indicator organism, but upon subsequent tests shows low or no risk, might be dismayed by a lack of response by government. A CBM qPCR monitoring system in recreational water would need to prioritize communication and understanding between regulators and CBM partners, and would likely function best when addressing specific objectives (Barzyk et al. 2018).

Whether a CBM qPCR monitoring system enables a more rapid response to hazards is yet to be seen; however, CBM qPCR monitoring certainly has the advantage of being able to generate data over a large geographic area and for numerous hazardous microorganisms. It could be adapted to measure organisms not typically considered in monitoring programs; as I have demonstrated in our study, the approach works equally well for eukaryotic hazards like parasitic organisms as it does for the more traditional prokaryotic targets like enteric bacteria. The flexibility inherent in CBM qPCR makes this an attractive and adaptive platform for governments and communities to answer management related questions for their watersheds.

My vision for the CBM qPCR monitoring system was that data analysis would not occur in the hands of CBM partners (Figure 2.1). Analysis of qPCR data, while not extremely complex, does require a more comprehensive understanding of qPCR, and data interpretation is typically the most erroneous component over CBM programs (Cohn 2008; Hunter et al. 2013). Despite our CBM partners desire for independence in data interpretation, I feel that a central 'expert' should still be responsible for data

interpretation in order to ensure quality in reporting. This could be a single laboratory or a network of QC partners, each responsible to train and assess accuracy of their own CBM partners. Our successes establishing a field laboratory for avian schistosomes and enteric bacteria monitoring in Michigan suggests that a CBM qPCR network could operate effectively within a framework that paired CBM volunteers with quality control partners that could also be operating remotely from the central agency.

Participants in our study expressed a desire to know how well they were performing the method. This highlights an important component of a large scale CBM monitoring program: a proficiency testing system which would test and train potential participants to ensure the method is being conducted appropriately. I believe this must include third party verification of a percentage of all samples tested. While verification is important to ensure CBM partners are generating reliable results, it is essential that communication be prioritized. This includes responding quickly to results reported by CBM partners when a potential hazard is detected. It also includes being honest with partners about their performance, and willingness by both the CBM and regulatory partners to collect and assess additional samples when clarification or confirmation is required.

To my knowledge, this is the first study to comprehensively test the accuracy of a CBM qPCR water monitoring approach in a real world context. The results show that when implemented in a controlled manner, such that a central body controls materials and protocols, results can be highly reproducible. Our study also suggests that CBM partners, whose buy in would be required for ensuring program longevity, value the method, the data, and what they could do with that data.

While CBM qPCR is certainly promising, there are a number of drawbacks to the method. Notably, this method does generate results that have higher variation, and thus we are more uncertain about the results. CBM qPCR ideally would empower and educate non-scientists; however, this method could potentially create undue concern amongst communities. For example, if they felt the results of their study suggested poor water

quality but government disagreed, this could result in conflict. Additionally, this method does require oversight, as volunteers lack the capacity to troubleshoot issues that could arise. Managing volunteers and projects is time consuming. The labour required to achieve a robust CBM qPCR program could be more costly (in terms of both dollars and cents and time) compared to other methods (i.e.: drone sampling, auto samplers (Doi et al. 2017; Searcy et al. 2018; Benson et al. 2019)) that are capable of generating water quality data across large temporal and spatial scales. Organizations interested in employing a method capable of generating data regarding concentrations of microbial hazards in water across spatiotemporal scales would need to assess their desire to engage with external groups and the associated costs of that approach versus the cost and convenience of employing a technology to accomplish a similar project.

CBM qPCR could process a large number of samples from a wide geographical area that could aid beach management for health and invasive species. CBM qPCR could act as a valuable component of an environmental monitoring surveillance system, but could also be a viable option for monitoring and management of rural drinking water systems. qPCR is a platform, and therefore a myriad of diagnostic tests could be deployed as needed in remote locations. While CBM qPCR programs may be more variable than traditional monitoring programs, they could serve as a comprehensive screening system for traditional monitoring programs. Large numbers of samples could be screened by CBM qPCR, and when issues are identified, this information could feed into the traditional monitoring system, which could take steps to further investigate the problem. In many contexts, CBM qPCR programs could support current environmental monitoring systems, foster good relationships with stakeholders in water in a variety of locations, and provide a wealth of information with which to better manage water resources. The next two chapters of this thesis are case studies demonstrating the monitoring projects I accomplished using the CBM methodology, and the scientific advancements made. Chapter 3 focuses on my work in Michigan, USA using the CBM qPCR methodology to advance swimmer's itch research and policy in that region, and Chapter 4 will focus on the bacterial CBM qPCR monitoring projects touched upon in this chapter.

2.4 Tables

Table 2.1 Comparison of standard curves performed on the Open qPCR thermocycler and the ABI 7500 thermocycler

	Copy number	Cycle threshold (average, st dev)		r2	Slope	Amplification factor
Cyanobacteria mcyE assay						
Open qPCR	5000	30.1	0.27	0.980	-3.05	2.100
	500	33.2	1			
	50	36.3	1.6			
ABI qPCR	5000	28.7	1.5	0.990	-3.700	1.800
	500	32.2	2.4			
	50	36.1	0.91			
Total cyanobacteria assay						
Open qPCR	50000	23.3	1.1	0.980	-3.37	1.87
	5000	26.3	1.3			
	500	29.9	3.5			
ABI qPCR	50000	23.8	0.62	0.98	-4.1	1.7
	5000	27.5	0.64			
	500	32	0.60			
Total *Pseudomonas* assay						
Open qPCR	5000	27.1	0.2	0.98	-3.9	1.8
	500	30.6	0.4			
	50	34.8	1.1			
ABI qPCR	5000	29.1	0.03	0.99	-3.26	2.02
	500	32.2	0.39			
	50	35.6	0.23			
Human-associated *Bacteroides* (HF183) assay						
Open qPCR	5000	25.6	0.25	0.99	-3.46	1.94
	500	29.2	0.24			
	50	32.9	0.28			
ABI qPCR	5000	24.8	0.13	0.98	-4.4	1.87
	500	28.1	0.31			

	50	33.7	0.26			
Enterococcus spp. assay						
Open qPCR	5000	28.9	0.28	0.98	-2.3	2.6
	500	31.4	0.48			
	50	33.7	0.85			
ABI qPCR	5000	28.8	0.30	0.99	-3.05	2.12
	500	31.5	0.64			
	50	34.9	1.1			
D. polymorpha assay						
Open qPCR	5000	25.500	0.40	0.99	-3.45	1.94
	500	29.200	0.30			
	50	33.700	0.80			
ABI qPCR	5000	25.200	0.47	0.98	-3.45	1.94
	500	28.700	0.47			
	50	32.200	0.52			
Pan-avian schistosome assay						
Open qPCR	5000	27.3	0.41	0.99	-3.03	2.1
	500	30.3	0.64			
	50	33.3	0.52			
ABI qPCR	5000	26.9	0.52	0.99	-3.03	2.1
	500	30.5	0.47			
	50	32.9	0.54			

Table 2.2 Comparison in reproducibility of CBM partner samples and expert samples

	Comparison Of Partner-Extracted DNA Samples Performed On The Open qPCR Versus The Quantstudio 3/ABI 7500				
qPCR Test	**Interclass correlation coefficient**	**Lower 95% CI**	**Upper 95% CI**	**Maximum log difference***	**N**
Toxic cyanobacteria 2018	0.57	0.1	0.86	1.2	12
Toxic cyanobacteria 2019	0.6	0.24	0.8	1.5	40
Avian schistosomes 2017	0.88	0.85	0.9	1	255
Avian schistosomes 2018	0.76	0.56	0.87	1	47

Avian schistosomes 2019	0.9	0.75	0.96	0.44	83
	Comparison Of Partner-Extracted And Expert-Extracted Split Samples				
	Interclass correlation coefficient	**Lower 95% CI**	**Upper 95% CI**	**Maximum log difference***	**N**
Toxic cyanobacteria 2018	0.65	-0.25	0.9	1.4	12
Toxic cyanobacteria 2019	0.67	0.366	0.83	1.3	39
Avian schistosomes 2017	0.54	0.32	0.68	1.4	255
Avian schistosomes 2018	0.59	0.34	0.75	1.3	70
Avian schistosomes 2019**	NA	NA	NA	NA	NA

Table 2.3 Comparison in reproducibility of CBM partner samples and expert samples of methods implemented for the first time in 2019.

Comparison Of Partner-Extracted DNA Samples Performed On The Open qPCR Versus The Quantstudio 3/ABI 7500					
qPCR Test	**Interclass correlation coefficient**	**Lower 95% CI**	**Upper 95% CI**	**Maximum log difference**	**N**
Total cyanobacteria 2019	0.6	0.138	0.65	1.4	40
Total *Pseudomonas* 2019	0.211	-1.4	0.74	2.22	13
Enterococcus spp. 2019	0.54	0.35	0.68	1.19	127
Comparison Of Partner-Extracted And Expert-Extracted Split Samples					
qPCR Test	**Interclass correlation coefficient**	**Lower 95% CI**	**Upper 95% CI**	**Maximum log difference**	**N**
Total cyanobacteria 2019	0.116	-0.68	0.53	3.96	39
Total *Pseudomonas* 2019	0.18	-0.179	0.529	3.69	22

Enterococcus spp. 2019	0.192	-0.131	0.423	2.17	137

Table 2.4: Questions asked of CBM partners

Section 1. Prior Experience

1. How would you rank your knowledge with molecular biology,PCR (polymerase chain reaction),eDNA or DNA based detection in general prior to the use of the qPCR field method

- o High prior knowledge
- o Some prior knowledge
- o Low prior knowledge
- o No prior knowledge

2. Had you performed PCR (polymerase chain reaction) before attempting the qPCR field method? (highlight your response)

- o Yes
- o No
- o I do not remember

Section 2. Training

3. Was the training on the qPCR field-testing sufficient?

3a. Did you utilize the written protocol/video?

3b. Was the in-person training valuable?

Section 3. DNA extraction

4a. Was the DNA extraction protocol simple?

4b.What could be improved?

Section 4. Operating the thermocycler

5. Did you find the operation of the Chai Bio Open qPCR (the thermocycler and computer) simple?

What could be improved?

6. What method of results reporting would you have liked to see?

Section 5.

7. Was the time spent on the method too long or just right?

8. Did the portable field qPCR unit fit well within your/your organizations normal monitoring tasks?

9. Did the portable field system meet your expectations?

10. Did you find the results obtained by qPCR valuable? What could make these results more valuable?

11. Do you think that field qPCR is appropriate to answer the questions your organization sought to answer in agreeing to participate in this trial?

12. Do you see any value for continued use of the field qPCR method within your organization?

13. Why were you interested in participating in this trial to begin with? Have your views on the use of field qPCR changed since then?

Table 2.5: Equipment used in the field laboratories.

Name	**Manufacturer/Catalogue Number**
Biomeme™ MI DNA extraction kit	Biomeme™
DNAeasy™ DNA extraction kit	Qiagen™ (69506)
0.4 uM filters	Pall™ (FMFNL1050)
Vaccum pump	Vaccubrand™ ME 1C
PCR tubes and caps	Axygen™ (PCR-02-FCP-C and PCR-0108-LP-C)
Microcentrifuge tubes	Eppendorf (Z666548-250EA)
Open qPCR thermocycler (single channel)	ChaiBio ™.
20 µL Micropipette	VWR (470231-608)
Maximum Recovery™ Filter tips	Axygen (TF-20-L-R-S)
Pelican Storm Case	Pelican™ IM2450
20uM Plankton Tow	Acquatic Research Instruments
Primetime™ Gene Expression Master Mix	IDTdna (1055772)
Computers	Google Chromebook™ and Acer Switch One™
DNAeasy DNA extraction equipment- found in the field laboratory	
Spectrafuge™ 24D	Dot scientific (C2400)
VorTemp™ 56 Shaking Incubator	Dot scientific (S2056)
Mortexer™	Dot scientific (BV-1005)
Mortexer ™ sample head	Dot scientific (BV1000-H15)

Table 2.6 Relevant excerpts from CBM partners surveys and the codes and themes.

THEME	**CODE**	**RELEVANT EXAMPLE**

Rapidly responding to hazards		Rapid response	"..implemented as an invasive species monitoring plans (sic) that poses to be 'faster' than traditional methods"
		Rapid method	"yes the time requirement [spent on the method] was good"
Triangulation of training		In-person training	"the in-person training went a long way in creating and(sic) increased comfort and confidence in the machine"
		Troubleshooting	"we had some software issues but they were resolved."
		Written protocol	"around 2-3 runs of the machine this resource was no longer needed"
		Videos	"yes, the training videos were really useful!"
Independence and verification of the CBM monitoring system	Quality control		"…a visual that compared our results to yours so we have some idea of if we were capturing the results accurately"
	Independence		"…more detail is always nice to see in the results in the sense that you might be able to explain what is going on or provide a reasonable account at least"

	Learning*	"I am always up for learning new methodologies to answering scientific questions"
	Communication	"…if there was an online form we could fill in as we go so there is no confusion about what we were doing.."
	Value	"used in the right way and right circumstance can be extremely valuable."

2.5 Figures

Figure 2.1 Overview of the implementation of the qPCR CBM program.

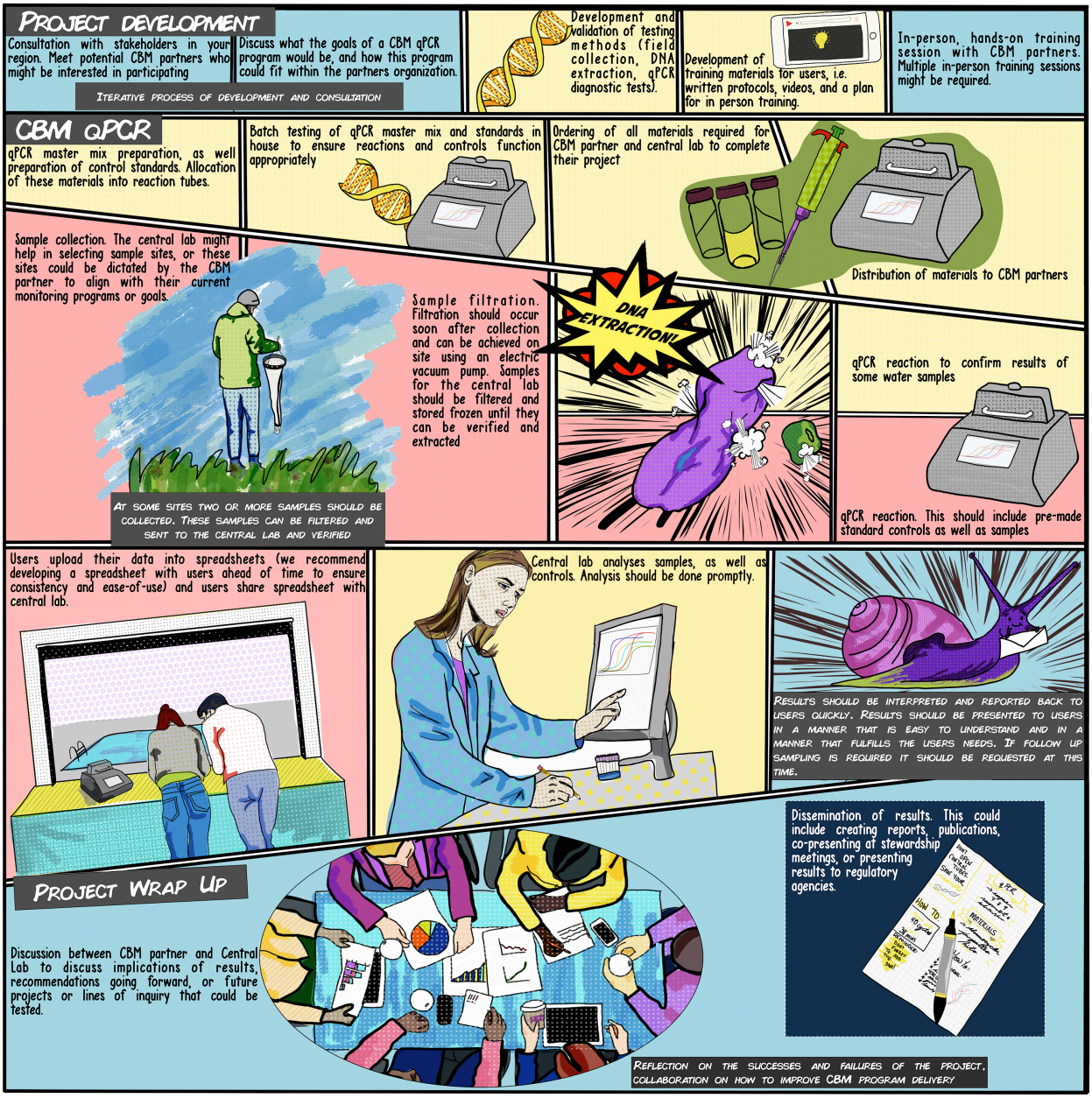
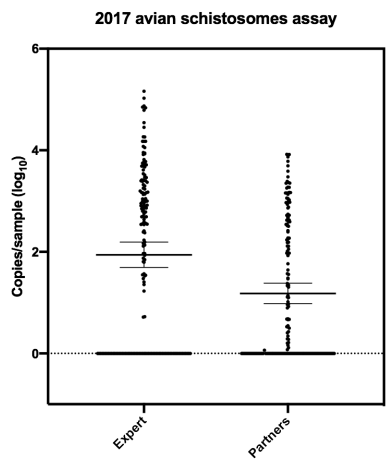
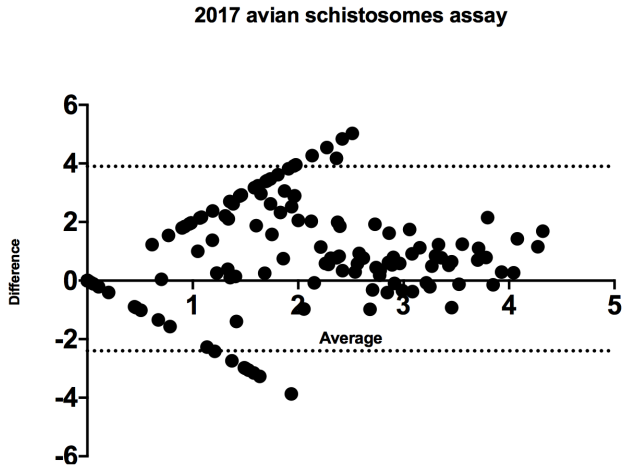
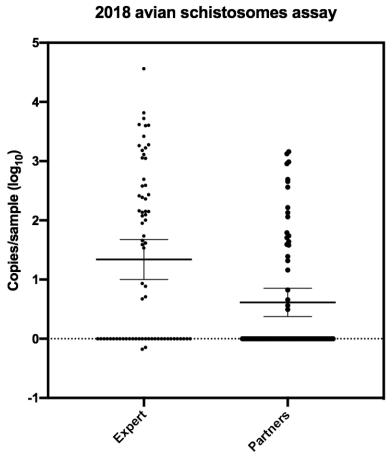
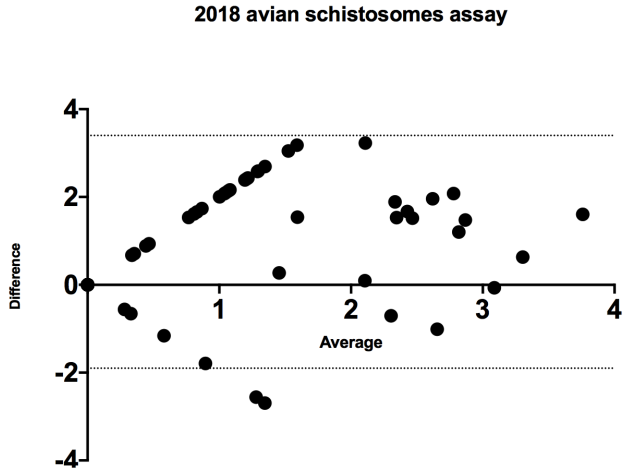


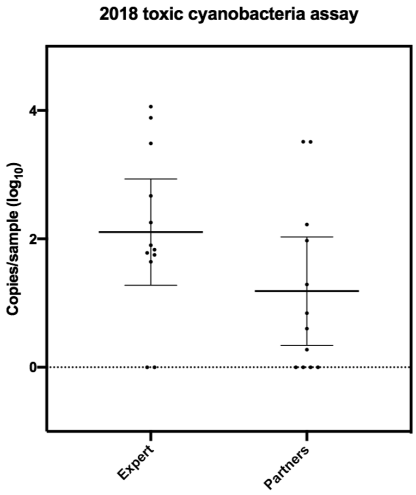
Figure 2.2 Analysis of agreement between CBM partner extracted samples and expert extracted samples.

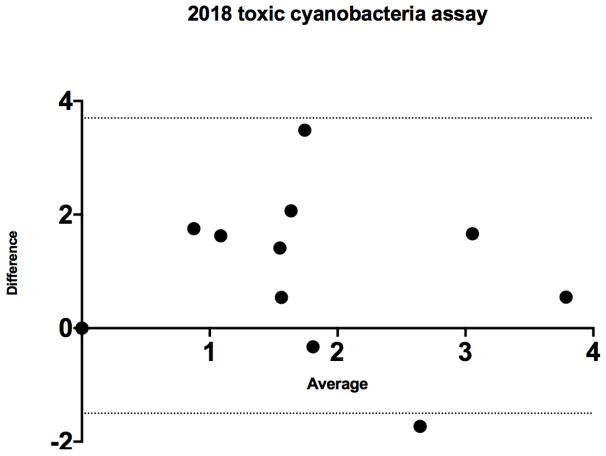


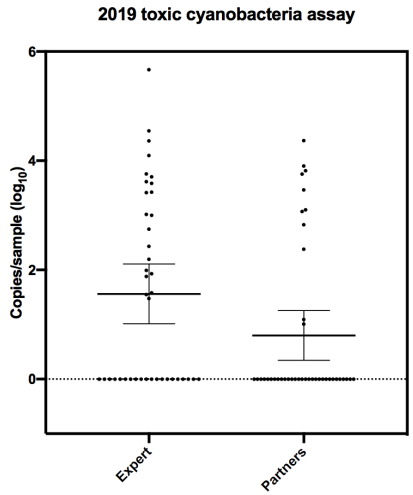







2018 toxic cyanobacteria assay
Copies/sample (log10)
4
2
0
Expert
Partners


2018 toxic cyanobacteria assay
Difference
4
2
0
-2
1
2
3
4
Average


2019 toxic cyanobacteria assay
Copies/sample (log10)
6
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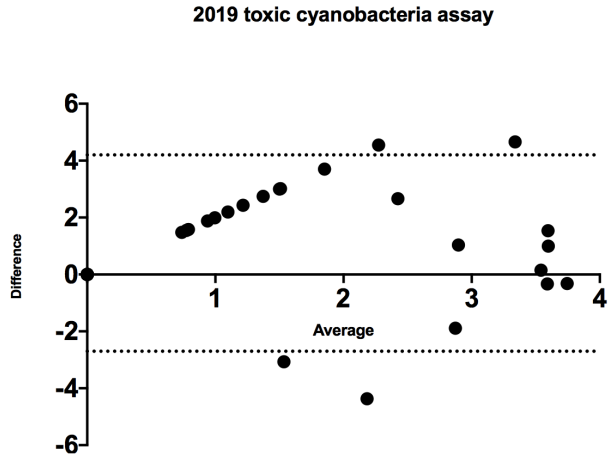
Expert
Partners


2019 toxic cyanobacteria assay
Difference
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4
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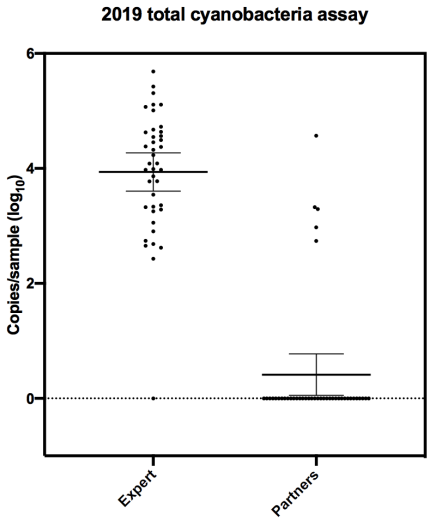
0
-2
-4
-6
1
2
3
4
Average


2019 total cyanobacteria assay
Copies/sample (log10)
6
4
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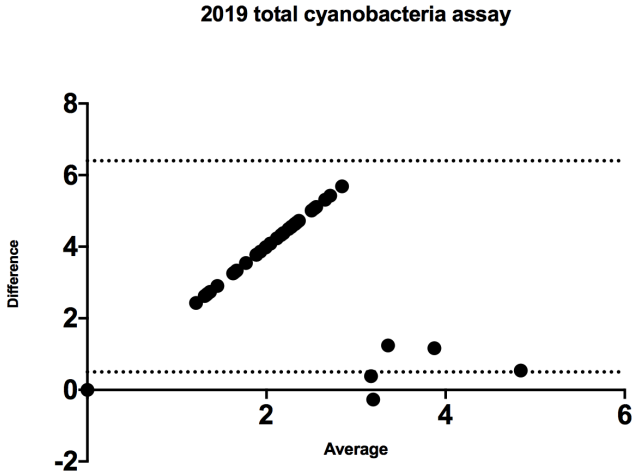
Expert
Partners


2019 total cyanobacteria assay
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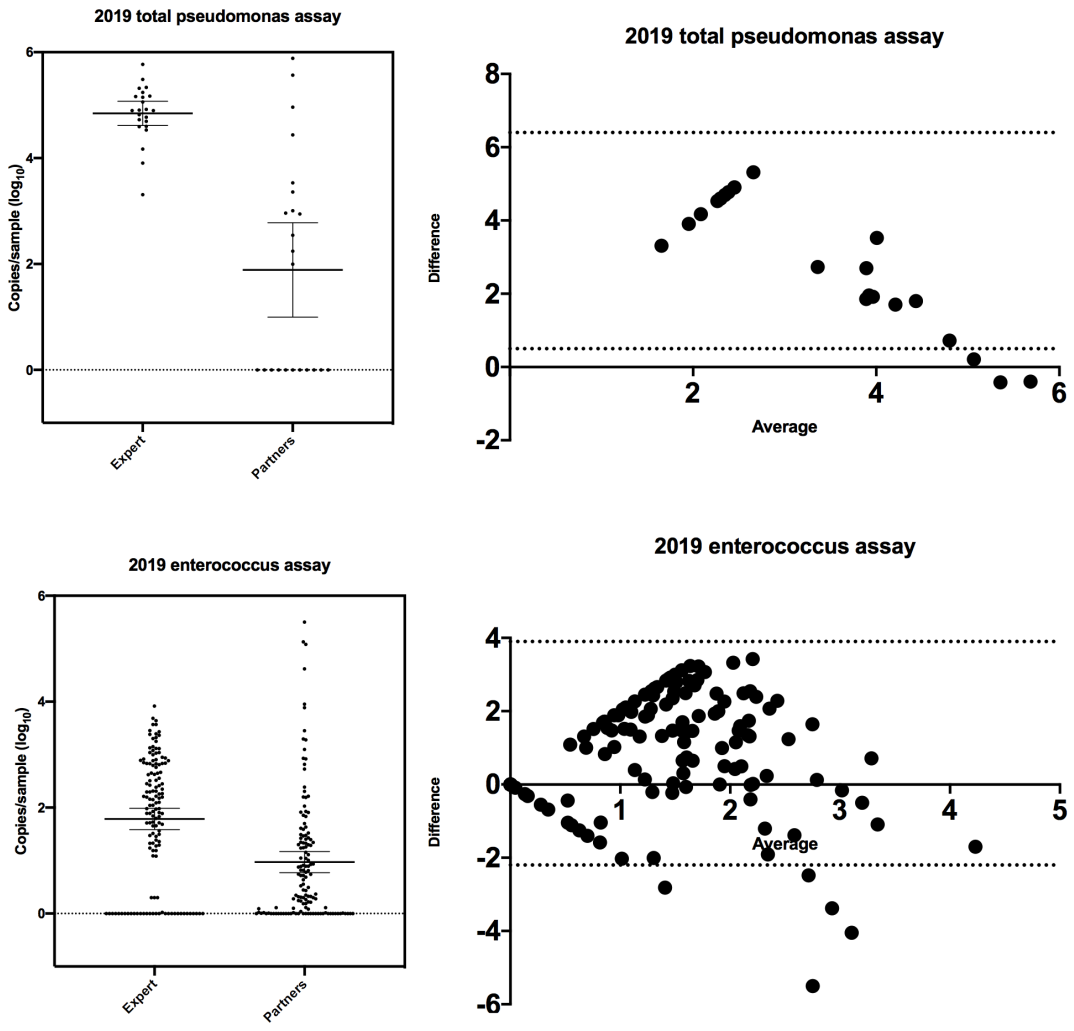
6
4
2
0
-2
2
4
6
Average



On the left log transformed samples from partners and experts samples are plotted and the mean and 95% confidence intervals of the data sets are shown. Partner samples on average demonstrate lower copy numbers than expert samples. On the right, Bland altman graphs demonstrate the reproducibility of the methods. Dashed lines are the 2 standard deviations from the mean.

Chapter 3: Swimmer's itch control in Michigan, USA: a case study in the power of Community Based Monitoring.

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3.1 Introduction

This chapter presents a series of studies that demonstrate how a CBM approach can be successfully implemented to address large scale water monitoring needs while also advancing research questions related to the target organism. The goal of this chapter is to both highlight the advantages that CBM can bring to monitoring and research while also detailing the scientific advancements made with my CBM partners in Michigan. This series of studies focuses on a comprehensive swimmer's itch monitoring program that I implemented with our CBM partners Ronald Reimink and Kelsey Froelich in northern Michigan, USA. In conjunction with these CBM partners, we assessed the effect of environmental variables on larval swimmer's itch parasite (cercariae) concentrations in lakes and found that cercariae move with the bulk flow of the water, and when this direction is towards the shore, cercariae concentrations are increased in these areas. Based on this observation, I hypothesized that targeted application of copper sulfate (which is a control strategy permitted and used commonly in the Michigan area) would be ineffective in reducing cercariae concentrations because cercariae shed from snails from neighboring beaches not affected by the copper sulfate would drift into the treatment area. Therefore, we assessed the efficacy of copper sulfate application to control cercariae concentrations in water. While the pan avian schistosomes assay was useful for assessing total cercariae abundance, I eventually I developed avian schistosomes source-tracking qPCR assays to delineate what species of schistosomes (and their hosts) are contributing parasites to the lakes of northern Michigan and used this data to assess temporal changes in avian schistosomes communities in 2019.

Trematodes from the family Schistosomatidae utilize primarily avian or mammalian (often rodent) definitive hosts and snail intermediate hosts. Family Schistosomatidae also includes genera *Schistosoma*, members of which cause the human disease schistosomiasis (Hoeffler 1974; Brant and Loker 2009a; Colley et al. 2014). Embryonated eggs leave the definitive host and hatch into free-living miracidia, which actively seek out a snail host. Upon finding a snail, they penetrate the snail intermediate

host. Inside the snail, miracidia develop into sporocysts, which produce cercariae asexually. The free-living cercariae are then shed from the snail host back into the water where they search for their definitive host. In the case of members of *Schistosoma* spp, this is a human, but trematodes of the genera *Trichobilharzia, Anserobilharzia, Gigantobilharzia, Dendritobilharzia* (known colloquially as the avian schistosomes) *and Schistosomatium* seek out avian or small mammal hosts to complete their lifecycle (Cort 1928; Price, Helen 1931; Strohmt et al. 1981; Brant et al. 2011). Accidental contact and penetration by these members of Schistosomatidae into human skin may cause the allergic condition known as cercarial dermatitis or swimmer's itch (Cort 1928; J and Jr. 1956; Baird and Wear 1987; Kolářová et al. 1999; Zbikowska 2004; Verbrugge et al. 2004; Coady et al. 2006). Swimmer's itch is caused by epidermal penetration of bathers by larval schistosome cercariae in surface waters (Brant and Loker 2009a). The resultant immune response leaves itchy papules that can last for weeks (Baird and Wear 1987; Verbrugge et al. 2004; Soldánová et al. 2013). Compared to other parasites like *Cryptosporidium parvum* or *Giardia llamblia,* which could be encountered in recreational water, swimmer's itch poses a relatively small health risk; thus, governments and regulators largely ignore swimmer's itch.

Returning to the issues discussed in the Chapter 1 of this thesis: there are far too many organisms in recreational water to monitor for, and often organisms are selected based on convenience and historical precedence. As Chapter 1 establishes, historically, bacteria were considered the riskiest group of organisms in water, and it was largely assumed that the absence of bacteria was indicative of safe water (National Research Council 2004). Swimmer's itch water monitoring is not undertaken by most regulatory agencies; instead, reporting typically occurs if individuals visit their doctor about their swimmer's itch (Gordy et al. 2018). Michigan is an exception to this as, for 7 decades, water users in Northern Michigan have refused to accept the itchy bumps caused by these parasites and have waged a war against avian schistosomes since the late 1940s (Blankespoor and Reimink 1991).

Cercariae were identified as the causative agent of swimmer's itch by William Cort on Douglas Lake, Michigan, in 1928 (Cort 1928). Since then, there has been prolific swimmer's itch research conducted in the area. The discoveries of *T. physellae, T. szidati,* and *T. stagniocolae,* all species of schistosomes capable of causing swimmer's itch, were made in Michigan (Talbot, Benton 1936). By 1941, Michigan's public health department had created a pilot program to chemically treat waters infested with swimmer's itch-causing trematodes using copper sulfate, and by 1943, Michigan had implemented a program that provided state supported funding for the treatment of public beaches, created an advisory assistance program, and began an extensive research program at the University of Michigan Biological Station on Douglas Lake (Blankespoor and Reimink 1991).

Since the mid 1900s there have been a variety of programs implemented to try and curtail the swimmer's itch problem in Michigan. The most prolific was the application of copper sulfate ($CuSO_4$) to lakeshores. Starting in 1939, copper sulfate was used primarily as a molluscicide as part of the state public health department's control program. If a lake could demonstrate that snails in the area were infected with cercaria, they could receive a permit to apply copper sulfate to designated beaches (Blankespoor and Reimink 1991). Between 1947 and 1973, it is estimated that over 1.67 million pounds of copper sulfate was applied to inland lakes in the State of Michigan (Blankespoor and Reimink 1991). Funding for this program was discontinued at a state level in the early 1970s, and responsibility was shifted to lake associations and communities to fund control efforts. Interestingly, even today, swimmer's itch is explicitly mentioned in both the Michigan Public Health Act and the Michigan Environmental Health Act, and lakes can still apply for control permits to use copper sulfate to control swimmer's itch (Michigan Legislature 1978; Michigan Legislature 1994; Francis and Haas 2006) . Copper sulfate is both a cercariacide, and a molluscicide. Its application has been used to control snail intermediate host *Planorbella trivolvis*, the intermediate host of *Bolbophorus* sp., a trematode that causes increased mortality in farmed channel catfish (Wise et al., 2005). However, $CuSO_4$ is also effective at killing free-swimming cercariae. Studies conducted on cercariae of a different digenetic trematode species of the genus *Echinostoma* found that concentrations as low as 0.01%

killed 100% of cercariae within 2 hours (Reddy et al., 2004). This research suggests copper sulfate should be an effective chemical for controlling swimmer's itch; however, chemical controls on large recreational lakes may be ineffective due to the relatively short toxicity period of Cu^{+} ions before precipitation as $CuCO_3$ (<24h) and the planktonic nature of cercariae allowing wind and water currents to move and concentrate cercariae.

