Applying a community-based monitoring framework to enteric bacteria monitoring and microbial source tracking

by

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Abstract

Using microbial source tracking (MST) to find possible causes of fecal contamination in freshwater systems via quantitative polymerase chain reaction (qPCR) is a robust field with ever increasing application. Rural lakes have been less studied using this technique but there is high potential for impact in these communities where fecal contamination has rarely been investigated. Using a community-based monitoring approach, we supported five lake associations in the northern region of the lower peninsula of Michigan as they sought to understand fecal contamination levels in both a freshwater stream system and five freshwater lakes. Working together to formulate questions, we aimed to gain understanding about the dynamics between septic systems, stormwater runoff and enteric bacteria levels, including using MST for human fecal contamination. We found a high prevalence of human fecal contamination in three study lakes with a high density of septic systems and one stream system, which was partially due to rain events. We also found a positive trend between high usage of septic systems and appearance of enteric bacteria in corresponding well water samples. This information adds to previous knowledge about the correlation between human MST markers and density and abundance of septic systems and could help make wastewater management decisions for these areas and the state of Michigan in the future.

Preface

The work in this thesis started in 2018 when three lake associations in Northern Michigan had ideas and passions to pursue water quality testing for enteric bacteria around their lakes. With continued interest and funding, this project blossomed into a five-year enteric bacteria testing project, and I became the graduate student in charge of it in 2020. Two discreet projects emerged from this work. The first is titled "Assessing fecal pollution source in a Northern Michigan Lake using qPCR and a community-based monitoring framework" and took place on Crystal Lake, Beulah, Michigan and is currently submitted to the journal *EcoHealth*. The second, larger, project encompasses results from 2018-2022 on Glen Lake, Lime Lake, Little Traverse Lake, and Lake Leelanau and is titled "Freshwater lakes with high density of septic systems show high levels of fecal contamination with possible link to septic system usage.". It is currently submitted to *Environmental Health and Assessment*. As the laboratory technician for Freshwater Solutions, LLC in 2018, I extracted all water samples and ran *Enterococcus* qPCR as a community partner to Dr. Hanington's lab at the University of Alberta (UA). Confirmation of results and microbial source tracking (MST) was completed by Dr. Sydney Rudko in 2018-19 and Ceilidh Welch in 2020-2022 at UA. Samples for all parts of this project were collected by trained lake biologists and volunteers.

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"The information furnished by quantitative bacteriology as to the antecedents of a water is in the nature of circumstantial evidence and requires judicial interpretation. No absolute standards of purity can be established which shall rigidly separate the good from the bad. In this respect the terms "test" and "analysis" so universally used are in a sense inappropriate. Some scientific problems are so simple that they can be definitely settled by a test. The tensile strength of a given steel bar, for example, is a property which can be absolutely determined. In sanitary water examination, however, the factors involved are so complex, and the evidence necessarily so indirect, that the process of reasoning much more resembles a doctor's diagnosis than an engineering test."

Samuel Cate Prescott & Charles- Edward Amory Winslow
"Elements of Water Bacteriology"
1904

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Chapter 1: Literature Review

Use of Enteric Bacteria Testing for Water Quality Monitoring

Since 1849, when John Snow first hypothesized that the 'poison' of cholera was transported via the water wells of London and ingested to impact the alimentary canal directly (Snow, 1849), the volume of knowledge about bacteria in and around water has greatly expanded. Much work has been done to increase the understanding of enteric bacteria (i.e., bacteria found in the intestines of animals) and improve the ability to test water for the presence of such bacteria. Certain enteric bacteria have been termed fecal indicator bacteria (FIB) because they signify the presence of fecal matter in a water source while not necessarily being pathogenic to humans on their own. Thus, their presence signifies that pathogens that could negatively impact human health may also be present in the water. Currently, the most commonly used fecal indicators are *Escherichia coli* and *Enterococcus*, although, historically, coliform bacteria, fecal Streptococci and other bacteria have also been monitored (U.S. Environmental Protection Agency, 2002c). The use of these bacteria has evolved as more is learned about the risk posed to humans through water contaminated with these bacteria.

The initial selection criteria for a FIB were based on a bacterium's need to be "present where pathogens are", "unable to grow in aquatic environments", "more resistant to disinfection than pathogens" and "easy to isolate and enumerate" (Dufour, 1984). Scientists now look for FIB that also have negligible to no reproduction in the water but survive long enough to be detected, are found only in fecal or sewage pollution, are not pathogenic, are as resistant to environmental conditions, water purification and disinfection processes as pathogens, and can be identified at low concentrations consistently through cost-effective, simple, quick methods (Grabow, 1996; Motlagh & Yang, 2019; Payment & Locas, 2011). Each indicator bacterium has different benefits and detriments of use and many factors may influence the persistence of FIB in the environment, including nutrient levels, temperature changes, UV light exposure and biotic interactions (Byappanahalli et al., 2012; Korajkic et al., 2019).

The use of FIB as a measure of water quality dates back to the 1880s, when *Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis* were first described for this purpose (Geldreich, 1978). It was not until the 1890s that bacteria in water were linked to potentially dangerous

pollutants (Davis, 1891). In the early 1900s, scientists began quantifying bacterial proliferation, using gas production as the measure of growth (McCrady, 1915). During this time, *Bacterium coli* (later named *E. coli*) (Castellani & Chalmers, 1919) was discovered to be of fecal origin and used for water testing (Escherich, 1885). During the 1940s, colony counts began to be employed for quantifying bacteria in water samples with the introduction of membrane filters, but it was not until the 1950s that it became a widely adopted method (Waite, 1985).

The first drinking water testing protocols were established in the early 1900s and used a multiple-tube lactose fermentation process to find the most probable number (MPN) of coliform bacteria. This test took nearly two days and was non-specific to fecal indicator species (McCrady, 1915). Starting in the 1960s and up until the publication of the US Environmental Protection Agency's (EPA) Ambient Water Quality Criteria for Bacteria in 1986, it was recommended that fecal coliforms should be used for water quality testing (U.S. Environmental Protection Agency, 2012b).

Further research in the field of bacterial water testing led to the use of fecal Streptococci for water quality testing due to the large number of these organisms excreted by humans and other endothermic animals, its presence in wastewater, and its inability to multiply in the environment (Ashbolt et al., 2001). Through further research, it was discovered that Streptococci was a highly diverse genus of bacteria, all of which were not fully enteric in nature, thus, it was split into four divisions based on antigen presentation and metabolic activities (Sherman, 1937). At this time, Enterococci was one of the four divisions presented of Streptococci. It was not until 1984 that the *Streptococcus* genus officially split into three genera: *Streptococcus*, *Lactococcus* and *Enterococcus* (Schleifer et al., 1985; Schleifer & Kilpper-Balz, 1984). Currently, there are three commonly tested FIB: fecal coliforms, *E. coli* and Enterococci.

Coliform bacteria

Coliform bacteria are a large group of bacteria present in animals' digestive tracts. The presence of coliform bacteria may indicate the presence of fecal material in a water source. Specifically, fecal coliforms are said to be only found in the gut of endothermic animals. Thus, these coliforms allow for more targeted water testing (Dufour, 1984). Fecal coliforms were largely negated for testing recreational water after a series of studies showed no correlation between

fecal coliform levels and gastrointestinal illness (Cabelli, 1983; Dufour, 1984). However, the most specific method to assess fecal coliform bacteria as a fecal indicator is to measure *E. coli*, as it has been associated with gastrointestinal diseases contracted during swimming (U.S. Environmental Protection Agency, 1986).

E. coli

In many jurisdictions, *E. coli* has become the standard indicator of water quality due to its high abundance in the gastrointestinal system of animals (Dufour, 1977). Testing for *E. coli* is commonly used to detect water and food contamination (Halkman & Halkman, 2014). It has recently been shown that there are several 'cryptic clades' of *Escherichia*, named such because standard biochemical testing does not differentiate them from *E. coli*, although other phylogenetic, genomic and transcriptomic data support a split from *E. coli* (Walk, 2015). There is also evidence that these subspecies of *E. coli* have been historically understudied, but should be explored to help understand the natural history of these enteric bacteria (Yu et al., 2021). *E. coli* can be quite persistent in environments other than the gut of animals (Zhi et al., 2019) and *E. coli* O157 has shown the ability to grow in a low carbon, freshwater environment (Vital et al., 2008). A review by Van Elsas et al. (2011) compiled evidence that the rate of survival of *E. coli* in nature declines based on a variety of factors, including high temperatures, high oxygen availability, high pH, and low dissolved organic carbon. The survival of *E. coli* outside the enteric environment and the potential for subspecies of *E. coli* to be found in the environment could confound the use of *E. coli* as a fecal indicator species.

Enterococcus

Enterococcus bacteria are a diverse genus of commonly used FIB. There are over 40 different *Enterococcus* species (Švec & Franz, 2014). Historically, Enterococci were thought to be almost exclusively found in the gut of humans and animals (Wheeler et al., 2002) however, evidence has shown that Enterococci can persist in extraenteric environments (Badgley et al., 2010; Byappanahalli et al., 2012; Whitman et al., 2003). Enterococci have been recommended for use as FIB since 1984 (Dufour, 1984). They are an appealing indicator organism for use in research and health-based monitoring efforts because Enterococci have shown the strongest link to gastrointestinal illness since early testing for this correlation (Cabelli et al., 1982). More recently,

Enterococcus was closely linked to negative human health outcomes following exposure to fecal contamination in recreational waters (Wade et al., 2008).

Current Fecal Indicator Bacteria Testing

There are several available methods for the testing of FIB. One of the most commonly used culture-based, membrane filtration methods for *E. coli* is Method 1603 (U.S. Environmental Protection Agency, 2002a). In this process, recently collected water samples are filtered through a membrane that catches the bacteria. The membrane is then put on mTEC agar, which contains a chromogen that is metabolized by *E. coli* to produce a red or magenta color. The agar, with filter, is left to incubate for 24 hours, enabling bacterial growth, and colonies are subsequently enumerated on the agar. Results are reported in colony forming units (CFU) per 100 mL of sample. There are a variety of other methods to find CFU results. For example, Method 1604 uses a medium with two different compounds that are metabolized differently by coliform bacteria and *E. coli* so that both can be enumerated simultaneously (U.S. Environmental Protection Agency, 2002b). Method 1600 uses mEI agar, which Enterococci metabolize to turn blue for counting purposes (U.S. Environmental Protection Agency, 2009a).

Another way to enumerate bacterial density is to use the fermentation tube test to estimate the MPN. In these methods, a water sample is put through a dilution series and mixed with broth, then allowed to grow, after which each tube is measured for growth or lack of growth. The MPN is found based on a calculation using the ratio of positive tubes for each of three dilutions (American Public Health Association et al., 1999). Both the culture-based method and MPN technique require the growth of bacteria for 18-24 hours. These methods also require bacteria to be viable and actively growing to detect them.

Since the invention of polymerase chain reaction (PCR) (Mullis et al., 1986; Saiki et al., 1985) and further, quantitative PCR (qPCR) (Mutter & Pomponio, 1991), nucleic acid sequences have been used to test for FIB in water samples. Although there have been a variety of techniques used to quantify PCR products (Cross, 1995), the work in this thesis relies on qPCR, which uses primer assays that are complementary to the DNA sequence exclusive to the species of interest, along with a probe that emits a fluorescent dye when attached to the DNA of interest (Holland et

al., 1991). The level of fluorescence can be measured and compared to a set of standard solutions to quantify the number of DNA copies in a water sample.

Within qPCR testing, it is important to understand both sensitivity and specificity. Sensitivity (i.e. analytical sensitivity) is the smallest amount of a nucleic acid sequence of interest that can be detected in a given sample (Saah & Hoover, 1997), in this case, via qPCR. It is sometimes called the limit of detection. Many qPCR assays are theoretically sensitive enough to detect single copies of the target DNA (Bartlett & Stirling, 2003). Specificity (i.e. analytical specificity), when applied to qPCR, refers to the ability of an assay to target a unique species of interest while giving a negative result when applied to other species (Saah & Hoover, 1997).

A benefit to using qPCR as a measurement tool is that it will capture results from culturable, viable, non-culturable and non-viable bacterial forms (Department of Environmental Quality, 2012). Bacteria could be non-culturable because they are dead or have been forced into a viable but non-culturable (VBNC) state, in which they are not dividing. It is thought that they enter this state when encountering environmental stresses (Higgins et al., 2007). qPCR can thus be beneficial when monitoring for a transient marker, as it increases the chance of a detectable DNA signal remaining in the water for longer. Another advantage of employing qPCR is the rapidity of obtaining results, which can be achieved within a few hours after sample collection, as opposed to the day or more required by culture-based approaches. It has been shown that results from culture-based methods significantly vary among diurnal testing more often than qPCR method results do (Wymer et al., 2021). When considering closing beaches or swim areas to protect human health, this can make a large impact and rapid results are important.

One area of concern with the use of qPCR is geographical sequence diversity, for example, of the human crAssphage, a bacteriophage which infects human enteric bacteria (Edwards et al., 2019), which could impact results if mutations in the sequence of interest cause assays to less effectively amplify the intended target in different regions of the world. Another concern when using qPCR is interference by other substances in the water, such as proteins, divalent cations (*DNeasy Blood & Tissue Handbook*, 2023), decaying organic matter and tannins (Hunter et al., 2019). These inhibitors may hinder DNA capture processes, reduce the enzymatic activity of Taq DNA polymerase, and/or deter primers and probes from joining with DNA of interest or

enzymes. It is recommended that water samples be consistently tested for this interference. Method 1611 is an US EPA-approved method for extracting DNA and running it through qPCR to test for *Enterococcus*, in which it is required that inhibition assessments are run for every sample (U.S. Environmental Protection Agency, 2012a). By using an extraction kit, such as Qiagen DNeasy Blood and Tissue Kit, the chance of inhibition can be greatly reduced. Clarifying steps in the extraction process are meant to lower inhibition levels (*DNeasy Blood & Tissue Handbook*, 2023), although DNA extraction efficiency can also be reduced when using these methods compared to Method 1611.