In the 1980s, and again in the early 2000s, studies were conducted looking at the efficacy of treating mallards and common mergansers, respectively, with praziquantel, an anti-helminthic drug used to treat infection with trematodes. The studies suggested that praziquantel was effective at reducing the infection prevalence of schistosomes in birds and that yearly treatment of birds at specific sites could reduce swimmers itch in localized areas (Reimink et al. 1995; Blankespoor et al. 2001). This was never a feasible method of reducing swimmer's itch, however, due to the labor required to trap and administer praziquantel to birds. These studies also included bird surveys which found adult avian schistosomes in Canada geese, mallard ducks, common grackles, and common mergansers (Reimink et al. 1995; Blankespoor et al. 2001). Avian schistosome infection prevalence in the common merganser was found to be the highest amongst the birds in both studies. Common mergansers are currently the only described definitive host for the avian schistosome (and swimmer's itch causing parasite) *Trichobilharzia stagnicolae* (Reimink et al. 1995; Blankespoor et al. 2001).

Likely due to the high infection prevalence of *T. stagnicolae* in the common merganser, this bird became the focus of much of the swimmer's itch research in the area, and it provoked the ire of lakeside residents. Despite the presence of numerous bird species (Michigan sits on both the Mississippi and Atlantic flyways, and during bird migration seasons, over 57 bird species are observed each year) that are known to harbor avian schistosome species present in the area, the common merganser became the scapegoat of all of the swimmer's itch issues in the region (Pardieck, K.L., D.J. Ziolkowski Jr. 2018). The lifecycle of the *T, stagnicolae* and other related avian schistosomes present in Michigan are outlined in Figure 3.1. In 2014, the Michigan Department of Natural

Resources (DNR) approved a 3 year pilot research study on Higgins Lake to relocate common merganser bird broods. A previous study had demonstrated that hatch year birds have a higher infection prevalence than post hatch year birds, and it was thought that these birds drove the swimmer's itch lifecycle on lakes. Common merganser birds were relocated to a specific relocation site on Lake Michigan with the hopes that by reducing the presence of the definitive hosts on the lake, swimmer's itch would be reduced (Department of Natural Resources 2018). There was some controversy during this time amongst lake associations and riparian owners about trapping and relocating mergansers versus harassing and hunting the birds.

It was in 2016 that I began working with a small contractor company in Michigan to validate the avian schistosomes qPCR assay. The company had been hired by the Higgins lake association to relocate mergansers as part of the State-supported pilot study. They were interested in a simpler method of measuring swimmer's itch that didn't involve snail specimen collection. Monitoring efforts for swimmer's itch typically rely on assessments of infection prevalence in snails. This method requires collecting many snails from multiple sites around a lake and allowing the snails to shed parasites when exposed to light the next morning. Shed cercariae are then morphologically identified using a stereomicroscope. While this method assesses the success of a specific parasite species at infecting a snail species such as *Stagnicola emarginata,* it has several downsides. Notably, avian schistosomes typically show a low infection prevalence (0.5-3%) in snail intermediate hosts (Crews and Esch 1986; Brown et al. 1988; Gordy et al. 2016), meaning thousands of snails need to be collected to assess infection prevalence. What's more, sites can only be compared if snail density is also measured. Clearly, these are laborious tasks, but additionally, the techniquetechnique is unable to determine the number of cercariae that may be encountered while recreating. Since cercariae are essential for the natural transmission of the parasite and cause swimmer's itch, quantifying their abundance in the water is key to understanding both the ecological and public health impacts of this parasite. Part of this chapter details the development and validation of a pan-avian schistosome *18s* rDNA targeting qPCR assay. This method, called qPCR cercariometry (the

quantification of cercariae in an environmental matrix), offers a notable advantage to traditional snail infection prevalence methodologies, especially when the end goal is assessment of swimmer's itch risk. To date, all known species of avian schistosomes are capable of causing swimmer's itch, making an assay targeting this clade advantageous. Additionally, cercariae concentration and distribution in the water body may be influenced by environmental factors such as wind and water movement, or predation, inactivation due to UV light, or death (Haas 1994; McCarthy 1999). As qPCR cercariometry enables the quantification of cercariae in the water at recreation sites, it measures the potential exposure of bathers to cercariae, which is not possible by conducting snail surveys of infection prevalence.

Working in conjunction with the local contractor company, I validated qPCR cercariometry as a method of assessing swimmer's itch in the region. Results from this 2016 season were promising; common merganser brood relocation seemed to be reducing the number of *T. stagnicolae* cercariae present across the lake as measured by the infection prevalence of the parasites in snails. Furthermore, we validated the qPCR assay, which the company was interested in continuing on with as part of its assessments of swimmer's itch.

In 2017, the pilot study was expanded by the DNR to four more lakes: Crystal Lake, Lake Leelanau, Glen Lake, and Lime Lake, and the state set aside a $250,000 appropriation toward research and control efforts on these lakes (Department of Natural Resources 2018). At this time, the contractor company in Michigan wanted to use solely the qPCR method to measure cercariae in the water, as this was simpler and more cost effective than snail collections while providing a finer level of detail for the lakeside communities. We strengthened our partnership with this company in 2017, bringing them on as community based monitoring partners and beginning a research collaboration with them. In this capacity, our partners not only performed sample collection but also filtered water samples on site and performed DNA extraction and qPCR on water samples. In 2017, we deployed the qPCR cercariometry method within the CBM framework, and the contractor

company (helmed by Ron, his daughter Kelsey, and her husband Chris) were collecting water samples, filtering them, splitting the sample, extracting half of the sample, and performing their own qPCR in the field. I validated the split samples and performed data analysis from the University of Alberta.

Results of the relocation study became more complicated in 2017 as the qPCR results suggested that some lakes with control programs were not experiencing the expected decline in swimmers itch. These results are detailed below, but nonetheless, in 2018, the Michigan DNR decided that lakes could obtain permits to either destroy merganser eggs and nests, to perform harassment with lethal reinforcement on the birds, or to trap and relocate them (Department of Natural Resources 2018). To obtain any one of these permits, a lake would need to demonstrate "1. Presence of the swimmer's itch parasite and evidence that Common Mergansers are the host associated with the parasite's lifecycle on the lake. 2. Documentation of Common Merganser broods on the lake. and 3. Evidence of increasing swimmer's itch cases or severity"(Michigan Department of Natural Resources 2018). To demonstrate evidence of increasing swimmer's itch cases or severity, lakes could show that the infection prevalence of *T. stagnicolae* in *S. emarginata* snails was greater than 0.5% with a minimum sample size of 1,000 snails. Alternatively, lakes could use the pan avian trematodes qPCR assay I worked to validate, and if the water samples demonstrated that 5 sampling locations on a lake had at least 50 cercariae/25L of water, lakes would be approved for a relocation permit.

In 2018, I, along with our CBM collaborators in Michigan, worked to understand the ecological dynamics of the various avian schistosomes on lakes in Michigan, and have demonstrated that more species of bird and trematodes are responsible for the swimmer's itch problems in the region. Following this, we developed new qPCR source-tracking assays to allow us to detect these parasites from a water sample. This work also contributed to our discovery of a new species of avian schistosome, which uses a snail intermediate host that was not previously thought to harbour trematodes of the genera *Schistosomatidae.* We also demonstrated why copper sulfate treatment of beaches is

ineffective for controlling swimmer's itch when applied at localized beaches.

All the work summarized above is elaborated in detail in the following chapter. It is the culmination of three published manuscripts and one manuscript that is currently being formatted for publication. None of it would have been possible without the CBM qPCR program. CBM is a powerful methodology for conducting large scale ecological and monitoring research. I was lucky enough to work with incredibly motivated partners in Michigan, who took no issue with sampling more than 18,000 snails and collecting thousands of water samples a year. Ron and Kelsey also had scientific questions of their own that they wanted to answer. This partnership has proved incredibly fruitful as it enabled all of us to contribute applied science knowledge and affect water monitoring policy in Michigan, but also to answer basic scientific questions about the ecology of avian schistosomes in natural environments.

3.2 Results

3.2.1 Snail and trematode species identifications

Two surveys of trematodes and snails were performed in 2016 and 2018. Mitochondrial *cox1 (CO1)* DNA sequences confirmed *T. stagnicolae* (99.4-100% matches to GenBank vouchers) to be the common schistosome emerging from *S. emarginata* snails collected at Higgins Lake, Crystal Lake, and Lake Leelanau in 2016. Samples of miracidia found in merganser feces confirmed the presence of *T. stagnicolae* among hatch year mergansers on North Lake Leelanau and Crystal Lake. Miracidia from fecal samples derived from hatch year mallards at South Lake Leelanau were identified as a *Dendritobilharzia sp.,* another avian schistosome species capable of causing swimmer's itch (Table 3.1)(Brant et al. 2011). In 2018 another survey was conducted. Cercariae that were putatively identified as avian schistosomes based on gross morphology (i.e.: eyespots and a bifurcated tail) and the observation of phototactic behavior were characterized using CO1 barcoding. Miracidia samples collected from fresh feces from hatch year waterfowl (to ensure the infection was contracted on the sampled lake) were also

sequenced via the CO1 region. From these results I identified *T. physellae* (shed from *Physa parkeri), T. stagnicolae* (*Stagnicola emarginata), T. szidati* (*Lymnaea* sp.*),* and *A. brantae (Gyralus* sp.) Additionally, I found miracidia from both *T. stagnicolae* and *T. physellae* in the feces of common mergansers*, T. physellae* and an unknown member of the Schistosomatidae family miracida in the feces of mallards. *T. stagnicolae* was the most common species of cercariae identified (Table 3.1).

3.2.2 The qPCR assay is specific for avian schistosomes

The qPCR assay (for primers and probes see Table 6.1) was tested against a library of 25 North American trematode species including a member of *Trichobilharzia* (Gordy et al. 2016). Although it is important to note that this assay had been also been validated previously against 20 avian schistosomes species in the original publication of this assay (Narayanan et al. 2015). Amplification was only observed in samples containing *Trichobilharzia* spp. (Table 3.2)

3.2.3 Extraction efficiency

DNA losses occur during any DNA extraction due to inefficient lysis or DNA absorbance. DNA extraction efficiency using the DNeasy kit ranged from 0.1-7%, and averaged 4.2%. DNA extraction efficiency using the Biomeme™ eDNA kit employed during the 2017 collection year averaged 4.1%, and ranged from 0.4-21%. These values are not significantly different (p *[two tailed]*=0.6, df =19). Copy numbers were adjusted to reflect a recovery rate of 4.2% (Table 3.3).

3.2.4 Accuracy and precision of the qPCR method

Sampling variability was assessed by three samples taken consecutively of 25L water samples at ten beaches on the same day on August 1, 2016 from Crystal Lake. The sites with the highest dispersion between consecutive samples (Sites B13, P7, Y13) differed by 10, 8 and 12 cercariae each, respectively (Figure 3.2B). Additionally, a single blinded experiment was performed wherein known cercariae stocks were shipped from Michigan, USA, to the laboratory in Alberta, Canada to be analyzed. The DNeasy extraction, qPCR, coupled with analysis using Equation 1 correctly predicted the number of cercariae in each stock all three times the experiment was performed (Figure 3.2C). The copy number in a single *T. stagnicolae* cercaria averages 55,851 copies (σ_x=65.85) (Figure 3.2A).

3.2.5 Avian schistosome concentrations vary in the water column

The vertical distribution of cercariae in the water column was assessed and demonstrates that the majority of swimmer's itch-causing cercariae position themselves in the top 30cm of the water column (Figure 3.2D).

3.2.6 Time of day sampling

Eight (25L) samples were collected at 4 time points throughout the day on 25 July 2017, to determine if the time of sample collection influences cercariae counts. On average, the highest numbers of cercariae were detected in the water at 8am, and average numbers of cercariae at the same sampling point declined throughout the day (Figure 3.2E).

3.2.7 Water monitoring results from 2016/2017 show high variability in cercariae concentrations at specific sites at 6 lakes in northern Michigan

Monitoring projects were conducted in 2016 and 2017. In 2016, Higgins Lake, Crystal Lake, and Lake Leelanau were monitored, and in 2017, Lake Leelanau, Lime Lake,

and Glen Lake were monitored. Sampling sites were selected based on local knowledge—places where property owners commonly reported swimmer's itch were sampled. Peaks in avian schistosome concentrations occur in all lakes at the end of July, or early August in both years. A smaller peak in concentration can also be seen in late June. South Lake Leelanau, Glen Lake, and Crystal Lake saw the highest numbers of cercariae detected in the water throughout their respective sampling periods, while Higgins Lake, Lime Lake, and North Lake Leelanau have lower numbers of cercariae. It should be noted that both Higgins Lake and Lime Lake instituted control measures for swimmer's itch in 2015 so lower cercariae numbers were expected. It is important to note the high degree of variability in cercariae concentrations amongst all lakes; even lakes with lower concentrations of cercariae occasionally see extreme peaks in cercariae concentration, likely due to environmental factors such as wind or beach hydrology, which may concentrate cercariae in certain locations (Figure 3.3).

3.2.8 Wind direction relative to the shoreline predicts cercariae concentrations

The effect of the factors time of day, wind direction relative to the shore, and week (standardized between years, for exact dates see Figure 2), was tested on cercariae concentrations. These effects were tested using a generalized linear model (Table 3.4). Results indicate that onshore, and alongshore winds are predictive of higher concentrations of cercariae. The data also show that cercariae concentration changes temporally over the summer months, with Weeks 4 and 8 of the sampling period predicting higher numbers of cercariae.

3.2.9 Copper sulfate is effective at reducing snail populations within the study site

Copper sulfate was applied at a concentration of 51.11 ppm in a single dose. The site area is depicted in Figure 3.4. It was effective at decreasing snail densities inside the treatment area (Table 3.5). Snails from the genera *Stagnicola*, *Physella*, *Campeloma*, *Helisoma*, *Pleurocera*, and *Marstonia* were found prior to treatment. Counts of all species

except for *Pleurocera sp.* decreased significantly after treatment ($p<0.0001$, $df = 5$). The two snails that act as hosts for avian schistosomes, *Stagnicola sp.*, and *Physa sp.*, decreased by 93% and 89%, respectively (Table 3.5). As snail densities were counted post-treatment, all living *Stagnicola sp.* and *Physa sp.* were collected and brought back to the laboratory. A total of 23 *Physa sp.* and 1 *Stagnicola sp.* were collected post treatment. These snails were then isolated and shed for cercariae identification. None showed patent infections.

3.2.10 Copper sulfate is not effective at reducing cercariae concentrations within the study site

Copper sulfate treatment was not effective at significantly reducing the number of cercariae in surface waters within the treatment area (Table 3.6, Figure 3.5, Figure 3.6). On June 23 (pre-treatment) only the north inside treatment location had detectable numbers of cercariae in the water (26.9 cercariae/25L). On the next sampling date, June 28, there was no change in cercariae number. On June 29, 26.3 cercariae/25L were detected at the north treatment point, and at the midpoint inside and south inside sampling points we detected 2 cercariae/25 L. After July 29, cercariae numbers continued to increase for all sites before beginning to decrease towards the end of the sampling period (Figure 3.6).

3.2.11 Species-specific qPCR assay design

Assays were designed to the CO1 region of *T. physellae* (Assay limit of detection $(LOD)_{95}$: 12 copies/5uL), *T. stagnicolae* (Assay LOD_{95}: 27 copies/5uL), *T. szidati* (LOD_{95}: 7.9 copies/5uL), and *A. brantae* (LOD_{95}: 57.8 copies/5uL) (Wilrich and Wilrich 2009) (Table 6.2, Figure 3.7). Specificity was assessed in silico and experimentally. CO1 regions of each species targeted were aligned against other related avian trematodes species, and diagnostic tests targeted the regions that were most different, in the case of *T. physellae* locked nucleic acids were added to key nucleotides to ensure specificity. Cross reactivity

was tested against each *Trichobilharzia* and *Anserobilharzia* species, as well as non-avian trematode species which were selected because they were the most prevalent species identified during our snail survey (Table 3.7). No cross reactivity was observed.

3.2.12 Water monitoring in 2018 suggests that swimmer's itch-causing parasites are still present in high abundance in water samples collected from lakes with active Common merganser relocation programs.

Of the seven lakes studied, four lakes, Lime Lake, Big and Little Glen Lake, and North Lake Leelanau, had merganser relocation programs in 2017 and 2018 in an attempt to control swimmer's itch. South Lake Leelanau, Long Lake, Elk Lake, Skegemog Lake, Lake Charlevoix and Walloon Lake did not have control programs. 25L water samples were taken mid-July from sites around each lake to assess cercariae concentrations. Elk Lake, Walloon Lake, North Lake Leelanau and Glen Lake had the highest numbers of avian schistosome cercariae per litre (Figure 3.8).

3.2.13 Deployment of species-specific qPCR assays suggests that *T. stagnicolae* continues to dominate lakes in northern Michigan

Water samples from each lake were randomly selected to be tested in the species-specific qPCR analyses. Samples that were negative for the 18S avian schistosome assay were excluded. Water samples from different locations and dates were tested using the species-specific qPCR assays and results were pooled by lake to understand the relative contribution of each species to the cercarial burden of each lake. *Trichobilharzia stagnicolae* was the dominant avian schistosome species present in the water samples. However, Big and Little Glen Lake, North Lake Leelanau, Lake Charlevoix, Elk Lake, South Lake Leelanau, and Walloon Lake, also had detectable quantities of *T. physellae*. Lake

Charlevoix was the only lake to have detectable amounts of *A. brantae* present in the water, while *T. szidati* was found at North Lake Leelanau (Figure 3.9).

Snail diversity data collected during the study indicates that *S. emarginata*. and *P. gyrina* snails are present at every lake sampled. Lake Charlevoix, the only lake found to have detectable levels of *A. brantae*, also had *Gyralus* sp. snails, as did Elk, Glen, Long, Lime, Walloon and North Lake Leelanau. Interestingly, we did not find any *Lymnaea* sp. snails in North Lake Leelanau, despite the presence of *T. szidati* in the water. Likely our surveys did not capture *Lymnaea* sp. snails as we had few collection sites on North Lake Leelanau, and we typically find *Lymnaea* sp. snails at low abundance in Northern Michigan (Table 3.8). Shoreline waterfowl surveys were also conducted for each lake. Mallard ducks were the most abundant bird recorded at all lakes, regardless of whether a merganser control program was in place, followed by Canada geese (Table 3.9).

3.2.14 Observation of a schistosome-like cercariae emerging from a freshwater *Planorbella trivolvis* (=*Heliosoma trivolvis*) snail that is detected by the 18s pan-avian schistosome qPCR assay

The 2018 field season included a comprehensive collection and archiving of over 11,000 snails and their cercariae. During this study, Kelsey observed an eye-spotted, phototactic cercaria emerging from snails of the genera *Planorbella trivolvis* (=*Heliosoma trivolvis*). Her field notes are quoted below:

"Wedn. June 13 [2018]: Look at snails from Glen Lake. Found 3 Helisoma [Planorbella] that had what we think are schistos. I saw them first and was alone. Called dad and Chris, found another.

<u>Description of Helisoma [Planorbella] Schistosomes</u>: Head stick to surface of water, split tail, go towards light, big head curls when swims, elongates at rest."

"Fri. June 15 [2018]: Analyzed Walloon snails. Found Helisoma [Planorbella] that had same 'schisto' cercariae."

"Wedn. 6/27 [2018]: Look @ Long Lake snails. -2 'schisto' going to run through qPCR. Ran qPCR samples. Found 2 'positive' schisto Helisoma [Planorbella] so I extracted the DNA & ran it through qPCR. Found to be very high positives. Emailed Patrick & he thinks it might be a turtle trematode that is showing problematic [cross reactivity]."

"Thurs 7/12 [2018]: Looked at SLL snails. No schisto, but saw what we think is spirorchid in Helisoma [Planorbella]. Patrick looked & agreed that they look like schist. He'll run DNA in Canada when we send samples."

Results of CO1 barcoding showed that the Michigan *P. trivolvis* schistosome-like cercariae sequenced showed 100% similarity to a species shed from a *Planorbella* snail identified by former Hanington Lab member Michelle Gordy here in Alberta during her Ph.D (Gordy et al. 2018). Based on Michelle's phylogeny, this avian schistosome is not a member of Spirorchidae (intermediate host: *P. trivolvis*, definitive host: Freshwater turtles), and instead groups within the avian schistosomes. The ability of the parasite to cause swimmer's itch was confirmed by placing live cercariae on a volunteer and the observation of a localized rash.

3.2.15 Development of a species-specific qPCR assay for the unknown *P. trivolvis* cercariae

A CO1 gene-targeting assay specific for the *P. trivolvis* schistosome was developed. Multiple specimens of the cercariae were collected from Michigan in 2018 were barcoded and aligned to find a conserved region. The target was also aligned against related avian schistosomes to ensure there would not be cross reactivity (Figure 3.10). The *P. trivolvis* cercariaes' CO1 sequence most closely resembled *T. stagnicolae, T. physellae, T. franki and*

T. querquedulae, and therefore the alignment focuses on these species. However, cross reactivity was also tested experimentally against *T. physellae, T. stagnicolae, T. szidati* and *A. brantae* (Table 3.7) and no cross reactivity was observed. The LOD95 of this assay is 89.9 copies/5uL (upper: 181, lower: 44.6).

3.2.16 Longitudinal assessment of five species of avian schistosomes in 3 lakes in northern Michigan

In 2019 I utilized the five avian schistosome source-tracking assays to conduct a longitudinal analysis of the avian schistosome community. Water samples were first screened via the *18s rDNA* pan-avian schistosomes assay, and samples negative via this assay were excluded from source-tracking. Walloon Lake, South Lake Leelanau, North Lake Leelanau, and Glen Lake were assessed. The *P. trivolvis* avian schistosome was found to be the most abundant parasite at both Walloon and South Lake Leelanau, while *T. stagnicolae* was the dominant parasite at North Lake Leelanau and Glen Lake. When the data is displayed by site, *T. stagnicolae* and the *Planorbella* cercariae co-occur at many sites and times (Figure 3.11, 3,12).

3.2.17 Swimmer's itch monitoring as an example of a successful community based monitoring program

A three year monitoring and swimmer's itch research program was accomplished via the community based monitoring framework established in Chapter 1. In 2017 and 2018 this project consisted of a team of three people on the ground in Michigan conducting fieldwork and performing qPCR, with oversight from two program coordinators (myself and Patrick Hanington) from the University of Alberta. In 2017, this consisted of 241 water samples collected and analyzed weekly from 28 sample sites at 3 lakes. In 2018, 280 samples were collected, but the scope expanded to include 11 lakes with 162 sampling sites.

In 2019 this program expanded considerably, to include 16 lakes, and 8 merganser brood relocation sites on Lake Michigan, for a total of 193 lake sampling points, and 966 samples, or, roughly a geographical area half the size of New York City. Clearly three people on the ground would be insufficient for this magnitude of sampling and therefore our CBM partners recruited an additional 3 staff members and recruited over 25 volunteers at each lake to collect water samples from between 9 and 10 am every Tuesday and drive these samples to our CBM partner's field laboratory, located at Lime Lake. Samples then immediately underwent DNA extraction, and were assessed via qPCR over the following week.

3.3 Discussion

3.3.1 Advances in swimmer's itch control research

qPCR has become a well vetted, well established method for recreational water monitoring for enteric bacteria (USEPA 2012); however, it is still an emerging methodology in parasitology. Molecular methods for the detection of the environmental stages of parasites have become more common in recent years and can be applied to fill crucial knowledge gaps related to the environmental transmission stages. As a review by Bass et al. (2015) points out, environmental sampling for parasites, coupled with molecular methods, can reduce sampling bias incurred by specimen-based sampling, because hosts are often unevenly distributed in an environment. Additionally, molecular parasitological methods have been demonstrated to be more cost effective and less labor intensive than specimen based detection (Huver et al. 2015). In 2015, a qPCR assay for the detection of swimmer's itch causing avian schistosomes was published (Narayanan et al. 2015). This paper did not include validation of sampling methodologies, a limit of detection 95 in gene copy numbers, or an estimate of DNA extraction efficiency. It also did not provide an estimate of gene copy number of the target (*18S rDNA),* which would enable estimation of organismal number. Validation of all these components would be essential to create a reproducible and useful method. Additionally, avian schistosomes are eukaryotic, and

eukaryotic targets in qPCR can be more challenging to validate and quantify because they are multicellular (Weiss et al.; Bik et al. 2013). Ribosomal gene copy numbers (common barcoding targets in eukaryotes for distinguishing different species) are known to vary by orders of magnitude in bacteria, fungi, and eukaryotes, and apparent copy numbers quantified by qPCR may also be influenced by downstream methods such as losses due to DNA extraction (Cankar et al. 2006; Adamska et al. 2010; Green and Field 2012). Given this, quantitation to an organismal level for a eukaryotic target must include a conversion between gene copy number and organismal number, which is predicated on an assessment of DNA losses due to extraction.

My validation of this method estimated the LOD95 of the qPCR assay to be 3.4 copies per reaction (upper limit 7.9, lower limit 1.5). The average *18S* rRNA gene copy number in a single cercaria is 55,851 copies; however, my conversion between gene copy number and cercariae number utilizes the equation: $x = [(y + 56521) / 57736]$(Figure 3.2). I opted to use a standard curve to predict the number of cercariae rather than using a single number to estimate as interpolating based on a standard curve better accounts for uncertainty and variability in the target measurement compared to using a single point measurement. Extraction efficiencies between 0.5-4% have been reported for bacteria (Hwang et al. 2012). Therefore, it is not surprising that DNA extraction efficiencies ranged from between 1 and 7 percent (Table 3.3). These results demonstrate how imperative it is to quantify losses through the extraction procedure in order to avoid underestimations in target concentrations.

Accuracy of the qPCR methods was tested in a single blinded study. Using the conversion equation and an estimation extraction efficiency of 4.2%, the actual number of cercariae were estimated correctly in each vial, suggesting that under ideal circumstances (i.e.: purified cercariae in a clean matrix), this method of estimating cercariae concentrations from qPCR copy numbers is accurate and robust (Figure 3.2). Water sampling was conducted by collecting and concentrating 20L of water through a 20uM plankton tow net. Sample collection was performed across the beach (roughly 1m deep, 1L

samples) from the top 30 cm of the water. Laboratory studies have demonstrated that cercariae of *Trichobilharzia* are positively phototactic and negatively geotactic, and the parasites are typically shed in the morning (Feiler and Haas 1988; Haas et al. 2008). Given these biological determinants of cercariae behavior, it could be expected that avian schistosome cercariae will be more likely to be found during the morning and in the topmost column of the water (Feiler and Haas 1988). Results of the water depth study demonstrated that the majority of cercariae reside in the top 15-20cm of the water (Figure 3.2D), which confirms observations made by others in the laboratory (Feiler and Haas 1988). The time of day experiment shows that, on average, more cercariae are found in the water earlier in the day, between 8 A.M. and 12 P.M., and gradually decline throughout the day (Figure 3.2E). However, most samples collected on this day were collected during onshore or alongshore winds; therefore, it remained unclear if wind or the time of sample collection may be responsible for the higher concentrations of cercariae observed between 8 A.M. and 12 P.M.

Precision was tested in the field by collecting 3 consecutive 25L water samples at ten beaches from Crystal Lake in 2016. Variation between samples in this dynamic natural environment is low, with the highest samples exhibiting variation of between 8 and 12 cercariae in between replicate samples. These results demonstrate that the assay provides a precise estimate of cercariae concentration over time in a natural environment (Figure 1B), but that multiple samples will result in more robust estimation of cercariae concentrations at a particular location (Figure 3.2).

With the qPCR cercariometry method validated, it was deployed for water monitoring at lakes in northern Michigan in 2017. It was also used to test the effect of environmental and seasonal drivers of cercariae abundance in a whole lake environment.

To elucidate the biological and environmental drivers of cercariae abundance, a generalized linear model was performed on the entire dataset from 2016 and 2017. Results of the generalized linear model demonstrated the importance of onshore and alongshore wind in explaining higher cercariae concentrations. Time of sampling (morning or

afternoon) did not explain higher cercariae concentrations. There were two weeks, corresponding to early July and early August, which also predicted higher numbers of cercariae that could be due to seasonal trends in patent infections among snails (Table 3.4). Wind conditions likely blow cercariae closer to shore; as such, it is intuitive that onshore and alongshore winds would increase concentrations of cercariae along the shore. This is intuitive given that our study has also confirmed laboratory experiments that cercariae position themselves in the topmost portion of the water body and, therefore, their movement in the water is likely influenced greatly by surface winds, akin to the movement of surface cyanobacteria scums (Kanoshina et al. 2003; Kahru et al. 2007). Wind direction has also been previously implicated in moving *Trichobilharzia* cercariae (Leighton et al. 2000). Our model suggests that time of day does not have a strong relationship with cercariae abundance. However, as avian trematodes do leave their snail hosts in the morning, early morning recreators may still be more likely to encounter high concentrations of cercariae. There are numerous reports in the literature of strong seasonal trends in snails shedding cercariae; this likely explains the higher overall cercariae concentrations found in Weeks 4 and 8. Numerous studies have reported seasonal trends in patent infections in snails (Crews and Esch 1986; Brown et al. 1988; Gordy et al. 2016). It must be acknowledged that we tested but a few factors which might affect cercariae concentrations—however, there are a number of potentially important variables which might influence cercariae concentrations, including water temperature, unpredictable cercariae shedding from snails, and shoreline bird abundance (Lo and Lee 1996; Abrous et al. 1999; Byers et al. 2008; Soldánová et al. 2016). Furthermore, qPCR as a DNA-based molecular method will detect both live and dead cercariae in the water column and therefore may overestimate live cercariae abundance in some instances (Bass et al. 2015).

The observation that cercariae move with the bulk flow of water, and the knowledge that some lake associations in Michigan still apply copper sulfate to localized areas of shoreline, led us to conduct a study measuring the effectiveness of copper sulfate at reducing cercariae concentrations. I hypothesized that localized application of this

compound would be ineffective at reducing cercariae as other cercariae shed from snails outside the treatment area would simply be blown back into the treatment area once the concentration of copper sulfate decreased, as copper sulfate has a relatively short toxicity period of Cu^{+} ions before precipitation as $CuCO_3$ (<24h).

Copper sulfate applied at a concentration of 51.11 ppm in a single dose was effective at decreasing snail densities inside the treatment area (Table 3.5). Six species of snails were found in the study area prior to copper sulfate treatment. Each was identified by gross morphology. Snails from the genera *Stagnicola*, *Physella*, *Campeloma*, *Helisoma*, *Pleurocera*, and *Marstonia* were found. Counts of all species except for *Pleurocera sp.* decreased significantly after treatment ($p<0.0001$, $df = 5$). Divers did not sample deeper than 1.5 m, yet it was noted that the deep side diver found significantly more snails than diver in the shallow area ($X^2 = 66.01$, $p<0.00001$). The two snails that act as hosts for avian schistosomes, *Stagnicola sp.*, and *Physa sp.*, decreased by 93% and 89%, respectively (Table 3.5). As snail densities were counted post-treatment, all living *Stagnicola sp.* and *Physa sp.* were collected and brought back to the laboratory. A total of 23 *Physa sp.* and 1 *Stagnicola sp.* were collected, isolated, and shed for cercariae identification using the methods of Blankespoor et al. (1998). None showed patent infections.

Copper sulfate treatment was not effective at significantly reducing the number of cercariae in the surface waters within the treatment area (Table 3.6, Figure 3.5, Figure 3.6). On June 23 (pre-treatment), only the north inside treatment location had detectable numbers of cercariae in the water (26.9 cercariae/25L). On the next sampling date, June 28, there was no change in cercariae number; we detected 26.3 cercariae/25L, and at the midpoint inside and south inside sampling points we detected 2 cercariae/25 L. After July 29, cercariae numbers continued to increase for all sites before beginning to decrease towards the end of the sampling period. (Figure 3.6). This cyclic pattern of cercariae abundance is consistent with previous findings both from water sample analysis and shedding snails (Figure 3.6).

This study suggested that $CuSO_4$ applied in local areas of a larger water body, while effective at reducing local snail populations and possibly the existing cercariae population, are, ultimately, ineffective at controlling cercariae concentrations beyond the day of application. Planktonic schistosome cercariae move with the bulk flow of water and survive entering the treated area shortly after lethal concentrations of copper are dissipated. Additionally, this study demonstrated the importance of utilizing qPCR cercariometry of ambient water to assess swimmer's itch risk, since an absence of snails does not necessarily equate to the absence of cercariae present in the water.

The snail and cercariae survey conducted in 2017 showed that there was still *T. stagnicolae* being shed from snails even at lakes with control program (Table 3.1). This isn't surprising, as there are many avian schistosomes capable of causing swimmer's itch, and there would be no reason to think that many of them wouldn't be present in northern Michigan—despite the fact that the prevailing public opinion in the region is that only *T. stagnicolae* causes swimmer's itch in their area. In fact, other species of avian schistosome have been found in surveys previously (Blankespoor et al. 2001). Additionally, Despite this fact,in 2018, the Michigan DNR approved a policy to allow lakes to apply for permits to destroy merganser eggs and nests, to harass or kill, or trap and relocate mergansers (Department of Natural Resources 2018). Mergansers are a top-level predator of fish in this region, and relocation of the species could have drastic ecological consequences--not only for fish and other birds, but also for the parasite community. The opportunity to document the consequences of this large-scale relocation meant that, in 2018, we undertook an extensive snail and cercariae survey.

Between June and August of 2018, 11,309 snails were collected from the lakes in Northern Michigan. Snail surveys of avian schistosomes parasites followed by CO1 barcoding identified three species of *Trichobilharzia T. szidati, T. stagnicolae,* and *T. physellae*, and *Anserobilharzia brantae,* infecting four species of snail at the lakes studied (Table 3.1). The presence of other swimmer's itch causing parasites in the area and their hosts may undermine the merganser relocation control effort, as it suggests that the

swimmer's itch issues stem from multiple species, rather than just one. This observation prompted us to hypothesize that perhaps one of these other species of swimmer's itch-causing avian schistosomes could now be present at a high relative abundance and be dominating cases of swimmer's itch in the region. To answer this question, we developed species-specific qPCR assays for the four species of avian trematodes identified in our survey.

qPCR tests were developed to the CO1 gene region of each species. Tests were validated both *in silco,* by performing alignments of the target regions to closely related avian schistosomes species, and by testing the assays experimentally against each other and other non-avian trematode cercaria present at high abundance in Michigan (Figure 3.7). The utilization of these tests to identify the species contributing to swimmer's itch in our study lakes has yielded a never before seen level of resolution The results of the species-specific qPCR assays show that while *T. physellae* also cycles at many lakes, they are still observed in far lower abundance than *T. stagnicolae* (Figure 3.8), which is contrary to the hypothesis that merganser relocation should be reducing *T. stagnicolae* abundance. Despite the high density of Canada geese present at all the lakes studied, *A. brantae* was only detected at Lake Charlevoix, and, again, despite the large number of mallards observed on the lakes, *T. szidati* was only detected at North Lake Leelanau.

The high relative abundance of *T. stagnicolae* at all the lakes in question, even those (Big and Little Glen Lake, North Lake Leelanau, Lime Lake) that have active control programs, suggest that these programs are not effective. These data suggest two hypotheses: either that a second bird host, sympatric with *M. merganser* could also host parasite *T. stagnicolae,* or that that the *T. stagnicolae* population is being driven by nonresident, non-hatch year merganser populations that are likely comprised of migratory birds in the fall or spring (i.e.: those that are not targeted by the merganser brood relocation programs). While I identified *T. stagnicolae* miracidia in the feces of *M. merganser,* it is possible another host exists that is also contributing *T. stagnicolae* to the ecosystem. *T. stagnicolae* is capable of infecting canaries (Brant and Loker 2009b), and

other passerine bird species have also been documented with *Gigantobilharzia* infections; it follows that these species could also be host to other avian schistosomes such as *T. stagnicolae*. Based on our data, I can only speculate on other potential hosts; 57 species of bird were observed in Michigan during the North American Breeding Bird Survey (BBS) from 2016 to 2017 alone, with over 25 species of water bird identified (Pardiek et al., 2018).

Based on the results of this study, in my final field season (2019), I wanted to investigate the potential for spring and fall migrant birds to be contributing parasites into the ecosystem prior to when broods emerge onto the lake. Additionally, despite the evidence that *T. stagnicolae* continued to be dominant in water samples, I was skeptical of this result. The 2018 field season included the identification of a species that, upon visual inspection, looked like an avian schistosomes cercariae (i.e.: possessed a bifurcated tail, eyespots, displayed phototactic behavior) being shed from a *Planorbella trivolvis* (=*H. trivolvis)* snail. A pan-avian schistosome species of trematode called a *Spirorchid* (intermediate host: *Planorbella* spp. and *Physellae* snails; definitive host: freshwater turtles (Holliman 1971; Brown et al. 1988; Kraus et al. 2014)) is also eye-spotted, and we believed this could be the cercariae we had found. Nonetheless, the DNA of these cercariae were amplified by the *18s rDNA* pan-avian schistosome qPCR assay. A former PhD student in the Hanington lab, Michelle Gordy, had reported in a previous publication a spirorchid-like cercariae being shed from a *P. trivolvis* snail collected in Alberta that phylogenetically grouped within the avian schistosomes (Gordy and Hanington 2019). I performed barcoding on the purified cercariae and found it was a 100% match to the sequences reported by Michelle in her paper, suggesting that perhaps there was a new species of avian schistosomes parasite hosted by a *P. trivolvis* snail found both in Alberta, and now in Michigan. Another student in the Hanington lab is now undertaking phylogenetic and life cycle analysis of this new species of parasite. Nonetheless, I also developed a species-specific qPCR assay specific to this *P. trivolvis* parasite, as I was curious about its abundance in water samples collected from Michigan (Figure 3.10).

Source tracking using the five species specific assays was performed on samples from Walloon Lake, South Lake Leelanau, North Lake Leelanau, and Glen Lake. This was done both to test the hypothesis that spring and fall migrant birds are driving the parasite populations at lakes, and to determine the relative contribution of the *Planorbella* cercariae species at these lakes. I hypothesized that if fall migrants are driving parasite populations at a given lake, I would observe higher concentrations of cercariae in September. The Common Merganser begins migration as early as August, so I would expect snails infected with parasites by these birds to begin shedding parasites in September if we are operating under the assumption that it takes approximately 30 days for a parasite infection in a snail to develop into a patent infection (Brant and Loker 2009b; Sauer et al. 2014; Pearce et al. 2015). Snails infected in the fall will likely overwinter in the sediments and then emerge from their estivation period in the spring. Thus, I expected to see a peak in cercariae concentrations in the water once water temperatures reach daytime temperatures between 17 and 20 degrees (ideal temperatures for snails to shed parasites), likely around the first week of June (Feiler and Haas 1988). As these snails would have overwintered, I would not suspect them to survive very long as typically, when snails come out of estivation, they mate and die shortly thereafter. The Common Merganser begins its migration north in mid April, ending as late as the end of May (Sauer et al. 2014; Pearce et al. 2015). Therefore, I hypothesized that in a lake driven primarily by spring migrants, I would see cercariae concentrations begin to rise in late June, or early July. As snails may shed cercariae indefinitely, I could expect to see these spring migrant infected snails continue shedding through July, likely resulting in a peak in cercariae concentrations in late July, that gradually declines (due to snail death) until September, when cercariae concentrations should begin to rise again due to contributions from early fall migrants. This hypothetical model is depicted in Figure 3.13.

Walloon Lake does not have a merganser brood relocation program, nor does South Lake Leelanau; however, merganser broods do not nest on South Lake Leelanau. North Lake Leelanau has had an active merganser brood relocation program for 3 years, as has Glen Lake. *T. stagnicolae* continues to dominate Glen and North Lake Leelanau,

despite the active removal of merganser broods from the lakes. These lakes also have the highest density of *S. emarginata* snails, the intermediate host of *T. stagnicolae* (Table 3.10). This suggests that intermediate host density plays an important role in how many cercariae eventually end up in the water column. The peaks seen in Figure 3.11 coincide quite well with my hypothetical model (Figure 3.13), though the peaks in cercariae concentrations seem delayed by two weeks in the real world data, suggesting that perhaps the time from infection to patency is closer to five or six weeks.

South Lake Leelanau does not typically attract merganser broods to the lake, likely due to a lack of appropriate nesting habitat; this lake does not have a merganser relocation program. This portion (which is connected to North Lake Leelanau via a small channel referred to as "the narrows") has far less *T. stagnicolae* cercariae than North Lake Leelanau and has far less *T. stagnicolae.* South Lake Leelanau also has a far higher relative abundance of the *Planorbella* cercariae than North Lake Leelanau (Table 3.10, Figure 3.11). I initially presumed this was because of a higher density of *P. trivolvis* snails on the lake; however, this does not seem to be the case as all four lakes have similar densities of *P. trivolvis.* One hypothesis for this observation could be that in lakes with a lower *T. stagnicolae density,* the miracidia of the *Planorbella* avian schistsosome are more likely to find their host *P. trivolvis* (i.e.: intermediate host dilution). Alternatively, competition between definitive hosts in the spring and fall could also drive this trend. Lakes without broods may have greater numbers of fish in the late summer and early fall; these lakes may attract more spring and fall migrant mergansers, which could in turn drive higher concentrations of cercariae in the water and drive a less competitive bird species that hosts the *Planorbella* avian schistosome to lakes without control programs, where resident merganser broods have decreased the number of fish in the water. Additionally, we don't know the complete lifecycle of the *Planorbella* avian schistosomes, or if its host is a migratory or nonmigratory bird, or if it has a two or three host lifecycle. The presence of a secondary intermediate host may also explain the differences in the abundance of this parasite between lakes. In short, there is likely some very complex ecological dynamic in

place here involving the interaction between biotic bird and mollusk hosts and abiotic environmental factors.

Interestingly, lakes without control programs have similar peaks in cercariae concentrations, as well as similar magnitudes of cercariae present, to lakes with control programs (Figure 3.11). I argue that this suggests that spring and fall migrant birds are driving cercariae concentrations in these lakes, not resident broods. The data demonstrated here not only support this, but biologically, the belief that has pervaded this region for nearly a decade—that merganser broods drive parasite populations on these lakes—is innately flawed if we consider the biology of both breeding merganser birds and parasite development within the snail host. This belief was based on an observation that hatch year birds have higher numbers of miracidia in their feces, therefore contributing more parasites into the lake (Blankespoor and Reimink 1991). However, given that mother mergansers lay eggs in May or early June, with broods appearing on the lake after a 26 day incubation period and a 1 day nesting period for the hatchlings, this puts baby birds on the lake between mid June and mid July. This is also when residents being to report mergansers to our CBM partners (Pearce et al. 2015). Assuming the hatchlings are infected soon after their arrival on the water, and an incubation period inside the bird (at which time a male and female parasite migrate through the skin, through the circulatory system to the intestines, mature into adult worms, and then produce ova) of between two weeks to a month before that bird sheds miracidia (Brant and Loker 2009b). We know infected snails have an incubation period of between 4 and 5 weeks before patency; one might see a peak in cercariae concentrations in the water from mid August to late September from bird broods—not during July and August when residents report the most problems with swimmer's itch (Brant and Loker 2009b). Therefore, infections that hatch year birds acquire on Michigan lakes (if these infections are not cleared during migration) have a greater effect on swimmer's itch at their overwintering sites in South America than they have on cercariae concentrations during peak recreation times in Michigan.

The site by site data has interesting implications for swimmer's itch control (Figure 3.12). This assessment demonstrates that certain sites are more heavily impacted by cercariae than others, likely due to wind direction and snail habitat. This type of assessment could prove highly practical for residents of this lake in choosing swimming locations or areas where it might be beneficial to create a public swimming area. Such assessments could also be very useful in regard to a wide variety of organisms, including cyanobacteria blooms, which are also known to be influenced by surface currents and wind direction, or when considering the potential for fecal contamination at sites.

3.3.2 Swimmer's itch as an example of a CBM qPCR monitoring program

In the introduction of this thesis the evolution of bacterial water monitoring is discussed. To reiterate, monitoring for indicator organisms in drinking water began in the early 20th century, using culture based methods, which involved growing bacteria isolated from water samples on agar plates and counting the colonies present in that sample. In the last 100 years, this field has progressed to monitoring via *Enterrococcus* qPCR (a better indicator of human health risk (Wade et al. 2010)); the same samples used for *Enterrococcus* qPCR can then undergo source-tracking via a variety of bacterial markers, including the human-associated *Bacteroides* or bird-associated *Catellicoccus marimammalliun* (Lee et al. 2013). The study above details four years of swimmer's itch research in northern Michigan, in which we have moved away from a primary method of detection that relied upon laboriously collecting hundreds of snails from an area, individually sorting the snails into separate dishes, and 24 hours later assessing morphologically via microscopy the families of trematodes present to a qPCR method with species level resoltuon. In four years, we have moved from the equivalent of culture-based methods in microbiology to species-specific assays to detect specific parasites in the water, which can aid us in answering questions about the human affliction of swimmer's itch, as well as ecological questions about host distribution, snail shedding, and parasite abundance throughout the year.

None of this would have been possible without the community partners and volunteers in Michigan. CBM partners in Michigan were part of the study design process, and I answered research questions they were interested in and that were developed collaboratively. Each year, they worked to collect thousands of water samples from sites across the region. They also collected thousands of snails while documented anything they found interesting or noteworthy. This resulted in the discovery of a new species, and an unprecedented amount of data about avian schistosomes concentrations in recreational water. Additionally, the buy in from partners meant that volunteer sample collection was accomplished for zero dollars. I bring this up not just to acknowledge their work, but also to highlight the power of community based monitoring as a methodology. However, the success and scale of this project was only possible due to a CBM approach that was driven from both the "top-down" and the "bottom-up".

From 2017 to 2019, the pan-avian schistosomes assay was performed entirely in the field in Michigan. From sample collection and filtration, to DNA extraction (first via the Biomeme ™ DNA extraction kits, and then via the DNAeasy™ DNA extraction method), to performing qPCR on the ChaiBio Open qPCR, results were first obtained on the ground in Michigan before being validated by myself here in Edmonton (this data is shown in Chapter 2). CBM is often touted as a large-scale method to conduct ecological research but is also sometimes criticized as producing unreliable or unpublishable data. I think this project in Michigan has disproved this. This CBM partnership has influenced policy by establishing qPCR as a valid method (as accepted by the state of Michigan DNR) for measuring swimmer's itch, has produced (to date) three manuscripts, has created six new methodological tools for measuring avian schistosomes in environmental water samples (methods which could surely be applied to similar parasites with environmental transmission through water), has resulted in the discovery of a new species of avian schistosomes which passes through a snail host previously believed to be incapable of harboring avian schistosome infections, and whose data has hopefully helped to clear the name of the common merganser. Our program expanded considerably in just three years, from 241 water samples collected by three people on the ground, to 966 samples collected

at 16 lakes by over 25 volunteers on these lakes. This case study illustrates the power of CBM as a methodology academically, but also practically by influencing policy and delivering relevant results to the people and communities who need them.

3.4 Tables

Table 3.1 Cercaria, snail and miracidia survey data 2016 and 2018.

Sequences were obtained by CO1 gene barcoding, and classifications were based on 98% or above sequence identity to an existing organism in the GenBank database. Sequences from 2016 were deposited into Genbank under accession numbers: MG964019 to MG964043. Sequences from 2018 were deposited into Genbank under accession numbers: MK433243 to MK433252

Identification	Year	Specimen	Location
Diplostomum sp*. 1*	2016	Cercariae	Crystal Lake
Plagiorchis sp.	2016	Cercariae	Crystal Lake
Apatemon sp. 1	2016	Cercariae	Crystal Lake
Trichobilharzia stagnicolae	2016	Cercariae	Crystal Lake
Diplostomum sp. 4	2016	Cercariae	Higgins Lake
Apatemon sp. 1	2016	Cercariae	Higgins Lake
Plagiorchis sp	2016	Cercariae	Higgins Lake
Trichobilharzia stagnicolae	2016	Cercariae	Lake Leelanau
Physella ancillaria	2016	Snail	Crystal Lake
Unidentified Planorbidae	2016	Snail	Crystal Lake
Elimia livescens	2016	Snail	Higgins Lake
Campeloma decisum	2016	Snail	Higgins Lake
Stagnicola elodes	2016	Snail	Higgins Lake
Marstonia lustrica	2016	Snail	Higgins Lake
Unidentified Planorbidae	2016	Snail	Lake Leelanau

Stagnicola elodes	2016	Snail	Lake Leelanau
Physella gyrina	2016	Snail	Lake Leelanau
Pleurocera catenaria	2016	Snail	Lake Leelanau
Unidentified Planorbidae –	2016	Snail	Lake Leelanau
*Trichobilharzia stagnicolae f*rom Hatch-year common merganser brood	2016	Miracidia	Crystal Lake
Trichobilharzia stagnicolae from hatch-year common merganser brood	2016	Miracidia	Crystal Lake
Trichobilharzia stagnicolae from hatch-year common merganser	2016	Miracidia	Crystal Lake
*Trichobilharzia stagnicolae f*rom hatch-year common merganser	2016	Miracidia	Higgins Lake
Trichobilharzia stagnicolae from hatch-year common merganser	2016	Miracidia	Lake Leelanau
Dendritobilharzia sp. from hatch-year mallard	2016	Miracidia	Lake Leelanau
Schistosomatidae gen. sp from Canada goose.	2016	Miracidia	Lake Leelanau
T. physellae	2018	Cercariae	South Lake Leelanau
T. stagnicolae	2018	Cercariae	Walloon Lake
T. stagnicolae	2018	Cercariae	Walloon Lake
T. stagnicolae	2018	Cercariae	Elk Lake
T. stagnicolae	2018	Cercariae	Elk Lake
T. stagnicolae	2018	Cercariae	Elk Lake
A. brantae	2018	Cercariae	Lime Lake
T. szidati	2018	Cercariae	Lime Lake
T. stagnicolae	2018	Cercariae	Lime Lake
A. brantae	2018	Cercariae	Lime Lake

T. physellae	2018	Cercariae	Lake Charlevoix
T. stagnicolae	2018	Cercariae	North Lake Leelanau
T. stagnicolae	2018	Cercariae	North Lake Leelanau
T. stagnicolae	2018	Cercariae	North Lake Leelanau
T. physellae	2018	Cercariae	Walloon Lake
T. physellae	2018	Cercariae	Long Lake
Avian schistosomatid	2018	Cercariae	Long Lake
T. stagnicolae	2018	Cercariae	Elk Lake
T. stagnicolae	2018	Cercariae	Lake Skegemog
T. stagnicolae	2018	Cercariae	Lake Skegemog

Table 3.2 The qPCR Assay is Specific to Swimmer's Itch Causing Cercariae.

The qPCR assay was tested against a library of purified trematode DNA from across North America. Asterisks indicate swimmer's itch causing species. (+) indicates the target product amplified, while (−) indicates that no target was amplified.

Species	**qPCR result**
*Trichobilharzia stagnicolae**	**+**
*Trichobilharzia szidati**	**+**
Cotylurus sp.	**–**
Diplostomum baeri	**–**
Diplostomum huronense	**–**
Diplostomum indistinctum	**–**
Diplostomum sp. 1	**–**
Diplostomum sp. 2	**–**
Diplostomum sp. 3	**–**
Diplostomum sp. 8	**–**
Drepanocephalus auritus	**–**
Echinostoma caproni	**–**
Echinostoma trivolvis	**–**
Neodiplostomum americanum	**–**
Notocotylidae sp.	**–**
Haematoloechus sp.	**–**
Icthyocotylurus sp. 3	**–**

Ornithodiplostomum sp. 8	–
Plagiorchis sp.	–
Pseudopsilostoma varium	–

Table 3.3 Extraction efficiencies of the Qiagen DNeasy DNA extraction kit, and the Biomeme™ eDNA field extraction kit.

Trial	Loss (%)	Recovery (%)
Qiagen DNeasy		
1	99.99	0.01
2	99.2	0.8
3	99.9	0.1
4	98.9	1.1
5	98.7	1.3
6	92.2	7.8
7	92	8
8	99.7	0.3
9	99.7	0.3
10	98	2
11	98	2
12	92.8	7.2
13	94.3	5.7
14	92.9	7.1
15	92.9	7.1
Biomeme™ eDNA		
1	98.1	1.9
2	96.1	3.9
3	98.6	1.4
4	78.8	21
5	96.6	3.4
6	99	1
7	98.7	1.3
8	99.6	0.4

Table 3.4. Generalized linear model of environmental factors influencing cercariae concentrations.

Asterisk (*) denote statistical significance.

Main effects			***P* value**
Wind direction			<0.0001*
Lake			<0.0001*
Week			<0.0001*
Time of day (am/pm)			0.59
Parameter Estimates			
Parameter	**β**	**Confidence Interval**	***P* value**
Wind			
Onshore wind	0.89	0.23 – 1.5	0.008*
Alongshore wind	1.2	0.57 – 1.8	<0.0001*
Offshore wind	0.48	-0.24 – 1.2	0.19
Lake			
North Leelanau	-1.213	-1.7 – -0.7	<0.0001*
Lime Lake	-1.825	-2.3 – -1.3	<0.0001*
Higgins	-1.406	-0.77 – -0.72	<0.0001*
Glen Lake	-0.077	-0.47 – 0.14	0.70
South Leelanau	-0.045	-0.42 – 0.50	0.85
Crystal	0.045	-0.42 – 0.50	0.85
Week			
Week 1	-0.74	-1.5 – 0.05	0.07
Week 2	-0.58	-1.2 – 0.70	0.08
Week 3	-0.13	-0.75 – 0.50	0.70
Week 4	0.63	0.03 – 1.23	0.04*
Week 5	-0.12	-0.8 – 0.50	0.72
Week 6	-0.42	-1.2 – 0.30	0.25
Week 7	0.38	-0.43 – 1.2	0.36
Week 8	2.53	1.9 – 3.2	<0.0001*
Week 9	0.738	-0.58 – 0.9	0.07
Time of Day			
Morning (8am-12pm)	0.08	-0.20 – 0.36	0.60
Afternoon (12pm-8am)	0.08	-0.20 – 0.36	0.60

Table 3.5 Results of Snail Survey

Total snail numbers, and in brackets, the average snail densities (snails/ m^2) found in entire treatment area pre and post-treatment.

Treatment	**Snail genera**					
	Stagnicola	***Physella***	***Campeloma***	***Helisoma***	***Pleurocera***	***Marstonia***
Pre $CuSO_4$	14 (0.07)	122 (0.61)	89 (0.445)	95 (0.475)	150 (0.75)	55 (0.275)
Post $CuSO_4$	1 (0.005)	14 (0.07)	4 (0.02)	22 (0.11)	307 (1.535)	10 (0.05)
% Change	-92.86%	-88.52%	-95.51%	-76.84%	104.67%	-81.82%

Table 3.6 Number of cercariae (per 25L) sample as determined by qPCR, pre and post-treatment.

			Site		
Date	**Outside N**	**Outside S**	**Inside A**	**Inside B**	**Inside C**
18-06-18	0	0	0	3.15	0
18-06-18	0	0	0	0	0
18-06-21	305.16	N/A	88.64	26.76	4.71
18-06-22	0	0	0.74	4.52	4.47
18-06-23	0.69	0	26.9	0	0
18-06-25	Copper sulfate application				
18-06-28	0	0	26.33	2.65	2.36
18-06-29	0.92	0	0.51	16.51	0
18-07-07	0	100.51	0.85	21.97	96.35
18-07-13	N/A	18.68	5.22	59.67	30.76
18-07-17	0	5.62	12.17	18.16	1.2
18-07-27	4.31	1.12	0	3.53	1.59

Table 3.7 Specificity of the species-specific diagnostics.

Each assay was tested for cross reactivity against purified DNA extracts of other trematode species. *Non-avian schistosome genera tested were: *Australapatemon* sp*., Cotylurus* sp., *Diplostomum* sp., *Notocotylus* sp., *Plagiorchis* sp., *Proterometra* sp., and *Uvulifer* sp.

Target \ Assay	*T. stagnicolae*	*A. brantae*	*T. szidati*	*T. physellae*	*P. trivolvis cercariae*	*Non-avian schistosomes* *
T. stagnicolae	+	-	-	-	-	-
A. brantae	-	+	-	-	-	-
T. szidati	-	-	+	-	-	-
T. physellae	-	-	-	+	-	-
P. trivolvis cercariae	-	-	-	-	+	-

Table 3.8. Snail genera observed in each lake.

Snail \ Lake	Charlevoix	Elk	Glen	South Leelanau	North Leelanau	Lime	Long	Skegemog	Walloon
S. emgarinata	+	+	+	+	+	+	+	+	+
P. parkeri	+	+	+	+	+	+	+	+	+
Lymnaea sp.						+			+
Gyraulus sp.	+	+	+		+	+	+		+
Pleurocera sp.	+	+	+	+	+	+	+	+	+
P. trivolvis	+	+	+	+	+	+	+	+	+
Campeloma decisum.			+	+				+	+
Marstonia lustrica	+		+	+	+	+	+	+	+
Viviparus sp.							+		

Table 3.9 Waterfowl densities by lake

Complete shoreline waterfowl surveys conducted in mid-July determined the definitive host diversity on each lake. Data are in birds/shoreline mile. Asterisks (*) indicate lakes were common mergansers are actively relocated.

Bird / Lake	Mallard	Canada Goose	Mute Swan	Common Merganser	Hooded Merganser	Red Breasted Merganser
Lake Charlevoix	7.08	2.78	0.10	1.12	0.30	0.00
Elk Lake	5.25	1.54	0.14	0.54	0.00	0.21
Big Glen Lake	8.70	0.65	0.37	0.28*	0.00	0.00
Little Glen Lake	9.22	1.25	0.00	4.22*	0.00	0.00
North Lake Leelanau	6.27	0.87	0.40	0.07*	0.00	0.00
South Lake Leelanau	14.12	1.72	0.00	0.00	0.08	0.00
Lime Lake	8.33	0.95	0.00	0.48*	0.00	0.00
Long Lake	6.47	0.66	0.00	0.00	0.06	0.00
Lake Skegemog	2.73	1.20	0.93	0.00	0.00	0.00
Walloon Lake	1.61	1.34	0.00	1.80	0.00	0.00

Table 3.10 Snail densities in 2019

Snail density and infection status was assessed in 2019 in parallel with water sampling efforts.

	S. emarginata	*P. parkeri*	*Gyraulus sp.*	*Pleurocera sp.*	*P. trivolvis*	Campeloma sp.	Marstonia sp.	Totals
North Lake Leelanau								
Snails Examined	856	14	39	105	70	n/a	5	**1089**
Density (sq.m.)	10.04	0.12	0.5	2.1	0.32	n/a	0.1	**13.18**
Schistosome Infections	5	0	0	n/a	1	n/a	n/a	**6**
% Schistosome infections	0.58%	0.00%	0.00%	n/a	1.43%	n/a	n/a	**1.01%**
Trematode infections	319	2	4	n/a	4	n/a	n/a	**329**
% trematode infections	37.27%	14.29%	10.26%	n/a	5.71%	n/a	n/a	**16.88%**
South Lake Leelanau								
Snails Examined	82	157	456	221	331	1	46	**1295**
Density (sq.m.)	0.04	1.02	6.18	4.42	1.6	0.02	0.92	**14.2**
Schistosome Infections	0	2	0	n/a	72	n/a	n/a	**74**
% Schistosome infections	0.00%	1.27%	0.00%	n/a	21.75%	n/a	n/a	**11.51%**
Trematode infections	35	9	9	n/a	133	n/a	n/a	**186**
% trematode infections	42.68%	5.73%	1.97%	n/a	40.18%	n/a	n/a	**18.11%**
Glen Lake								
Snails Examined	1045	385	77	604	301	1	2	**2415**
Density (sq.m.)	2.77	2.79	0.62	6.16	1.55	0.01	0.02	**13.92**
Schistosome Infections	2	0	0	n/a	1	n/a	n/a	**3**
% Schistosome infections	0.19%	0.00%	0.00%	n/a	0.33%	n/a	n/a	**0.26%**
Trematode infections	97	9	1	n/a	31	n/a	n/a	**138**
% trematode infections	9.28%	2.34%	1.30%	n/a	10.30%	n/a	n/a	**5.80%**
Walloon Lake								
Snails Examined	501	123	42	218	84	10	6	**984**
Density (sq.m.)	1.79	0.32	0.15	2.18	1.79	0.1	0.04	**6.37**
Schistosome Infections	5	2	0	n/a	50	n/a	n/a	**57**
% Schistosome infections	1.00%	1.63%	0.00%	n/a	59.52%	n/a	n/a	**20.72%**
Trematode infections	36	26	3	n/a	61	n/a	n/a	**126**
% trematode infections	7.19%	21.14%	7.14%	n/a	72.62%	n/a	n/a	**27.02%**

3.5 Figures

Figure 3.1 Lifecycles of avian schistosomes cycling at lakes in northern Michigan.

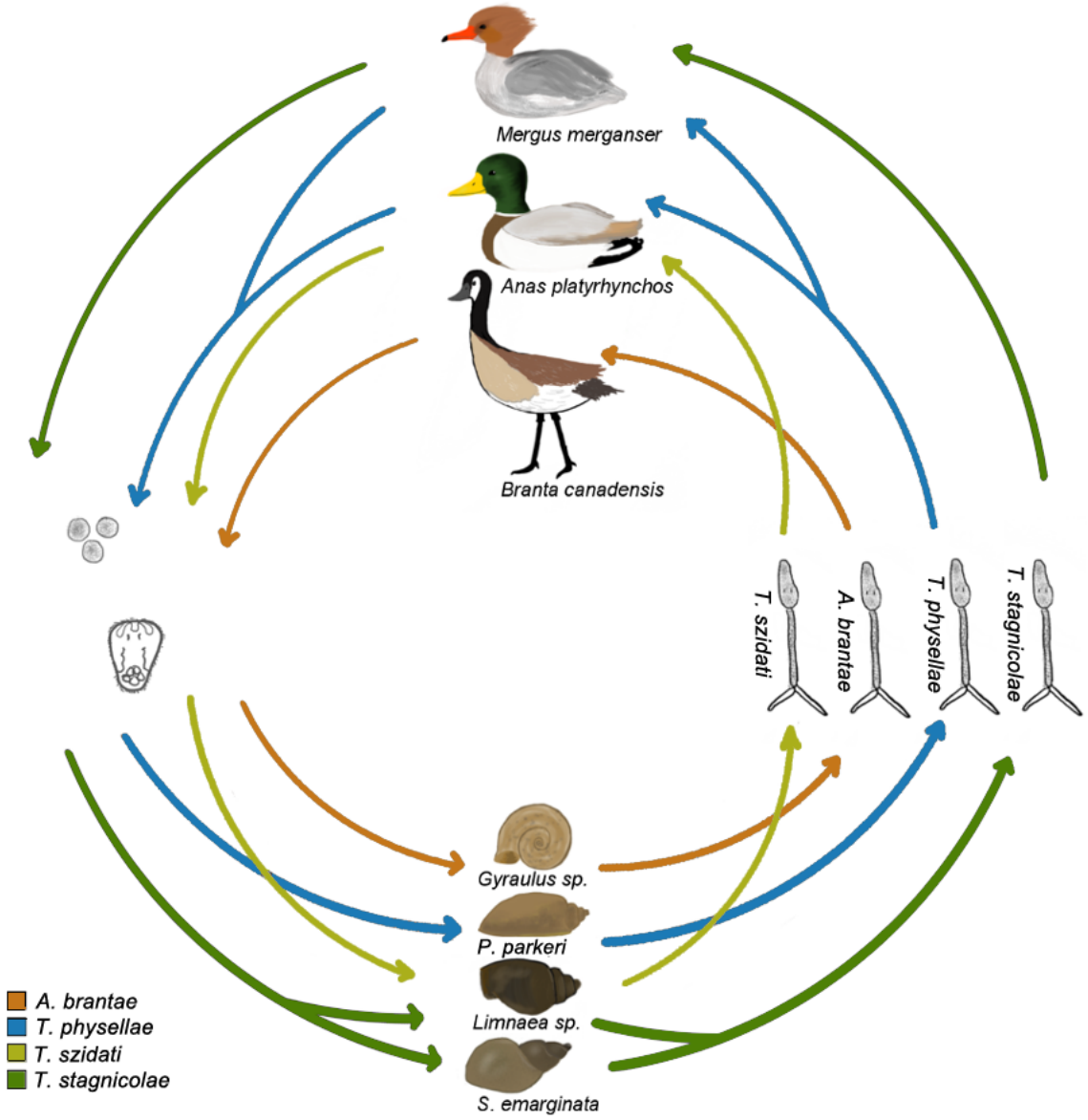
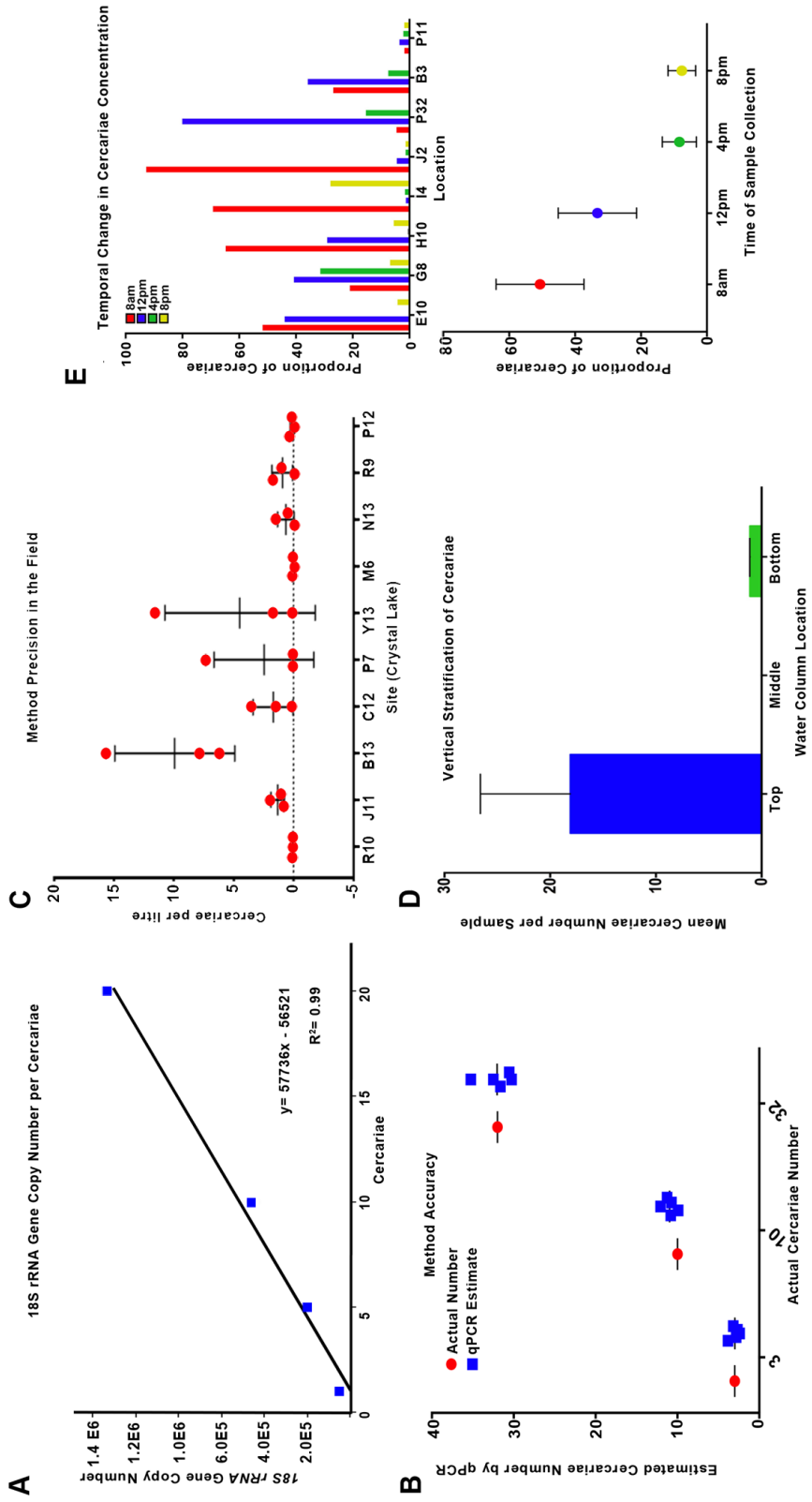
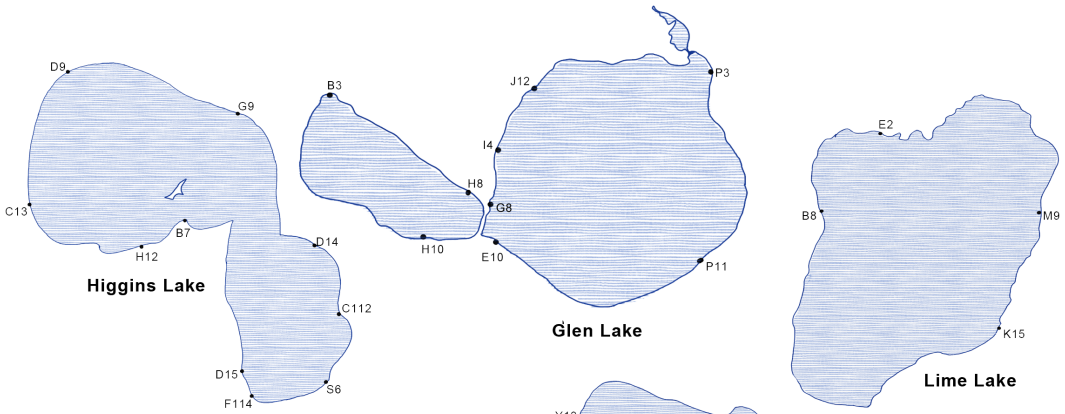


Figure 3.2 . qPCR Method Validation.

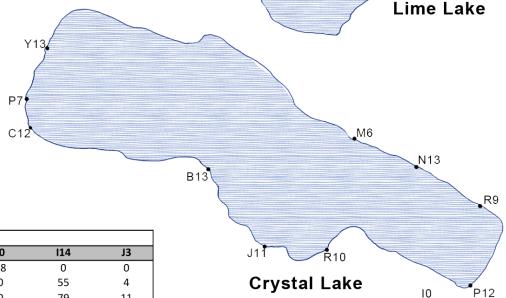


A. Cercariae standard curve used to derive conversion equation for the conversion of qPCR copy number to number of cercariae. B. Accuracy of the qPCR method. Actual number of cercariae, hand counted via microscopy, and the qPCR estimate of cercariae based on five replicate qPCR reactions. C. Precision of the method in the field. Replicate 25L water samples were analyzed at different sites on Crystal lake in 2016. Error bars represent standard deviation around the mean. D. Vertical stratification of cercariae concentrations. Top = 0-30cm from surface, Middle = 50-100cm Bottom = 100-150cm. E. Daily changes in cercariae concentration. Top: Changes in proportion of cercariae at each site from 8am to 8pm. Bottom: Average proportion of cercariae of all sites at each time point.

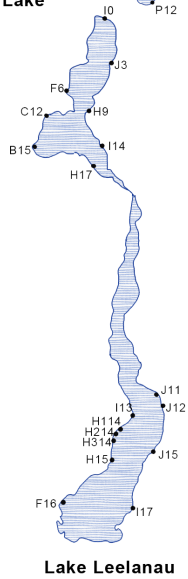
Figure 3.3 Monitoring results.