In 2012, it was recommended by the US EPA that culture-based methods of testing be used, but they mention the use of qPCR as an alternative if local testing of protocols are confirmed to be effective in ambient waters (U.S. Environmental Protection Agency, 2012b). The US EPA (U.S. Environmental Protection Agency, 2012b) recommends using either *Enterococcus* or *E. coli* for recreational water testing. *Enterococcus* can be tested via traditional plating methods or qPCR, while *E. coli* should only be tested using plating methods. *Enterococcus* was the focus of the National Epidemiological and Environmental Assessment of Recreational Water (NEEAR) studies, which showed a strong correlation between gastrointestinal illness and *Enterococcus* levels via qPCR testing. Thus, *Enterococcus* became the accepted qPCR target organism (Wade et al., 2006, 2008, 2010). Since the NEEAR studies, assays for *E. coli* have been developed for use in qPCR (Walker et al., 2017), but have not been validated by the US EPA nor been correlated to gastrointestinal illness.

A statistical analysis study completed by Gonzalez and Noble (2014), comparing qPCR to culture-based methods of water testing showed that using culture-based methods only correctly predicted management decisions at a slightly higher rate than qPCR did. However, the group found inhibition to be a major problem within their qPCR work. This study, along with the fact that qPCR for water testing is fast, highly sensitive, and can be decentralized (Froelich et al., 2019; Rudko, 2020; Rudko et al., 2020, 2022), creates a strong argument for the use of qPCR in water quality research aimed at identifying sources of enteric bacteria in water.

Indicator species in water quality testing have long been used to signify the risk of gastrointestinal illness. Over 20 years ago, Prüss (1998) reviewed 22 water quality studies and

showed a correlation between Enterococci, fecal Streptococci, and *E. coli* in freshwater and adverse health outcomes. The US EPA further reiterated this in their 2012 Water Quality Guidelines (U.S. Environmental Protection Agency, 2012b). As mentioned above, Wade et al. (2006, 2008, 2010) found a statistically significant correlation between fresh and marine waters contaminated with *Enterococcus* and gastrointestinal illness in primary contact recreators, using qPCR (Method 1611) as the testing method.

For human health, we are most concerned with human fecal contamination because it is most likely to carry disease-causing agents such as Norovirus, Rotavirus, Adenovirus, *Giardia lamblia* (Soller et al., 2010), human-infectious Cryptosporidium species (Centers for Disease Control and Prevention, 2022) and pathogenic bacteria, such as *E. coli* 0157: H7 (Ameer et al., 2023). Few groups have found a correlation between FIB and disease-causing viruses in water (Gersberg et al., 2006), while many have found that FIB did not correlate well with the presence of such viruses (Baggi et al., 2001; Espinosa et al., 2009; Gerba et al., 1979; Korajkic et al., 2018; LaBelle et al., 1980).

While the US EPA relies on individual states within the US to set water quality standards (WQS), many, including Michigan, have yet to do so for *Enterococcus* using qPCR. Michigan has developed a WQS for *E. coli* of 130 *E. coli*/100 mL as the maximum acceptable level of a 30-day geometric mean. At the same time, a single-day WQS value cannot exceed 300 *E. coli*/100 mL without eliciting action at the sampling location (Department of Environmental Quality, 2006). Comparatively, for *Enterococcus* values measured via qPCR, the US EPA has set a statistical threshold value (STV) for an estimated illness rate of 32/1000 primary contact recreators of 1,280 calibrator cell equivalents (CCE) /genome equivalents (GE) per 100mL for single day sampling, or 300 CCE/GE per 100 mL for a 30 day geometric mean (U.S. Environmental Protection Agency, 2012b). The STV is a single day measure of bacterial levels in tested water and can be used in conjuncture with a 30 day geometric mean, or as a stand-alone value (Department of Environmental Quality, 2012). The US EPA also advises a WQS value for *E. coli* of 100 CFU/100 mL for a 30 day geometric mean or STV of 320 CFU/100 mL to achieve the same level of health protection as the illness rate associated with *Enterococcus* testing (32/1000 primary contact recreators) (U.S. Environmental Protection Agency, 2012b).

Microbial Source Tracking

A major advantage of qPCR-based nucleic acid water testing is the ability to apply microbial source tracking (MST) to water samples, a technique that has gained traction in the 21st century because of its ability to determine the source of fecal contamination (Hagedorn et al., 2011). MST is used to test water samples for specific nucleic acid markers that link detected FIB to a human, or other, source. This is especially important if the contamination is of human origin, as it could signify the presence of pathogenic microorganisms responsible for human enteric disease (Fewtrell & Bartram, 2001). While qPCR analysis of water samples for general FIB may indicate how much fecal contamination is present, it does not identify the specific source. *E. coli* and Enterococci are found in the intestinal tracts of various animals and thus do not point to a clear culprit responsible for water pollution. Herein lies the benefit of MST to further test samples with high levels of FIB to reveal the source of fecal pollution. Numerous studies have employed MST to gain insights into fecal contributors in watersheds, with a primary focus on assessing human fecal contamination (Kongprajug et al., 2021; Li et al., 2019; McQuaig et al., 2012; Nguyen et al., 2018). Furthermore, a diverse array of highly specific and sensitive MST assays are utilized via qPCR.

Human (HF183) Marker

The HF183 Taqman assay is one of the most frequently employed MST tests for human fecal pollution. HF183 is a 16S rRNA genetic marker of *Bacteroides* spp. (Bernhard & Field, 2000), which is one of the most common intestinal bacteria in humans (Holdeman et al., 1976). Specifically, HF183 has been linked to *B. dorei* (Haugland et al., 2010). *Bacteroides* spp. make good target species due to their anaerobic behavior that does not allow regrowth in the environment while being highly concentrated in fecal material (Fiksdal et al., 1985). Due to their anaerobic nature, *Bacteroides* spp. are not useful as culturable FIB because they quickly die once they exit the gastrointestinal environment (within 72 hrs). *Bacteroides* spp. may be impacted by temperature or oxygen concentration changes. Thus, we observe seasonal changes in the persistence of these bacteria in nature, depending on the species (Ballesté & Blanch, 2010). However, in a laboratory setting using natural river water, the nucleic acid signature can persist for up to two weeks, depending on the temperature (Kreader, 1998).

The HF183 marker was first developed for PCR use (Bernhard & Field, 2000) and later adapted for qPCR analysis using SYBR Green I detection (Seurinck et al., 2005). It was further modified for the use of TaqMan® chemistry-based qPCR assays (Haugland et al., 2010). Although HF183 presence has been linked to FIB levels (specifically *E. coli*) (Fremaux et al., 2009), it has also been shown to have no direct correlation with FIB levels (Staley et al., 2015). The HF183 marker does not display 100% specificity to human hosts. It has shown low cross-reactivity to dog, chicken and bird feces (Ahmed et al., 2012), and dog and cat feces (Kildare et al., 2007; McQuaig et al., 2009), however, it has been found in much higher amounts and is more consistently present in human feces than in other hosts (Haugland et al., 2010). Possible cross-reactivity must be carefully considered when drawing conclusions regarding HF183.

Other Human Markers

Many other human MST markers exist, including the commonly used BacH (Reischer et al., 2007), BacHum (Kildare et al., 2007), humbac (Viau et al., 2011), HumM2 (Shanks et al., 2009) and *Bacteroides theta* (Yampara-Iquise et al., 2008). These markers have been tested against each other and other human MST markers. In one study, HF183, BacHum and BacH all showed higher than 94% specificities, while HuBac and Human-Bac (two other human fecal markers than those listed above) had specificities of 63% and 79%, respectively (Ahmed et al., 2009). Another study showed that HF183 and BacHum both positively quantified 100% of sewage samples they were tested against, but BacHum had cross-reactivity to cat, dog, gull and raccoon feces, while HF183 only cross-reacted with cat feces (Van De Werfhorst et al., 2011). Nshimyimana et al. (2017) showed that HF813, BacHum and BacH all were cross-reactive with rabbit fecal material, and *B. theta* had the highest sensitivity (69% in human stool, 100% in sewage) and specificity (98%) of the five human-specific fecal markers tested. BacHum cannot fully discriminate between human and dog samples (Kildare et al., 2007). Shanks et al. (2010) compared the cross-reactivity of 10 qPCR assays of target genes known to be found in *Bacteroidales* associated with human fecal material with DNA extracts from 23 different animal species. Of note, they found that HF183 cross reacted with dog and chicken feces, while HumM2 cross-reacted with elk and sheep. Overall, there is varying evidence about the best MST for human fecal contamination, as some claim HF183 to be the most specific and sensitive (Layton et al., 2013; Schiaffino et al., 2020; Van De Werfhorst et al., 2011). In contrast, others find

BacHum (Haramoto & Osada, 2018; Malla et al., 2018; Odagiri et al., 2015) or *B. theta* (Aslan & Rose, 2013; Nshimyimana et al., 2017) to be best. Still, others found them comparable in their metrics (Ahmed et al., 2009). Although there are diverse results from bacterial human MST assay testing, these assays maintain the unique ability to track human fecal contributions to a water system.

In a study done on an urban river in Chile, concentrations of a human bacteriophage MST marker (CrAssphage) were measured and showed a highly linear relationship with HF183 concentrations in water samples (Jennings et al., 2020), which is a reason some may choose to measure human enteric viruses during MST. However, specificity and sensitivity can be low in these tests. Because of the low concentrations of these markers in fecally contaminated waters, there can be high inter-laboratory variation in results (Harwood et al., 2013). If the main goal of a study is to assess the risk of adverse human health outcomes, enteric viral testing may be advantageous over the use of bacterial MST markers.

There is varying evidence that FIB specific to human enteric bacteria are more closely associated with human-associated pathogens than general FIB. A recent study showed HF183 (a common human fecal marker) correlated significantly with five of six pathogenic viruses tested in untreated wastewater samples, the highest correlations occurring with human adenovirus 40/41 (spearman's correlation coefficient = 0.788), human norovirus GI + GII (spearman's correlation coefficient = 0.720), and enterovirus (spearman's correlation coefficient = 0.702) (Ahmed et al., 2022). Steele et al. (2018), however, showed no significant correlation between human fecal markers and norovirus, adenovirus, *Campylobacter*, or *Salmonella*. Viau et al. (2011) used generalized estimating equations to show that *E. coli* levels were negatively associated with adenovirus ($\beta = -0.73$, $p = 0.02$), while adenovirus and norovirus were found less often in the presence of humbac than in its absence (adenovirus: $\beta = -1.6$, $p = 0.02$; norovirus genogroup I: $\beta = -1.3$, $p = 0.02$). Humbac did have a positive association with *Campylobacter* ($\beta = 1.4$, $p = 0.02$), but showed no association with *Salmonella*, enterovirus and norovirus genogroup II (Viau et al., 2011). Savichtcheva et al. (2007) found total coliforms, fecal coliforms, and human-specific *Bacteroides* 16S rRNA genetic markers to all significantly correlate with *Salmonella* and pathogenic *E. coli* levels, while the human-specific marker did not correlate with *Clostridium perfringens, Shigella*, *Staphylococcus aureus,* or *Vibrio cholerae*. Schriewer et al. (2010) found

no association between human Bacteroidales and *Campylobacter*, *Cryptosporidium*, *Giardia*, pathogenic *E. coli*, *Salmonella* or *Vibrio cholerae*. When studying photoinactivation rates of FIB (both general and human specific), the only positive correlation found with enterovirus and FIB was with the human related *Bacteroidales* marker ($r_p = 0.23$, $p<0.05$) (Boehm et al., 2009).

Overall, there is increasing evidence that human fecal markers could be a measure of pathogen risk in water communities, but results are still inconsistent with research ongoing. Pathogens themselves may be transient in a community, thus, finding a specific pathogen cycling through the population is unlikely at any given sampling period. However, the presence of FIB, specifically of human origin, would indicate a contamination source to water and increase the probability of finding such viruses in the water. In this way, human FIB serve as potential risk indicators for human disease, albeit inconsistent. Thus, testing for FIB is still regarded as the best way to assess the level of general fecal pollution in the water (Payment & Locas, 2011), with the addition of human source tracking of positive samples if resources allow.

Canada Goose (CG0F1-Bac)

While there are MST markers that amplify a variety of avian species (Green et al., 2012), the CGOF1-Bac marker was designed for MST via qPCR of Canada geese (*Branta canadensis*) by Fremaux et al (2010). It has been found to infrequently cross-react with pigeon feces, but not with other animals (Fremaux et al., 2010; Krentz et al., 2013). It has a limit of quantification of less than 10 copies of target DNA per reaction (Fremaux et al., 2010), and has been used for water contamination assessment (Frey et al., 2015; Marti et al., 2013; Wilkes et al., 2013) and comparison with other MST markers (Kobayashi et al., 2013). While there are other MST markers for Canada geese (Lu et al., 2009), CGOF1-bac has performed best in comparison tests by not displaying the cross-reactivity frequently found with other assays (Krentz et al., 2013). Scientific interest in using a MST assay for Canada goose fecal pollution assessment stems from the potential of fecal contamination containing pathogens such as *Campylobacter jejuni*, *Salmonella typhimurium*, and *Listeria monocytogenes* (Gorham & Lee, 2016).

Dog (DG3)

The Dog MST marker (DG3) was developed in 2014 (Green et al.) and has been since used in fecal contamination monitoring (Li et al., 2019; Shrestha et al., 2020). Levels of DG3 have been linked to rainfall and Enterococci levels (Seymour et al., 2020). Other dog MST markers that have been developed include DogBact (Sinigalliano et al., 2010), DG37 (Green et al., 2014) and BacCan-UCD (Kildare et al., 2007).

Human Fecal Contributions to Freshwater Watersheds

Fecal contributors to a freshwater system can be diverse. FIB from naturally occurring wildlife, as well as neighboring agricultural animals, are certain to make contributions to an ecosystem, alongside inputs from human sources like septic systems and stormwater runoff. It has been well documented that waterfowl are major contributors to FIB in water (Edge & Hill, 2007; Kobayashi et al., 2022; Mathai et al., 2019; McLellan & Salmore, 2003). Once in a water source, Staley et al. (2012) hypothesize that submerged aquatic vegetation, sediments, and stormwater act as reservoirs of FIB and that resuspension of sediments would greatly impact FIB levels. Other studies have confirmed that aquatic vegetation (Mathai et al., 2019) and sediment (Kim & Wuertz, 2015; Rothenheber & Jones, 2018; Wheeler Alm et al., 2003) are sinks for FIB. Stormwater sewer systems have been well documented to carry high loads of FIB (Brownell et al., 2007; Petersen et al., 2005; Sidhu et al., 2013; Stein & Ackerman, 2007), including human fecal material (Ahmed et al., 2019; Sauer et al., 2011; Sercu et al., 2009; Sidhu et al., 2012; Staley et al., 2016). Using MST, it is possible to focus on the contribution of human fecal contamination to these freshwater systems.

A major contributor of human fecal material to watersheds is the improper functioning of onsite water treatment systems. In 1996, the US EPA named septic systems the third most reported groundwater contamination source after leaking underground storage tanks and landfills (Department of Environmental Quality, 1996). The problem continued to present itself in a 2004 report from the US EPA, which named municipal discharges/sewage (a category that includes septic systems) as the third leading source of contamination of bays and estuaries assessed, the sixth and seventh top sources of impairment in lakes and ponds, and reservoirs, rivers, and streams, respectively. Municipal discharges/sewage systems were also cited as the second leading source of shoreline impairment to the Great Lakes following historical pollution to the

sediment (U.S. Environmental Protection Agency, 2009b). Verhougstraete et al. (2015) have demonstrated that areas in Michigan with greater density of septic systems display a higher level of human enteric bacteria markers in river water. Similarly, Peed et al. (2011) monitored an Ohio stream system and found a correlation between septic system density and human MST markers in wet weather conditions.

Michigan is the only state in the United States that does not have a statewide septic code for preexisting septic systems. However, there are criteria for the size and design of new on-site wastewater treatment systems (Department of Environmental Quality, 2013). Four bills were recently introduced to the Michigan Legislature, which, if passed, would require septic system inspection every five years (*Michigan Legislature*, n.d.). This would be a significant undertaking as there are an estimated 1.3 million septic systems in Michigan (Department of Environment, Great Lakes, and Energy, 2023). Michigan also has no laws regulating bacteriological testing of well water (Michigan Department of Health & Human Services, 2023). Around 13% of people in the United States are estimated to drink well water (U.S. Department of Health and Human Services, 2022). A well water study done in Montana showed 40% of well water samples were contaminated with coliform bacteria (Bauder et al., 1991). Another study by the United States Geological Survey found that about one-third of all wells in the United States were contaminated with FIB (Desimone et al., 2009), while a study in Ohio of private well water showed 45% of water samples positive for coliform bacteria and 9% of water samples positive for *E. coli* (Won et al., 2013).

Decentralizing qPCR water testing using community-based monitoring

Community-based monitoring (CBM) is a relatively new but widely used tool for freshwater studies worldwide (Robinson et al., 2021). It is formally defined as "a process where concerned citizens, government agencies, industry, academia, community groups and local institutions collaborate to monitor, track, and respond to issues of common community concern" (Whitelaw et al., 2003). Using ideas and resources from engaged community members can have far-reaching impacts compared to a small group of scientists (Dickinson et al., 2012). While some large-scale studies have been done without using CBM (Li et al., 2019), CBM has enhanced many aquatic biology initiatives, including assessments of ecosystem health (Robinson et al.,

2021), macroinvertebrate classification (Storey & Wright-Stow, 2017), and parasite diversity (Rudko et al., 2020). CBM projects have been implemented worldwide (Carlson & Cohen, 2018), including within indigenous communities of Australia, New Mexico, and Canada (Wiseman & Bardsley, 2016), in Africa (Elijah et al., 2017; Rivett et al., 2013), and Asia (Zhang et al., 2017).

While some scientists interchangeably use the terms CBM and 'citizen science,' Muhamad Khair et al. (2021) argue for the uniqueness of CBM. Their stance emphasizes that CBM programs have a localized influence, as they engage the community in question formulation rooted in specific concerns or issues, ultimately leading to tangible changes within the community. Citizen science, comparatively, is national or worldwide, where volunteers collect data to increase those volunteers' scientific knowledge and interest. Based on these definitions, the work presented in this thesis can be called a CBM project.

Steps of the qPCR testing method can be used in a CBM context by decentralizing phases of the process from core laboratories with the use of volunteer samplers, trained individuals extracting DNA or the use of qPCR machines that are significantly cheaper and easier to use than traditional core qPCR instruments. Portable qPCR machines, like the Chai Open qPCR, cost less than $5000, are rugged and are easily portable. By having other, smaller laboratories sample and extract DNA, more sampling can be completed, with the extracted DNA being shipped to a core laboratory for qPCR analysis or by running it on a portable qPCR machine. Another benefit of CBM qPCR use is the flexibility of timing, compared to culture-based methods. Depending on the target species, water samples need to be filtered within hours or days of collection. However, a frozen filter can be stored at -20C for six months without a measurable loss of DNA during the extraction (Gilpin et al., 2013). With proper controls for quality and data reliability in place, such as highly specific training, checks for correct procedures and precise labelling, it has been shown that qPCR can be effectively used in a CBM program and will yield accurate results to answer various scientific questions that both the core laboratory and community partners desire to answer (Rudko, 2020).

According to a cross-Canadian study done by Carlson and Cohen (2018), there are three main reasons that a community may participate in CBM. They may be using CBM as: a) an

educational tool while contributing to a research program, b) to generate more data than can be accomplished by a single (often government-led) group to monitor the concerns of community members, or c) to provide information to all orders of government to create policy change. Similarly, Alender (2016) found that in the United States, the largest reasons for volunteering in a CBM program were to “enhance the environment,” “help the community,” “get outside or connect with nature,” and “contribute to scientific knowledge”.

Many CBM programs risk gathering more data than they can realistically process and analyze, so it is suggested that the end goal is established first and data is collected to inform that goal within an established and agreed-upon monitoring framework (Conrad & Daoust, 2008). The range of community involvement in CBM projects can vary substantially concerning study design and implementation, sometimes including high levels of engagement from community partners in study development and data collection (Conrad & Daoust, 2008).

Various CBM frameworks have been established to standardize data collection, promote active community involvement, and ensure the overall success of CBM programs (Muhamad Khair et al., 2020). Pollock and Whitelaw (2005) suggest a framework with two main phases: establishing CBM and implementing CBM. The establishment phase includes gathering all interested parties, assigning project champions to ensure longevity, fundraising and creating the overall organizational structure. This planning stage allows an understanding of each party's responsibilities while ensuring proper funds are in place. In the second phase, they suggest setting goals for the program, determining what skills the volunteers may have, monitoring, and then communicating influence and successes to the public.

Conrad and Daoust (2008) suggest a CBM framework that is more fluid, with many points of feedback where the main steps involve identifying stakeholders and their skills and resources available, developing both monitoring and communication plans and then implementing those plans. When results return from the monitoring phase, it is key to evaluate and give feedback to make any necessary changes to the plan. Finally, Gharesifare et al. (2019) recently reviewed CBM monitoring programs. They made a conceptual CBM framework in which CBM programmers are recommended to ask five main questions focusing on goals and objectives, technology, participation, results, and power dynamics. While there are a variety of frameworks

available for a CBM program, they all focus on clarifying who will do specific work, where funding will be attained, how collected data will be used, and how results and progress will be communicated.

It could be argued that CBM programs do not accumulate reliable data due to non-professionals performing the collection (Stokes et al., 1990). Evidence to contradict this assertion was presented by Storey and Wright-Stow (2017) as they found a strong correlation (Pearson correlation coefficient, $r = 0.88$ in the most correlated metric of measurement) between volunteer results from monitoring macroinvertebrates compared to professionals. McLaughlin and Hilts (1998) did find common errors committed by community participants when monitoring wetland species and suggested a more time-intensive training process. However, not all CBM projects are as technical as the wetland organism classification in their study. Having clear monitoring standards is key to a successful CBM program. Thus, training must be completed for all protocols (Muhamad Khair et al., 2020). Consistent qualitative and quantitative data records completed by different volunteers are also imperative to a properly functioning CBM program (Bliss et al., 2001).

Our group has reliably used CBM environmental sampling and DNA extraction methods to test for schistosome parasites (Froelich et al., 2019; Rudko et al., 2018, 2022; Soper et al., 2023) and enteric bacteria contamination (presented in this thesis) in freshwater systems. Through strong, long-term community partnerships with lake associations in Michigan, a profusion of water samples have been collected, mostly by volunteers, and extracted in a satellite laboratory by trained professionals in Leelanau County, MI. These extracted samples were then sent to the University of Alberta (UA) for qPCR analysis of general FIB and MST targets. CBM has been imperative to the success of these projects and to further our scientific knowledge about swimmer's itch and enteric bacteria contamination.

Research Overview

Our work takes place in Northern MI, a location where community members have an interest in water quality of inland lakes. We aimed to quantify FIB on five freshwater lakes, focusing on *Enterococcus*, but comparing data to *E. coli* values in some cases. We look for possible sources of contamination with a focus on human fecal contamination, using the HF183 assay. Our work

covers a five-year timespan, beginning in 2018 when we answered a question about the prevalence of HF183 contamination around three lakes. In subsequent years we aimed to answer questions about the source of HF183 contamination, including the analysis of stormwater runoff and septic system influences.

Chapter 2: Assessing fecal pollution source in a Northern Michigan Lake using qPCR and a community-based monitoring framework

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Abstract

Implementing quantitative polymerase chain reaction (qPCR) within a community-based research framework expands the scope and scale of community-driven monitoring and research efforts. The increasing accessibility of qPCR technology and methodology has allowed for incorporating community partners in numerous ways, ranging from sample collection to running qPCR tests. Here, we report on a community-driven study at Crystal Lake in Beulah, MI. Historically high levels of the enteric bacteria *Escherichia coli* in the inlet to Crystal Lake, Cold Creek, spurred an interest in understanding more about fecal pollution and its source. In this study, we monitored 17 sites in Cold Creek and around Crystal Lake and used qPCR to assess levels of *Enterococcus* while source-tracking all samples for human, dog, and Canada goose fecal markers (HF183, DG3 and CG0F1-Bac, respectively). Replicate samples were sent for *E. coli* culture-based testing. Results showed high fecal contamination (*E. coli* and *Enterococcus*) and consistent HF183, DG3 and CG0F1-Bac-positive samples at specific sample sites. Varying degrees of relatedness were found between *Enterococcus* levels grouped by precipitation amount. Due to the nature of the sampling sites, we hypothesize that human fecal contamination is due to stormwater outflows and septic system influences and not direct human contact with the water. A Cohen's Kappa analysis between the *Enterococcus* qPCR test results and *E. coli* culture-based test results indicated a moderately positive relationship. The historical *E. coli* dataset, now accompanied by the *Enterococcus*, HF183, DG3 and CG0F1-Bac data, confirms consistent and elevated levels of fecal pollution in Cold Creek and Crystal Lake that is likely related to human sources with stormwater outflows being a contributor to this contamination.

Introduction

Community-based monitoring (CBM) is a relatively new but widely used tool for freshwater studies worldwide (Conrad & Daoust, 2008). Also called "citizen science," using ideas and resources from engaged community members can have far-reaching impacts compared to a small group of scientists (Dickinson et al., 2012). CBM has enhanced many aquatic biology initiatives, including assessments of ecosystem health, macroinvertebrate classification, and parasite diversity (Robinson et al., 2021; Rudko et al., 2020; Storey & Wright-Stow, 2017). The range of community involvement in CBM projects can vary substantially, sometimes including high levels of engagement from community partners in study development and data collection (Conrad & Daoust, 2008). In this study, local community members approached scientists with concerns about the impact of fecal bacteria on water quality, and a research approach was designed around their concerns.

Fecal indicator bacteria (FIB) can indicate the presence of disease-causing pathogens in the water due to fecal contamination (Fewtrell & Bartram, 2001). *Escherichia coli* and *Enterococcus* are standard FIB recommended by the US Environmental Protection Agency (EPA) (Department of Environmental Quality, 2012b) as targets for water quality testing. *Enterococcus* can be assessed using traditional plating methods or via quantitative polymerase chain reaction (qPCR), while *E. coli* is frequently assessed using plating methods per the EPA guidelines (Department of Environmental Quality, 2012b). Culture-based plating methods require a 24-hour growth period and bacteria to be viable and actively growing for detection. In contrast, qPCR methods are faster (less than three hours), can detect DNA from live or dead organisms or environmental (e)DNA, and can be used to source track detected FIB contaminants in water samples (Noble et al., 2010). A statistical analysis study conducted by Gonzalez and Noble (2014) comparing qPCR to culture-based water testing methods showed that using culture-based methods correctly predicted management decisions at a slightly higher rate than qPCR. However, another study by Wade et al. (2008) showed that *Enterococcus* measured by qPCR more accurately predicted GI illness than membrane filtration methods.