Lime Lake					
	Site	B8	E2	K15	M9
17-Jun	Week 1	9.1	1.2	1.8	2.3
23-Jun	Week 2	50	48	0	1.5
28-Jun	Week 3	28	0	0	0
03-Jul	Week 4	0	1.1	1.6	1.4
10-Jul	Week 5	20	0	0	1.1
20-Jul	Week 6		0	0	2.8
24-Jul	Week 7	120	84	12	2.4
31-Jul	Week 8	12	0	0	66
09-Aug	Week 9	10	1.3	1.2	0



North Lake Leelanau									
	Site	B15	C12	F6	H17	H9	I0	I14	J3
27-Jun	Week 3	16	4	17	2	0	38	0	0
07-Jul	Week 4	2	5	188	9	8	0	55	4
13-Jul	Week 5	0	3	2	0	0	0	79	11
20-Jul	Week 6	3	22	2	0	30	11	7	7
24-Jul	Week 7	23	1	33	28	68	87	20	3
31-Jul	Week 8	2	0	0	44	11	0	29	8
07-Aug	Week 9	0	4		7	0	0	1	0
29-Jun	Week 5	0	1	0	0	0	0	0	0
13-Jul	Week 6	1	1	0	0	0	1	11	0
26-Jul	Week 7	10	3	0	31	0	87	4	0
02-Aug	Week 8	2	0	0	0	0	1	I	29
04-Aug	Week 9	0	2	0	2	1	0	5	0



Glen Lake										
	Site	B3	E10	G8	H10	H8	I4	J2	P11	P3
15-Jun	Week 1	200	41	2	17	41		15	1	1
23-Jun	Week 2	0	0	3	3	2	9	1	15	10
26-Jun	Week 3	44	26	46	180	110	0	42	130	2
03-Jul	Week 4	2	170	0	1500	33		19	2	130
10-Jul	Week 5	7	35	2	110	32	0	48	24	210
18-Jul	Week 6	25	2	14	4	33	0	18	2	200
03-Aug	Week 8	350	200	220	390		500	78	120	170
08-Aug	Week 9	1	0	2	28		40	20	2	9

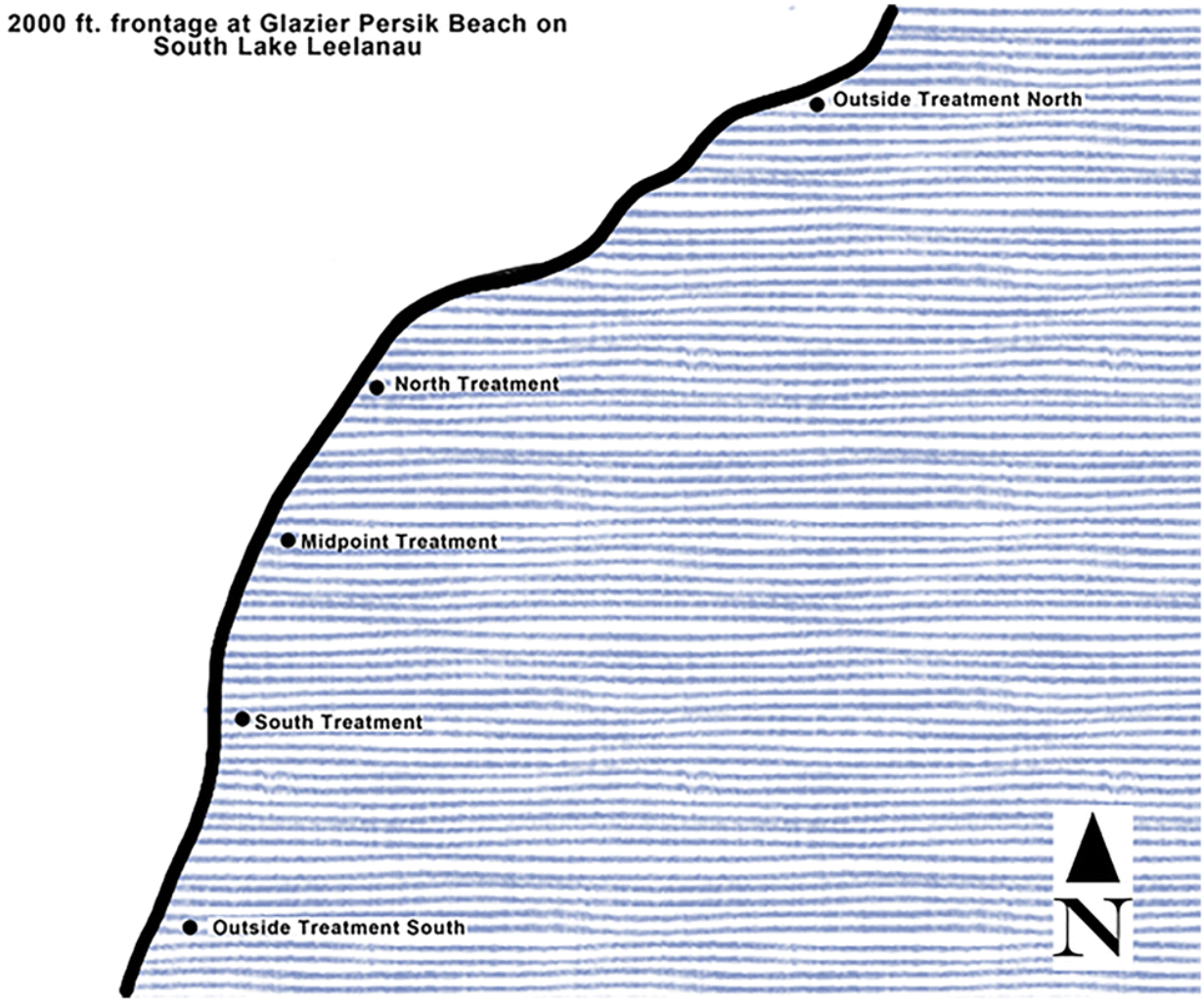
South Lake Leelanau											
	Site	F16	H114	H15	H214	H314	I13	I17	J11	J12	J15
17-Jun	Week 1	41	0	0	3	0	0	0	52	35	1
21-Jun	Week 2	11	110	0	36	9	460	230	12	37	170
28-Jun	Week 3	0	29	0	2	3	0	680	6	0	0
07-Jul	Week 4	0	2	200	26	140	0	17	43	70	2
13-Jul	Week 5	8	5	15	52	28	0	1	0	0	0
17-Jul	Week 6	5	12	7	15	1	0	10	34	8	0
27-Jul	Week 7	2	0	1	4	2	4	10	8	24	2
02-Aug	Week 8	270	820	200	380	170	500	3200	854	43	230
07-Aug	Week 9	200		1			7	0	11	7	1

Crystal Lake											
	Site	Y13	P12	B13	C12	M6	N13	J11	R9	R10	P12
28-Jun	Week 5	0	0	0		0	2	0	17		0
07-Jul	Week 6	47	1	32	27	4	0	17	1	1	185
19-Jul	Week 7	15	5	0	150	83	220	244	43	1	532
04-Aug	Week 8	2	7	66	1	0	0	0	0	1	0

Higgins Lake											
	Site	C112	D15	G9	F114	B7	D14	D9	S6	H12	C13
08-Jun	Week 1	0	0	0	0	0	0	17	0	0	0
16-Jun	Week 2	1	0	1	0	0	1	1	1	43	1
28-Jun	Week 3	0	0	7	0	3	2	2	10	0	0
11-Jul	Week 4	0	44	1	3	2	0	0	0	1	1
23-Jul	Week 5	185	0	11	1	0	0	0	73	0	0

Lakes in northern Michigan were monitored weekly throughout 2016 and 2017. Results are in cercariae/25L.

Figure 3.4 Water sampling sites.



The North, Midpoint and South treatment sites were treated with CuSO4 while the two outside sites were control areas. Water samples were collected at each of the five sites pre and post treatment

Figure 3.5 Mean number of cercariae found in waters samples inside and outside of the treatment area both before and after treatment as found by qPCR.

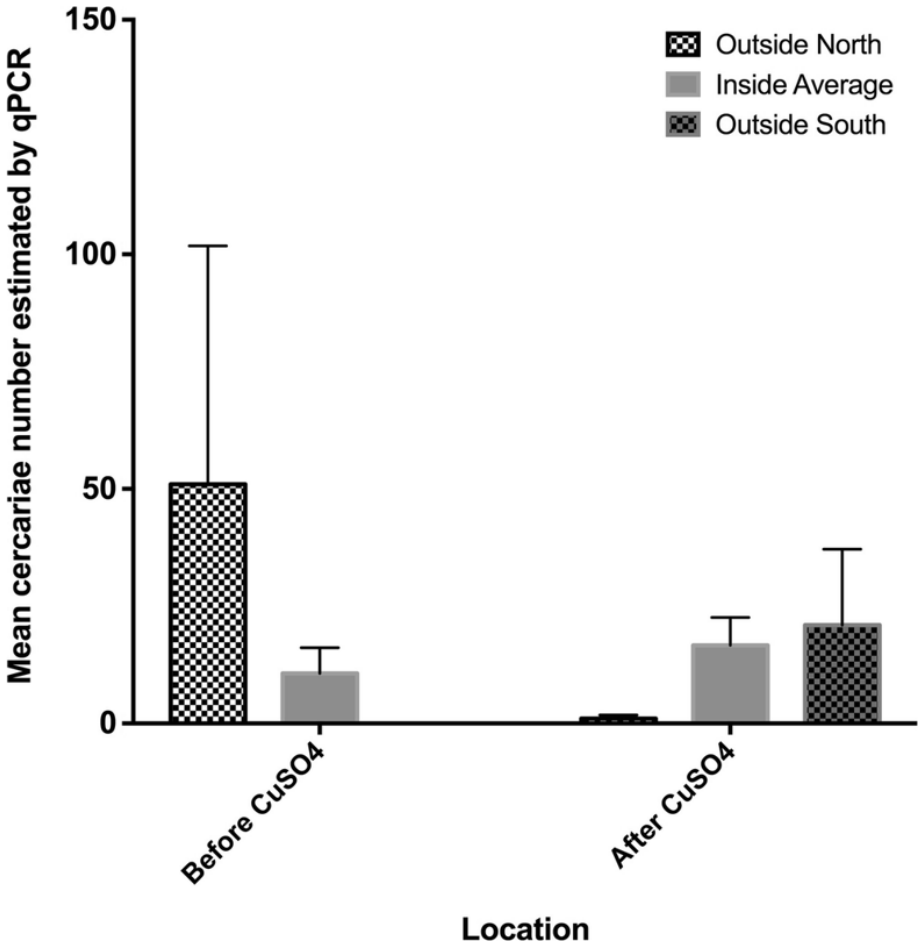


Figure 3.6 Number of cercariae per 25L sample as estimated by qPCR, both pre and post-treatment.

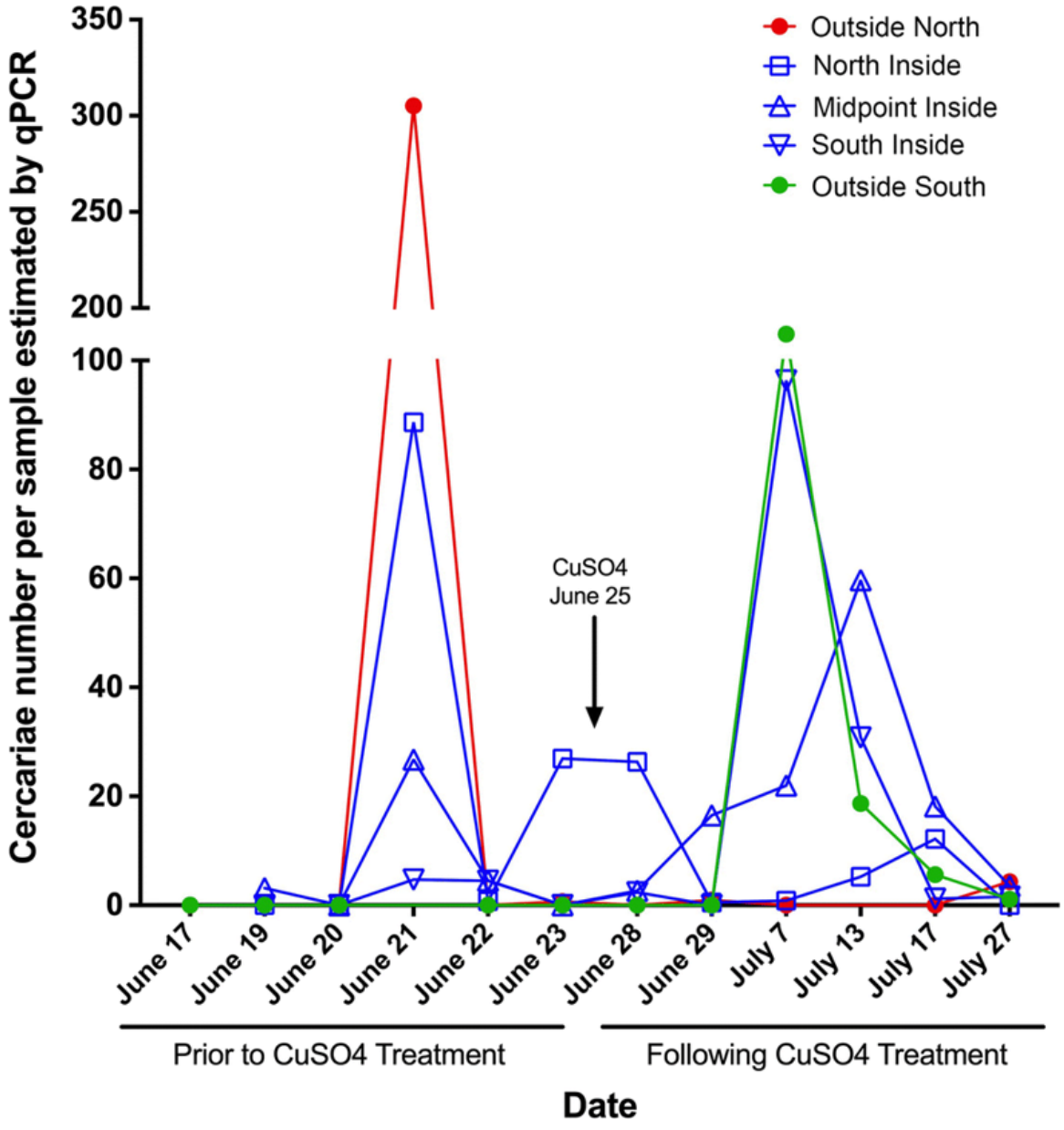
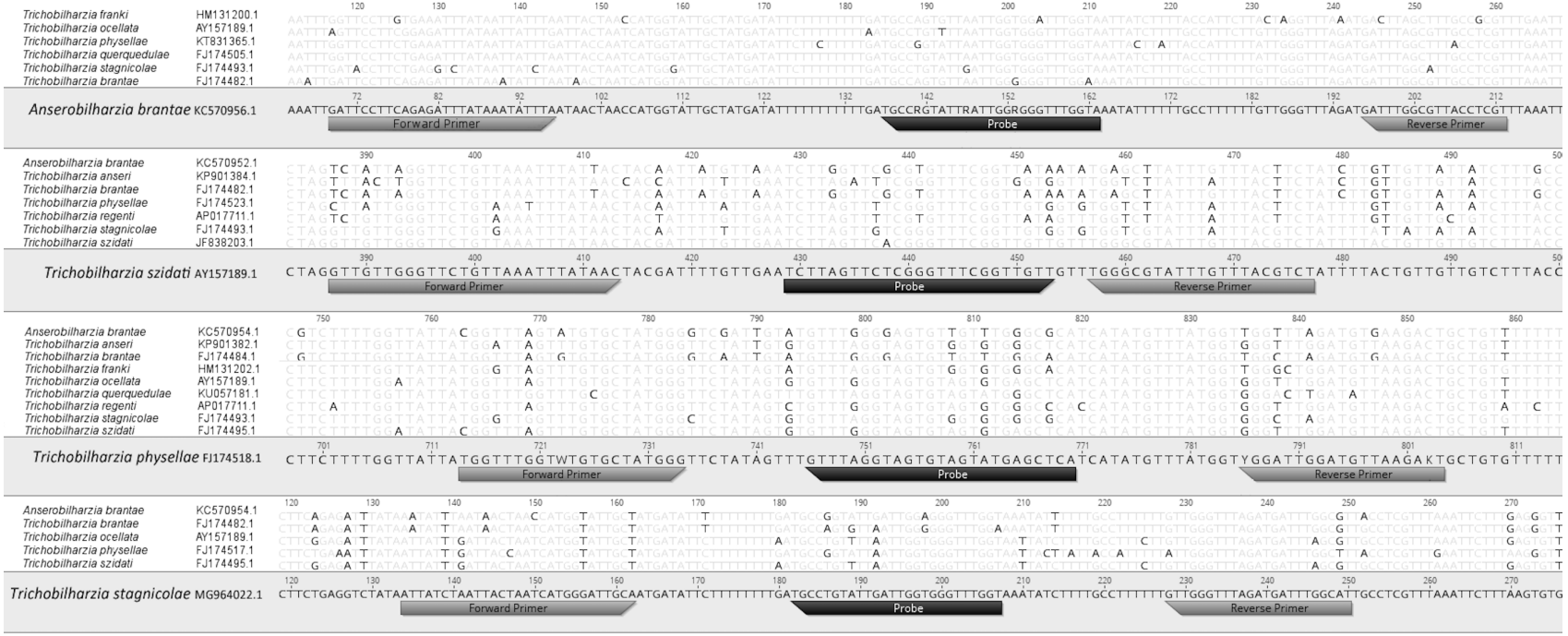


Figure 3.7. Primer and probe locations for each assay.


Trichobilharzia franki HM131200.1
Trichobilharzia ocellata AY157189.1
Trichobilharzia physellae KT831365.1
Trichobilharzia querquedulae FJ174505.1
Trichobilharzia stagnicolae FJ174493.1
Trichobilharzia brantae FJ174482.1
Anserobilharzia brantae KC570956.1
Forward Primer
Probe
Reverse Primer
Anserobilharzia brantae KC570952.1
Trichobilharzia anseri KP901384.1
Trichobilharzia brantae FJ174482.1
Trichobilharzia physellae FJ174523.1
Trichobilharzia regenti AP017711.1
Trichobilharzia stagnicolae FJ174493.1
Trichobilharzia szidati JF838203.1
Trichobilharzia szidati AY157189.1
Forward Primer
Probe
Reverse Primer
Anserobilharzia brantae KC570954.1
Trichobilharzia anseri KP901382.1
Trichobilharzia brantae FJ174484.1
Trichobilharzia franki HM131202.1
Trichobilharzia ocellata AY157189.1
Trichobilharzia querquedulae KU057181.1
Trichobilharzia regenti AP017711.1
Trichobilharzia stagnicolae FJ174493.1
Trichobilharzia szidati FJ174495.1
Trichobilharzia physellae FJ174518.1
Forward Primer
Probe
Reverse Primer
Anserobilharzia brantae KC570954.1
Trichobilharzia brantae FJ174482.1
Trichobilharzia ocellata AY157189.1
Trichobilharzia physellae FJ174517.1
Trichobilharzia szidati FJ174495.1
Trichobilharzia stagnicolae MG964022.1
Forward Primer
Probe
Reverse Primer

Alignments of the CO1 gene regions with the primer and probe locations for each assay.

Figure 3.8. Abundance of cercariae by sampling site. Water samples were obtained in mid-June and cercrariae abundance was determined using the pan-avian schistosomes qPCR.

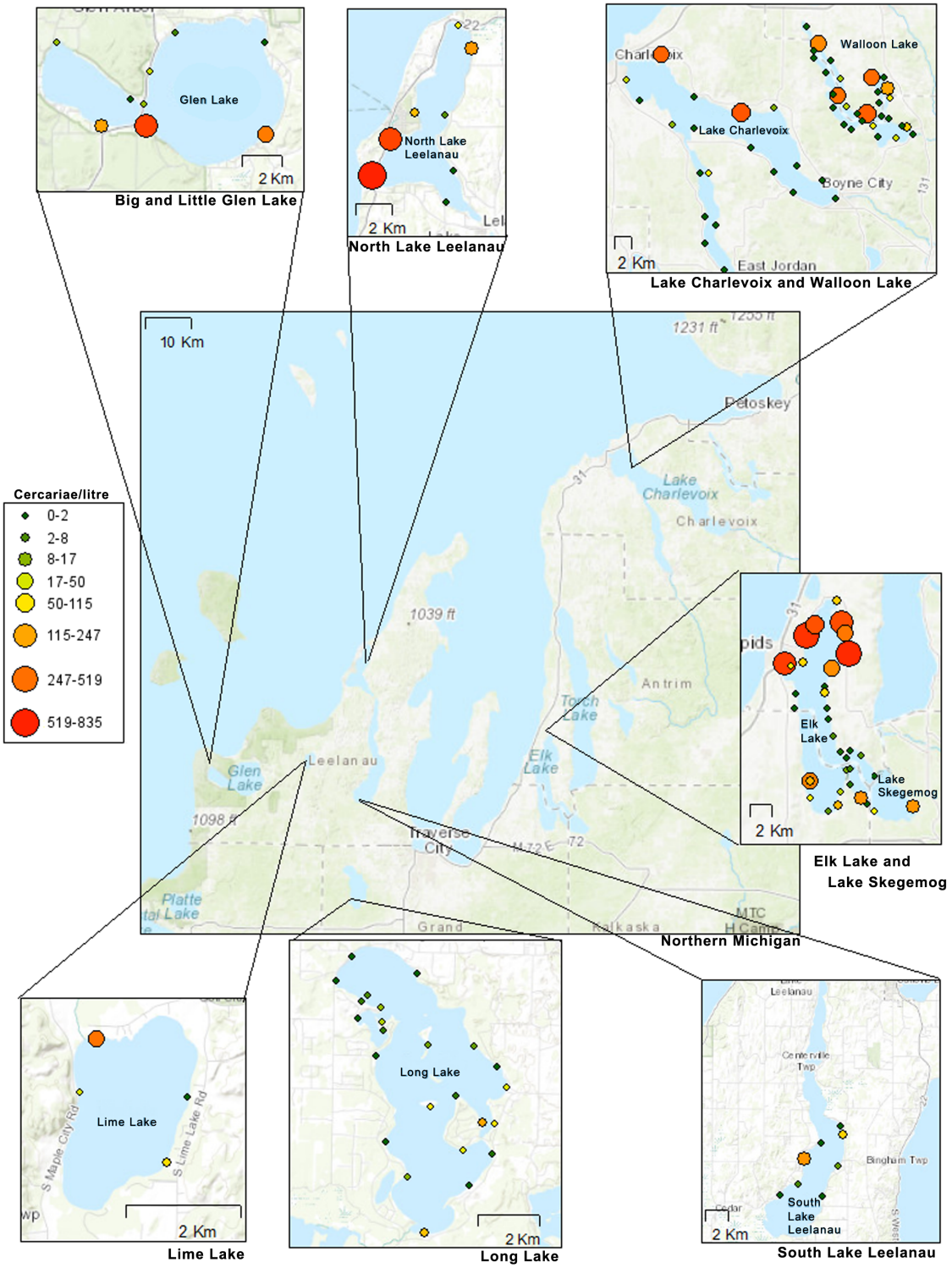
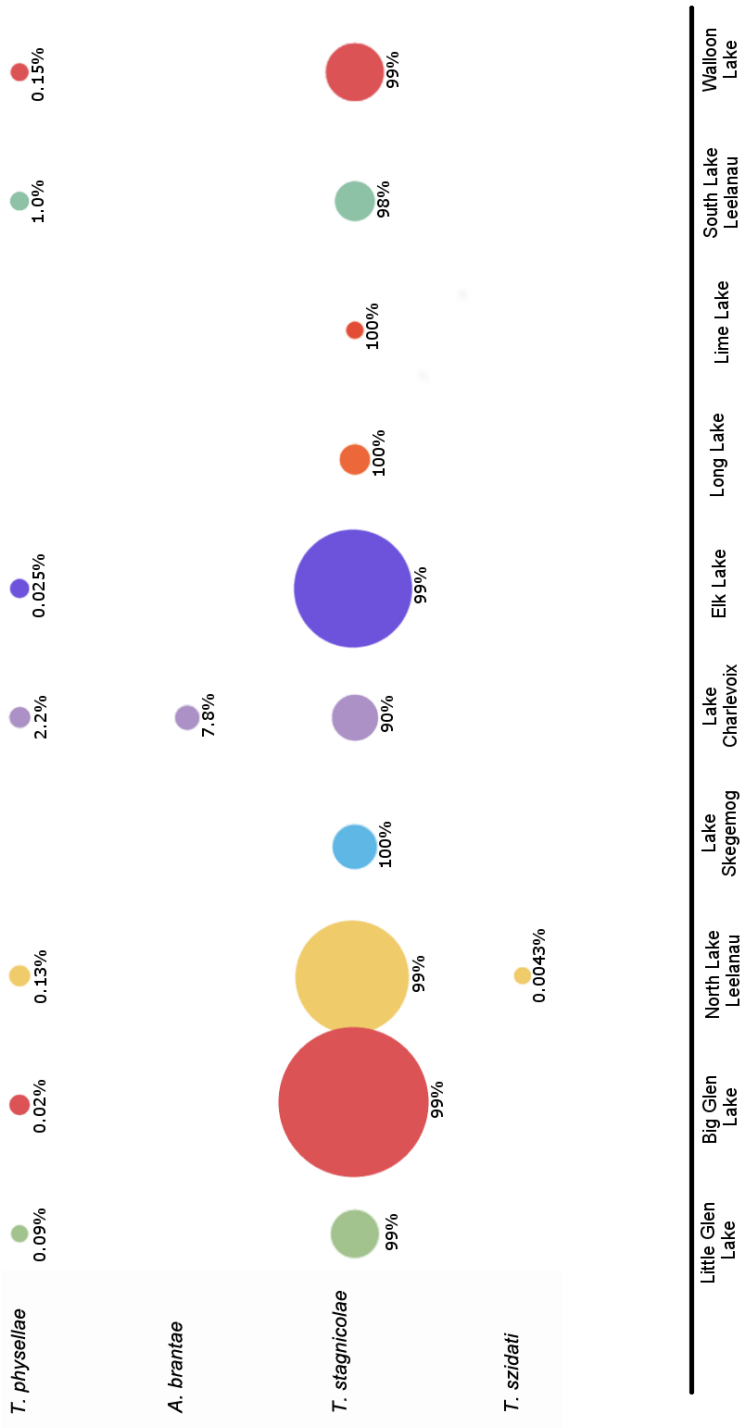
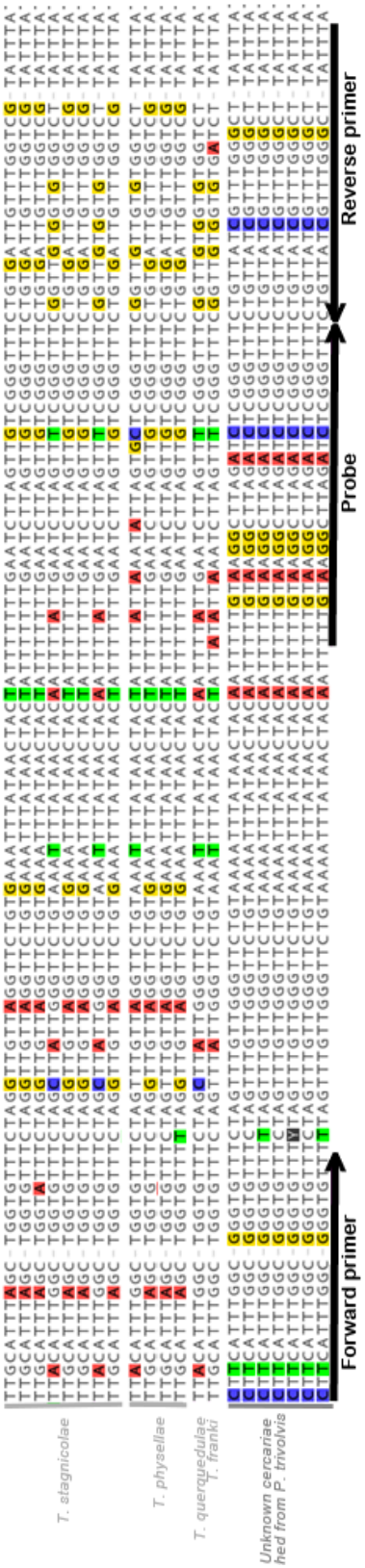


Figure 3.9 . Percent contribution *of T. stagnicolae, T. szidati, T. physellae* and *A. brantae* species to each lake.



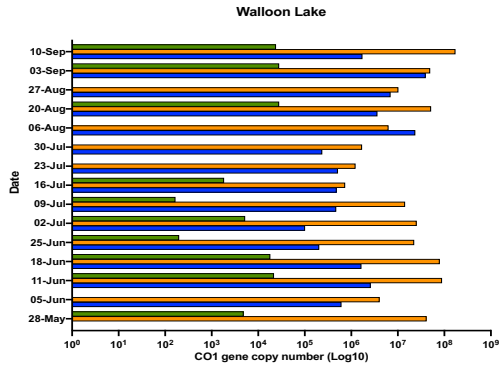
Water samples from different locations and dates were tested using the species-specific qPCR assay results were pooled by lake to understand the relative contribution overall of each species to each lake. The percent contribution (based on gene copy number) of each species was calculated. Diameters of the circles represent the percent contribution of the species both at a single lake, and across all lakes.

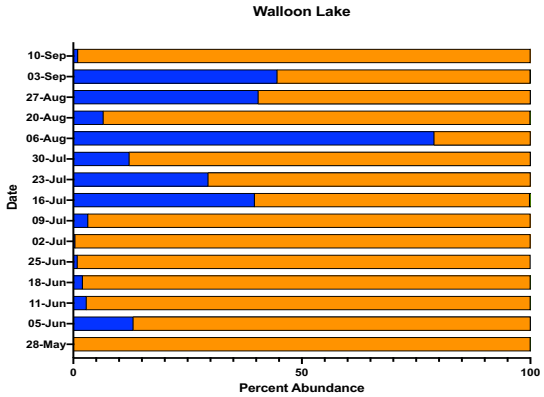
Figure 3.10 Primer and Probe locations for the *P. trivolvis* cercariae assay.

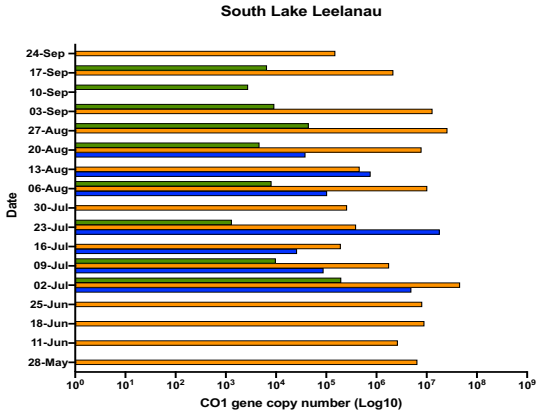


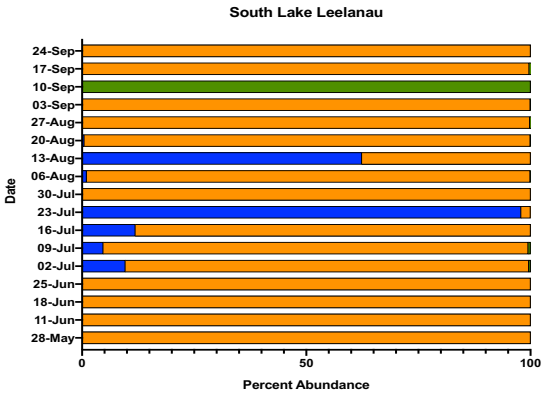
Alignment of the *P. trivolvis* cercariae CO1 region against the CO1 region of phylogenetically related species. Sequence dissimilarities are highlighted, and primer and probe sequences for the species-specific assay are indicated.

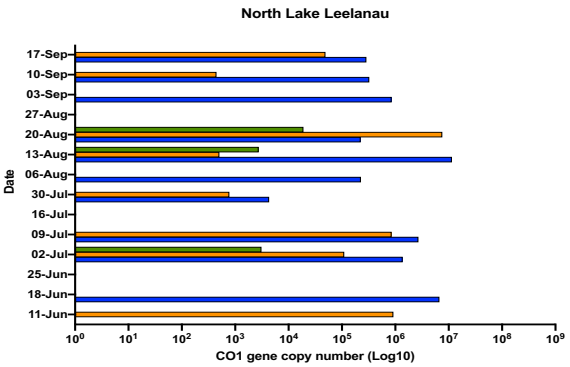
Figure 3.11 Longitudinal assessment of species contribution to four lakes in northern Michigan.