From the perspective of a community-driven project, qPCR confers advantages over a culture-based methodology. Filtration of a composite water sample and preservation of the filter for

future qPCR analysis is more accessible than the parallel process for culture analysis (U.S. Environmental Protection Agency, 2012b). qPCR analysis can even be made accessible to community partners, decentralizing the equipment required away from core laboratories and using relatively inexpensive, portable machines. This allows for more water samples to be taken in each area or time period and gives communities more control over the type of questions with which to engage (Rudko, 2020).

While *E. coli* and *Enterococcus* are commonly used indicators of fecal pollution, they are nonspecific to their source. Another significant benefits of using qPCR as the method for quantifying fecal contamination lies therein. Microbial source tracking (MST) can be performed using qPCR to identify the source of the fecal contamination. Of interest in this study is the contribution of human, dog, and Canada goose fecal pollution to the overall fecal contamination measured at a lake. The HF183 MST marker that targets human *Bacteroides* is a specific and reliable qPCR-based assay to measure human fecal pollution (Ahmed et al., 2007; Johnston et al., 2013). Dogs and Canada geese are also common in our study area and have MST markers that are reliably tested for (DG3 and CG0F1-Bac, respectively) (Fremaux et al., 2010; Green et al., 2014).

Stormwater sewer systems have been shown to carry high loads of FIB (Brownell et al., 2007; Petersen et al., 2005; Sidhu et al., 2013; Stein & Ackerman, 2007), including human fecal material (Ahmed et al., 2019; Sauer et al., 2011; Sercu et al., 2009; Sidhu et al., 2012; Staley et al., 2016), while in Michigan, fecal pollution of human origin is an issue that has been linked to the density of septic systems (Verhougstraete et al., 2015). Fecal contamination around the Village of Beulah and Cold Creek in Crystal Lake, MI, has been a growing concern of residents of the Betsie River/Crystal Lake watershed (McCauley et al., 2016). The Benzie/Leelanau Health Department has been monitoring Beulah Beach near the outlet of Cold Creek since 2013 and has closed the beach for full body contact for 20 days from 2013-2022 (Michigan Department of Environment, Great Lakes, and Energy, 2022b). The Crystal Lake Watershed Association (CLWA) and Benzie Conservation District (BCD), both primary caretakers of the Crystal Lake Watershed, have invested resources to collect and analyze water samples for enteric bacteria in the Cold Creek watershed annually since 2016. Before that, samples were analyzed periodically by the State of Michigan.

In this study, we sampled weekly at critical points along Cold Creek and near its mouth on Crystal Lake from 6/30/21 to 8/11/21, and biweekly from 6/1/22 to 8/24/22. Samples were analyzed using qPCR targeting *Enterococcus*, followed by source tracking all samples for human, dog and Canada goose fecal contamination (HF183, DG3, and CG0F1-Bac markers, respectively). Additionally, we compared qPCR results assessing *Enterococcus* with traditional plating techniques testing for *E. coli*. By working with community partners to answer a question of interest for them and furthering our scientific knowledge of MST and traditional bacterial testing methods, we exemplify the power of community-based monitoring to increase water quality interest by the public.

Methods

Sample Locations:

A map outlining the sampling sites for this project is presented in Figure 2-1. As further background to site selection, Beulah (Benzie County, Michigan, USA) maintains a public water and sanitary sewer system, with sanitary waste pumped into lagoons to the south of the village. There are three stormwater outflows onto the public beach and several that discharge into Cold Creek (which is the largest tributary of Crystal Lake). The topography of the commercial street that runs parallel to the public beach directs most surface stormwater to the beach area, primarily via impervious surfaces (McCauley et al., 2016). In 2021, 11 sampling locations were chosen in collaboration with CLWA board members and Benzie Conservation District staff as areas of high interest due to previous water sampling (Figure 2-1). Four of the chosen sites were from small inlets around Crystal Lake, and seven were within the Cold Creek Watershed. An additional three inlet sites were added on the last day of sampling per community partner requests. In 2022, sites were chosen based on data from the previous year and where additional information was needed to clarify results. Samples were taken along Cold Creek (CC), at Beulah Beach in Crystal Lake (BB-CL) and at the stormwater outflow (BB-SW), at the Crystal Avenue stormwater outflow (CAO), Shadko Creek (SC), Harris Creek (HC) and Bellow's Creek (BC). GR was also a small unnamed creek. Three sites were added in 2022 that had never been sampled before. After three sampling dates, one of those sites (CC-03+) was eliminated in favor of sampling CC-06, which was sampled in 2021, to measure contamination levels of the

north/middle branch of Cold Creek before it coalesced with the south branch of Cold Creek. In total, there were 17 sites sampled throughout the two-year study.

There are three branches of Cold Creek. CC-09 is the most upstream sampling site on the north branch of Cold Creek and feeds into CC-06. CC-05+ is the most upstream sampling site on the middle branch of Cold Creek, which feeds into CC-05. The north and middle branches of Cold Creek coalesce into CC-04. Thus, these sites collectively are called the north/middle branch of Cold Creek. CC-10 is on the opposite side of the road as CC-05 and feeds into the southern branch of Cold Creek. CC-03+ is the most upstream site on the south branch of Cold Creek, and it combines with CC-10 to feed into CC-03. Thus, these sites are called the south branch of Cold Creek. CC-03 and CC-04 both converge to enter a settling pond. CC-02 is at the outflow of the settling pond, and CC-01 is at the outflow of Cold Creek into Crystal Lake.

Sample Methods:

In 2021, duplicate samples were collected by a biologist from the Benzie Conservation District and a summer intern every week for seven weeks between 6/30/21 and 8/11/21 using the Method 1611 collection protocol (U.S. Environmental Protection Agency, 2012a). In 2022, duplicate samples were taken every two weeks between 6/1/22 and 8/24/22 and collected identically to 2021. Samples were kept on ice and immediately transported to the laboratory at Freshwater Solutions, LLC (FWS) in Cedar, MI, where they were filtered within six hours of collection. Filters were frozen at -20 °C until extraction could be complete the following day or extracted immediately. At each location sampled for each period, an additional sample was collected and sent to SOS Analytical in Traverse City, MI, a state testing laboratory, which uses standard colony counting techniques to enumerate *E. coli* (U.S. Environmental Protection Agency, 2002a). Precipitation data from the NOAA National Weather Service Beulah 7SSW, Michigan station were summed for seven days, 48 hours and 24 hours before each sampling event.

DNA extraction was performed using the Qiagen DNeasy Blood & Tissue kit per the manufacturer's directions with a physical disruption step after adding proteinase K and buffer AL. qPCR analysis for *Enterococcus* was completed as previously reported using a modified version of the US EPA Method 1611 that compares sample cycle threshold values to a known-quantity standard curve (Rudko et al., 2020). PCR inhibition was assessed following the protocol

described in US EPA Method 1611 (U.S. Environmental Protection Agency, 2012a). Samples were run in duplicate on the Applied Biosystems QuantStudio 3 qPCR thermocycler. All samples were assessed for the HF183, DG3 and CG0F1-Bac markers per published protocols (Fremaux et al., 2010; Green et al., 2014; Haugland et al., 2010). Assay sequences are presented in Table 2-1.

Technical replicates of each water sample were run during qPCR analysis. Any samples for which the technical replicates were incongruous were re-assessed for clarification and if again showed an incongruous result the two copy/reaction numbers were averaged and used to calculate GE/100 mL. No qPCR inhibition was detected in any of the samples.

Water Quality Standards:

While the EPA relies on individual states within the US to set water quality standards (WQS), many, including Michigan, have yet to do so for *Enterococcus* using qPCR. Michigan has developed a WQS for *E. coli* of 130 *E. coli*/100mL as the maximum acceptable level of a 30-day geometric mean. At the same time, a single-day value cannot exceed 300 *E. coli*/100 mL without eliciting action at the sampling location (Department of Environmental Quality, 2006). To compare the *E. coli* data to the *Enterococcus* qPCR, the US EPA statistical threshold value (STV) for an estimated illness rate (NGI) of 32/1000 primary contact recreators (1,280 calibrator cell equivalents/genome equivalents per 100 mL) was used (Department of Environmental Quality, 2012).

Statistical Analysis:

Duplicate sample data for each site on a given day were combined, and the highest *Enterococcus* value was taken for analysis. If either sample showed positive for human, dog, or Canada goose marker, they were labelled as 'positive.' We added 1 to all *E. coli* and *Enterococcus* data and log10 transformed them prior to analysis. For the comparison analysis between *E. coli* and *Enterococcus,* each were rated '1' if they fell below the single day WQS or a '2' if they fell above the single day WQS, and percent agreement was assessed for the data. Then, a Cohen's Kappa analysis was run on these data. Additionally, a Spearman's rank correlation coefficient (rho) test was used to analyze the relationship between $\log_{10}$ transformed *E. coli* levels (*E.*

coli/100 mL) and $\log_{10}$ transformed *Enterococcus* levels (GE/100 mL) due to the non-parametric nature of these data.

To investigate the relationship between precipitation amount and *Enterococcus* levels, a Kruskal-Wallis test was run on data based on groupings by precipitation from the previous 24 hours, 48 hours, and 7 days. Further, Dunn's multiple comparison tests were run to compare *Enterococcus* values of each group, based on level of precipitation, to *Enterococcus* values in the 0.0 cm precipitation group. All analyses were run in R Studio (Version 2022.12.0+353) (R Core Team, 2023) except for the Kruskal-Wallis analysis, which was performed in GraphPad Prism 10.0.0 (Boston, Massachusetts).

Results

From 2013-2015, 43 samples were taken by the Michigan Department of Environmental Quality (MDEQ) at one beach on Crystal Lake (Beulah Beach). Six of those samples (14%) yielded a WQS value of over 300 *E. coli*/100 mL. Between 2017 and 2020, there were 89 water samples taken in this area, and 27 of them (30%) were found to have *E. coli* values over the WQS of 300 *E. coli*/100 mL, while the rest of the samples had smaller though not zero values (Supplementary Table 2-1). In 2021 and 2022, 33% (62 out of the 187) of *E. coli* water samples were considered contaminated enough to signal a beach posting or closure.

In 2021 and 2022, only 3 of 184 water samples returned negative for *Enterococcus*. Forty eight percent (88 out of the 184) of samples showed an *Enterococcus* level that high enough to warrant source tracking, according to the STV (Figure 2-2). In 2021 there were 55 water samples over the 1,280 GE/100 mL STV, while in 2022 only 33 water samples were above this threshold (Figure 2-3).

Sites CC-09, CC-04, CC-06, CC-10, CC-05 and CC-01 all had median *Enterococcus* levels over the STV of 1,280 GE/100 mL. In the north and middle branches of Cold Creek (CC-09, CC-06, CC-05, CC-05+ and CC-04), 74% of samples (39 out of 53) were over the STV. In the South branch of Cold Creek (CC-10, CC-03+ and CC-03), 55% of samples were over the STV. CC-02 (outflow of the settling pond) had 57% of samples over the STV, while CC-01 (entrance of Cold Creek to Crystal Lake) had 71% of samples over the STV (Figure 2-3). According to *E. coli* data,

43% of samples from the middle branch of Cold Creek were above the STV (23 out of 53), and 33% from the south branch of Cold Creek were above the STV (10 out of 30).

Enterococcus levels, grouped by amount of precipitation for the previous 24 hours, 48 hours and 7 days showed a significant difference between groups, according to a Kruskal-Wallis test (Table 2-2). When these data were further tested with a Dunn's multiple comparison test, there was no significant relationship between higher levels of precipitation and more *Enterococcus* in water samples. When considering precipitation from the previous 24 hours, only one out of 5 precipitation levels (0.53 cm precipitation group), had *Enterococcus* levels that were significantly different than *Enterococcus* levels from the 0.0 cm precipitation group. Three precipitation levels out of 11 (0.13 cm, 0.53cm, and 1.07 cm groups) had *Enterococcus* levels that were significantly different from the 0.0 cm precipitation group when measuring the previous 48 hours of precipitation. Similarly, when measuring precipitation for the previous 7 days, four out of 13 groups (0.58 cm, 1.24 cm, 3.28 cm, and 4.24 cm) had *Enterococcus* levels significantly different than those of the 0.0 cm precipitation group. In all tests, there was no scientifically plausible explanation for the sporadic groups that had a significant relationship. This type of analysis could be impacted by the fact that this data is not homoscedastic, so additional analyses may be needed to further investigate the relationship between *Enterococcus* values and precipitation. The lowest precipitation day showed some of the highest *Enterococcus* values of the study period (Figure 2-3).

Of the 184 samples analyzed for *Enterococcus*, 26 were found positive for HF183. Of these positive samples, 15 were found at outflows into Crystal Lake, near the public beach. One site had four positive HF183 samples (CC-01), while three sites had three HF183 positive samples (BB-SW, CAO, and CC-10). Five had no positives for the marker (CC-02, CC-03+, CC-04, CC-05+, SC) (Figure 2-4). Notably, there are stormwater outflows upstream of CC-01 and at BB-SW and CAO, which had three of the four highest numbers of HF183 positive samples.

Fifty-six samples tested positive for dog fecal contamination, with CC-01, BB-SW, CAO and BB-CL having all but three of the positive results. Ten sites tested positive for Canada goose contamination, with a total of 38 positive samples. The three highest sites for Canada goose contamination were BC, CC-09 and HC (10, 8 and 6 positive samples, respectively).

The north/middle branch of Cold Creek had 8% of samples (4 of 53) positive for human fecal contamination, while the south branch of Cold Creek had 16% (5 of 31) positive for HF183. CC-02 (water leaving the settling pond) did not possess human fecal contamination in any of the samples, but CC-01 (inflow to Crystal Lake) showed 29% of samples (4 of 14) positive. Of the 17 sampling locations, 12 showed at least some amount of human fecal contamination.

In the percent agreement analysis comparing *Enterococcus* qPCR and *E. coli* culture data, 71% of samples were in agreement on whether the sample value would have yielded a beach management decision. Of the samples tested, 26% were above the single day WQS for both *E. coli* and *Enterococcus*, and 45% were below the single day WQS for both FIB (Figure 2-5). A Cohen's Kappa test of these data showed an unweighted kappa = 0.42, which implies a moderate agreement between these two variables. Additionally, the Spearman's rho analysis indicated a positive association between the *E. coli* and *Enterococcus* data (rho = 0.608, $p < 0.001$).

Discussion

Our study demonstrates the value of CBM to address monitoring and research questions that interest scientists and local community members. This community-based study provided insight into enteric bacteria levels at a historically contaminated area and clarified the source of that contamination as being partially of human origin. Human, dog, and Canada goose contamination were all assessed and found in water samples during our study. However, when considering results from a risk perspective, we focus on the 26 samples that were positive for human contamination, across 12 sampling sites, due to the increased chance of human fecal contamination carrying other human disease-causing agents (Department of Environmental Quality, 2012).

This study allowed us to compare two different FIB and use MST to assess the origin of the fecal contamination. We observed a moderate positive relationship between values when comparing culture-based *E. coli* monitoring methods to qPCR-based *Enterococcus* monitoring methods. This is unsurprising since much research has focused on comparing these two species. It has been shown that when comparing results from these two tests there is a low correlation between them (Kinzelman et al., 2003). *Enterococcus* is thought to persist longer in the environment, thus making it a more conservative indicator (Jin et al., 2004). It has also been shown that qPCR

results do not closely correlate with culture-based methods when measuring the same species, likely due to the fact that qPCR-based assessment will detect live (culturable), viable but not culturable (VBNC) and dead bacteria (Wade et al., 2008).

Standard *E. coli* testing protocols limit the ability to source track positive samples, leaving in question the source of this fecal contamination. If only *E. coli* culture data and *Enterococcus* qPCR data had been collected from the two branches of Cold Creek, we would have concluded that the north/ middle branch of Cold Creek displayed greater FIB contamination. We would have missed that the south branch of Cold Creek had a higher persistence of HF183 contamination. This would have left out a significant aspect of our conclusions and left uncertainty with respect to the source of the pollution in our results. For this study, being able to source track for human, dog and goose fecal contamination was vital to meeting the needs of community partners. qPCR results do overestimate the number of times a beach action would need to be taken when compared to *E. coli* data, perhaps due to DNA detection from non-viable *Enterococcus*. However, just because *Enterococcus* is not culturable does not mean it did not come from a source that could reflect a risk for GI illness (Wade et al., 2008). Thus, results should be carefully considered when examining culture-based *E. coli* testing alone.

Precipitation in the 24 hours, 48 hours and seven days preceding sample collection was assessed to determine whether precipitation influenced FIB, HF183, DG3 and CG0F1-Bac presence and abundance. Previous studies have demonstrated a relationship between FIB and precipitation (Celico et al., 2004; Islam et al., 2017; Laureano-Rosario et al., 2017; McKee et al., 2020; Noble et al., 2003; Walters et al., 2011). Precipitation measured over the previous 24 hours, 48 hours, or seven days did show a significant association with *Enterococcus* levels grouped by amount of precipitation, measured by a Kruskal-Wallis test, however a Dunn's multiple comparison test found no significant relationship between high precipitation levels and more bacteria in the water. Precipitation did not relate to HF183, dog or Canada goose positive samples. We conclude that the observed increases in bacterial levels in the water were not due to runoff events.

Enterococcus levels varied in specific branches of Cold Creek. CC-03 and CC-04 are located at the inflow to a settling pond, put in place to decrease the sediment that ultimately makes its way into Crystal Lake. CC-02 is a sampling location added in 2022 to help clarify the settling pond's

relationship with bacterial levels. CC-04 is the entry point of the north branch of Cold Creek into the settling pond and shows consistently high values for *Enterococcus* but no detectable contamination from human fecal material. CC-09, a part of the north branch of Cold Creek, has historically had a lot of goose activity and eight positive samples for goose fecal contamination. Thus, we can conclude that geese are likely a contributing source to the high *Enterococcus* values at this site. CC-03 is the entry point of the south branch of Cold Creek into the settling pond and shows much lower levels of *Enterococcus* than CC-04 but does show human fecal contamination. On the other side of the settling pond, CC-02 shows *Enterococcus* results to be a bit higher than CC-03 in 2022 but lower than CC-04, perhaps showing a mix of highly contaminated water with low-contaminated water. Of note is the sanitary waste lagoon with spray irrigation wastewater treatment system which receives sanitary waste from the city of Beulah and is located southwest of our sampling sites. This system, which includes six lagoons and 12 spray irrigation zones, has been deemed 'failing' and work is approved to upgrade the wastewater treatment plant (Michigan Department of Environment, Great Lakes, and Energy, 2022a). This failing system could contribute fecal material to the groundwater in our study, but the relationship is unknown at this time.

Human fecal pollution was more frequently observed in the south branch of Cold Creek than the north/middle branch of Cold Creek. Future research should focus on this area to better specify the source of this contamination. Interestingly, no human fecal material was found at the outflow of the settling pond. There has been conflicting conclusions about the effect of UV light on enteric bacteria measured by qPCR, with some studies showing no effect of UV light on *Enterococcus* levels (Chern et al., 2014; Dick et al., 2010) or HF183 levels (Korajkic et al., 2014; Walters & Field, 2009) as measured by qPCR, while others have shown sunlight to significantly decrease enteric bacteria levels (Walters et al., 2009). Culturable bacteria became unmeasurable much sooner than those measured by qPCR, suggesting that the DNA of dead bacteria may persist in the environment after viable bacteria have died (Walters & Field, 2009). Perhaps the HF183 marker decayed due to prolonged exposure to UV radiation while in the settling pond, so none was found at the outflow.

The use of HF183 as an MST indicator has had mixed results regarding the cross-reactivity with dog fecal contamination. One study showed no cross-reactivity (Ahmed et al., 2007), while

others have shown some cross-reactivity (Ahmed et al., 2012; Kildare et al., 2007; McQuaig et al., 2009). All water samples from sites CC-01, BB-SW, and CAO that were positive for HF183 were also positive for DG3. Because of the potential for cross-reactivity between the HF183 assay and dog feces, we cannot conclusively say the fecal contamination is of human origin. However, none of the water samples taken from sites BC, CC-03, CC-05, CC-09, CC-10, GR and HC that were positive for HF813 contamination were positive for DG3 contamination, which suggests that there is human fecal contamination in our study area. Site CC-06 had two water samples positive for HF183, but only one of them was also positive for DG3. BB-CL also showed high dog fecal contamination and had two positive HF183 samples. CC-01, BB-SW, CAO and BB-CL are in areas of public use with many impervious surfaces while also being the sites most greatly impacted by stormwater outflows.

Dominant sources of fecal contributions in a single area have been shown to vary depending on the time of year and location within a watershed (Stein & Ackerman, 2007; Whitlock et al., 2002). Thus, the presentation of dog, Canada goose, and human fecal pollution could have naturally varied over the sampling period. Due to this possibility and potential cross-reactivity with dog markers, it may be recommended to include caffeine sampling or another confirmatory test for human contamination (Sidhu et al., 2013).

Along with the outflow into Crystal Lake, the south branch of Cold Creek (specifically CC-10) should be examined for HF183 sources, as it had a consistent HF183 signal throughout the study and has low potential for cross-reactivity with the dog MST marker. BC, CC-09 and HC were the sites most greatly impacted by goose fecal contamination (6 or more positive samples). BC drains a public park that may be appropriate habitat for geese, while HC is a wooded area that drains some orchards at the head of the watershed. CC-09 is near a wetland area, with a pond upstream that could house geese during certain times of the year.

One drawback of using HF183 as an MST target is the low persistence in the environment compared to FIB (Liang et al., 2012; Walters & Field, 2009). The low number of samples that are found to be positive can be hard to analyze. This was found in one study examining sanitation issues in central Appalachia (Cantor et al., 2017). It was suggested that a general FIB should also be sampled along with HF183 to help clarify results. The HF183 marker gene is also

known to decay faster in the environment than pathogen genes do (Ahmed et al., 2021). Thus, the absence of HF183 markers in the water does not mean there is zero risk of infectious agents in water. Because of this, we suggest that any site with even one positive sample for HF183 found there would be considered to have human fecal contribution.

When considering recreational swimming, many factors may impact an individual's risk of developing gastrointestinal illness (GI). While this was not a focus of the study since many of our sample locations are not recreational swim locations, public interest in health risks are often pertinent. The most common pathogen shown to persist in contaminated water is norovirus, which can persist in ambient waters for up to 61 days (Seitz et al., 2011). This is much longer than the average persistence of HF183 (Walters & Field, 2009), meaning that if there is a contribution of human fecal material into a water source, there may be a risk of gastrointestinal illness many days after the HF183 marker is found (Boehm et al., 2015). While none of the sampling sites along Cold Creek have recreational use (including the inflow to Crystal Lake), the sampling site at Beulah Beach (BB-CL) has many swimmers throughout the summer. Interestingly, the Beulah Beach samples possessed significantly lower *Enterococcus* values than the Cold Creek samples but did have two HF183-positive samples. This is the only site in our study where detectable human fecal contamination was present in water in direct contact with swimmers, which may simultaneously be the source of human fecal contamination for that site.

Stormwater outflows and septic systems may more likely contribute to human fecal material at the sites without recreational swimming. Septic system density has been linked to increased fecal contamination (Sowah et al., 2014) in the water, specifically from human sources (Sowah et al., 2017; Verhougstraete et al., 2015). Stormwater systems have also been linked to high FIB and human MST numbers (Ahmed et al., 2019; Petersen et al., 2005; Sauer et al., 2011; Sercu et al., 2009; Sidhu et al., 2012; Staley et al., 2016; Stein & Ackerman, 2007). We noticed that the highest *Enterococcus* levels were found on a day without rain the previous seven days, not linking those bacterial levels to runoff due to precipitation. One study showed no link between precipitation and human fecal bacteria in stormwater outflows (Sauer et al., 2011), which is consistent with our findings. We hypothesize that these high values were due to point source contamination that consistently has input into the system but is generally more diluted with greater precipitation, especially because stormwater outflows in our study have continual flow,

not just during rain events. During low precipitation times, the bacteria would coalesce in higher levels in the water.

As a community, evidence from this study shows the need to further investigate bacterial contamination sources between the settling pond and outflow into Crystal Lake. *Enterococcus* was at lower levels when leaving the settling pond, with no HF183 found, than when it entered Crystal Lake. Thus, the water gained *Enterococcus*, human and dog fecal contamination between leaving the settling pond and getting to Crystal Lake. Three stormwater outflows between the settling pond and the sampling site entering Crystal Lake are likely sources of contamination. The stormwater sewer system for the city of Beulah is currently unmapped and mapping this system while checking the integrity of infrastructure would benefit the community to further clarify where the HF183 and DG3 influence may be coming from. While Beulah City is on a sewer system, several houses have individual septic systems that are not tied into the sewer system and could be contamination sources, although these are a less likely source.

While the use of qPCR has dramatically increased the speed and specificity of microbial water testing, there are still limitations in what target sequences are looked for and the amount of information provided from those specific targets. Using newer technology to test environmental DNA (eDNA) along with metabarcoding has shown great promise for the future of microbial and invasive species water testing (Garlapati et al., 2021; Nevers et al., 2018; Wu et al., 2022). This testing would allow a greater understanding of the bacterial community and its dynamics within a watershed. The ability to test for all bacterial species in a water sample would be beneficial as we would not be limited to a specific target. With this methodology, we may see species emerge as influential to water quality that have yet to be a focus of testing via DNA-based water monitoring. eDNA testing could improve microbial water testing for communities such as the one in this study, which are looking for source points of contamination and hoping to provide data to compel change in their community.

Although community-based monitoring is an effective way to collect numerous water samples over a short period (Rudko et al., 2020), studies have yet to be published in which this framework focuses on enteric bacteria monitoring to answer citizen questions. This community-based study showed results that correspond to current literature about contamination of water in

municipalities while collecting a plethora of samples with the help of community partners. Specifically, this study provided meaningful information for community members about the water quality of Cold Creek, with added value including the source of contamination.

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Table 2-1: Primer and probe sequences for the *Enterococcus*, HF183, DG3 and CG0F1-Bac qPCR assays used in this study.

Assay	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan Probe (5'-3')	Reference
Enterococcus	GAGAAATTCCAAACGAACTTG	CAGTGCTCTACCTCCATCATT	TGGTTCTCTCCGAAATAGCTTTAGGGCTA	U.S. Environmental Protection Agency (2012a)
HF183 (human)	ATCATGAGTTCACATGTCCG	CGTAGGAGTTTGGACCGTGT	CTGAGAGGAAGGTCCCCCACATTGGA	Haugland et al. (2010)
DG3 (dog)	TTTTCAGCCCCGTTGTTTCG	TGAGCGGGCATGGTCATATT	AGTCTACGCGGGCGTACT	Green et al. (2014)
CG0F1-Bac (Canada goose)	GTAGGCCGTGTTTTAAGTCAGC	AGTTCCGCCTGCCTTGTCTA	CCGTGCCGTTATACTGAGACACTTGAG	Fremaux et al. (2010)

Table 2-2: Results from Kruskal-Wallis test with Dunn's multiple comparison test for *Enterococcus* values grouped by precipitation amount.