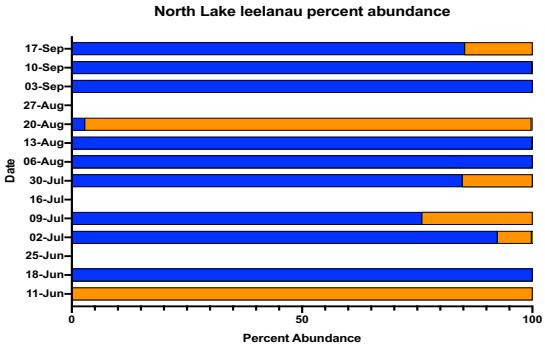

Walloon Lake
10-Sep
03-Sep
27-Aug
20-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
05-Jun
28-May
Date
CO1 gene copy number (Log10)

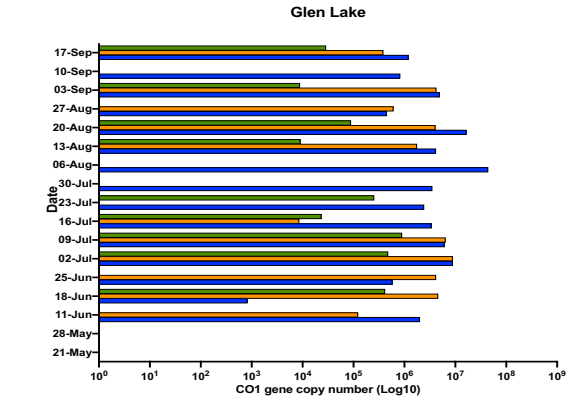

Walloon Lake
10-Sep
03-Sep
27-Aug
20-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
05-Jun
28-May
Date
Percent Abundance

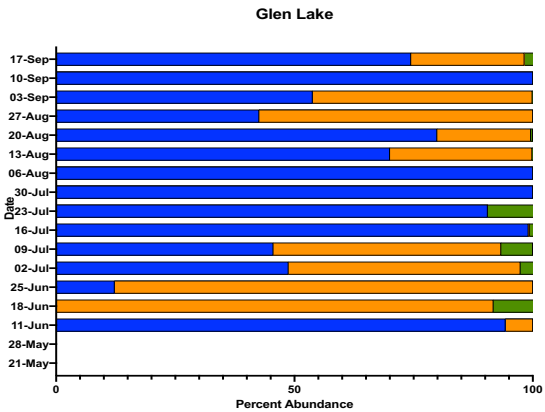

South Lake Leelanau
24-Sep
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
28-May
Date
CO1 gene copy number (Log10)


South Lake Leelanau
24-Sep
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
28-May
Date
Percent Abundance


North Lake Leelanau
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
Date
CO1 gene copy number (Log10)


North Lake leelanau percent abundance
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
Date
Percent Abundance

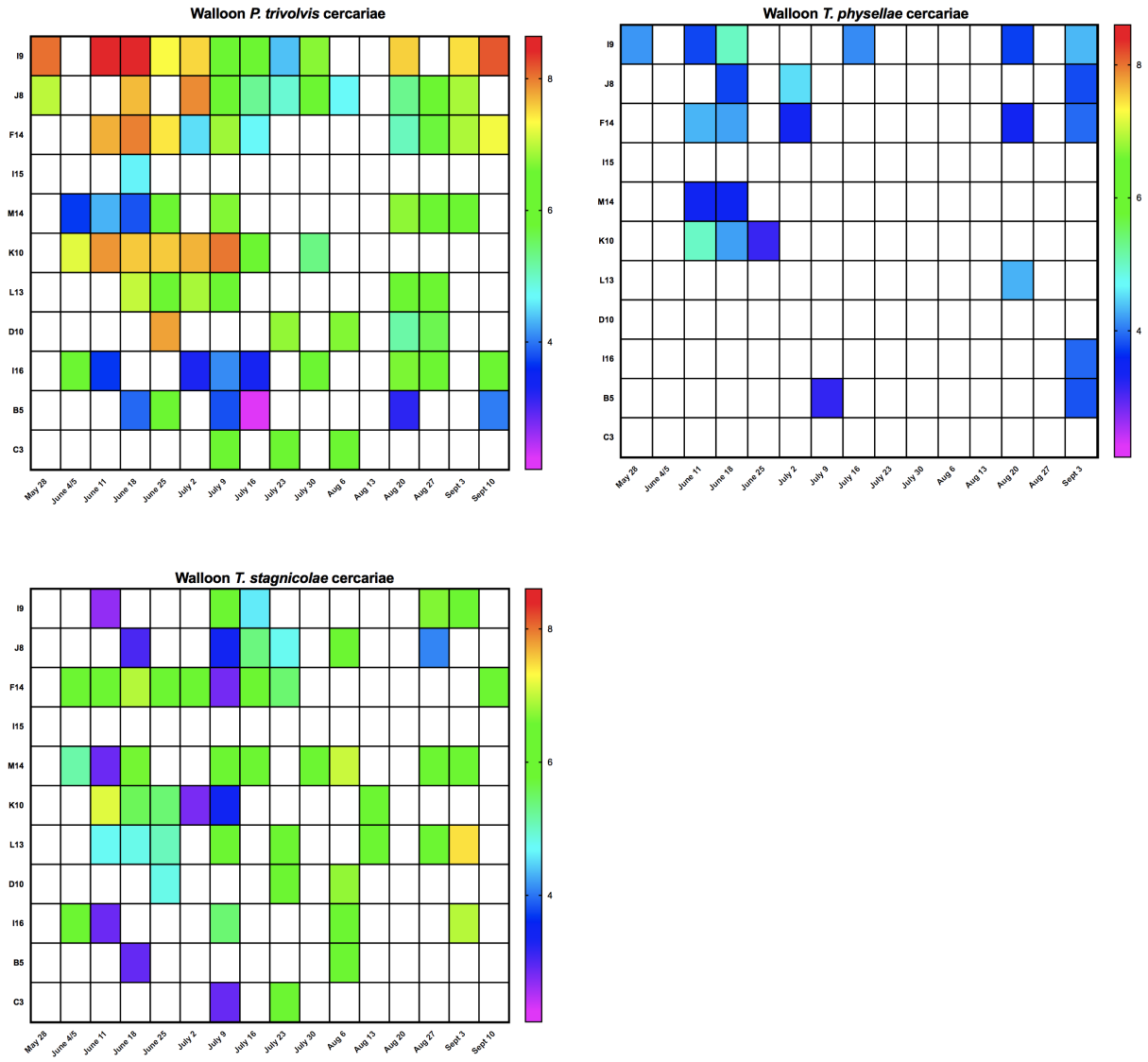

Glen Lake
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
28-May
21-May
Date
CO1 gene copy number (Log10)

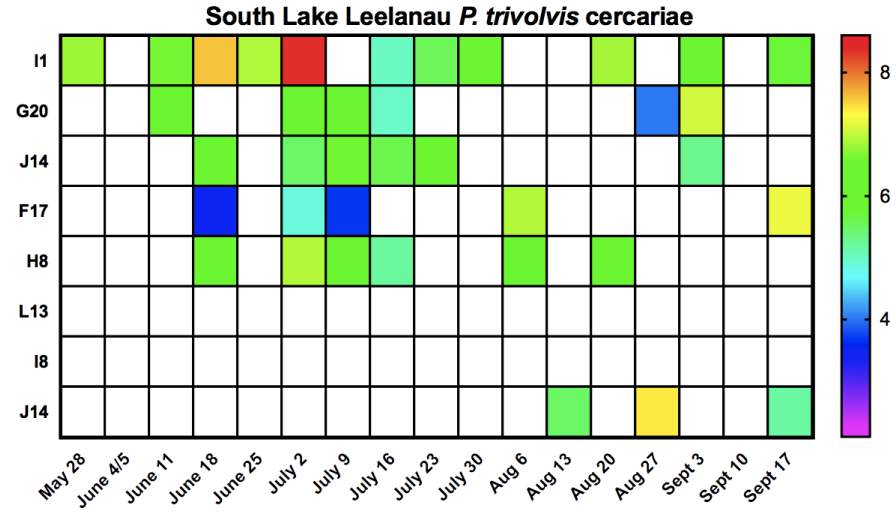

Glen Lake
17-Sep
10-Sep
03-Sep
27-Aug
20-Aug
13-Aug
06-Aug
30-Jul
23-Jul
16-Jul
09-Jul
02-Jul
25-Jun
18-Jun
11-Jun
28-May
21-May
Date
Percent Abundance

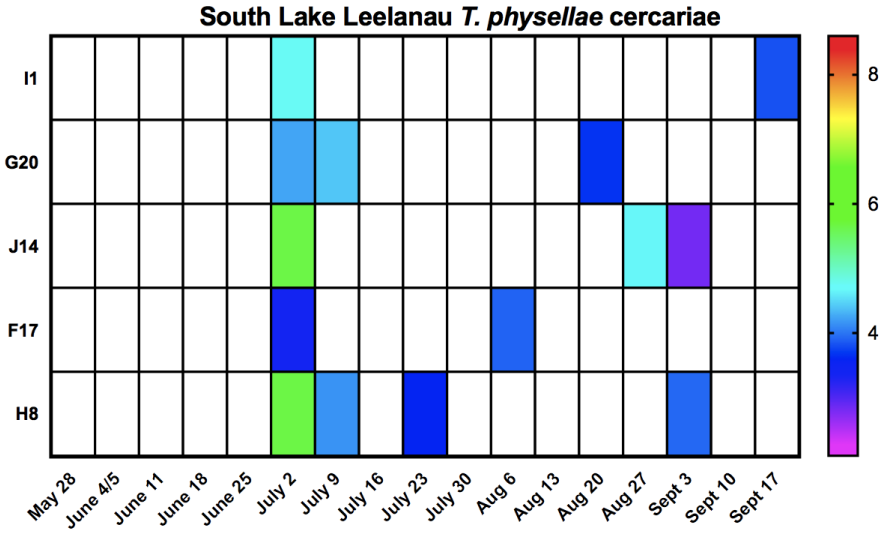
All sample sites across the lake were averaged to determine the average cercariae per litre across the lake on each sample date. Figures on the right show the average CO1 gene copy number (Log_{10} scale) as determined by the *P. trivolvis* qPCR assay for *T. stagnicolae* (blue), *T. physellae* (green), *and P. trivolvis* avian schistosomes (orange). *T. szidati* and *A. brantae* source-tracking was also performed but not found in any of the samples from these four lakes. Left figures show the same data but converted to a percent abundance, which better visualizes the relative contribution of each species to the lake on each date.

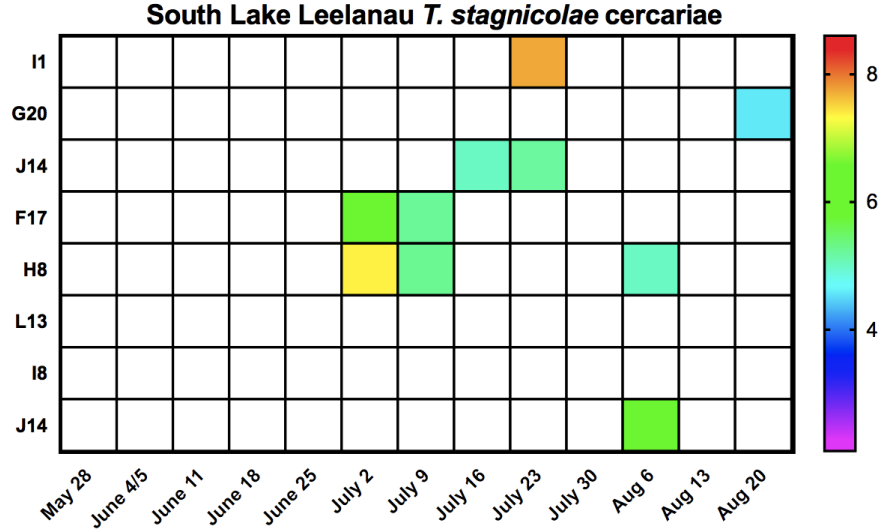
Figure 3.12 Site assessment of species specific source-tracking assays over time.

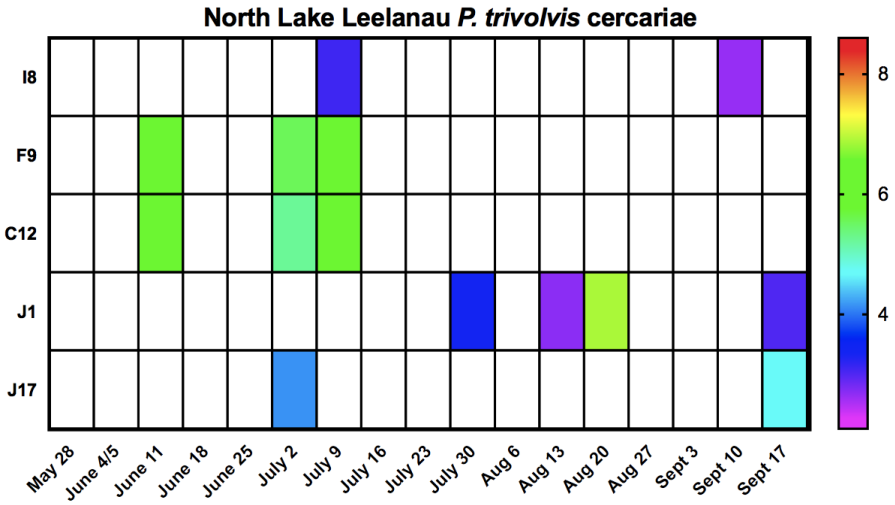
The x-axis shows the sample dates, while the y-axis lists the sample sites.

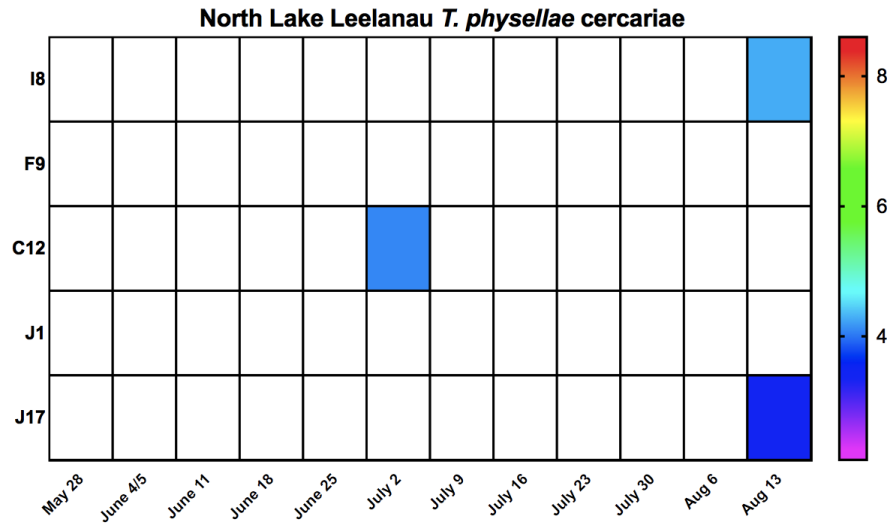



South Lake Leelanau P. trivolvis cercariae
I1
G20
J14
F17
H8
L13
I8
J14
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
Aug 6
Aug 13
Aug 20
Aug 27
Sept 3
Sept 10
Sept 17
8
6
4


South Lake Leelanau T. physellae cercariae
I1
G20
J14
F17
H8
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
Aug 6
Aug 13
Aug 20
Aug 27
Sept 3
Sept 10
Sept 17
8
6
4


South Lake Leelanau T. stagnicolae cercariae
I1
G20
J14
F17
H8
L13
I8
J14
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
Aug 6
Aug 13
Aug 20
8
6
4


North Lake Leelanau *P. trivolvis* cercariae
I8
F9
C12
J1
J17
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
Aug 6
Aug 13
Aug 20
Aug 27
Sept 3
Sept 10
Sept 17
8
6
4


North Lake Leelanau *T. physellae* cercariae
I8
F9
C12
J1
J17
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
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Aug 6
Aug 13
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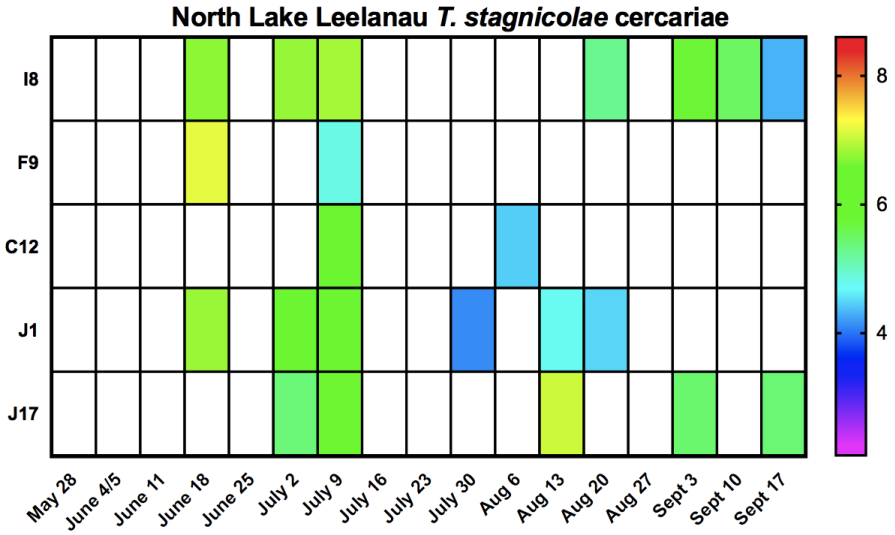
North Lake Leelanau *T. stagnicolae* cercariae
I8
F9
C12
J1
J17
May 28
June 4/5
June 11
June 18
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July 2
July 9
July 16
July 23
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Aug 27
Sept 3
Sept 10
Sept 17
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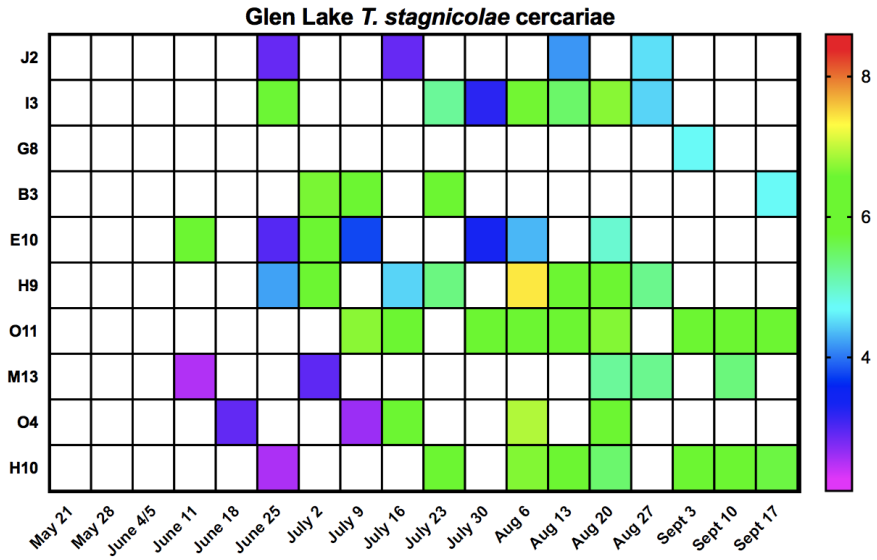
4


Glen Lake T. stagnicolae cercariae
J2
I3
G8
B3
E10
H9
O11
M13
O4
H10
May 21
May 28
June 4/5
June 11
June 18
June 25
July 2
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July 16
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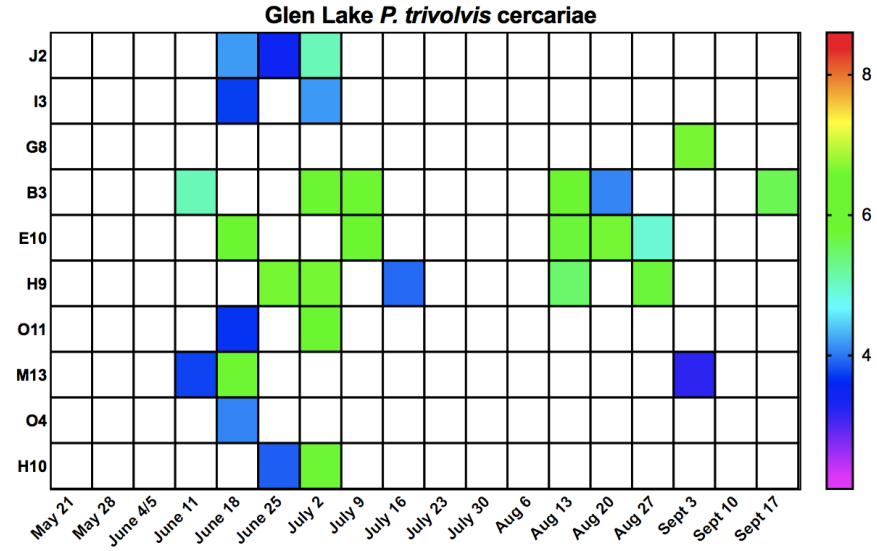
Aug 6
Aug 13
Aug 20
Aug 27
Sept 3
Sept 10
Sept 17
8
6
4


Glen Lake P. trivolvis cercariae
J2
I3
G8
B3
E10
H9
O11
M13
O4
H10
May 21
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
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Sept 3
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Sept 17
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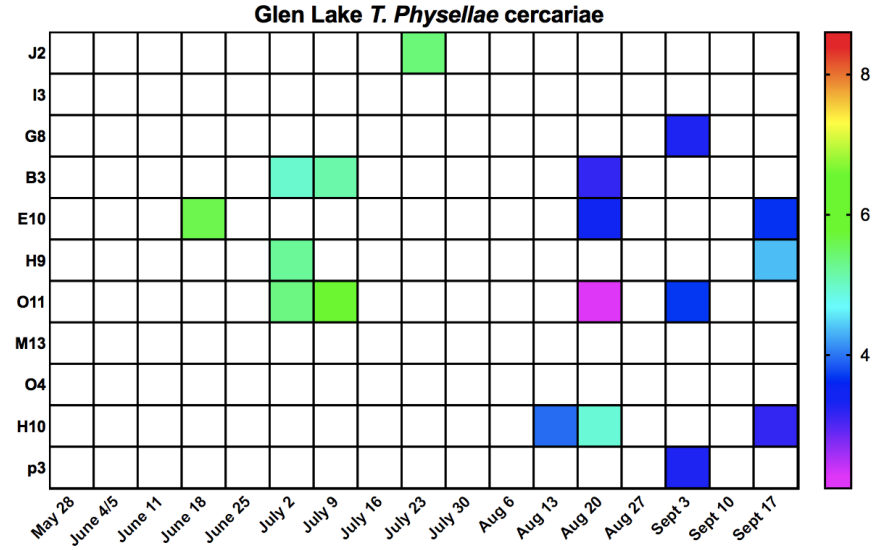
4


Glen Lake T. Physellae cercariae
J2
I3
G8
B3
E10
H9
O11
M13
O4
H10
p3
May 28
June 4/5
June 11
June 18
June 25
July 2
July 9
July 16
July 23
July 30
Aug 6
Aug 13
Aug 20
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Sept 3
Sept 10
Sept 17
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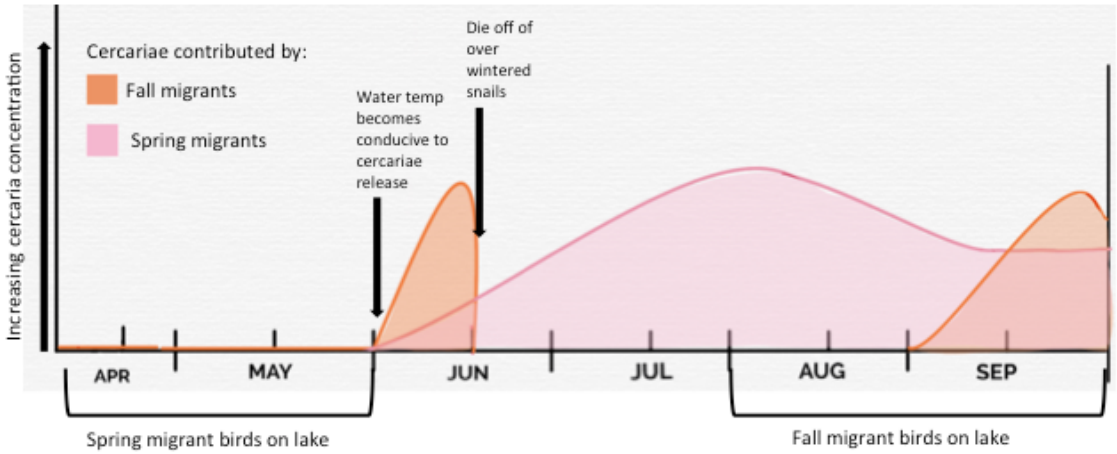
4

Figure 3.13 Theoretical model of fall and spring migrant birds and their contributions to cercariae abundance in lakes.



Chapter 4: Bacterial indicators of water quality

Authors: Sydney P Rudko, Kelsey Froelich, Ronald R Reimink, Bradley Peters, Cyndi Schlosser, Candis Scott, Patrick C. Hanington.

4.1 Introduction

In the introduction of this thesis I emphasized the participatory approach I took to this project, and described that in an attempt to attract partners to the project, I offered a variety of testing options and responded to partner requests for specific tests as best I could. This chapter describes the work conducted as part of the CBM qPCR program related to bacterial indicators used in recreational water. Some of the qPCR testing included in this chapter are indicators that are commonly used to measure the occurrence of fecal indicator bacteria in recreational water (i.e.: *Enterococcus*, and *Bacteroides* source tracking markers), while others are new qPCR tests for indicators and tests that I have also worked to validate for saprozoic bacteria, (i.e.: toxic and total cyanobacteria and total *P. aeruginosa* tests). The goal of this chapter is to illustrate the flexibility of CBM qPCR has an approach for water monitoring. Most of the CBM partners performed testing for multiple organisms, in a variety of different contexts. Some CBM partners were interested in the method for sentinel surveillance, but other groups had specific research questions in mind. This chapter serves to illustrate the breadth of projects CBM qPCR can be used for, and will discuss our findings from these three separate studies.

4.1.1 Cyanobacteria blooms, and toxin production

Cyanobacteria are a hazard to human and animal health due to the production of toxins. The most common toxin producing genera are *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nostoc and Oscillatoria (Planktothrix)*; however, over 40 genera have been demonstrated to produce toxins (Pick 2016). Cyanotoxins can be grouped into three main groups by the organs they affect: the hepatotoxins target the liver (microcystins, nodularins, and cylindrospermopsin), neurotoxins target the brain (anatoxin, anatoxin-a, and saxitoxins), and dermatoxins are

harmful to the skin (aplysiatoxin, lyngbya-toxin, and LPS) (Chorus and Bartram 1999). Surveys of blooms in Europe, Australia and the United States have found that between 27% and 83% were toxic; however, toxicity may vary in space and time, and some blooms may produce multiple toxins. This makes management of toxic blooms incredibly complex (Falconer 1999; Stewart et al. 2006).

qPCR is one of many tools that has been investigated for monitoring cyanobacteria blooms (Pearson and Neilan 2008). Routine monitoring for cyanobacteria blooms and toxins can use either cell counts performed via microscopy (with or without species level identification), or toxin levels can also be assayed via a protein phosphatase inhibition assay (specific for nodularins and microcystins), an ELISA, or with mass spectrometry (Yoshizawa et al. 1990; Zeck et al. 2001; Ibelings et al. 2014). Directly testing for toxins provides information about toxin presence, concentration and variety; however, these tests are often impractical due to their cost and the time required to complete testing. qPCR has a number of advantages compared to other approaches in that it is sensitive, specific, rapid, and can be cost effective for large numbers of samples (Kurmayer et al.).

The Canadian Recreational Water guidelines specify a regulatory guideline of 20 ug/L of total microcystin toxins or a total cyanobacteria count of 100,000 cells/mL (Federal Provincial Territorial Working Group 2012). This guideline suggests that when either of these values is exceeded, a beach should be closed as there may be a risk to human health. More generally, the Canadian Recreational Water Guideline suggests that contact with water where a bloom exists or has recently collapsed should be avoided (Federal Provincial Territorial Working Group 2012). In practice, most regulatory bodies can easily and cheaply conduct cell counts for cyanobacteria.

Total microcystin testing via ELISA or mass spectrometry often takes place less frequently because it is expensive and time consuming. In Alberta, both Alberta Health and the Alberta Lake Management society conduct total microcystin testing; however, few lakes are included in this testing. In practice, most cyanobacteria monitoring in Alberta is conducted on site via visual inspection. If a bloom is visually identified by Alberta Health

Services, the beach is posted with a public warning sign. However, samples are collected and undergo cell counts, the species of cyanobacteria are identified and samples are sent for toxin analysis, and all of these results are considered before a water quality advisory is issued (Alberta Health Services 2019). A CBM approach could compliment this policy by enabling communities to feel engaged in bloom management, and also collecting valuable data about cyanobacteria blooms in Alberta across a wide geographic area.

In this chapter I will discuss some of the ongoing work to establish qPCR testing for the microcystin gene within the CBM qPCR framework in current monitoring agencies within Alberta. Microcystin toxin is produced the *mcy* gene cluster. This cluster consists of genes *mcy A-J*. The *mcyA mcyB and mcyC* genes encode nonribosomal peptide biosynthesis (NRP) modules, while *mcyD* and *mcyE-G* encode and polyketide synthesis modules (PKS), and NRP-PKS hybrids. These NRP, PKS, and NRP-PKS modules are used by the cell (instead of the ribosome) to produce microcystin toxin. Microcystin toxin, and subsequently the *mcy* gene cluster were first discovered in *Microcystis aeruginosa.* Since then, it has been discovered that numerous species of cyanobacteria possess the *mcy* gene cluster, and can produce microcystin (Kurmayer et al.; Pearson and Neilan 2008).

The qPCR diagnostic test I deployed was developed by Alberta Health and the Provincial Labs for Public Health; it targets the *mcyE* gene of *M. aeruginosa* (Qiu et al. 2013). Initially in 2016 we had brought on Alberta Health Services and ALMS to perform this test as part of their routine monitoring. Alberta Health Services liked the system, and used it in 2016, but in 2017 declined to continue participating in our program. ALMS has been a continued partner since 2016 for toxic cyanobacteria testing. In 2018, I also worked to adapt a previously published qPCR assay for total cyanobacteria that targets the 16s rRNA gene. The work with ALMS was not conducted with a specific *a priori* hypothesis, and instead served to continue to the use of this test by government monitoring agencies as an indicator of bloom risk.

4.1.2 *Pseudomonas aeruginosa* in recreational bathing waters.

P. aeruginosa is an important saprozoic pathogen often found in recreational water (Rice et al. 2012b). Saprozonoses are infections transmitted to humans via largely abiotic environments. When referring to a saprozoic pathogen, we are referring to the plethora of bacteria, viruses or protozoa living in natural environments (i.e.: water, soil) that can initiate opportunistic infections with humans upon contact (Ashbolt 2015). *P. aeruginosa* is a ubiquitous bacteria of water and soil sediments. It can survive in nutrient poor environments and forms biofilms. It is the causative agent of folliculitis (a common skin infection in which the hair follicle becomes infected and inflamed causing a rash) and is the most common cause of otitis externa (Rice et al. 2012b). *P. aeruginosa* is commonly reported in outbreaks associated with recreational water (Craun et al. 2005; Hlavsa et al. 2015). While often characterized as an opportunistic pathogen, there is also evidence that *P. aeruginosa* may be a member of the skin microbiota; 10% of bathers and non-bathers carry the bacteria in their ears. *P aeruginosa* is particularly well suited for growth in hot tub/spa and swimming pool environments. It can grow at between 4 and 42 °C, and forms biofilms on plumbing surfaces (Lutz and Lee 2011; Mao et al. 2018). It is also intrinsically resistant to many antibiotics due to its low outer member permeability and the presence of numerous efflux pumps (Lutz and Lee 2011). A chlorine residual of above 0.4 mg/L prevents growth of *P. aeruginosa*, and this has been demonstrated in both laboratory experiments and *in situ*; however, the bacteria have demonstrated increased resistance to UV light, requiring between a 17-28% longer UV exposure to cause a 1-log reduction than *E. coli* (Seyfried and Fraser 1980; Dejung et al. 2007). Additionally, *P. aeruginosa* only demonstrated a 1-1.1 log reduction at UV doses of 54, 108, and 162 mJ/m^2 compared to a 3-log reduction of standard indicators (Rice et al. 2012b). Furthermore, and especially relevant to the natural swimming pool environment, UV disinfection has no residual activity, thus bacteria that survive can go on to reproduce after exposure (Guo et al. 2009). Relatively few studies have assessed *P. aeruginosa* concentrations in the absence of an outbreak, but when outbreaks occur they typically report densities of *P. aeruginosa* in the range of $10^2 – 10^7$ c.f.u. /mL (Roser et al. 2014; Roser et al. 2015).

Natural swimming pools (NSPs) are swimming pools that utilize biological controls, and filtration to clean the water, whereas traditional pools rely on chlorination and filtration to disinfect waters (Barna and Kádár 2012; Graciaa et al. 2018). There are, arguably, some disadvantages to traditional chemical disinfection agents—odor, and concerns over disinfection byproducts and an increased prevalence of bladder cancers in certain populations (Costet et al. 2011). However, natural treatment environments have not been well studied in their ability to remove hazardous microorganisms often found in swimming pool environments such as fecal indicator bacteria, environmental saprozoics, and protozoan parasites like *C. parvum/hominis and G. intestinalis* (Craun et al. 2005; Barna and Kádár 2012).

The Borden Park Natural Swimming Pool (NSP) was opened in Edmonton, Alberta in the summer of 2018. The facility manager was eager to participate in our CBM qPCR study. The Borden Park NSP is subject to the Alberta Recreational Water Guideline, which imposes mandatory testing for cyanobacteria and (as of 2018) fecal coliforms, and enterococcus (as of 2019); it does not include testing for human specific bacterial indicators or saprozoic pathogens like *P. aeruginosa, Legionella pneumophila,* or *Mycobacterium* spp (Hubálek 2003; Ashbolt 2015; Government of Alberta 2018). Borden Park therefore performed on-site testing for HF183 (2018), moving to *Enterococcus* spp. in 2019, and *P. aeruginosa* during both years.

I was particularly interested in the prevalence of *P. aeruginosa* in the system for several reasons. Notably, the natural treatment environment could be one that selects for colonization by saprozoic bacteria; perhaps this is particularly true for *P. aeruginosa* due to its ability, especially in the absence of a disinfectant, to form biofilms (Rice et al. 2012b).

Utilizing the CBM qPCR framework, the facility manager at Borden Park collected samples and tested for enteric indicators, and *P. aeruginosa*. In both 2018 and 2019, the pool had low or non-detectable levels of enteric indicators; as such, the results here will focus on the *P. aeruginosa* data. Using the CBM qPCR approach we were able to collect valuable information over two seasons at the facility. This data can provide valuable

information about the occurrence of *P. aeruginosa* in a natural swimming pool environment.

4.1.2 Monitoring for enteric indicator bacteria in Michigan USA.

Fecal contamination of recreational waters is often a concern of lake users and property owners. Methods for evaluating the occurrence and levels of fecal indicator bacteria (FIB) have been used for over a century to assess water quality (Ashbolt et al. 2001; Simpson et al. 2002; Noble et al. 2003). FIB bacteria are a broad class of bacteria that survive in the gut of humans and animals. FIB bacteria can be used as indicator organisms. Indicator organisms are not inherently dangerous, but increases in their prevalence in water bodies can indicate an increased risk of infection from a hazardous organism (i.e.: *E. coli O-157, Campylobacter jejuni, G. intestinalis),* and can also be used to identify locations of fecal contamination (Sinclair et al. 2012). In most locations recreational beaches undergo monitoring for FIB. Many locations still test for thermotolerant fecal coliforms; however, it is generally accepted that total enterococci measured by qPCR best correlate with risk of GI illness in recreational waters, especially those impacted by a point source of contamination (i.e.: a wastewater treatment plant) (Noble et al. 2003; Wymer et al. 2005; Wade et al. 2006).

The USEPA recommends a regulatory target of less than 1280 calibrator cell equivalents (cce) per mL, and the new Alberta Recreational Water Guideline has recently adopted this target as well. Exceedance of this limit triggers a source-tracking study. Source-tracking studies attempt to elucidate the source (i.e: human, cattle, bird, etc.) of the fecal contamination at a beach. If the source of contamination is neither human or cow, the enterococcus limit is raised to 6400 cce/100ml, which would lead to a beach closure regardless of the source of contamination (USEPA 2012).

In 2018, I offered CBM partners the HF183 test for human-associated *Bacteroides*. This test is commonly used as a source-tracking method to assess human contamination

at recreational beaches after an exceedance detected by the total *Enterococcus* qPCR test (Boehm et al. 2015; Nshimyimana et al. 2017; Ahmed et al. 2018). This test was utilized by my CBM partners in Michigan and by Borden Park. The rationale for offering this test instead of total *Enterococcus* was that CBM partners were specifically interested in human contamination. Additionally, a study published by Boehm et al. suggested that an HF183 gene copy number of 4200 correlates to an increased GI illness risk (Boehm et al. 2015). In Michigan, an extensive human contamination tracking study was undertaken in which volunteers and our CBM partners collected water samples every 150 meters along the shorelines of four lakes and assessed these samples for human contamination. In 2019 I piloted Method 1611 with the Michigan CBM partners. The lake associations that participated in the 2018 source-tracking study were curious about the effect rainfall had on enteric concentrations in their lakes and were interested in assessing the source of the contamination. As Chapter 2 discusses, the reproducibility of this method was poor in CBM partners' hands—however, I was able to utilize their collected samples to acquire the data they needed.

4.2 Results

4.2.1 Detection of total cyanobacteria

The *16S* total cyanobacteria assay was published by Al-Tebrineh *et al.* (2010), and was designed for use in water samples and targets a conserved 16S rRNA gene domain of cyanobacteria. I tested the cross reactivity of this assay against a host of common environmental bacteria (Table 4.1) and did not detect cross reactivity. The LOD95 of the 16S assay is 4 copies/ 5uL. This assay did amplify the DNA of field collected cyanobacteria blooms (data not shown).

4.2.2 Identification of microcystin positive cyanobacteria blooms in 2019

In 2019 ALMS performed screening for the microcystin E gene on lakes that took part in their Lakewatch program. Of the 16 lakes monitored, all but one, Elkwater Lake, had detectable *mcyE* genes during early August. Notably, Upper Man Lake had detectable

levels of *mcyE* as early as the beginning of June, and Wabamun, Laurier, and Little Beaver Lake were *mcyE* positive by the end of June (Figure 4.1).