		Dunn's Multiple Comparison Test			
Length of Measured Precipitation	Kruskal-Wallis results[1]	Precipitation (cm) grouping level	Mean rank difference compared to 0.0 cm precipitation level	Z-value[2]	p-value
24 hours before sampling	26.72, p < 0.0001*	0.03	50.93	2.21	0.14
		0.46	25.54	1.22	>0.9999
		0.53	59.19	2.84	0.02*
		1.22	51.55	2.47	0.07
		1.98	-54.97	2.51	0.06
48 hours before sampling	80.04, p < 0.0001*	0.25	12.69	0.55	>0.9999
		0.08	4.78	0.18	>0.9999
		0.13	109.40	4.70	<0.0001*
		0.53	69.29	2.98	0.03*

		0.56	61.04	2.42	0.17
		1.07	-71.69	2.84	0.05*
		1.40	-56.74	2.17	0.33
		1.52	61.66	2.65	0.09
		1.63	35.64	1.53	>0.9999
		1.73	52.91	2.25	0.27
		3.76	-44.87	1.86	0.70
7 days before sampling	95.52, p < 0.0001*	0.58	-124.6	4.20	<0.001*
		0.69	-48.13	1.58	>0.9999
		0.84	16.38	0.59	>0.9999
		1.24	-123	4.05	<0.001*
		1.30	8.749	0.31	>0.9999
		1.70	56.53	2.02	0.57
		2.00	-4.367	0.156	>0.9999
		2.31	-40.22	1.436	>0.9999
		3.28	-109.7	3.607	0.004*
		4.24	-97.78	3.4	0.009*
		4.93	-49.04	1.613	>0.9999
		5.54	8.129	0.274	>0.9999
		10.85	-17.27	0.6167	>0.9999

* Signifies significance at the 95% level
[1] Kruskal-Wallis value followed by p-value for significance
[2]Z-value is a comparison between the *Enterococcus* levels between that precipitation grouping level and the *Enterococcus* levels in the 0.0 cm precipitation group

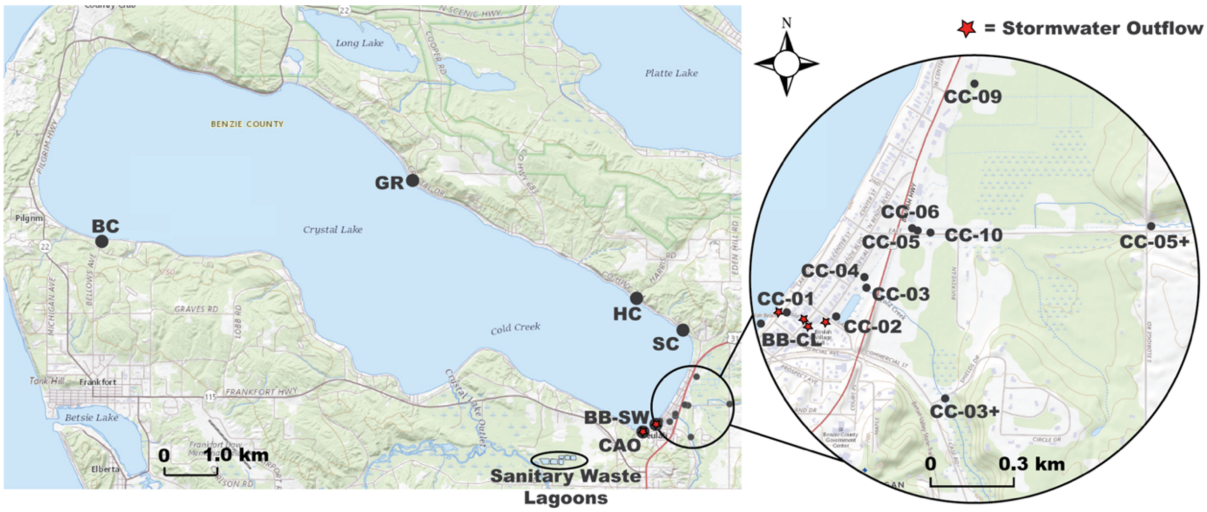

Figure 2-1: Map of sampling locations around Crystal Lake, Benzie County, MI. Zoomed image shows sampling sites along Cold Creek inlet. Fifty mL of water was collected at each site throughout the summers of 2020 (weekly) and 2021 (biweekly) and tested for *Enterococcus* and *E. coli*. Dots represent sample sites. Stars indicate stormwater outflow locations near or corresponding to our sampling sites.

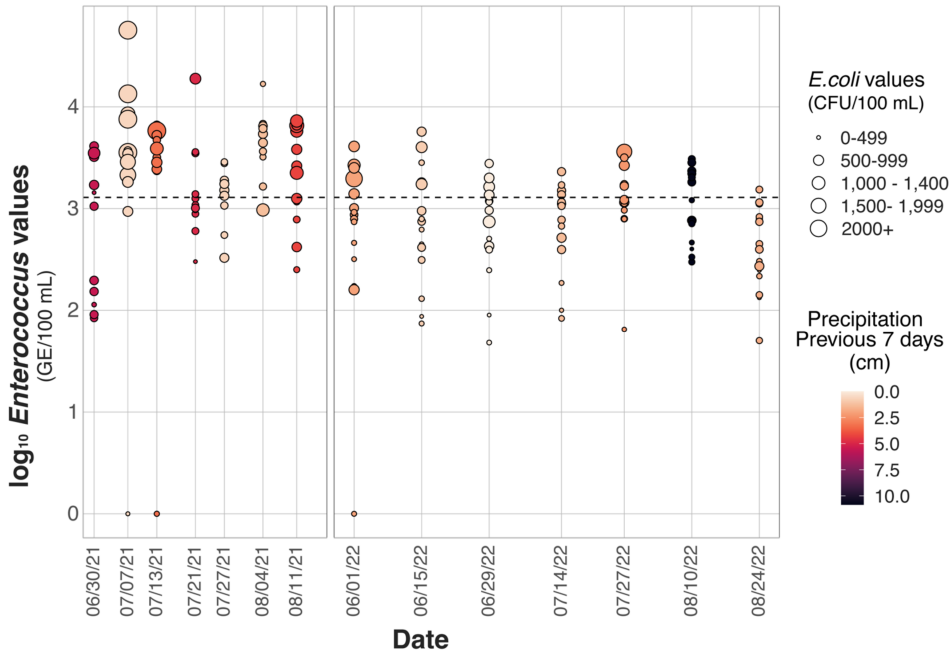

Figure 2-2: Log_{10} of *Enterococcus* values (GE/100 mL) compared to the sampling date. Coloring shows the total precipitation of the previous 7 days (cm). Size is based on *E. coli* results (CFU/100 mL). Dotted line signifies the statistical threshold value (STV) of 1,280 GE/100 mL for *Enterococcus*.

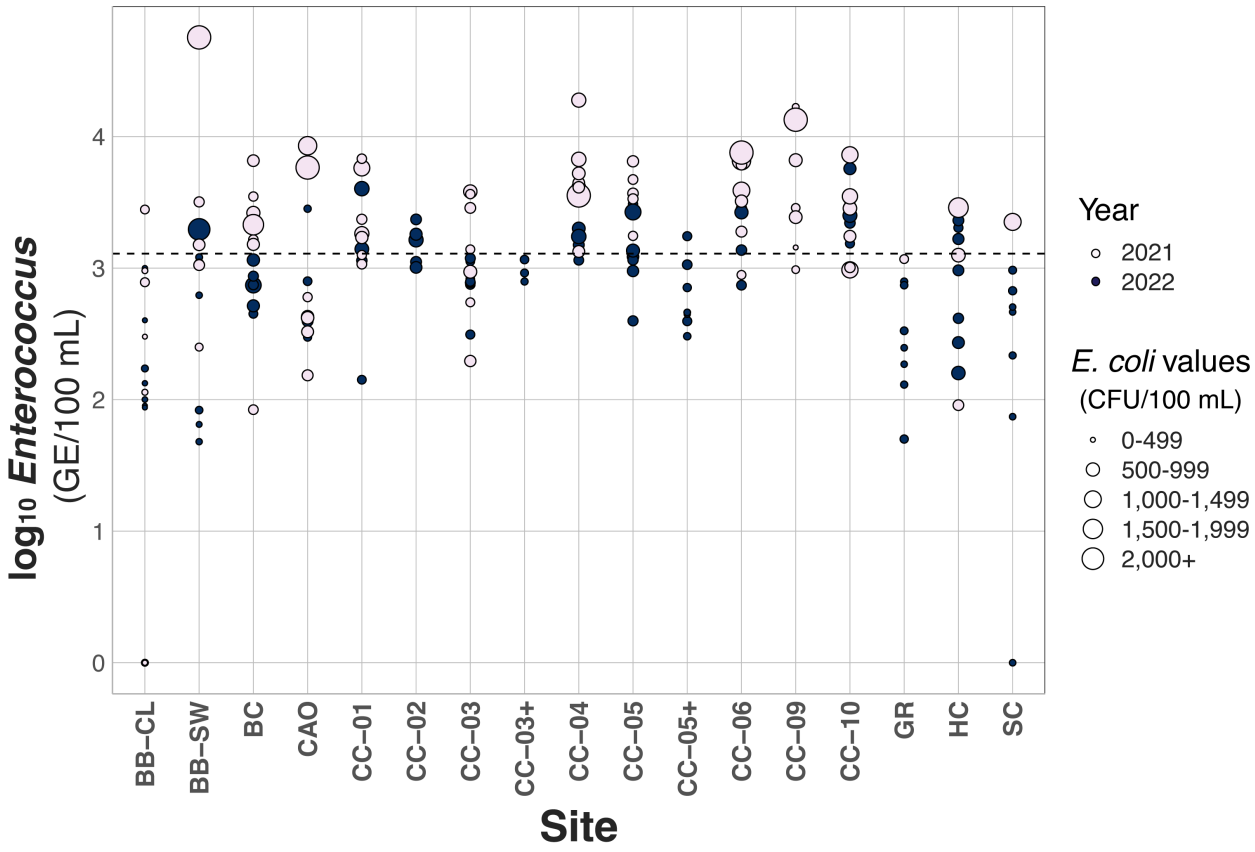

Figure 2-3: Log$_{10}$ *Enterococcus* values (GE/100 mL) at each sampling site on Cold Creek or Crystal Lake, MI colored by year of sampling and sized based on *E. coli* results (CFU/100 mL). Dotted line signifies the statistical threshold value (STV) of 1,280 GE/100 mL for *Enterococcus*.

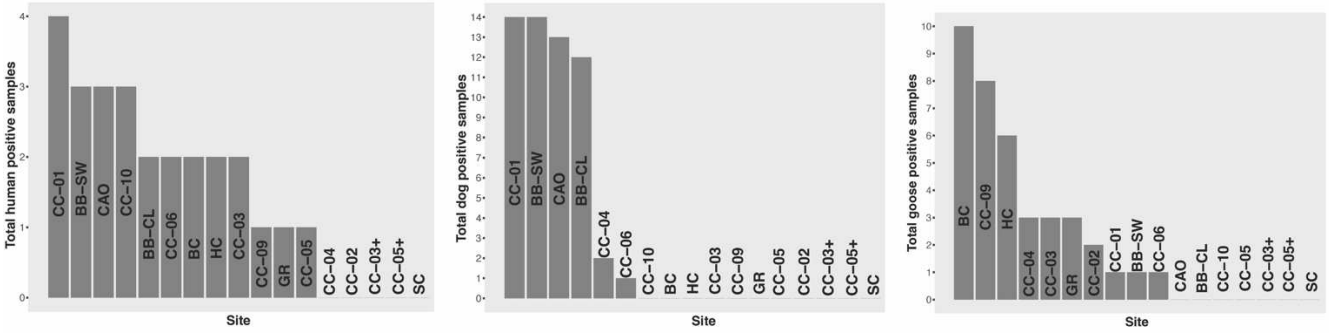

Figure 2-4: Number of samples that were positive for HF183 (human), Dog3 (dog), and CG0F1-Bac (Canada goose) at each sampling site. Note the different scales on the y-axis of each graph.

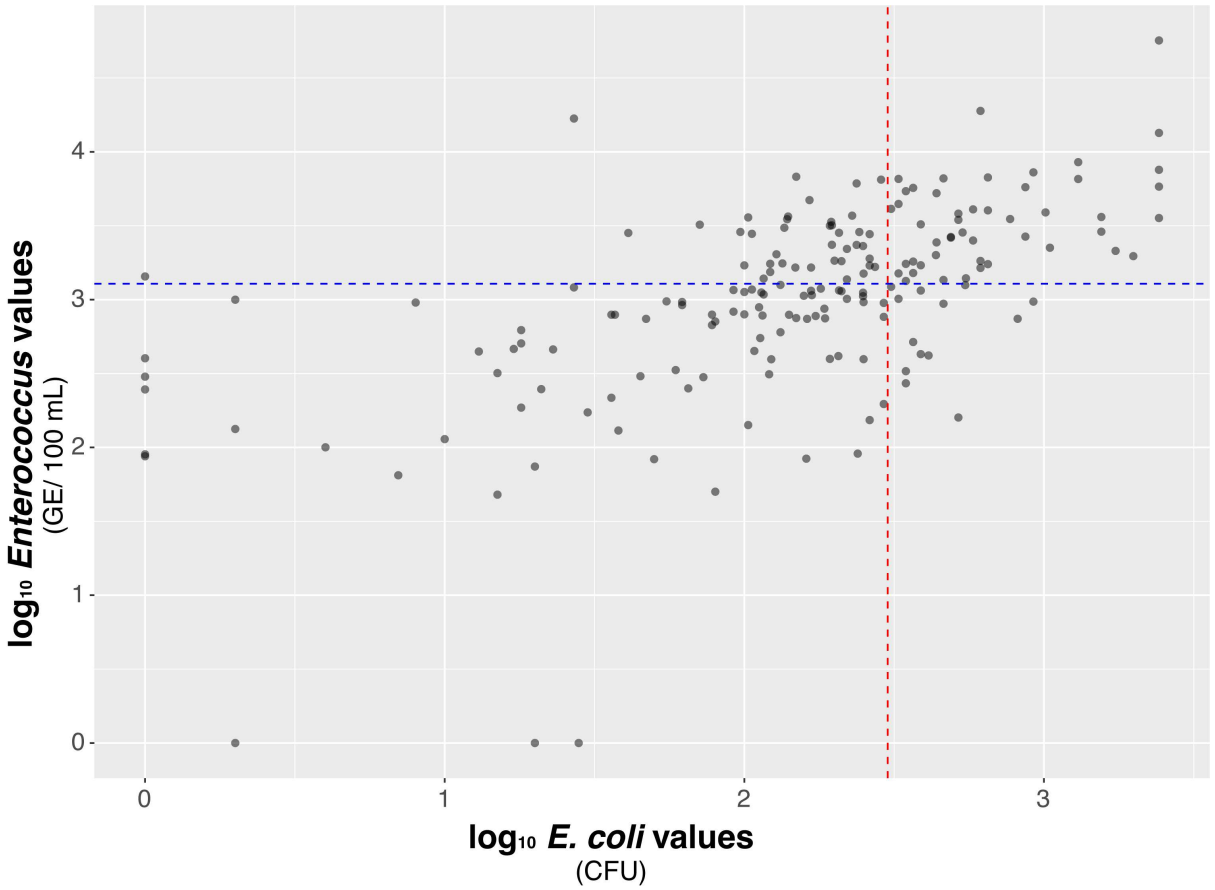

Figure 2-5: Comparison of log_{10} *Enterococcus* values (GE/100 mL) as measured with qPCR to log_{10} *E. coli* values (CFU/100 mL) as measured with culture-based methods. Blue line represents the standard threshold value (STV) for *Enterococcus* of 1,280 GE/100 mL. The red line represents the single day water quality standard (WQS) of 300 CFU/100 mL for *E. coli.*

Supplementary Table 2-1: Site GPS coordinates for Crystal Lake and Cold Creek.

Site	Latitude	Longitude
BB-CL	44.629	-86.097
BB-SW	44.62846	-86.097535
BC	44.660889	-86.232194
CAO	44.627306	-86.10075
CC-01	44.629361	-86.095861
CC-02	44.6291733	-86.093534
CC-03	44.630167	-86.092056
CC-03+	44.626506	-86.088372
CC-04	44.630573	-86.09209

CC-05	44.632111	-86.089694
CC-05+	44.632222	-86.078834
CC-06	44.63219	-86.08986
CC-09	44.637	-86.087
CC-10	44.632	-86.089
GR	44.67125	-86.156389
HC	44.65075	-86.101778
SC	44.645056	-86.090028

Note: CC stands for Cold Creek. CAO is the Crystal Avenue Stormwater Outflow. BB-CL is on Beulah Beach in Crystal Lake. BB-SW is the Stormwater Outflow at Beulah Beach. SC, HC, and BC were all small creek inlets, which are Shadko Creek, Harris Creek, and Bellow's Creek, respectively. The label GR represents the homeowner from whose property a sample was taken at a small inlet.

Supplementary Table 2-2: Historical (2017-2020) *E. coli* testing data from locations near Crystal Lake, MI, with 2021 and 2022 data.

Date	CC-01	CAO	CC-03	CC-04	CC-05	CC-06	CC-09	CC-10	BC
7/12/17	770		210	816					
8/14/17	204		80	435					
9/19/17	82		55		88	276			
11/1/17	31		37		66	57			
5/16/18	126		63		86	31			
6/13/18	1120		397		308	210			
7/17/18	461		276		613	488	866		
8/14/18	111		152		1414	56	272		
8/27/18	1300		2419		1120	1986	2419	2419	
10/15/18	112		59		59	127			
11/28/18	21		38		8	16			
6/3/19	66		68		68		50		34
7/22/19	344		197		410		205		249
8/20/19	161	20	121		248		160		93

9/23/19	613	649	517		411		179		517
5/13/20	21		57		15		11		24
6/27/20	119		144		56		69		74
7/22/20	238		186		961		387		206
8/20/20	59		58		75		33		167
8/26/20		2419							
9/28/20	162								119
6/30/21	387	261	461	308	194	387	0	770	160
7/7/21	613	1300	241	2419	228	>2419	>2419	517	1733
7/13/21	195	2419	115	437	164	1011	437	534	326
7/21/21	131	131	112	613	102	111	54	219	138
7/27/21	167	345	139	345	133	261	96	345	365
8/4/21	148	345	517	326	285	236	26	921	147
8/11/21	866	411		649	435	1300	461	921	488
6/1/22	548	14	140	579	866			579	184
6/15/22	649	40	120	649	291			365	185
6/29/22	727	387	179	435	461			261	816
7/14/22	210	249	172	249	206	219		99	365
7/27/22	166	99	99	1553	308	488		192	387
8/10/22	199	72	148	210	135	206		219	291
8/24/22	102	77	91	166	192	161		121	107

Note: Data presented are colony-forming units (CFU) with red cells indicating values over the single day water quality standard (WQS) of 300 CFU/100 mL. *E. coli* results were obtained from SOS Analytical in Traverse City, MI.

Chapter 3: Freshwater lakes with high density of septic systems show high levels of fecal contamination with possible link to septic system usage

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Abstract

Inadequate septic and sewage systems have long been contributors of fecal contamination to local ground and surface waters, particularly posing risks to local drinking and recreational water quality. Community members from four inland lakes in Northern Michigan were interested in analyzing enteric bacteria levels around their respective shoreline perimeters. In the first year of study, a "snapshot" approach was employed, involving intensive sampling over a three-day period to evaluate human fecal contamination, as measured by the HF183 *Bacteroides* spp. assay. The results revealed that over 28% of samples tested positive for this marker. In the subsequent year, inlets and control sites were sampled before and after rain events to understand their contribution to both general and human fecal contamination of surface water. Post-rain samples exhibited significantly higher *Enterococcus* levels than pre-rain samples, with many highly contaminated samples containing DNA from human fecal bacteria. For the final three years of the study, numerous samples of surface and well water were collected throughout the summer at lakeside residences and tested for *Enterococcus* and HF183 signals. These data were compared to septic system and well metrics, and residential usage logs. The findings highlighted the potential impact of usage patterns on *Enterococcus* positivity in well water samples, emphasizing the importance of not overusing a septic system. This research enhances our understanding of enteric bacteria levels within highly populated freshwater lake environments and underscores the critical role of properly maintained septic systems.

Introduction

Enteric bacteria are found in the gastrointestinal tract of many organisms and have long been used as fecal indicator bacteria (FIB) to test waters for potential pathogens (Ashbolt et al., 2001). Fecal indicator bacteria are not pathogenic at low levels but typically signify the presence of other gastrointestinal organisms that can cause human disease, such as *Salmonella* spp., enteric viruses, and parasites such as *Cryptosporidium* (Macler & Merkle, 2000).

Culture-based methods have historically been used for FIB testing (American Public Health Association et al., 1971), originally targeting fecal coliforms and continuing with the development of methods for testing Enterococci (Levin et al., 1975) and *Escherichia coli* (Dufour et al., 1981). Quantitative PCR (qPCR) is a DNA-based monitoring tool that amplifies and enumerates a DNA sequence specific to a genus or species such as *Enterococcus* or *E. coli* (Frahm & Obst, 2003). Although culture-based approaches to tracking enteric bacteria in water have a long history, the use of qPCR for water quality testing is quickly evolving since qPCR can capture results quickly (~4h) and, in many cases, with greater sensitivity (Bartlett & Stirling, 2003) compared to conventional culture-based methods.

Another benefit of qPCR is the ability to conduct microbial source tracking (MST) to gather information regarding the source of fecal contamination more efficiently than culture-based methods. Of interest in this study was the contribution of human fecal matter to freshwater ecosystems, for which we used the HF183 *Bacteroides* assay (Haugland et al., 2010). The anaerobic behavior of *Bacteroides* spp. does not allow regrowth in the environment while being highly concentrated in fecal material, making them a good monitoring target (Fiksdal et al., 1985). Although HF183 presence has been linked to FIB levels (specifically *E. coli*) (Fremaux et al., 2009), others have shown no direct correlation between the two measures (Staley et al., 2015).

Improper functioning of onsite wastewater treatment systems can contribute fecal material to watersheds. Michigan is the only state in the United States that does not have a statewide septic code. However, four bills were recently introduced in the Michigan Legislature, which, if passed, would require septic system inspection every five years (Michigan Legislature, n.d.). This would be a significant undertaking as there are an estimated 1.3 million septic systems in Michigan

(Department of Environment, Great Lakes, and Energy, 2023). In addition, Michigan lacks any laws regulating the microbiological quality of well water (Michigan Department of Health & Human Services, 2023). It is estimated that approximately 13% of people in the United States drink well water (U.S. Department of Health and Human Services, 2022). A previous study in another state (Montana) showed varying degrees of contamination of well water by FIB, including up to 40% of samples contaminated (Bauder et al., 1991). Another study by the United States Geological Survey found that about one-third of all wells in the United States were contaminated with FIB (Desimone et al., 2009).

Septic systems are designed to filter sewage using two parts: the septic tank and the drainfield. The septic tank takes initial sewage and allows for the separation of liquid and solid waste. Solid waste sinks to the bottom of the tank to decompose until the tank is emptied via pumping. Liquid waste (i.e., septic wastewater) is discharged through a series of pipes to a drainfield, where it is allowed to seep into the soil via perforated tubes. This liquid is treated while being filtered through the soil, allowing chemical and biological processes to occur. Water from the drainfield eventually reaches the groundwater. Septic systems can fail to properly clean sewage for a variety of reasons, the biggest being improper care for the system. If the solid waste in the septic tank is allowed to build up, it can be forced into the drainfield. An old drainfield may also become clogged with organic materials or pipes in the system can become clogged or broken (Vogel, 2005). These problems can cause an excess amount of fecally contaminated material to enter the groundwater and any nearby surface water.

A community-based monitoring (CBM) approach was used to assess enteric bacteria contamination in recreational waters in Leelanau County, MI. CBM is a widely used method of study (Conrad & Daoust, 2008), which can have far-reaching impacts compared to a small group of scientists (Dickinson et al., 2012). This approach can be implemented for water quality testing to allow volunteer participation at numerous stages, including water collection and sample filtration (Rudko et al., 2020). Rudko, et al (2020) have even shown the ability of community partners to have a more integrated level of engagement, in which they continue the water testing process past filtration by completing DNA extraction and qPCR analysis of samples. Government agencies monitor many beaches in MI, but due to limited resources, typically, only a few samples from the highest priority areas are collected. Sampling in this way does not allow

for a comprehensive monitoring project to the scale of an entire lake. The CBM model allows flexibility for scientists and community members involved while allowing for projects that meet the needs of both community members and researchers.

Glen Lake, Lime Lake, and Little Traverse Lake associations voiced interest in learning more about enteric bacteria levels in surface water and tributaries around their lakes, along with possible sources of contamination. This began a community partnership study in which we sought to answer these contamination questions with a research-oriented approach. We hypothesized that leaching of septic systems was contributing to enteric bacteria levels in the lakes, specifically of human origin. These lakes are all found in Leelanau County, in which the groundwater level is measured between 182 and 195 metres above sea level. The water level is measured to be between 7.5 and 35 metres deep below the surface. This land is very permeable to water as it is mainly composed of sand and gravel from glacial deposits (United States Geological Survey, 2023).

In our first year of study, using this CBM approach, we demonstrate that lakes with at least 75% of their shoreline developed and septic systems an average of 50 metres apart have frequent surface water fecal pollution of human origin, as previously demonstrated in rivers (Verhougstraete et al., 2015). Subsequently, we found that human fecal contribution was brought into the lakes via inlets after a rain event but hypothesized that these bacterial levels were not widespread enough to account for all contamination seen the previous year. In the final three years of the study, we focused on ground and surface water contamination due to potential septic system leakage. We found that septic system usage is important to consider when attempting to decrease or control human fecal contamination in a water body.

Methods

Study sites: Partnerships were formed with three lake communities in Leelanau County, MI, due to their high interest level and willingness to coordinate sampling efforts. Glen Lake, Little Traverse Lake, and Lime Lake are all glacially formed lakes with an array of native plant and animal species. Glen Lake comprises Big and Little Glen, connected by a natural canal. Big Glen Lake is oligotrophic, while Little Glen Lake is considered mesotrophic when analyzed by itself (Seites-Hettinger, 2010). Little Traverse Lake is a mesotrophic lake (Seites-Hettinger, 2014),

while Lime Lake is oligotrophic (Seites-Hettinger, 2011). In 2019, Lake Leelanau Lake Association expressed interest in participating in the study. Thus, they were added to that single year. Lake Leelanau has two large lakes (North Lake Leelanau and South Lake Leelanau) connected by a narrow channel. See Table 3-1 for all area and parcel information.

The average distance between septic systems was found by dividing the shoreline length by the number of parcels. Google Earth (Google Earth, n.d.) was used to estimate the percent of developed shoreline on each lake by measuring shorelines where there was no development, adding all measurements together for the lake, dividing by the total shoreline measurement and subtracting from one.

Sampling:

2018: All samples were collected following EPA Method 1611 protocol (U.S. Environmental Protection Agency, 2012a) every 153 metres around Glen Lake, Lime Lake, and Little Traverse Lakes. Samples were taken 15 m from shore. One sample was taken at the deepest point on Lime Lake, at the request of community partners. On July 16, 2018, and July 17, 2018, a group of four community volunteers and three scientists collected water samples around Big and Little Glen Lake. Water samples on Little Traverse Lake were collected on July 17, 2018, by three volunteers and one scientist. Water samples from Lime Lake were collected on July 9, 2018, by a group of four volunteers and two scientists. All volunteers were trained on-site to perform water collection, and a scientist was present at all water collection sites to ensure accuracy. GPS coordinates, water depth, water temperature, wind speed and wind direction at each sample site were collected on all lakes. Dissolved oxygen levels, pH, conductivity, turbidity, % saturation and shoreline features were collected only on July 16, 2018, for each site on Little Glen Lake using the Hydrolab Surveyor HLF Handheld with the Hydrolab HL4 (OTT HydroMet).