4.2.3. qPCR detection of total pseudomonas

The total *P. aeruginosa* qPCR assay is specific for the DNA gyrase B gene of *P. aeruginosa* and was first published *in* Lee et al. (2011). I tested the cross reactivity of this assay against a host of common environmental bacteria (Table 4.1), and did not observe cross reactivity. The LOD95 of this assay is 6 copies / 5uL (Table 6.2).

4.2.4 qPCR monitoring for *P. aeruginosa* in the Borden Park NSP.

In 2018 levels of *P. aeruginosa* in the main and kids basins of the NSP were consistent between the two basins and consistently contained around 3 log copies of the *gyrB* gene (Figure 2). In 2019 this level increased by 3-log (Figure 4.3), and both basins consistently contained roughly 6 log copies of the *gryB* gene (Figure 4.2).

4.2.5 Monitoring for human fecal contamination at four lakes in northern Michigan

In 2018 human-associated *Bacteroides* (*B. dorei)* marker HF183 was used to identify human fecal contamination at Glen Lake, Lime Lake and neighboring Little Traverse Lake. Samples were taken roughly every 150 m along the shoreline. A heatmap depicting the relative amounts of HF183 expressed in genome equivalents (ge) per 50ml is shown in Figure 4. The highest sample contained 139 ge/50ml *B. dorei*. In 2019 we used Method 1611 to assess *Enterococcus* levels at these lakes, and Lake Leelanau before and after a large rain event. *Enterococcus* levels increased at all lakes after the rainfall event (Figure 4.5). 10 of these samples exceeded *Enterococcus* guidelines based on a conversion from gene copy number to *E. faecacium* genome equivalents. Source tracking for human contamination of all samples was performed using the HF183 source tracking marker, and only one sample from Lake Leelanau had detectable levels of the marker. Source tracking for cow and ruminant contamination was performed using source-tracking marker Rum2Bac, and all samples were negative for this marker. Source-tracking *C. marimammalliun* (via the LeeSG

assay) feces was also conducted and samples were also negative for this marker (Raith et al. 2013; Sinigalliano et al. 2013; Boehm et al. 2015).

4.4 Discussion

4.4.1 Monitoring for the *mcyE* gene in cyanobacteria

qPCR is an adaptable and flexible methodology; coupled with a CBM framework, it can address an array of research and practical questions. I utilized a PAR framework that allowed CBM partners to select organisms they were interested in testing for, then collaborated with them to accomplish the goals of their organization.

The cyanobacteria testing was done in collaboration with the Alberta Lake Management Society. ALMS performed screening for the microcystin E gene on a variety of lakes as part of their LakeWatch program. The *mcyE* gene qPCR test was originally published in Sipari *et al.* 2010, and were further validated by Qiu *et al.* (2013) as part of a cyanobacteria study conducted by Alberta Health (Sipari et al. 2010; Qiu et al. 2013; Alberta Health 2014). As part of the monitoring conducted in this chapter we found microcystin E genes present as early as late June at some water bodies, with concentrations rising again in late July and August (Figure 1). Typically, microcystin toxin production is related to water temperature, with higher temperatures correlating with increases in toxin production (higher temperatures also correlate to increases in cyanobacterial bloom density) (Xu et al.; Dziallas and Grossart 2011). While qPCR of microcystin genes is likely not a reliable indicator of toxin abundance, in the absence of routine toxin analysis (Beversdorf et al. 2015), the microcystin qPCR provides practical data about lakes that could potentially harbor toxic cyanobacteria blooms and about those whose blooms are less hazardous (Ibelings et al. 2014). To my knowledge, no comprehensive study of other cyanotoxins (i.e.: anatoxins, cylindrospermopsins) has never been undertaken in Alberta. Arguably, a CBM qPCR approach could be an excellent

way to screen for these other toxin groups, especially considering that Alberta's highly eutrophic lakes are already prone to cyanobacteria blooms and that climate change is poised to provide more favorable conditions for toxic blooms (Pick 2016).

4.4.2 *P. aeruginosa* testing at the Borden Park NSP

The Borden Park NSP is a unique and singular attraction in Alberta, and in Canada. As a swimming environment that is not chemically treated, it also potentially represents a unique risk to health (Craun et al. 2005; Hlavsa et al. 2015). The NSP treats water first through a zooplankton filter (estimated log reduction of bacteria: 0-log), then through a Neptune filter (estimated log reduction bacteria: 2-log) , and through a hydrobotanic filter, through a submerged substrate filter (estimated log reduction bacteria: 1-log), and finally through UV irradiation (25mJ/cm^{2,}, estimated log reduction bacteria: 5-log).

P. aeruginosa is a unique bacteria to study in the NSP environment as it has the ability to form biofilms on surfaces, and is capable of surviving both wide range of temperatures and in a low nutrient environment. *P. aeruginosa* is also a common cause of infection in bathing water environments (Rice et al. 2012b). In 2011 an assay to detect specifically *P. aeruginosa* in water matrices via the DNA gyrase subunit B (*gyrB*) gene was developed (Lee et al. 2011). This paper tested the specificity of this assay against a multitude of other pseudomonads (*P. agarici, P. alcaligenes, P. alcaliphila, P. amygdali,P. anguilliseptica, P. asplenii, P. avellanae, P. azotoformans, P. balearica, P. brassicacearum, P. caricapapayae, P. chloritidismutan, P. chlororaphi, P. cichorii, P. citronellolis, P. corrugate, P. entomophilla, P. fiscuserectae, P. fluorescens, P. fragi, P. frederiksbergensis, P. fulva, P. iners, P. jessenii, P. kilonensis, P. koreensis, P. lubricans, P. marginalis, P. marginata, P. mediterranea, P. mendocina, P. mohnii, P. monteilii, P. moorei, P. mosselii, P. mucidolens, P. oleovorans, P. oryzihabitans, P. parafulva, P. plecoglossicida, P. putida, P. reinekei, P. savastanoi, P. stanieri, P. straminea, P. stutzeri, P. synxantha, P. syringae. P. taetrolens, P. taiwanensis, P. thivervalensis, P. tolaasii, P. umsongensis, P. vancouverensis, P. viridiflava,* and *P. xanthomarina*) *in silico*, and based on alignments of over 611 sequences, the authors designed the primers and probe for specificity of *P. aeruginosa*. This paper also tested

cross reactivity experimentally against *P. putida,* and *P. stutzeri*, and against *Salmonella enterica,* three species of *Bacillus*, two species of *Bacteroides*, and a species of *Prevotella melaninogenica*, and found no cross reactivity (Lee et al. 2011). I also experimentally tested the cross reactivity of this assay, and its ability to detect environmental isolates isolated from Borden Park. I found no cross reactivity against a host of bacteria species, and I found that the assay was able to detect environmental isolates of *P. aeruginosa* from Borden Park (Table 4.1). The *gyrB* gene is a favourable target as it has a higher molecular evolution rate and low horizontal gene transfer (Kasai 1998; Yamamoto et al. 2000). The copy number per genome of *gryB* in *P. aeruginosa* has not been characterized; however, it is *gyrB* is a single copy gene in *Borrellia burgdoferi, B. fragilis,* and *Vibrio parahaemolyticus* (Samuels et al. 1994; Venkateswaran et al. 1998; Lee and Lee 2010).

In Chapter 2 I discussed some of the challenges related to the CBM qPCR implementation at Borden Park; notably samples had poor agreement with my own extracts. This data was therefore compiled using my own analysis of the samples, not that of my partners at Borden Parks. From 2018 to 2019 I report a 3-log increase in *gyrB* copy numbers in both the main basin and the children's basin (Figure 3). Sampling from July and August of each of these basins reveals that the copy number between both basins stays relatively consistent, which is unsurprising as water from the children's basin and main basin are mixed together into a secondary basin before treatment through the filtration systems.

P. aeruginosa has been demonstrated to have some resistance to UV disinfection. Additionally, *P. aeruginosa* (which may slough off as a biofilm, and go through the UV light in this form) may show increased resistance to UV disinfection, futhermore, Borden Park does not typically leave its' UV light running continuously due to the increased cost of operation (Dejung et al. 2007; Rice et al. 2012b). Grab sampling (as we have been performing for *P. aeruginosa* testing), will only capture planktonic *P. aeruginosa*, and given that this sampling is occurring in a swimming pool environment which would favor biofilm development, it is likely that this method has underestimated the number of *P.*

aeruginosa actually present in this environment as a biofilm (Price and Ahearn 1988; Uhl and Hartmann 2005; Rice et al. 2012a).

Typically *P. aeruginosa* levels are kept in control by residual chlorine (Mao et al. 2018), but the NSP does not have any form of chemical disinfection. Based on this data in the upcoming 2020 pool season, I would recommend continuing to monitor for *P. aeruginosa* both via the qPCR assay, and via a culture based method to better elucidate viability. *P. aeruginosa* concentrations in the range of 10^2-10^7 cfu/100ml have been demonstrated to cause outbreaks of folliculitis (Roser et al. 2014). Additionally, it may be worthwhile to visually inspect certain areas (such as the basins) and any other accessible areas for evidence of a biofilm and mechanically removing any biofilms that are found (Price and Ahearn 1988; Uhl and Hartmann 2005; Rice et al. 2012b).

This is also an example of how a CBM program can be used to screen for potentially hazardous conditions. While our routine monitoring for fecal indicator bacteria (*Enterococcus* and human-associated *Bacteroides*) suggests that hazardous events haven't occurred in regards to these organisms (and perhaps that the pools work to educate the public on not swimming within two weeks of GI illness and enforcing showering before entering are effective), this CBM qPCR program has detected a potential hazardous scenario in regards to a saprozoic organism.

4.4.3 The Michigan enteric bacteria study

The Michigan enteric study took place in 2018 and 2019. In 2018, CBM partners performed extensive sampling on three lakes in northern Michigan, and we utilized human-associated *Bacteroides* marker HF183 to assess for human contamination. We identified a low level of human contamination from various locations around all three lakes, which was unsurprising as all three lakes boast numerous summer villages (Figure 4). In 2019, we performed *Enterococcus* testing before and after a heavy rainfall event, sampling on inlet streams. Three samples were taken, one directly at the stream mouth

and two samples 50 feet to the left and right of the inlet. Average *Enterococcus* spp. expressed as genome equivalents/100mL of water are reported. This differs from Method 1611, which quantitates qPCR results to calibrator cell equivalents (i.e.: based on live cell culture equivalents) (USEPA 2012). This change was made because it would be inappropriate to give community partners live and potentially hazardous bacteria culture. Rainfall increased the amount of enterococcus detected at most sites (Figure 4.5). This was expected, as rainfall events are well known to cause increases in fecal indicator bacteria, as bacteria deposited on land from the surrounding watershed are washed into lakes and rivers (Coulliette et al. 2009; Stidson et al. 2012). Samples that exceeded 1280 ge/100ml underwent source-tracking for cattle (via ruminant marker Rum2Bac (Raith et al. 2013)), and gull contamination (Lee et al. 2013), and all samples were tested for the HF183 marker (*B. dorei)*. One sample at Lake Leelanau was positive for low levels of the HF183, but this sample did not exceed the *Enterococcus* benchmark of 1280 g/100ml. Two samples at Glen lake were positive for less than 10 copies of the HF183 marker, and one site at Lime Lake was also positive for less than 5 copies of the 1f183 marker. Otherwise none of the samples were positive for the ruminant, nor the gull marker.

While enterococcus is a widely used indicator of fecal contamination, and in locations with point source fecal contamination has been demonstrated to be predictive of gastrointestinal illness, its' use in locations where nonpoint source contamination can be more challenging because there can be numerous sources of enterococci, including animal feces or environmental reservoirs (Wymer et al. 2005; Fleisher et al. 2010; Sinigalliano et al. 2010; Heaney et al. 2012). Source tracking of samples is performed to see if the likely source of the contamination is human or cattle excrement as these are the riskiest sources of human contamination (Simpson et al. 2002). Birds and waterfowl can also contribute enterococcus into a water body, in addition to many other animals. When enterococcus leaves the gastrointestinal tract, it is subject to a host of environmental factors that have been demonstrated to gradually reduce its concentration over time. Sunlight (i.e.: UV irradiation) reduces concentrations of enterococci and other FIB (Fujioka et al. 1981; Sinton et al. 1999; Noble et al. 2004). Additionally enterococci typically grow poorly in

nutrient poor environments like oligotrophic lakes (Sinclair and Alexander 1984; Heim et al. 2002). Nonetheless, environmental sinks of enterococci exist. Although environmental conditions outside the gut are largely unfavorable, there is a growing body of evidence that some members of *Enterococcus* spp. seem to persist, and perhaps even grow in extraenteric environments (Anderson et al. 2005; Lee et al. 2006; Sinton et al. 2007). *Enterococcus* spp. have been shown to persist in aquatic and terrestrial vegetation (Mundt 1961; Whitman et al. 2003). Numerous studies have found enterococci as components of beach sand (Hartz et al. 2008; Yamahara et al. 2009; Heaney et al. 2012), and recent evidence of enterococci associated with sediment biofilms suggests that at least some populations are resident (Piggot et al. 2012). Furthermore, studies have found enterococci growth associated with intermittent wetting of sand (Yamahara et al. 2009).

In a stream bed environment, it's possible to imagine a scenario in which enterococcus enter the stream environment due to animal contamination, a farm, or a septic field, but under drought conditions persist in the soil or sediment of the stream bed, only to be released into a lake environment after a heavy rainfall suspends sediment (Le Fevre and Lewis 2003; Jin et al. 2004). This could explain our inability to determine the source of fecal contamination, as perhaps the species-specific source-tracking markers have disappeared from the environment while the enterococci have persisted. Additionally, nearly every warm blooded mammal might contribute enterococci into the environment, making it possible that I simply haven't found the source yet. In the future I would recommend a sanitary survey be conducted of the streambeds that exceed the 1280 ge/100ml guideline to determine possible sources of contamination (i.e.: agriculture, septic fields), and sampling closer to these sites to assess potential contamination. Sediment sampling could also be conducted in these locations.

Regardless, the advantage to using total enterococcus as a measure of fecal contamination at these locations is its clear guidelines with regards to management decisions, even in the absence of a known source of contamination. An enterococcus value

over 6400 ge/100mL suggests that there is a health risk regardless of the source of contamination, and one of our samples did exceed this guideline after a rain event.

These three case studies highlight the main advantage of a CBM qPCR method, which is the ability to capture a wide variety of information on a large array of organisms over a large geographic area. Even in instances where the CBM qPCR testing method was imperfect because we collect a duplicate sample, we were still able to obtain a wealth of information that was relevant to our CBM partners in their various contexts and have generated a wide array of hypotheses and interesting observations to follow up upon in upcoming years. In the case of toxic cyanobacteria monitoring in Alberta, we've been able to differentiate potentially "riskier" blooms from those without the potential to produce cyanotoxin microcystin. In the presence of programmatic and managerial infrastructure, this information could be used to target specific blooms for toxin analysis or could be used to elevate the risk level of certain lakes (i.e.: from low risk to medium risk). In collaboration with ALMS, we've also demonstrated that the microcystin gene can be present in water bodies as early as June. At the Borden Park NSP, I have struck a balance between providing the fecal indicator tests the group wants to proactively protect bathers while also advancing a large gap in the scientific literature regarding levels of *P. aeruginosa* in recreational water during non-outbreak times. Our data has also captured a large increase in *P. aeruginosa* concentrations since the first year the pool opened which can be followed up upon to assess if this is a risk, or if the population of *P. aeruginosa* stabilizes in the coming years. This partnership with our research group, the Ashbolt group, and the City of Edmonton also yielded us the opportunity to conduct a full scale spiking experiment at the NSP to assess the log reduction of various surrogates through the NSP treatment environment, This study, while outside the scope of this thesis, shows the value of collaboration with community partners in research. The Michigan enteric study is perhaps the best example of how large scale volunteer buy in to CBM projects can accomplish an incredible wealth of data. In 2018, our CBM partners and their volunteers sampled every 150m of shoreline at three lakes in northern Michigan—an incredible feat and one that would have been impossible without community partners. In 2019, although

our study was smaller, volunteers around the lake still collected samples between 9 and 10 am before and after a large rainfall event, and transported these samples to a field laboratory on Lime Lake for processing. This sampling was accomplished for only the cost of materials and would have been impossible without the efforts of community volunteers.

4.5 Tables

Table 4.1 Specificity of the *16S* cyanobacteria and the *gyrB* gene *P. aeruginosa* assays

Species	Amplified by 16S gene cyanobacteria assay	Amplified by *P. aeruginosa* gyrB gene assay
E. coli 25922	--	--
Campylobacter jejuni	--	--
E. coli O157:H7	--	--
Shigella sonnei	--	--
Klebsiella oxytoca	--	--
Enterococcus faecalis	--	--
Edwardsiella tarda	--	--
Enterobacter hormaechei	--	--
Stenotrophomonas maltophilic	--	--
Ochrobactrum anthropic	--	--
Proteus vulgari 6380	--	--
Elizabethkingia meningoseptica 13253	--	--
Staphylococcus epidermidis	--	--
Acinetobacter baumannii	--	--
Environmental *P. aeruginosa isolate 1* from Borden Park NSP	--	+
Environmental *P. aeruginosa isolate 2* from Borden Park NSP	--	+
P. aeruginosa PAO1	--	+

4.5 Figures

Figure 4.1. *McyE* gene copy numbers from 15 lakes across Alberta in the summer of 2019.

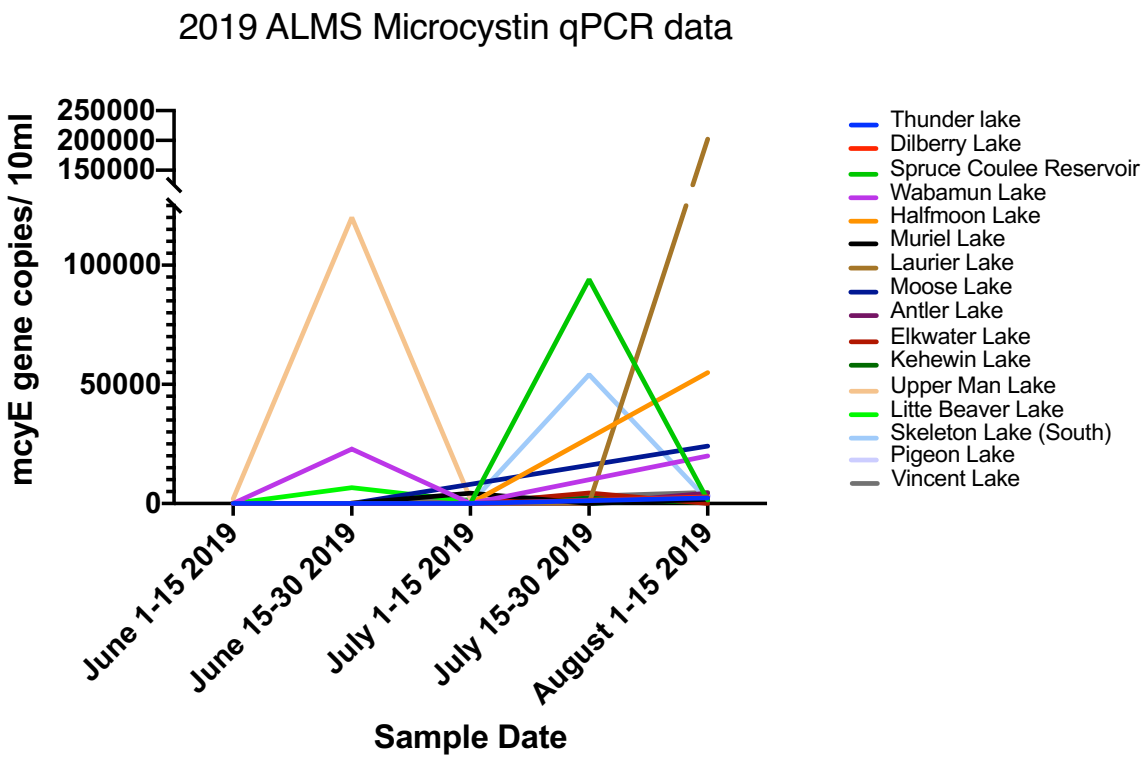


Figure 4.2: Concentrations of *P. aeruginosa gyrB* gene in the kids and main basins at the Borden Park Natural Swimming Environment

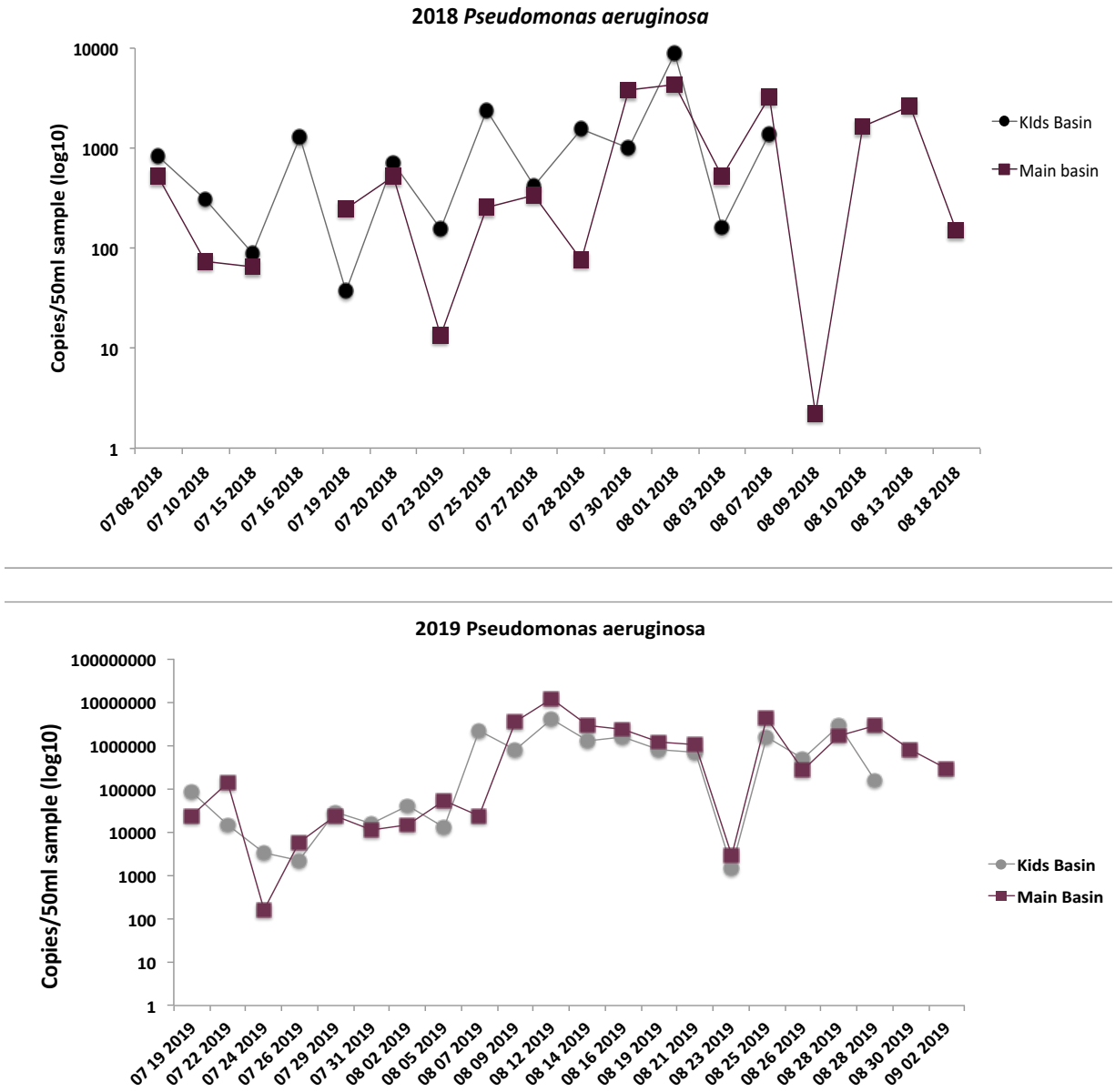
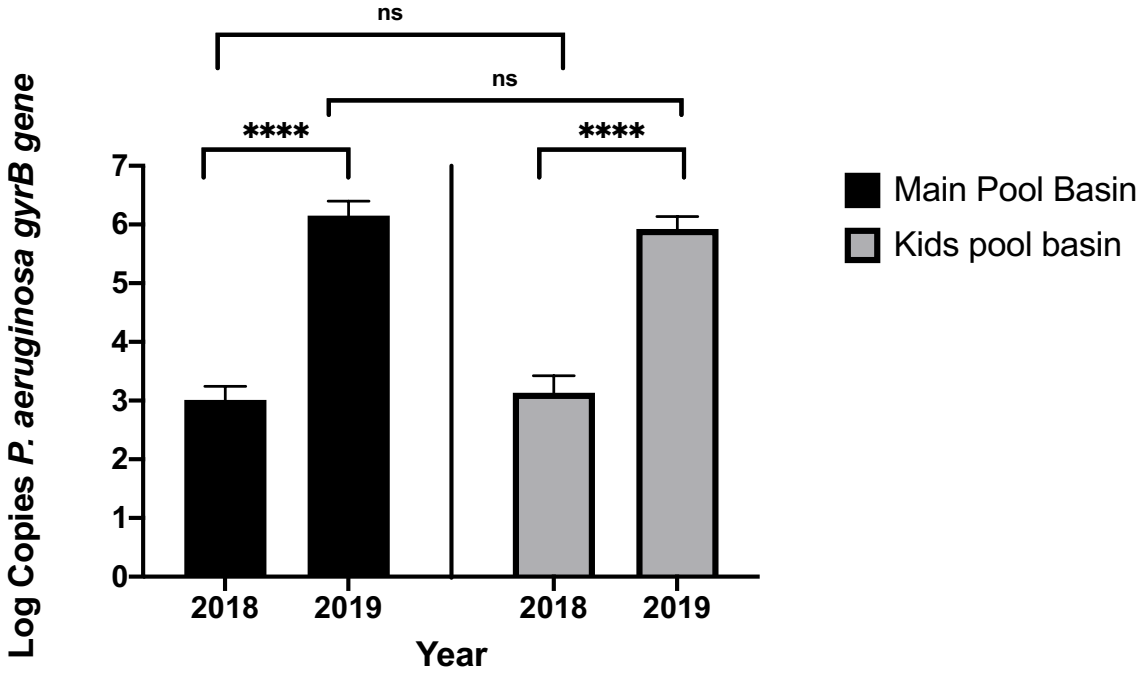
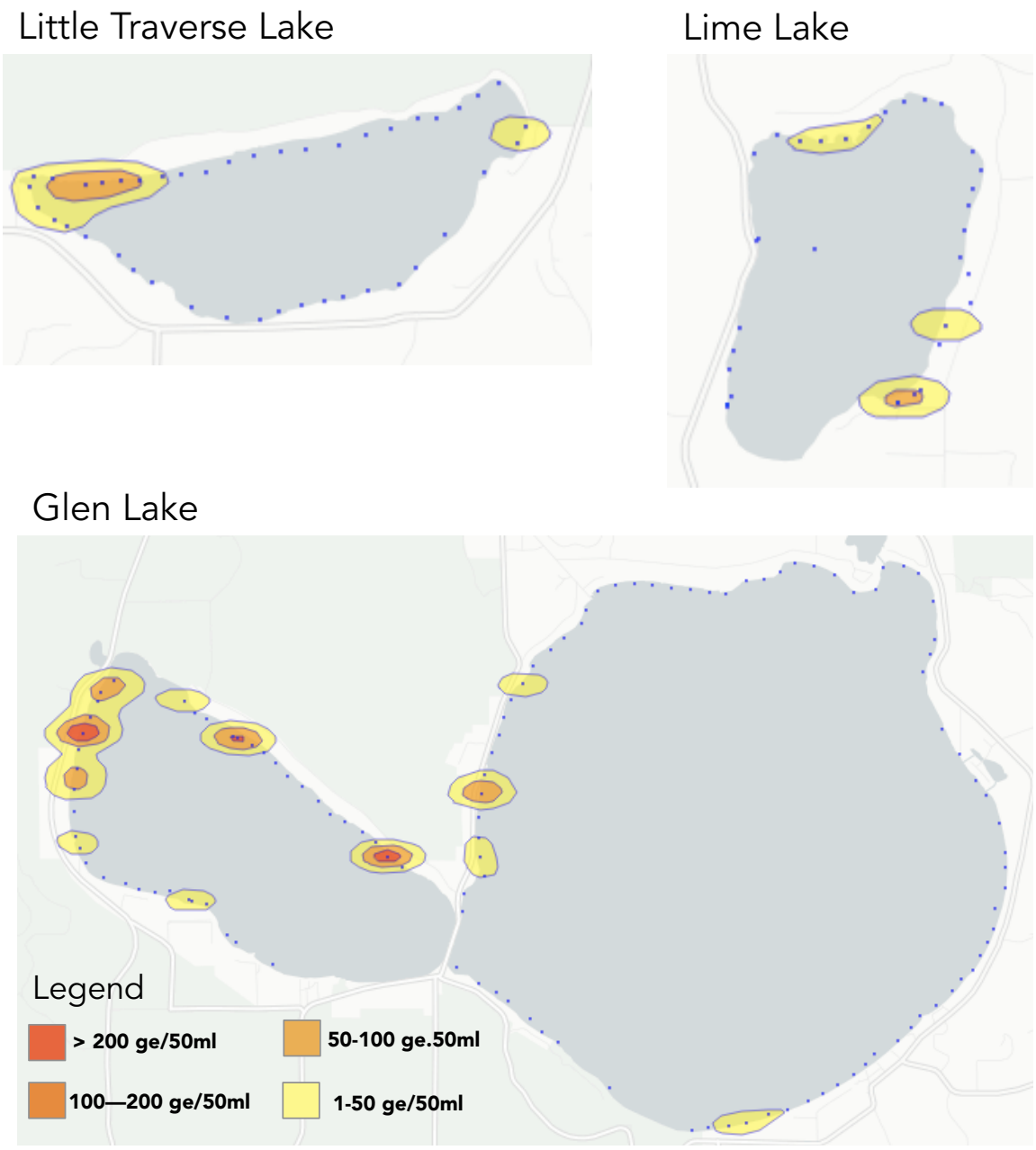


Figure 4.3. Average concentration of *P. aeruginosa gyrB* gene in each basin in 2018 and 2019.



There is a statistically significant difference between the average copy number in both basins in 2018 and 2019. Stars denote statistical significance ($p<0.001$).

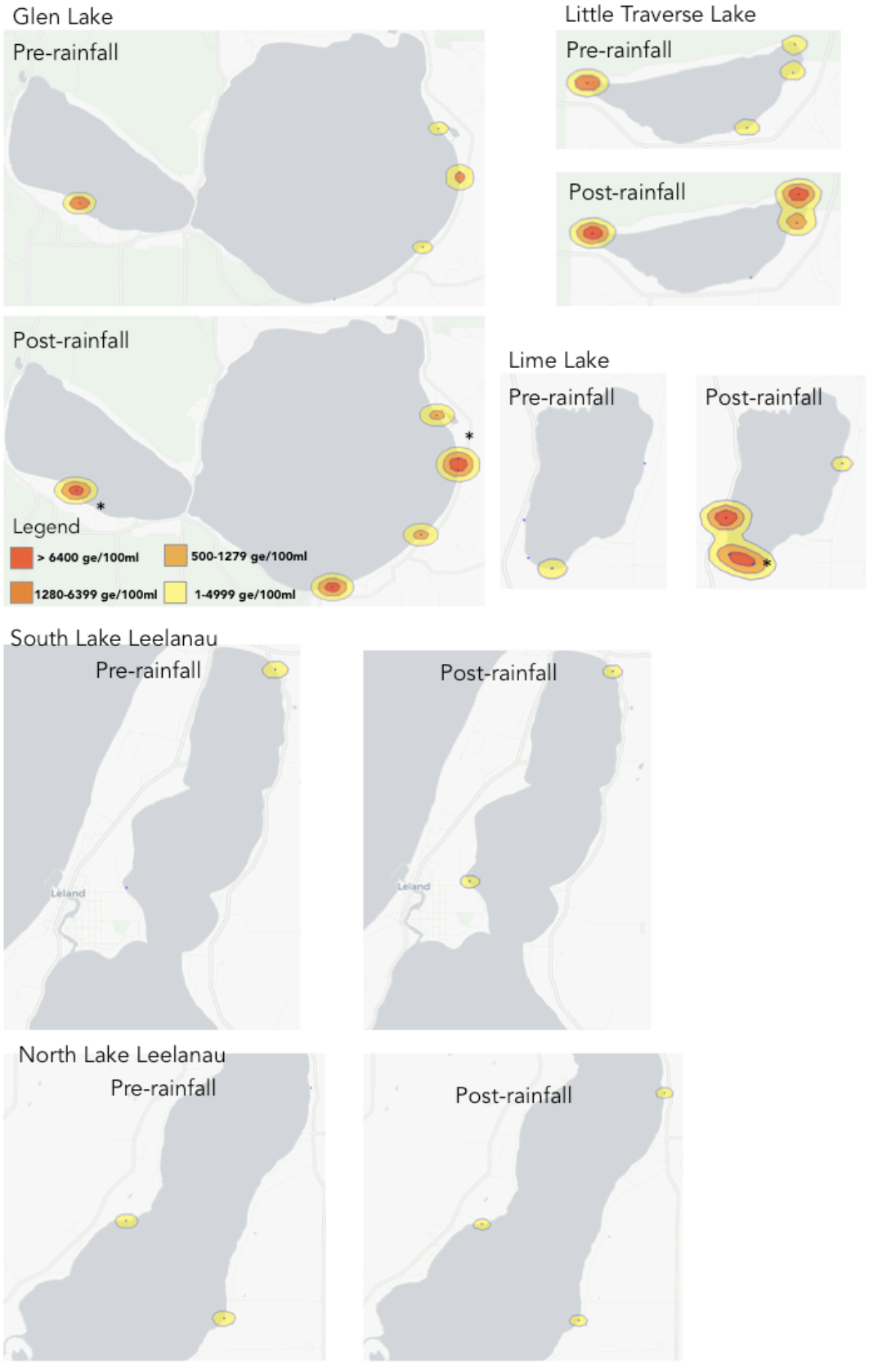
Figure 4.4. Monitoring for human-associated *bacteroides* (via marker HF183) of Lime, Little Traverse and Glen lakes in Michigan in 2018.



Samples that had no detectable levels of HF183 are displayed as single points on the map.

Data are expressed in genome equivalents per 50ml (ge).

Figure 4.5. Monitoring for *Enterococcus* at Lime, Little Traverse, Glen Lakes and South and North Lake Leenlanau in Michigan, 2019.


Glen Lake
Pre-rainfall
Post-rainfall
Legend
> 6400 ge/100ml
500-1279 ge/100ml
1280-6399 ge/100ml
1-4999 ge/100ml
Little Traverse Lake
Pre-rainfall
Post-rainfall
Lime Lake
Pre-rainfall
Post-rainfall
South Lake Leelanau
Pre-rainfall
Post-rainfall
Leland
North Lake Leelanau
Pre-rainfall
Post-rainfall

Samples that had no detectable levels of *Enterococcus* are displayed as single points on the map. Data are expressed in genome equivalents per 100ml (ge/100ml). Points marked with asterisks' were positive for human-associated *bacteroides* marker HF183.

Chapter 5: Methods

Authors: Sydney P Rudko

5.1 Community based monitoring methods

5.1.1 Implementation study design

I first connected with relevant stakeholders of recreational water in Alberta, and worked with them to determine their monitoring or policy goals. Using a participatory research approach, we then developed qPCR tests and testing methodologies that would fill these needs. Since the goal of this study was to measure the effectiveness of a CBM monitoring program in a real world context, participants in the study were instructed to collect a duplicate sample or cut the filter membrane in half after filtration and send this to the university lab. Samples in our lab would be processed in an identical fashion to the field user to compare novice versus expert methodologies. Additionally, CBM partners sent their extracted DNA to our lab, which enabled us to also perform qPCR on their DNA extracts and to perform inhibition reactions.

When the planning for this project began there were two portable thermocyclers on the market available from two small start-up companies. One was the ChaiBio Open qPCR™, a larger thermocycler with 16 wells that requires a laptop to access its onboard CPU to, and Biomeme™, a company with a hand held three well system that ran off an iPhone. While the Biomeme™ device was extremely portable, our CBM partners expressed a desire to run more than three samples at once, and additionally the Biomeme™ device meant that it would not be possible to run the suite of controls I had planned. What's more, none of our CBM partners actually wanted to perform qPCR in the field, they wanted to essentially establish satellite labs at their offices or in their garages, collect samples, and bring them back to some kind of make shift lab to actually process them. As such, the ChaiBio seemed the best fit, and was the thermocycler I selected. However, I did test the Biomeme™ system as I worked on validation of a zebra mussel assay, and did

experience some technical issues with this thermocycler. Notably, software failure that resulted in deletion of the results was quite common. The software freezing after the initialization of a run was also common.

At the time the study was undertaken, there was only one commercial DNA extraction kit available that was "field ready" and didn't require the use of a centrifuge, or heating block. Ultimately this product, the Biomeme M1 sample prep kit™, was the DNA extraction kit our CBM partners would use.

There was also a company located at the University of Alberta that had a proprietary gel-based master mix that could amplify from crude lysates of water and fecal samples. While I trialed this particular option for our field kits for some time, the variation in copy number between internal replicates of the same sample would often differ by a log or more on a purified DNA sample, and I felt this was an unacceptable level of variation (Figure 6.1).

At the same time, IDT had introduced a liquid temperature and light stable master mix, and I felt this would be a good fit for our CBM partners, as most had expressed a desire to perform testing at a central location, thus I could store master mix in a fridge or freezer, but not be concerned that the qPCR master mix could spoil if perhaps a CBM partner was slow at placing DNA into the reaction tubes.

5.1.2 Sample collection

Specific water collection methods are detailed below for each target of interest; regardless of the volume collected, all samples were then filtered through a 0.4 µm polycarbonate filter (Pall FMFNL1050) using an electric vacuum pump (Vaccubrand®). CBM partners had the option of either collecting and filtering a duplicate water sample for analysis, or cutting their filter membranes in half to be analyzed at the university lab.

5.1.3 Avian schistosome monitoring

Sample collection was conducted as described in Rudko et al 2018. Briefly, 25L water samples, collected one litre at a time across a shoreline up to ~1m deep were passed through a 20µm plankton tow. Debris from inside were washed down using well water followed by a 95% ethanol wash and collection in sterile 50-mL conical tubes.

5.1.4 Toxin-producing cyanobacteria monitoring

Sample collection was conducted from watercraft operated by CBM partners on various lakes. Samples were collected through a one-way foot valve attached to weighted 3/4" Nalgene tubing. Samples were only collected from the euphotic zone as determined by a Secchi disk measurement at each lake's deepest point. Ten sampling locations were selected for each lake, with water being composted from each sampling location into a central container. Water from this container was then poured into 50-mL conical tubes.

5.1.5 HF183 monitoring:

All samples were collected by scooping two 50ml samples in sterile, conical, collection tubes from the surface water 15m from shore every 150m along the entire perimeter of each participating lake.

5.1.6 *Enterococcus* spp. monitoring

All samples were collected by scooping two 50ml samples in sterile, conical, collection tubes.

5.1.7 *P. aeruginosa* monitoring

All samples were collected by scooping two 50ml samples in sterile, conical, collection tubes from the pool deck near the drain that removes water from the basin.

5.1.8 In-field and in-lab DNA extraction

DNA extraction was conducted using the M1 Sample Prep Kit (Biomeme™) according to the manufacturers' instructions. The M1 sample prep kit is designed to function in the field. Lysis is accomplished by placing a filter in the lysis buffer and

shaking for one minute. Next, the solution is passed through a syringe unit fitted with a DNA binding column. The column undergoes two washes to remove proteins and salts, and then is dried using an acetone buffer before elution. In 2018, the avian schistosomes monitoring group was interested in transitioning to a DNA extraction method that would allow for batch processing of samples. We therefore opted to transition their program to the DNAeasy™ DNA extraction kit (Rudko et al. 2018). To set up this remote laboratory in a cost-effective manner, equipment (centrifuge, heating block, and vortex) were sourced from Dot Scientific (Table 6.3), and pipettes were from VWR.

DNA extraction for Enterococcus sampling in 2019 was conducted using USEPA Method 1611. Briefly, 0.2ug/mL salmon testes DNA was mixed with Qiagen buffer AE. 600uL of this solution was added to Generite bead tubes containing Sigma #G-1277 glass beads. Filters were added to the tubes and bead beat in a vortexer with a bead beating attachment, and were beat for 1 minute. Samples were then centrifuged at 12,000 x g for 1 minute, afterwhich 350uL of supernatant was from the beadtube was pipetted into a new snap cap tube, centrifuged again for 5 minutes at 12,000 x g until finally another 200uL of supernatant was pipetted into a final snap cap tube.

5.1.9 Maintaining workflows

All master mix components were mixed in a clean room located at the University of Alberta and aliquoted into 0.2 mL thin wall PCR tubes (Axygen). All plasmid dilutions and preparation of positive controls occurred in a deadbox. Standards and reaction tubes were prepared independently to prevent cross contamination.

5.1.10 In-lab qPCR method

Samples were quantitated relative to a plasmid standard curve which contained 50,000, 5000, 500, 50, 5 and 0.5 copies. Each of the gene targets below was synthesized into a puc19 plasmid vector (Genscript). Thermocycling was performed on the ABI 7500 Fast or the QuantStudio 3 using a standard, 40 cycle, two-step reaction. The thermocycling parameters were a 30 second hold at 95 degrees, followed by a 30 second

denaturation cycle at 95 degrees, and a 10 second 60 degrees annealing cycle. Each qPCR reaction had a final volume of 20uL, and we added 5uL of DNA to each reaction. All probes used in the CBM study were fluorescein labeled. Primers and probes are listed in Table 6.1, and the detection limits of each assay are listed in Table 6.3.

5.1.11 Avian schistosomes monitoring

The *18S* avian schistosomes targeting qPCR assay was performed as described in Narayaran et al. 2015 and Rudko et al. 2017, and are described Methods 6.2.10(Narayanan et al. 2015; Rudko et al. 2018). The LOD_{95} of this technique is 3.4 gene copies/ rxn (Table 6.2). qPCR master mix (IDT DNA) containing 1x Master mix, and 200nm forward reverse primer and fluorescein-labeled probe was used.

5.1.12 *McyE* gene cyanobacteria, and total cyanobacteria monitoring

The *mcyE* gene targeting qPCR assay was performed as described in Qiu et al. (2013) and Sipari et al. (2010). The LOD95 of this technique is 7 copies/5uL. qPCR master mix (IDT DNA), containing 1x Master mix, and 200nm forward reverse primer and 125nm fluorescein-labeled probe was used. The 16S total cyanobacteria assay was published in Al Tebrineh *et al.* (Al-Tebrineh et al.; Al-Tebrineh et al. 2010; Sipari et al. 2010; Qiu et al. 2013). The LOD95 of this technique is 4 copies/5uL.

5.1.13 HF183 human-associated *Bacteroides* monitoring

This *16S* rRNA gene-targeting assay was performed as described in Haugland et al. 2010. The LOD95 of this technique is 7.2 gene copies/rxn. qPCR master mix (IDT DNA), containing 1x Master mix, and 100nm forward reverse primer and 80nm fluorescein-labeled probe was used. Data are expressed in geneome equivalents based on a 16S copy number of 7(Stoddard et al. 2015).

5.1.14 *P. aeruginosa* monitoring

The *gyrB* gene-targeting assay was performed as described in Lee *et al.* (2011). qPCR master mix (IDT DNA), containing 1x Master mix, and 200nm forward reverse primer and 200nm fluorescein-labeled probe was used. The LOD95 of this technique is 6 copies/5uL

5.1.15 *Enterococcus* monitoring

The 23S rRNA gene assay for *Enterococcus* spp. was performed according to Method 1611 (USEPA 2012), except samples were not quantitated using the ΔΔCCE (calibrator cell equivalent) method because this method requires the parallel extraction of purified, filtered *E. faecalis*, which would be inappropriate to give to CBM partners. Therefore, samples were quantitated to a standard curve, and data were analyzed to reflect genome equivalents based on 4 copies of 23S rRNA gene expected in *E. faecalis.* Of course, 23S is a multicopy gene and different species (and life-cycle stages) of *Enterococcus* have different copy numbers of 23S rRNA gene (Lobritz et al. 2003).

5.1.16 In-field qPCR method

Mastermix components and concentrations were unchanged between the lab method and the field method, nor were the thermocycling parameters.

5.1.17 Inhibition controls

Inhibition controls were performed as described in Rudko et al. (2017). Plasmid control DNA was spiked in excess into qPCR reactions containing 5uL of water sample DNA, and inhibition was defined as a 3-ct (i.e. 1 log) shift in amplification. Inhibition controls for *Enterococcus* samples were performed according to USEPA Method 1611 which uses the SKETA assay to detect salmon testes DNA(USEPA 2012).

5.1.18 Creation of the field kits

Field kits given to CBM partners contained: the M1 DNA extraction kit (Biomeme™), 1.5 mL snap-cap tubes, sample collection vials (Corning), a 20 micron plankton tow (Acquatic Research Instruments), 0.45 uM polycarbonate filter funnels (Pall,

FMFNL 1050), a 20 uL pipette, a box of pipette tips, PCR tubes, a laptop, and ChaiBio™ Open qPCR thermocycler, all the necessary cables, and reaction strips (Table 6.3)

5.1.19 Training of CBM partners

CBM partners were provided with a training video, and a written protocol. Additionally, they were provided with two in-person training session. Typically we would demonstrate the method in our laboratory, and the second training session would on-site at their location, where the CBM partner would run their first samples.

5.1.20 Capturing CBM partners perceptions of the method

CBM partners (6 in total) were administered a survey with open-ended questions regarding the implementation of the method. All 6 CBM partners submitted a completed survey. Surveys were blinded from the researchers to encourage honesty from participants; a research associate received the surveys via email and edited them to remove any personal identifiers before sending them to the analyst. Data were analyzed using deductive thematic analysis. Open coding was used, and codes were developed and modified as the analysis took place. Analyzing the codes enabled the identification of initial themes; these preliminary themes were refined to demonstrate interesting patterns in the data that were important to the successes or failures of the implementation. Themes were realized semantically (i.e. the explicit or surface meaning of the data), and latently, to identify and examine underlying ideas and assumptions that inform the semantic content of the data (Braun and Clarke 2006).

5.1.21 Bland-Altman plots

Bland-Altman plots were created in GraphPad Prism 8 on the log transformed copy number per 5uL data. Log transformation was performed prior to conducting the analysis because this method assumes that the SD of method differences is uniform across measurements, but it has been documented that variability in measurement becomes greater when a larger value or amount of analyte is being measured (Martin Bland and Altman 1986; Bartlett and Frost 2008).

5.1.22 Statistics used to assess the accuracy of the CBM program

Statistical analyses were conducted in SPSS (version 25). Graphs were made in GraphPad Prism 8. Limit of Detections were calculated using the POD/LOD calculator(Wilrich and Wilrich 2009). Maximum log difference was calculated by calculating the upper limit of the 95% confidence interval of the average log difference between paired samples for each target. Interclass correlation analysis was performed in SPSS on the log-transformed data using a two-way random effects model with average measures, and a type c model with a consistency definition. A two-way random effects model was selected because it models both an effect of operator and the sample, and assumes that both are drawn randomly from larger populations.

5.2 Avian schistosome methods

5.2.1 Water sampling

Twenty-five-liter water samples were collected at each location and passed through a 20 cm x 80 cm x 20 micron zooplankton net (Aquatic Research Instruments). Samples were collected (1L at a time) across the entire swath of the beach, in approximately waist deep water, with the goal of maximizing the likelihood of capturing free-swimming cercariae. Debris from inside the net were washed down using well water followed by a 95% ethanol wash and collection in 50mL conical tubes. This 50mL sample was then passed through a 0.45µM polycarbonate filter (Pall), the filter paper removed from the bed, and stored in 1ml of 95% ethanol for transport to the University of Alberta (Canada) for DNA extraction and qPCR analysis.

5.2.2 Environmental variable measurements

Wind direction, and effect (ie, onshore, offshore, along shore), as well as with time of day were also recorded at each sampling site.

5.2.3 Snail, miracidia and cercariae collection from Lakes in Michigan

Cercariae, miracidia and snails were collected from Higgins Lake, Lake Leelanau, and Crystal lake in 2016 and 2017. **In 2018, s**even lakes in Northern Michigan were selected for study: Big and Little Glen, Lime, North and South Lake Leelanau (Leelanau County); Long, Elk/Skegemog (Grand Traverse County); Charlevoix and Walloon Lakes (Charlevoix County). The lakes monitored were selected because they had either been removing and relocating common mergansers for the last 1-3 years, were attempting to qualify for a trap and relocation permit or were simply interested in conducting a full assessment to determine the extent of their swimmers itch problem.

To collect snails, 1m² weighted hoops were randomly tossed throughout each shoreline collection site, collection sites were selected with the help of local riparian's and were places where people reported contracting swimmer's itch. All snails within the hoops were collected using specially designed snail scoops with attached mesh bag nets. Following collection, snails were isolated into individual plastic wells filled with well water, housed throughout the day and night, and exposed to bright natural and fluorescent light the next morning, as described in Blankespoor and Reimink (1998). Snail genera were identified morphologically. Trematode cercariae shed from snails were pipetted into collection tubes and preserved with 95% ethanol (schistosomes were noted). Ethanol preserved samples were stored at ~ -20C and then transported to the University of Alberta for further processing.

Tissue from each representative snail species was also preserved in 95% ethanol. Miracidia were collected from fresh bird feces deposited on the beach by diluting fecal samples in well water, hatching the miracidia under fluorescent lights, and pipetting into 95% ethanol. The species and approximate age of the bird from which the feces were deposited were recorded at the time of collection.

5.2.4 DNA barcoding of avian schistosomes and non-avian schistosome cercariae

The *CO1* region was PCR amplified from the DNA extracted from purified cercariae/miracidia samples. If a cercaria had been morphologically characterized as an

avian schistosome, the *CO1* region was amplified using the pan-schistosome barboding primers listed in Table 6.1(Brant and Loker 2009b). If a cercaria was not morphologically identified as an avian schistosomes, the pan-invertebrate primers (the Folmer primers) were used for sequencing (Table 6.1). The same primers that were first used to amplify from the *CO1* gene were also used to amplify and barcode snail tissues(Folmer et al. 1994). PCR products were Sanger sequenced (Macrogen Inc, South Korea). Forward and reverse sequences were trimmed prior to being pairwise aligned using Geneious.

5.2.5 DNA extraction from cercariae

Ethanol (95%) preserved cercariae were extracted by first removing the ethanol using a vacuum centrifuge. Once dried, DNA extraction was performed using the Qiagen DNAeasy™ Blood and Tissues kit. The standard tissue lysis protocol was followed, but only using 25% of the buffer volume recommend in the protocol.

5.2.6 Extraction of schistosome DNA from water samples

Filters were placed in 200µl of lysis buffer AL (Qiagen) and 20µL of proteinase K were then added to the tube containing the filter, along with 1mm silica carbide beads (in 2019, this changed to 1mm glass beads), and bead beat for 10 minutes on high using a vortex, or for 2 minutes using the Beadmill homogenizer. before DNA extraction using the DNeasy Blood and Tissues Extraction Kit [Qiagen]. In 2017, DNA extraction was accomplished in Michigan using the Biomeme™ Environmental DNA extraction kit (Biomeme™, Philadephia, PA, USA) according to the manufacturers instructions, with one deviation—samples were eluted into 100uL of elution buffer. This change was made to reduce costs associated with shipping samples as the Biomeme™ extraction can be accomplished lakeside in Michigan. In 2018 and 2019, Michigan also performed DNA extraction using the DNA easy protocol.

5.2.7 Method robustness

Three consecutive samples (25L each), at 10 sampling locations on Crystal Lake were collected and evaluated to assess the precision of the entire method in the field.

Additionally, three samples containing an unknown number of cercariae were sent from researchers in Michigan to the University of Alberta where qPCR was performed in a single blind trial to assess the accuracy of the qPCR assay in predicting the true number of cercariae in a sample.

5.2.8 Time of day sampling

Eight locations were selected on Big and Little Glen Lakes for sampling on 25 July 2017. Two teams collected water samples at each of 4 locations at approximately 8:00, 8:15, 8:30, and 8:45 a.m. The 4-site collection sequence was repeated beginning at 12:00 p.m., 4:00 p.m. and 8:00 p.m.

5.2.9 Depth of water sampling

A stacked water column trap was designed and built to capture stacked subsamples of water. The water column trap can be used at depths up to 1.5m deep. The trap consists of 3, 4-inch PVC ball valves connected in series by 2, 4x6 inch threaded nipples. (Menards #MA0013H, #CC08350). Each section corresponded to a depth of 50cm. The column trap was lowered into the water with valves open. Each valve was then manually closed, trapping 550 mL of lake water in each connector nipple. The subsamples were then emptied into separate buckets by opening the valves, one by one beginning at the bottom. A total of 30 full columns were collected. The buckets were then emptied into a 20um mesh plankton tow, the net washed with 95% ethanol, and the sample concentrated to ~50mL. The depth of the water where sampling occurred was approximately 1.2 meters. Therefore the top most section of the trap was not completely covered by the water, and the top most section of the trap only collected the top 30cm of the water column (Figure 6.2).

5.2.10 qPCR detection of schistosome 18S rRNA

The *18S* rRNA qPCR assay was run according to the method described in Narayanan et al (2015) (Table 6.1) Unlike the original method, samples were quantified to copies of plasmid DNA using a standard curve that consisted of 50,000, 5000, 500, 50 and

0.5 copies of cloned puc57 plasmid DNA (Genscript, New Jersey, USA) containing the avian schistosome *18S* rRNA gene. The LOD_{95} of this technique is 3.4 copies per reaction (upper limit 7.9, lower limit 1.5) (Table 6.1, Table 6.2) (Wilrich and Wilrich). Samples and standards were performed in triplicate, with inhibition controls run for each sample in duplicate. Inhibition controls were performed as described in Rudko et al. (2017), where a plasmid inhibition control was spiked into water samples and subsequently detected by qPCR (Table 6.1). Inhibition was defined as a 3-cycle threshold shift (USEPA 2012). Thermocycling (qPCR) was performed in a post amplification room using the ABI 7500 fast Real Time qPCR system.

5.2.11 Quantitation of plasmid copies per cercaria

Hand counted stocks (stereo microscope [Zeiss]) of *T. stagnicolae* cercariae (1, 5, 10 & 20 cercaria(e)) were counted, and DNA extracted, according to (Webster 2009). The average *18S* rRNA copy number was determined using qPCR. Results were used to generate a standard curve to convert DNA copies per sample to cercariae per sample. conversion equation: $x = [(y + 56521) / 57736]$ was derived and used to calculate the number of cercariae per sample. *X* is the number of cercariae and *y* is the copy number per sample.

5.2.12 Extraction efficiency

Extraction efficiency was determined by spiking 5 *T. stagnicolae* cercariae onto a clean 0.45µM filter membrane and preserving the samples in 100% ethanol. Samples were analyzed in the same manner as described above for the water samples. conversion equation was applied to determine the number of cercariae recovered through the method under ideal circumstances.

5.2.13 Statistics to assess the effect of environmental variables on cercariae concentrations

The change in cercariae concentrations relative to the time of day, and wind direction relative to the shoreline, were assessed using a generalized linear model with a

log link function, based on the negative binomial distribution. The outcome variable was cercariae concentration. Explanatory variables were lake, week (standardized to account for different days during the same week across years), time of day (morning or afternoon), and wind direction (on shore, off shore, and along shore). In the initial model, the interaction effect of time of day and wind direction was also tested, but this interaction was found non-significant and was removed to improve model fit. Additionally, site on the lake, year, and lake were modeled as random effect variables in the original model with independent correlation matrices, but were found to be insignificant and were removed from the final model. All statistical analyses were performed using SPSS (version 24, IBM, Armonk, North Castle, New York, USA). T-tests and graphs were executed using PRISM (ver. 7.0, GraphPad)

5.2.14 Study Site Copper Sulfate Study

South Lake Leelanau (SLL) is a mesotrophic lake with a surface area of 5,932 acres and a mean depth of 7.2 m. A submerged riverbed connects it to North Lake Leelanau, which measures 2,914.3 surface acres with a mean depth of 12.2 m. The combined shoreline of the lakes is about 66.3 km (Breck, 2004. The study area was in the Glazier-Persik community on the southwest shore of SLL. This lakefront runs approximately 625 meters and is densely populated with residences used as year-round homes, summer homes and rental properties.

Three water collection sites were selected within the copper sulfate treatment area, one in the middle and one near each end. Two control sites were selected outside the treatment area, one 600 m to the north and one 300 m to the south, both far enough removed so no residual copper could impact the snails or cercariae. Current flow and prevailing winds consistently move the planktonic community in the direction from south to north at this location.

Research was conducted in late June and July to assure water temperatures and photoperiods were conducive to cercariae shedding, and to provide enough pre- and post-sampling dates for collecting statistically significant data.

5.2.15 Copper sulfate application

A total of 90.13 kg of fine granular copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was applied evenly by a line of volunteers wading across the treatment area. Copper sulfate was applied by hand using small handheld scoops on the evening of June 25 2017 under clear and calm conditions. Conditions remained calm and stable throughout the night and into the next day, well past the stage when the toxic copper had all precipitated as non-toxic $CuCO_3$.The area measured approximately 625 m long X 32 m wide with an average depth of 0.9 m. The total water volume within the treatment area was estimated at 18,200 m^3. The average copper concentration was 51.11 ppm, which is well above the recommended concentration of 20 ppm. Water alkalinity plays a role in how quickly copper precipitates as $CuCO_3$, but based on the alkalinity of most lakes in Michigan, lethal concentrations of the copper ion are expected to last for less than 24 hours (Michigan Department of Environmental Quality, 2014).

5.2.16 Snail collections before and after copper sulfate application

Four days prior to the 25 June copper sulfate application, snail densities were determined for all snail species. Using snorkel equipment and wet suits, two researchers randomly tossed weighted 0.5 m^2 vegetation sampling hoops (Forestry Suppliers #78503) and recorded the number of snails of each species in each hoop. Each field biologist threw the hoop 200 times, which resulted in a total sample area of 200m^2. A zigzag pattern through the length of the treatment area assured all water depths were sampled. Only mature snails were counted. The identical procedure was repeated 10 days after copper sulfate treatment. Additionally, snail densities were assessed 50 m on either end of the application area to serve as a control, with a total of 50 m^2 sampled in each control zone.

5.2.17 Water collection before and after copper sulfate treatment

Pre-treatment water samples were collected at the three experimental and two control locations daily from June 19—23. The first week post-treatment, water samples were taken two different days (June 28 & 29) and then once a week for the next four weeks.

5.2.18 Statistics to assess efficacy of copper sulfate treatment

A two-way ANOVA was used to compare pre and post treatment cercariae abundance. A chi-square test was used to compare number of snails found on each side of the treatment area.

5.2.19 Bird Surveys

Diversity of the avian community within each lake ecosystem was measured by conducting a complete shoreline waterfowl survey, traversing the near-shore perimeter slowly by boat. All waterfowl were observed with the aid of field glasses and recorded by species, location, sex, and age (hatch-year and after hatch-year). Whole-lake surveys were conducted in mid-July when most broods had hatched yet had not begun flying.

5.2.20 Design and validation of the species-specific qPCR assays

Species-specific qPCR assays (primers and Taqman probe) were designed specific to the CO1 genes *of T. stagnicolae, T. szidati (=T. ocellaea), T. physellae,* and *Anserobilharzia brantae (=T. brantae)*, and an newly discovered yet unnamed species of avian schistoome shed from a *Planorbella trivolvis* snail (Table 6.1). The detection limits for these assays are listed in Table 6.2.

5.2.21 Avian schistosome qPCR and species-specific qPCR

The 18S pan-avian schistosome qPCR was carried out as described in Rudko et al. 2018. To calculate absolute numbers of cercariae based on the amount of DNA detected, we performed the method from Rudko et al. 2018, briefly a percent recovery of 4% was assumed, and the formulae $x = [(y + 56521)/57736]$. The species-specific qPCR assays were

performed as follows; 200nm each of primer and probe were added to Prime Time Gene Expression Master Mix (IDT) in a clean room. Samples were run in duplicate and copy numbers were ascertained for each run by performing a standard curve consisting of 50,000, 5,000, 500, 50, 5 and 0.5 copies of purified plasmid DNA cloned with the CO1 gene of each respective species. Genes were synthesized by IDT, and cloned into pJET 1.2. Fast cycling was performed on the Quantstudio 3 (Applied Biosystems), using the following protocol: 95° hold (20 seconds), 95° denaturation (1 second), 60° annealing (20 seconds).

5.2.22 Data analysis

Calculations (ie: cercariae abundance and waterfowl density) were performed in Microsoft Excel, and heat maps were made in ArcMap (10.6).

5.2.23 2019 Community based monitoring sampling for swimmer's itch

Community partners in Michigan collected samples for CBM partners in 2019. Volunteers collected swimmer's itch samples every Tuesday between 9 and 10 am. Samples from each lake were then transported by volunteers to the CBM partners field laboratory where they were extracted using the methods described above.

5.3 Bacterial community based monitoring

5.3.1 Specificity of the 16S total cyanobacteria and *P. aeruginosa* specific assays against common environmental bacteria

Glycerol stocks of *E. coli* 25922, *Campylobacter jejuni, E. coli* O157:H7, *Shigella sonnei, Klebsiella oxytoca*, *Enterococcus faecalis*, *Edwardsiella tarda*, *Enterobacter hormaechei*, *Stenotrophomonas maltophilic*, *Ochrobactrum anthropic*, *Proteus vulgaris* 6380, *Elizabethkingia meningoseptica* 13253, *Staphylococcus epidermidis*, *Acinetobacter baumannii*, Environmental *P. aeruginosa* isolate 1, and Environmental *P. aeruginosa* isolate 2, and *P. aeruginosa* PAO1, were grown overnight on selective agar. Single colonies were picked and mixed into water in a PCR plate, this task was accomplished by another scientist and the plate was blinded from me. This plate was

boiled for 5 minutes prior to 5uL of lysate being added to the qPCR master mix containing the *16s* total cyanobacteria assay, and the qPCR master mix containing the *gyrB P. aeruginosa* assay.

5.3.3 Sampling for cyanobacteria

As described in 6.1.12.

5.3.4 Sampling the Borden Park Natural Swimming Pool

As described in 6.1.14

5.3.5 Sampling for enteric bacteria

As described in and 6.1.14, 6.1.15

5.3.6 qPCR methods for cyanobacteria and *P. aeruginosa*

As described in 6.1.12

5.3.7 DNA extraction of Enterococcus samples

As described in 6.1.15

5.3.8 Bacterial source-tracking methods

C. marimamallian source-tracking was performed using 0.25 uM of forward and reverse primers (Table 6.1), and 0.125 uM fluorescein labeled probe(Lee et al. 2013). Ruminant source-tracking was performed using 0.2 uM of both primers and fluorescein labeled probe. Human associated *bacteroides* source-tracking via HF183 was accomplished using 1 uM of each primer and 0.08 uM fluorescein labeled probe(Boehm et al. 2015). All assays were performed using a fast-cycling 2-step qPCR (95C [30 sec], 40x, 95C [30 sec], 60c [10 sec]). In 2018, sample volumes were 50ml samples, and thus 5uL of extracted DNA was used to perform HF183 testing. In 2019, sampling volumes were reduced to 30ml, and therefore 10uL of extracted DNA was used.

5.3.9 Statistics assessing bacterial CBM projects

Statistical analyses (log reduction values and Welsch's t-test) were performed in PRISM (ver. 8), and heatmap intensity value were coded my hand, and maps were made using HeatMapper (Babicki et al. 2016).

5.4. *Dreissena polymorpha* community based monitoring

5.4.1 Sample collection for *D. polymorpha* larvae

Samples were collected with a Wisconsin net (20 cm mouth opening, approximately 70 cm long, with 63 µm mesh). A vertical haul was used to obtain an integrated depth sample. The collected samples from each haul were decanted into a 1 L bottle, 50ml of this was subsampled for DNA analysis.

5.4.2 Extraction of DNA from plankton tow samples

50 mL samples were centrifuged at 4000 g for 20 minutes to pellet the debris. Supernatants were decanted, and 500uL subsamples of the pellet were extracted using the DNAeasy™ DNA extraction, tissues protocol, with no modifications.

5.4.3 Folmer region PCR on native bivalves

Native mussels were individually removed from specimen jars and placed into a 2ml snap-cap tube containing buffer ATL (lysis buffer from the Qiagen DNAeasy™ Blood and Tissues kit), they were crushed using a pestle against the bottom of the tube, the samples were then processed according to the kit protocol. Barcoding was performed using the Folmer primers according to the method described in 5.2.4(Folmer et al. 1994). Samples were analyzed using BLAST, and a greater than 95% match was considered a species level match, while a less than 95 but greater than 90% match was considered a genus level match.

5.4.4 *D. polymorpha* qPCR assay

The primers for the *Dreissena polymorpha* assay were initially published in Ventura et al. 2017(De Ventura et al. 2017) This assay targets the CO1 gene and has been previously validated for cross reactivity again the Quagga mussel (*Dreissena bugensis*), I desgined a fluorescein labeled probe that bound in the middle of the primers to improve specificity (Table 6.1). The probe was designed first by aligning CO1 genes of a variety of representative sequences of *D. polymorpha* CO1 from NCBI Genbank. The assay was testing against water samples collected in Michigan that were known to be infested with *D. polymorpha* mussels, *D. polymorpha* mussels isolated from boat inspection stops in Alberta, and also was tested against the entire ABMI native mussels collection (no cross reactivity was observed). qPCR was performed using 0.2 uM of both primers and probe, and were performed using a fast-cycling 2-step qPCR (95C [30 sec], 40x, 95C [30 sec], 60c [10 sec]).

5.3.5 *D. polymorpha* data analysis

Heat map intensity value were coded my hand, and maps were made using HeatMapper (Babicki et al. 2016).

5.4 Tables

Table 5.1 Primers and probes used in this study

Assay		Sequence 5'-3'
18s rRNA avian schistosomes (Narayanan et al. 2015)	Forward	AGCCTTTCAGCCGTATCTGT
	Reverse	TCGGGAGCGGACGGCATCTTTA
	Probe	/FAM/AGGCC/ZEN/TGCCTTGAGCACT/IABkFQ/
T. stagnicolae CO1 (Rudko et al. 2019)	Forward	ATTATCTAATTACTAATCATGGGATTGCA
	Reverse	ATGCCAAATCATCTAAACCCAAC
	Probe	/FAM/ACCAAACCC/ZEN/ACCAATCAATACAGGCA/IABkFQ/
T. szidati CO1 (Rudko et al. 2019)	Forward	GTTGTTGGGTTCTGTTAAATTTATAAC
	Reverse	AGACGTAAACAAATACGCCCA
	Probe	/FAM/TCTTAGTTC/ZEN/TCGGGTTTCGGTTGTTGTT/IABkFQ/
T. physellae CO1 (Rudko et al. 2019)	Forward	TGGTTTGGTWTGTGCTATGGG
	Reverse	AKTCTTAACATCCAATCCY
	Probe	/FAM/TGAGC+TCA+TACTACACTACC+TAAAC/IABkFQ/

A. brantae CO1(Rudko et al. 2019)	Forward	GATTCCTTCAGAGATTTATAAATATTTA
	Reverse	ACGAGGTAACGCCAAATC
	Probe	/FAM/TACCAAACC/ZEN/CRCCAATRAACACRGGCA/IABkFQ/
P. trivolvis tricho CO1(Rudko et al. 2019)	Forward	CTTCATTTGGCGGGTGTT
	Reverse	AAATAAGCCCAAACGATAACAG
	Probe	/TEX615/TTTGTAAGGCTTAGATCTCGGGTTT/IAbRQSp/
Pan-intervebrate CO1 (barcoding primers LCO/HCO)(Folmer et al. 1994)	Forward	GGTCAACAAATCATAAAGATATTGG
	Reverse	TAAACTTCAGGGTGACCAAAAAATCA
Pan-schistosome CO1 (barcoding primers)(Brant and Loker 2009a)	Forward	TGAGCWAYHACAAAYCAHGTATC
	Reverse	TTTNTYTCTTTRGATCATAAGC
16S cyanobacteria (Al-Tebrineh et al.)	Forward	AGCCACACTGGGACTGAGACA
	Reverse	TCGCCCATTGCGGAAA
	Probe	FAM/CCTACGGG/ZEN/AGGCAGCAGTGGG/IABkfq
mcyE cyanobacteria assay (Sipari et al. 2010; Qiu et al. 2013)	Forward	AAGCAAACTGCTCCCGGTATC
	Reverse	CAATGGGAGCATAACGAGTCAA
	Probe	/FAM/CAATGGTTAT/ZEN/CGAATTGACCCCGGAGAAAT /IABkFQ
P. aeruginosa gyrB assay(Lee et al. 2011)	Forward	GGCGTGGGTGTGGAAGTC
	Reverse	TGGTGAAGCAGAGCAGGTTCT
	Probe	/FAM/TGC+AGTG+GA+ACGACA/IABkFQ
Enterococcus 23S rRNA(USEPA 2012)	Forward	GAGAAATTCCAAACGAACTTG
	Reverse	CAGTGCTCTACCTCCATCATT
	Probe	FAM/TGGTTCTCTCCGAAATAGCTTTAGGGCTA/IABkFQ
HF183(Cao et al. 2018)	Forward	ATCATGAGTTCACATGTCCG
	Reverse	CGTAGGAGTTTGGACCGTGT
	Probe	FAM/CTGAG/ZEN/AGGAAGGTCCCCCACATTGGA/IABkFQ
Rum2Bac (Raith et al. 2013)	Forward	ACAGCCCGCGATTGATACTGGTAA
	Reverse	CAATCGGAGTTCTTCGTGAT
	Probe	FAM/ATGAGGTGGATGGAATTCGTGGTGT /IABkFQ
LeeSeaGull (Lee et al. 2013)	Forward	AGGTGCTAATACCGCATAATACAGAG
	Reverse	GCCGTTACCTCACCGTCTA
	Probe	FAM/TTCTCTGTTGAAAGGCGCTT/IABkFQ
D. polymorpha (De Ventura et al. 2017)	Forward	GCTAAGGGCACCTGGAAGCGT
	Reverse	CACCCCCGAATCCTCCTTCCCT
	Probe	FAM/ACCAATCAA/ZEN/TTTCCGAATCCCCCCA/IABkFQ
Inhibition control (Rudko et al. 2017)	Forward	GGTTACGTTACCGATTGTGTTAT
	Reverse	GCACGGAGAAGTATTCTTGATATAG
	Probe	FAM/TCGCATACTATGACCGAGACCCTGT /IABkFQ

SKETA salmon sperm inhibition control(USEPA 2012)	Forward	GGTTTCCGCAGCTGGG
	Reverse	CCGAGCCGTCCTGGTC
	Probe	FAM/AGTCGCAGGCGGCCACCGT/IABkFQ

FAM = fluorescein; TEX615 = Texas Red 615; IABkFQ = Iowa Black fluorescent quencher; ZEN= ZEN fluorscent quencher; +(A/T/C/G) = Locked Nucleic Acid

Table 5.2 Assay detection limits (with 95% confidence)

Assay	LOD95 (copies/5uL)	Upper 95	Lower 95
18s avian schistosom*e*	3	8	1
T. stagnicolae CO1	27	35	20
T. szidati CO1	7.9	10	2
T. physellae CO1	12	15	6
A. brantae CO1	58	60	52
P. trivolvis tricho CO1	89	100	32
16S rRNA cyanobacteria	4	10	2
mcyE cyanobacteria assay	7	12	2
P. aeruginosa gyrB assay	6	12	3
Enterococcus spp. *23S*	3	6	1
HF183	7	10	5
Rum2Bac	10	16	6
LeeSeaGull	6	10	4
D. polymorpha	4	9	2

5.5 Figures

Figure 5.1 Performance of gel-based field stable master mix on copy numbers of extracted DNA.

Gel based master mix is shown in red, while the same sample performed using IDT master mix is in teal.

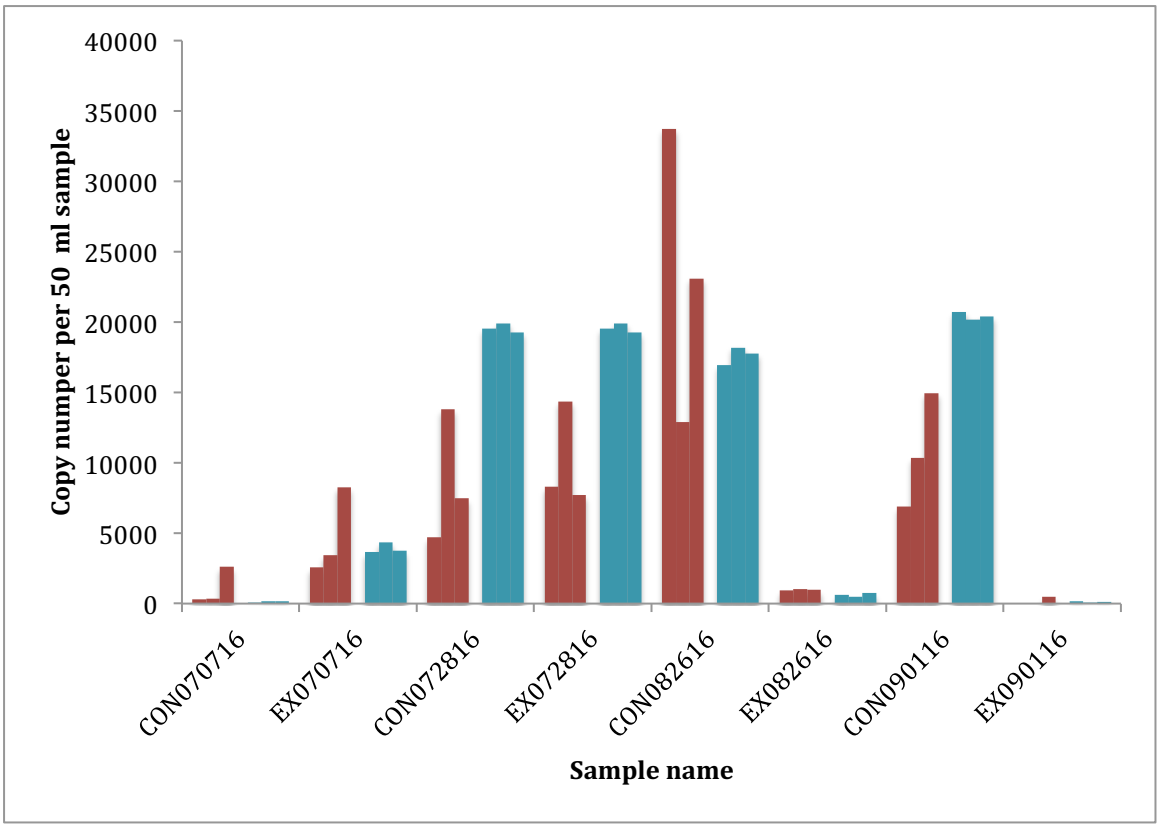
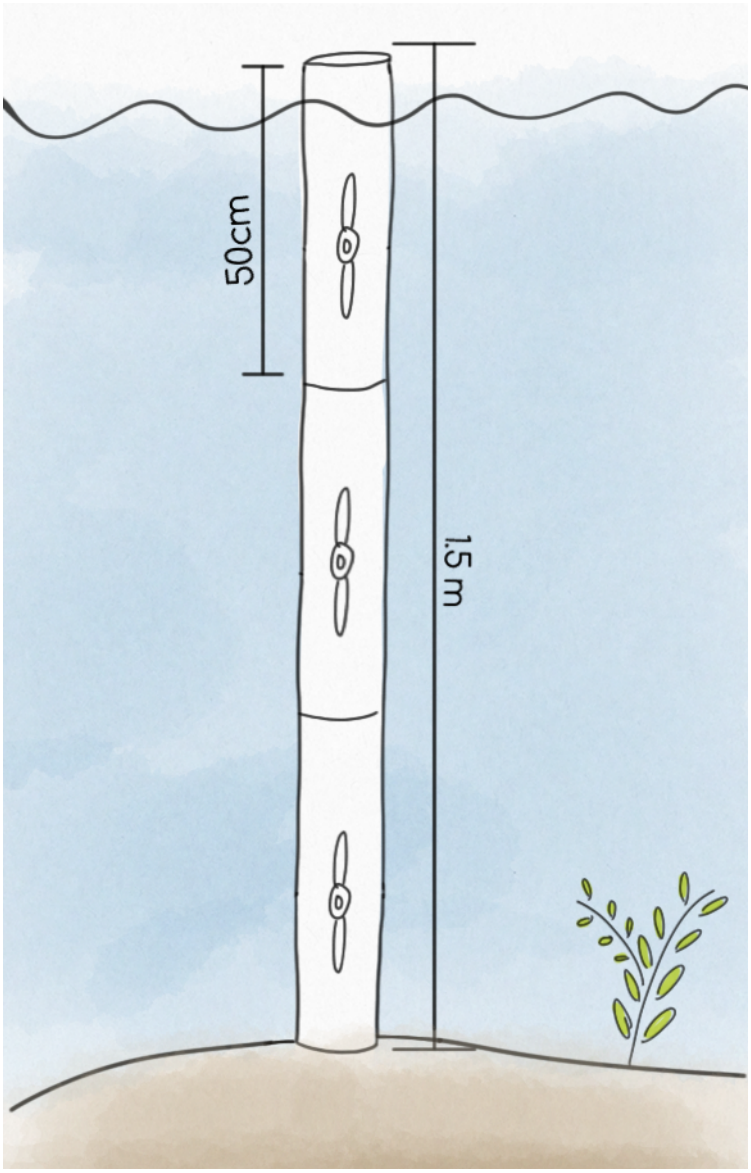


Figure 5.2 Design of the stacked V trap.



Chapter 6: Discussion

Author: Sydney Rudko

6.1 Community based monitoring is integral to the future of recreational water monitoring

Recreational water is a social, cultural and economic asset to society. It provides fresh water for drinking, social areas for gathering, and places for people to commune with nature (Klessig 2001). Recreational water also supports bustling summer villages and campgrounds, bringing economic prosperity to rural areas (McNaughton 1994). Water is an important natural resource and its' protection is essential for preserving drinking water and for preserving the various ecosystems that it supports. Monitoring and surveillance of natural water bodies is essential for their protection, but recreational water can be challenging to monitor. It is dispersed geographically, making routine sampling difficult and sample transport costly and labourious. Additionally, there are numerous stakeholders involved in recreational water at any given location. Property owners, industry, provincial parks, or First Nations communities, for example, may all utilize a particular lake or contribute to a watershed, and they may have different perspectives on what is a concern and how to manage the water body and the surrounding watershed. Furthermore and as was discussed earlier in the introduction, there are a myriad of potential organisms to monitor for.

In 1972, Alvin Weinberg coined what he called "trans-science", a term meant to highlight the fact that there are questions about science that can be asked of science, but cannot be answered by science (Weinberg 1972). He acknowledges the fact that there is a grey area between science and policy, and that there are some questions that, when asked of science, requires scientists to look past the facts and make determinations based on other values (i.e.: social values, environmental values). Weinberg's contemporaries have expanded upon this idea, arguing for the inclusion of other forms of knowledge and expertise in policy and management decision making. Such expertise may be called "lay

expertise", or "local knowledge" (Carolan 2006). Carolan (2006) calls for the inclusion of "public expertise", which he defines as the explicit incorporation of societal and stakeholder values in the "doing" of environmental science and resource management. In environmental decision-making there are different forms of expertise (Carolan 2006). An academic studying the ecological interactions in a watershed has a different type of expertise than a local fisherman noticing declines in an important fish population. Likewise, a policy expert may be well versed on the specific laws and regulations for a watershed, while a local beach manager might understand the specific on-the-ground factors that promote or inhibit the execution of a particular policy. To summarize, all stakeholders have different expertise to contribute to a particular problem, and the best solution is likely only possible through collaboration (Conrad and Hilchey 2011b).

Public participation in environmental monitoring is increasing, likely due to the dramatic increase in environmental consciousness that has happened in recent decades and that the CBM approach to environmental management and monitoring has been widely advocated for globally (Conrad and Hilchey 2011b; Conrad and Hilchey 2011a). Additionally, the ability for all levels of government to effectively monitor the environment has decreased in part due to the increasing complexity of environmental issues, as well as due to funding cuts (Conrad and Daoust 2008; Fernandez-Gimenez et al. 2008). CBM has been demonstrated to be a cost effective way for governments to increase their monitoring capacity (Yarnell and Gayton 2003). In addition, studies have found that CBM improves collaboration and knowledge about the environment for participants (Trumbull et al. 2000; Fernandez-Gimenez et al. 2008; Buyx et al. 2017). qPCR can be used within CBM approaches to water monitoring. qPCR is a platform that can be used for monitoring for a variety of environmental hazards, including hazards effecting health, ecology, and the economy. qPCR testing can be unified around a single collection method (i.e.: grab sampling, composite sampling, or plankton tow sampling), and a single sample collected can be used for multiple qPCR tests. As this thesis demonstrated, qPCR can also be deployed across geographical distances in the form of a comprehensive decentralized monitoring system.

While there are limitations to qPCR (notably, an inability to distinguish live cells from dead cells), CBM qPCR could function as a passive surveillance system for a variety of biological targets at key locations. This could complement the traditional centralized environmental monitoring systems that already exist, providing data on potentially hazardous areas while eliminating costs associated with transporting and testing negative samples. Moreover, CBM qPCR could also be deployed more specifically as a sentinel surveillance tool. As a flexible platform capable of detecting virtually any organism with DNA, assuming a test and sample collection method is available and established, CBM qPCR could be deployed quickly and specifically to assess the presence or concentration of an organism. CBM qPCR's applicability goes far beyond the sphere of recreational monitoring. The method could potentially be adapted for small scale, rural drinking water treatment plants, for water monitoring activities on First Nation's reserves, or even incorporating water monitoring into school curricula.

To my knowledge, this study is the first of its kind to deploy a portable qPCR method amongst a variety of partners. In the field of water monitoring more broadly, it contributes a new methodology to this field and demonstrates conditions by which a CBM qPCR project can be successful. This work has contributed significantly to the world of swimmer's itch research and more broadly the monitoring of environmental stages of parasites. The studies contained in this thesis have moved monitoring for avian schistosomes from using microscopy to identify schistosomes morphologically to utilizing qPCR cercariometry to determine the number of cercariae in the water (something that was previously impossible using snail collection and morphological characterization of species). This test has gone on to become a test accepted by the Michigan Department of Natural Resources as a legitimate means of measuring swimmer's itch infestation at beaches in Michigan. I also utilized a generalized linear model to predict when concentrations of cercariae are likely to be higher for recreators, providing useful information to communicate to recreators who wish to reduce their risk of contracting the skin condition (Rudko et al. 2018). Furthermore, I worked to demonstrate that copper sulfate application applied to specific beaches is ineffective at reducing the number of

cercariae present in the water column (Froelich et al. 2019). Finally, I developed source-tracking assays that enabled the longitudinal monitoring of three species of avian schistosomes at four lakes in Michigan, which clearly demonstrated ecologically that spring and fall migrants contribute the majority of swimmer's itch causing parasites that plague recreators during July and August; in the near future, this will hopefully end the ecologically disruptive, expensive, and ineffective which relocates common merganser broods from lakes in northern Michigan to control swimmer's itch (Rudko et al. 2019). The fact that this work was all conducted in a short period of only three years is further testament to the power of the community based monitoring we've been conducting in collaboration with a contractor firm and many of the lake associations in Michigan.

In Alberta I have worked to integrate CBM qPCR testing into a variety of different recreational water contexts, including monitoring recreational lakes for toxin-producing cyanobacteria, monitoring a swimming pool for *P. aeruginosa,* and monitoring for invasive zebra mussel species. These studies have generated numerous interesting observations and hypotheses about the incidence and occurrence of these pathogens and pathogen indicators in a variety of different contexts. These studies serve to demonstrate the flexibility and adaptability of CBM qPCR.

Overall all of these studies work in concert to demonstrate the broad utility of CBM qPCR. My analyses show that when deployed in a controlled manner, CBM qPCR can generate robust data that, while more variable than traditional laboratory methods, agrees with results produced by central labs. While not every implementation of every test went perfectly, this thesis also highlights the importance of training and ongoing support for CBM partners. Additionally, these studies support ongoing compliance testing of CBM partners: this could involve giving CBM partners a sample spiked with a known concentration of organism and having them perform the qPCR method themselves and evaluating them. These studies together contribute to a growing body of scientific literature suggesting that we can effectively train and trust "citizen scientists".

6.2 A vision for the future of CBM qPCR

CBM programs historically suffer from poor quality, fragmented data, and sustained volunteer buy in can be difficult. The perceived rigor and acceptability of CBM studies remains on the fringes of validity as a true scientific method.

In this study I was able to achieve incredible buy in from CBM partners (volunteers) because I embraced a philosophy that allowed CBM partners to contribute and collect data they needed for their specific context. In general, CBM programs will be more effective if "volunteers" are viewed as partners in an equal power relationship with researchers or government. I argue that part of this relationship means helping partners create their own research questions and designing a project such that all partners will benefit and obtain the data they need as opposed to simply using partners as volunteer data collectors. Disrupting the typical power dynamic in of the "expert" and "lay person" in CBM studies may also go far in legitimizing CBM as an established methodology in environmental monitoring. For example referring to CBM partners as "partner" or "collaborator" or "community collaborator" instead of "volunteer" or "citizen/community scientist" implies collaboration and contribution on the part of the partner, and respect and trust on the part of the scientist or expert. Additionally, emphasizing the specific expertise of the community partner and their contribution to research papers and communications (including adding partners as authors on manuscripts) will also go a long way to legitimize this methodology within the scientific community.

Many scholars have suggested that training is key to the success of CBM programs. My study highlighted cases both where training was highly effective, and cases where training failed and resulted in low agreement between partner and expert extracted samples. I don't believe training alone is enough to ensure data integrity; ongoing compliance testing of prepared samples and partners' water samples is important to ensure methods are being followed and to ensure equipment is performing properly. However, willingness to adapt to newer and more easily operated technologies would also help to improve data integrity. Ensuring CBM partners data needs are being met is

quintessential to ensuring high quality data. CBM partners who care about the projects they are taking part in and have a vested interest in the results will be more committed to producing high quality data that is impactful. Additionally, CBM partners should be embedded within a program that has the structure and capacity to support partners through training, data acquisition, analysis and any end of project deliverables that might need to be put together. This should include some aspect of training, but should also include regular "check ins" with partners to ensure that their workload hasn't increased to an unmanageable degree or to ensure that locations where sampling might be occurring are still accessible. In summation, improving data quality in CBM partnerships involves a coordinated effort between training, ensuring partners are invested in the project, and ensuring that practical constraints are reasonable.

6.3 Concluding remarks

We are currently entering a new era of environmental protection and monitoring—an era in which in light of the consequences of climate change we must find robust, efficient means of monitoring the environment that can result in changes to improve environmental health. Community based monitoring as a collaborative framework for applied environmental research has the potential to bring groups together to facilitate collaboration and cooperation. The inclusion of qPCR and other rapid methods within community monitoring programs can strengthen data acquisition, and can complement existing monitoring structures, and, as a platform, qPCR can be adapted to a variety of different contexts and research questions, saving time and money. CBM qPCR should be a method utilized by governments to conduct monitoring in an equitable manner that serves the people.

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Appendix 1: Monitoring for *Dreissena polymorpha* using a CBM framework in Southern Alberta

A1.1 Introduction

Invasive mussels are an imminent threat to Alberta's waterways. *Dreissena polymorpha* (the zebra mussel) is a fresh and brakish water mussel whose ancestral home is the Black and Caspian seas of Russia (Karatayev et al. 2015). In the last 200 years this species has spread, along with *D. rostriformis bugensis* (quagga mussels), across Europe and North America. Since their arrival in North America in the mid eighties, the zebra mussels have infested the great lakes, and have progressed westward as far as Lake Winnipeg. Dreissenid mussels have a reproductive strategy that differs greatly from other freshwater mussels and is more akin to that of marine mussels (Ackerman et al. 1994). Dreissenids spawn externally, releasing eggs and sperm freely into the water. Successful fertilizations result in the development of larval veliger's, which develop planktonically, eventually settling and adhering to hard substrates (Ackerman et al. 1994). In contrast, freshwater native mussels in Alberta follow one of two reproductive strategies, members of the family Unionidae undergo internal fertilization, where the fertilized gametes mature into their first larval stage known as the glochidia. Female mussels then release glochidia into the water where they attach to the gills, fins or skin of a host fish. A cyst forms quickly around the glochidia, and a few weeks later it falls off as a juvenile mussel (Watters and O'Dee 1998; Graf 2013). Molluscs of the family Sphaeriidae undergo internal fertilization, and juvenile mussels develop within their mother (Graf 2013).

The reproductive strategy of the dreissenids results in more offspring than native mussels (Ram et al. 1996). Most places with a dreissenid infestation find veligers present in the water all year. However, spawning time is related to water temperature, with lower temperatures triggering the release of gametes and sperm, and veliger development. Therefore, larval veliger production is highest in early spring, and late summer/early fall

(Fraleigh, P. C., Klerks, P. L., Gubanich, G., Matisoff, G., & Stevenson 1993). Part of the success of the dreissenid also owes to the production of byssal thread which enables the mussels to adhere to almost any substrate, and adherence is important for the mussels to develop into adults. Often dreissenids will adhere to native mussels, and this adherence via the byssal thread can be lethal to native mussels. (Thomas F. Nalepa 1992).

The production of the byssal threads enables the zebra mussel to adhere to a wide variety of substrates. Typically the zebra mussels prefers hard substrates for adherence, but interestingly, zebra mussels have also been reported at high densities in the silty bottom environments of lake eerie, adhering to muddy and silty sediments directly, and then creating a positive feedback loop in which further mussels adhere to the already adhering mussels, amplifying the success of the mussel in these areas (Coakley et al. 1997; Berkman et al. 1998). This adherence is incredibly strong, and as more mussels begin to colonize a surface, it becomes nearly impossible to physically remove the mussels. This trait leads to biofouling of pipe infrastructure and billions of dollars in damages to water utilities (Strayer 2009). Zebra mussels can also adhere to other organisms in the water. The Unionid mussels, native to North America, are especially impacted by zebra mussel invasions, as they provide an excellent hard substrate for zebra mussels to adhere to, and this inevitably leads to their death(Ricciardi et al. 1998).

Zebra mussels have largely been transmitted by human activities; their spread through the Great Lakes has been attributed to the ballast water of ships. They can also be spread via virtually any piece of equipment or recreational gear that has had contact with water. Veliger larvae cannot survive desiccation, which is why public awareness campaigns have focused on the message "Clean, Drain, Dry" to stop the spread of invasive species (Strayer 2009).

The work in this chapter was completed in collaboration with Aquality Environmental Consulting, Alberta Environment and Parks (AEP), and the Alberta Biodiversity Monitoring Institute (ABMI). AEP contracts to Aquality Environmental to conduct invasive mussel sampling of reservoirs in Southern Alberta, these samples are then assessed via microscopy for the presence of veligers. Aquality reserved half of the sample for me to do qPCR detection of veligers. This involved the development of a qPCR assay for zebra mussels. In 2018, this method was rolled out as a CBM methodology, and Aquality also performed their own qPCR testing on the water samples.

A1.2 Results

A1.2.1. Validation of the Ventura qPCR assay

The qPCR assay was validated both *in silico* and experimentally. I consulted the Alberta Government website, as their Open Data portal has a comprehensive dataset which contains species of mussels known to occur in Alberta (Environment and Parks and Alberta 2017). Table A1.1 shows the species of mussels known to occur in Alberta, their taxonomic and common name, and includes a brief description of their fertilization style. The CO1 genes of these Alberta native mussel species were obtained from NCBI Genbank. While there were CO1 sequences available for the vast majority of species on the list, there were some species of *Musculium, Pisidium*, and *Sphaerium* that lacked sequences that had been resolved down to a species level of identification—therefore, a number of representative species which were resolved to a genus level were downloaded in lieu of finer taxonomic resolution. The alignment showed that the sequences of *D. polymorpha* have a high dissimilarity than those of the native Alberta mussels, and there are numerous nucleotides in the primer and probe regions that would prevent cross reactivity. The entire ABMI native juvenile mussel collection was tested against the *D. polymorpha* assay,

and no cross reactivity was detected. The ABMI native mussel collection is not fully identified as it is very difficult to discern the species of juvenile mussels based on morphology and there has not been any sequencing or barcoding performed on this collection. Therefore, I attempted barcoding on the collection.

A1.2.2 Native mussel identifications

Barcoding of the Fulmer (CO1-targetting) region was conducted on all samples that contained more than one juvenile mussel. Barcoding was also attempted with a mussel specific barcoding assay, and a pan-eukaryote barcoding assay, but these PCR reactions didn't yield enough product for sequencing. Using the Fulmer assay I was able to identify only five of the 81 specimens. Two were identified as *Sphaeridae* gen. sp., unsurprising as the majority of the native mussels in Alberta are of this family. One showed a 99% nucleotide similarity to *Pisidium obtusale,* and one was identified as of the order Veneroida.

A1.2.3 Monitoring of Southern Alberta reservoirs

In June 2017 we identified *D. polymorpha* mussel DNA in 9 sampling sites in southern Alberta reservoirs. These sites were positive for between a few hundred to a few thousand copies of the CO1 gene. By July 2017, the intensity and number of positive sites had decreased, and by August 2017 we did not detect any positive samples. A team from Alberta Environment and Parks investigated the reservoirs in June of 2018 and did not find any adult zebra mussels (Figure A1.1).

Monitoring continued in 2018, but based on recommendations by Alberta Environment and Parks sampling was targeted to late August when it is suspected that established adult zebra mussels would be spawning in the environment. This time we

utilized the CBM qPCR framework, whereby Aquality also performed DNA extractions and qPCR in parallel with myself. All samples collected at this time were negative.

A1.4 Discussion

Our results suggest that the zebra mussel assay, with the Taqman probe, is specific for *D. polymorpha* based on both *in silico*, and experimental results. When this assay was deployed in water samples to assay for larval zebra mussel samples in 2017, it detected positive water samples in June and July.

Zebra mussel invasions often occur via the spread of larval mussels in the ballast water of boats or ships; however larval mussels may also adhere to virtually any water equipment. Waterfowl can also spread larvae as they migrate, and zebra mussels may begin releasing veligers in early spring (Strayer 2009). I suspect that in these early samples we were detecting larval mussels that had entered these popular recreation reservoirs, which are also used for agricultural irrigation, via ballast water from recreational boats. While Alberta has a comprehensive and mandatory watercraft-monitoring program, coupled with an extensive "Clean, drain, dry" public awareness campaign, it is highly likely that some water users don't comply, and monitoring programs will likely miss some watercraft, or water equipment, especially during high-use times.

In general, there is a high cost of invasion for an alien species, and it is normal for a species to undertake multiple "invasion attempts" before successfully establishing (Jiménez-Valverde et al. 2011). However, *D. polymorpha*, and also *D. rostriformis* have proven to be extremely adept at invading a variety of environments, including the silty, muddy-bottomed environment of Lake Erie where zebra mussel colonization levels have been reported of up to 20,000 live mussels per meter squared—suggesting that the silty bottom, lakes of Alberta shouldn't provide much of a hindrance for either species(Coakley et al. 1997). Calcium concentrations are important for the establishment of invasive mussels, these organisms prefer high calcium environments, with free calcium concentrations in excess of 20mg/L(Whittier et al. 2008). Alberta lakes are alkaline, and

typically have free calcium concentrations well above 25 mg/L (Alberta Environment and Parks 2019) . Taken together, this suggests that monitoring and public awareness campaigns need to be maintained, if not expanded, to ensure Alberta stay invasive mussel free, as we now have evidence that invasion attempts have occurred.

A1.5 Tables

Table A1.1 Native Mussels of Alberta and their Reproductive strategies

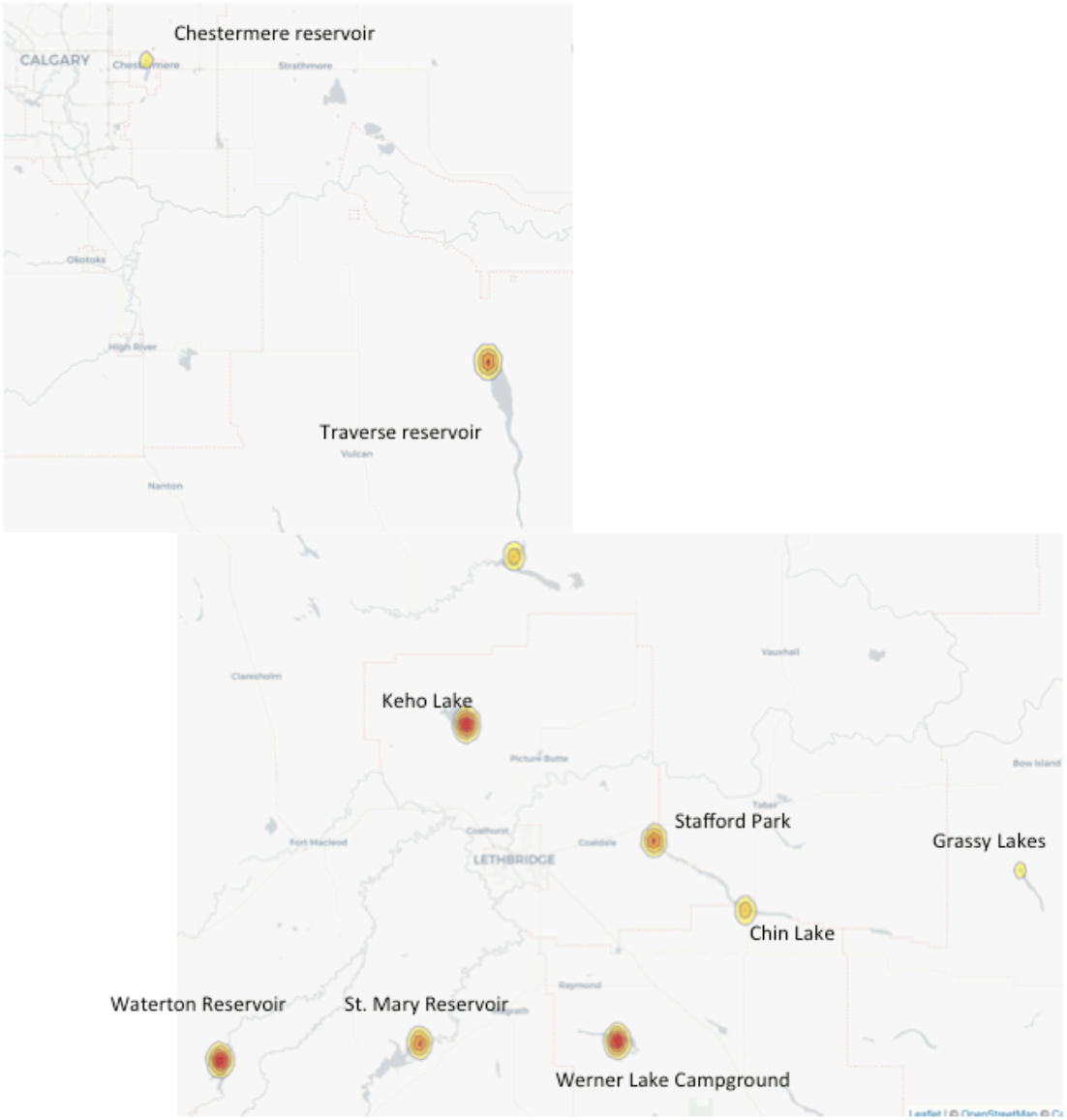
Species	Common name	Fertilization style
Anodonta kennerlyi	Western Floater	Glonchidia parasitic larvae
Anodontoides ferussacianus	Cylindrical Papershell	Glonchidia parasitic larvae
Lampsilis siliquoidea	Fatmucket	Glonchidia parasitic larvae
Lasmigona complanata	White Heelsplitter	Glonchidia parasitic larvae
Lasmigona compressa	Creek Heelsplitter	Glonchidia parasitic larvae
Musculium lacustre	Lake Fingernailclam	Hermaphrodite, internal fertilization
Musculium partumeium	Swamp Fingernailclam	Hermaphrodite, internal fertilization
Musculium securis	Pond Fingernailclam	Hermaphrodite, internal fertilization
Musculium transversum	Long Fingernailclam	Hermaphrodite, internal fertilization
Pisidium casertanum	Ubiquitous Peaclam	Hermaphrodite, internal fertilization
Pisidium cf conventus	Alpine Peaclam	Hermaphrodite, internal fertilization
Pisidium compressum	Ridgedbeak Peaclam	Hermaphrodite, internal fertilization
Pisidium fallax	River Peaclam	Hermaphrodite, internal fertilization
Pisidium ferrugineum	Rusty Peaclam	Hermaphrodite, internal fertilization
Pisidium idahoense	Giant Northern Peaclam	Hermaphrodite, internal fertilization
Pisidium lilljeborgi	Lilljeborg Peaclam	Hermaphrodite, internal fertilization
Pisidium milium	Quadrangular Pillclam	Hermaphrodite, internal fertilization
Pisidium nitidum	Shiny Peaclam	Hermaphrodite, internal fertilization

Pisidium rotundatum	Fat Peaclam	Hermaphrodite, internal fertilization
Pisidium subtruncatum	Shortended Peaclam	Hermaphrodite, internal fertilization
Pisidium variabile	Triangular Peaclam	Hermaphrodite, internal fertilization
Pisidium ventricosum	Globular Peaclam	Hermaphrodite, internal fertilization
Pisidium walkeri	Walker Peaclam	Hermaphrodite, internal fertilization
Pyganodon grandis	Giant Floater	Glonchidia parasitic larvae
Pyganodon grandis grandis	Common Floater	Glonchidia parasitic larvae
Sphaerium nitidum	Arctic Fingernailclam	Hermaphrodite, internal fertilization
Sphaerium rhomboideum	Rhomboid Fingernailclam	Hermaphrodite, internal fertilization
Sphaerium simile	Grooved Fingernailclam	Hermaphrodite, internal fertilization
Sphaerium striatinum	Striated Fingernailclam	Hermaphrodite, internal fertilization

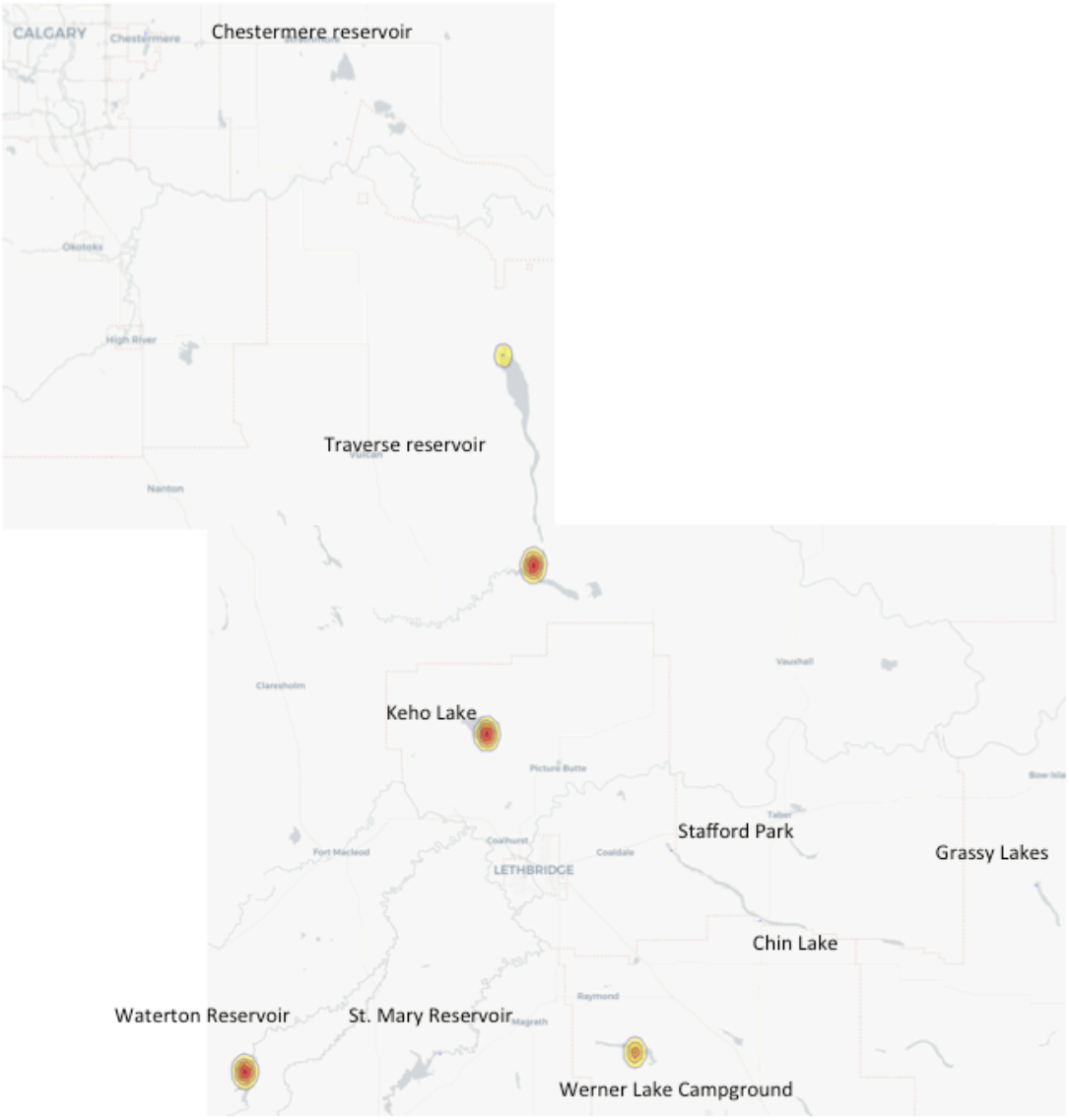
A1.6 Figures

A1.1. Map of where *D. polymorpha* was detected in Southern Alberta Reservoirs

June 2017


Chestermere reservoir
CALGARY
Strathmore
Okotoks
High River
Traverse reservoir
Vulcan
Nanton
Vauxhall
Claresholm
Keho Lake
Picture Butte
Bow Island
Taber
Stafford Park
Grassy Lakes
Fort Macleod
Coalhurst
Coaldale
LETHBRIDGE
Chin Lake
Raymond
Waterton Reservoir
St. Mary Reservoir
Werner Lake Campground

July 2017



Colors and sizes represent the relative intensity of the qPCR signal found at each side. Dark red indicates the highest copy numbers, while yellow represent the lowest.