Samples were delivered to the Freshwater Solutions, LLC (FWS) lab in Cedar, Michigan and filtered within six hours of collection. Filters were either immediately extracted or frozen in a -4 °C freezer until extractions could occur the following day. Extractions were completed using Qiagen DNEasy Blood & Tissue kit per the manufacturer's directions with the addition of a physical disruption step after adding proteinase K and buffer AL. Extracted DNA was frozen in a -4 °C freezer until shipping. Later it was shipped overnight to the University of Alberta (UA) for

testing using the HF183 qPCR assay, which was implemented following the published protocol (Haugland et al., 2010).

2019: Water samples were taken on July 10, 2019, and July 16, 2019, at lake inlets and control sites before and after a rain event that exceeded 3.5 cm of rain in a 24-hour period on July 15, 2019. At each inlet, a sample was taken directly at the inlet and 10 meters to the left and right of each inlet. Control sites were chosen by community partners as sites not directly impacted by water entering the lake via any inlet. Samples were collected by either lake biologists or volunteers. All samplers were trained to collect surface water samples using the EPA 1611 method (U.S. Environmental Protection Agency, 2012a) on a day before sample collection and given the opportunity to practice sample collection in the presence of a scientist. Precipitation data was obtained from the NOAA National Weather Service Traverse City Cherry Capital AP, Michigan station. Samples were taken on the same three lakes as in 2018 with the addition of North and South Lake Leelanau. Water samples were collected and extracted using the same protocols as the previous year and extracted DNA was analyzed for *Enterococcus* using the primer and probe sequences identified in Method 1611: Enterococci in Water by TaqMan Quantitative Polymerase Chain Reaction (QPCR) Assay (2012). Any sample that exceeded the value of 1,280 GE/100 mL for *Enterococcus* was then analyzed for HF183.

2020-2022: Between 2020 and 2022, a call for volunteer riparian area residents who were willing to have sampling done on the well and surface waters associated with their properties was put out by lake associations on Lime Lake, Little Traverse Lake, and Glen Lake.

Samples for Glen Lake were collected on two consecutive days in June, July, and August, while Lime and Little Traverse collections were done on a single day during those months. On Glen Lake, samples were taken by a lake biologist and student interns. A group of five volunteers sampled Little Traverse Lake and a lake biologist sampled Lime Lake. Samplers were trained identically to the previous year, with the addition of showing volunteers how to collect well water samples. All volunteers were given written directions to have with them in the field. Well water samples were taken by letting the wellhead run for 5 minutes to eliminate wellhead contamination, then turning water off and cleaning the nozzle with a disinfectant wipe. Water was again turned on and two 50 mL samples were taken. In 2021 and 2022, duplicate samples were collected at well and surface water locations during the three sampling dates. All filtration

and extraction methods mirrored those of 2018. Extracted DNA was shipped to UA for *Enterococcus* testing along with assessment of human fecal contamination via the HF183 assay.

Use Logs:

All volunteers participating in the 2020-2022 well-surface water study were asked to keep a nightly record of the number of people at their residences. Numbers were compiled and analyzed for June and July of each year for individual volunteers. We call this measure 'people nights' to represent the number of people that spent the night at each residence throughout the months of June and July.

Septic and Well Information:

Age of the septic system and distance from shore, along with the age of the well, depth and distance from the septic field were found using county records (Benzie-Leelanau District Health Department, n.d.). Records were found to be incomplete in some instances and missing in others. In those cases, homeowners were contacted to obtain the required information. Information that the homeowners provided was added to our data set. When the homeowner could not provide information, it was left as unknown and not included in subsequent analyses.

qPCR Analyses:

qPCR analyses conducted at UA for *Enterococcus* and HF183 testing were performed using the Applied Biosystems QuantStudio 3. IDT DNA PrimeTime Gene Expression Master Mix was used for qPCR reactions. *Enterococcus* assay primers and probe sequences were published by US EPA (2012). As described in Method 1611 (U.S. Environmental Protection Agency, 2012a), qPCR master mix contained 1x master mix (IDT), 1uM forward and reverse primers, and 0.08uM fluorescein-labeled probe. HF183 assay primers and probe sequences were published by Haugland et al. (2010). qPCR master mix contained 1x master mix (IDT), 1uM forward and reverse primers, and 0.08uM fluorescein-labeled probe as described in Haugland et al. (2010). All primers and probes can be found in Table 3-2.

Samples were quantitated to a standard curve of synthesized plasmids containing the target sequence. Standard curve concentrations consisted of 50000, 5000, 500, 50, and 5 copies of the target sequence per reaction. *Enterococcus* genome equivalents (GE) were calculated based on 4 target sequence copies per genome. No-template controls with molecular grade water were

included in each instrument run. Each reaction contained 15uL of mastermix and 5uL of template.

Precipitation Data:

Precipitation data were retrieved from the NOAA National Weather Service Maple City 1E, Michigan station for the 24 hours prior to sampling. Precipitation data was unavailable for June 28, 2020, June 21-28, 2021, and July 12-13, 2021.

Statistical Analysis:

Software:

All analyses were run in R Studio (Version 2022.12.0+353) (R Core Team, 2023) unless otherwise noted.

2018:

All HF183 copy values were $\log_{10}$ transformed. Mean values for each lake were calculated, separating Big and Little Glen Lakes for analysis.

2019:

A Kruskal-Wallis test with a Dunn's multiple comparison test was run on pre- and post-rain samples at each site using GraphPad Prism 10.0.0 (Boston, Massachusetts).

2020-2022:

When duplicate samples were taken on the same day in 2021 and 2022, the sample with the highest measured value was used for analysis of *Enterococcus*. If either duplicate sample was positive for the HF183 marker, it was labeled 'positive'.

Septic use logs were categorized as low (<300 people nights) or high (≥300 people nights) as a predictor variable to assess the relationship between septic use and well water *Enterococcus* detection (yes/no) using logistic regression or *Enterococcus* levels ($\log_{10}$ *Enterococcus* (GE/100 mL) using the Wilcoxon rank sum test. Regression models were checked for the impacts of clustering by running multilevel models with a random intercept for well site to account for clustering. The logistic regression models were assessed for goodness of fit using the Pearson Chi-square and Hosmer-Lemeshow tests. Akaike's Information Criteria and Bayesian Information Criteria were used to assess the relative fit of single level versus multilevel models,

with lower levels indicating better fit. All tests were assessed for statistical significance using $p \leq 0.05$. These analyses were run on Stata/IC 15.1 (College Station, Texas).

To perform a Monte Carlo bootstrap resampling analysis on the well *Enterococcus* data, we subsampled our data set, 18 counts at a time, with replacement. We assumed a uniform distribution, i.e., the chance of selecting any given data point was uniform across the data set. We did this 10,000 times and, each time, calculated the proportion of positive samples. The average proportion of positive samples was found for all 10,000 drawn samples.

Due to the non-parametric nature of our data, a Spearman's rank correlation coefficient (rho) analysis was used to analyze the relationship between surface water sample *Enterococcus* levels with well age, septic age and use logs. Similarly, a Spearman's rho test was used to analyze the relationship between well water sample *Enterococcus* levels and well age, septic system age, use logs, well depth and well distance from septic. To compare HF183 positivity to these same variables, a Wilcoxon rank-sum test was used with groupings for HF183 positive and negative samples.

Due to the clustered natured of our precipitation values, we ran a Kruskal-Wallis test with a Dunn's multiple comparison test using well water and surface water *Enterococcus* data at each level of precipitation. We compared *Enterococcus* values at each level of precipitation to the *Enterococcus* values in the group that had no precipitation. This test was completed on GraphPad Prism 10.0.0 (Boston, Massachusetts).

Results

2018: Spatial assessment of human fecal pollution

Of the 211 water samples collected on three lakes in 2018, 60 (28.43%) yielded an HF183 signal. The mean HF183 ($\log_{10}$ copies/100 mL) level on Big Glen Lake was 0.73, on Little Glen Lake was 1.21, on Lime Lake was 0.67 and on Little Traverse Lake was 0.71. The highest value on Big Glen Lake was 3.7 $\log_{10}$ copies/100 mL, on Little Glen Lake was 4.2 $\log_{10}$ copies/100 mL, on Lime Lake was 3.3 $\log_{10}$ copies/100 mL and on Little Traverse Lake was 3.4 $\log_{10}$ copies/100 mL (Figure 3-1). These lakes are highly populated with, on average, a septic system every 37 metres on Glen Lake, every 58 metres on Lime Lake and every 44 metres on Little Traverse Lake.

2019: Pre- and post-rain enteric bacteria levels

To discern if inlets were a means by which the human fecal pollution detected in 2018 entered the lakes, 64 water samples were collected before and after a 2019 rain event and tested for *Enterococcus* and HF183 (Supplementary Figure 3-1). Of the pre-rain event samples, only one of the 64 samples (1.56%) exceeded 1,280 GE/100 mL. After the rain event, 13 of the 64 (20.31%) were above this threshold. When running a Kruskal-Wallis test to compare *Enterococcus* values based on location, the inlets showed significantly more *Enterococcus* after the rain event than before ($p = 0.037$) and the inlets after the rain event showed significantly more *Enterococcus* than the control sites after the rain event ($p < 0.001$) (Figure 3-2). When assessment of human fecal pollution using the HF183 marker was conducted on samples that were above the 1,280 GE/100 mL threshold, 5 of the 14 (35.71%) samples were positive. These five samples ranged from 2.71-3.07 $\log_{10}$ copies/100 mL. Of the samples positive for HF183, 2 were at control sites, 2 were at inlet sites and 1 was to the right of an inlet (Supplementary Table 3-1).

2020-2023: Groundwater and surface water testing

A total of 291 well water and 300 surface water samples were collected over the three-year study. There were slightly fewer well water samples collected due to homeowners forgetting to turn their water on or having the well inaccessible for a sampling date. In 2020, there were 32 sites sampled each month. In 2021, there were 39 sites sampled each month, and in 2022, there were 31 sites sampled each month. Of the 291 well water samples collected, 110 were positive for *Enterococcus*, and 16 were positive for HF183. Of the 300 surface water samples collected, 171 were positive for *Enterococcus*, and 20 positive for HF183. All samples that showed positivity for HF183 had only one positive technical replicate and thus could not be quantified. They are still considered positive for the purposes of this study due to the amplification of DNA in one instance. The HF183 assay in use has a limit of detection of 10 copies of DNA per sample (Green et al., 2014), while the limit of detection on the qPCR machine in use has been reported previously as 7.2 gene copies per reaction for the HF183 assay (Rudko, 2020). Thus, the samples with only one positive technical replicate are assumed to be near this limit of detection with very low copy numbers of HF183.

No statistically significant associations were observed between well water or surface water *Enterococcus* levels compared to septic system age, well age, well depth, well distance from the septic, or usage logs according to the Spearman's rho test (Table 3-3). Results from the Wilcoxon rank-sum tests showed no significant relationship between HF183 positivity and well age, septic system age, use logs or precipitation levels from the previous 24 hours in surface water samples (Table 3-4). Similarly, this same test showed no significant relationship between HF183 positivity and well age, use logs, precipitation from the previous 24 hours, well depth or well distance from septic. There was a significant relationship between septic system age grouped by HF183 positivity ($p = 0.04$) with a mean septic age in the negative HF183 group being 1994.83 and 2001.36 in the positive HF183 group (Table 3-5). No sites were positive for both the well and surface water HF183 marker on the same day.

Although a Spearman's rho analysis showed no association between use logs and *Enterococcus* levels in well or surface water samples, when analyzing usage log data compared to well water *Enterococcus* levels, we noticed a low number of *Enterococcus* negative samples in the high use log group (Figure 3-3). When we further compared the proportion of positive samples for these same sites based on groupings of use logs by 100 people nights, we saw an increase in the proportion of positive samples when use logs were above 300 (Figure 3-4). In this top level of usage, there was consistent use throughout the summer in all properties, often with peak usage days scattered throughout the summer. In the 300+ people nights group, we observed that the proportion of positive well samples was 78%. All samples above 400 people nights were positive for *Enterococcus*. Because our sample size in this 300+ people nights category was small (18 samples at 6 sites), we wanted to test if this proportion of positive well samples was just an abnormality due to the low sample number. To test this, we ran a Monte Carlo bootstrap model as described above. In this modelled data set, 5.38% of replicate drawn samples were over 78% positive. Thus, we are 94.62% confident that this trend is not due to chance. This same trend did not emerge when analyzing the surface water or HF183 data. When this data was further analyzed we found that the risk of *Enterococcus* detection was not significantly different between wells on properties with septic use more or less than 300 people nights (Table 3-5, $p=0.12$) according to a Wilcoxon rank sum test. Model fit parameters for the logistic regression model did not support a significant amount of clustering of results by well site. There was no

significant difference between the $\log_{10}$ *Enterococcus* levels from wells between the properties with septic use more or less than 300 people nights (Wilcoxon rank sum p=0.22, Table 3-5).

When comparing *Enterococcus* levels to precipitation from the previous 24 hours, the surface water *Enterococcus* levels showed a significant difference between precipitation groups according to a Kruskal-Wallis test (P<0.0001) while the well water *Enterococcus* levels showed no difference between precipitation groups (p=0.72) (Table 3-6). When this data was further analyzed with a Dunn's multiple comparison test, the surface water *Enterococcus* levels in three of the seven precipitation groups (0.25 cm, 0.48 cm, and 1.17 cm groups) of precipitation significantly differed from *Enterococcus* levels in the 0.0 cm precipitation group. There was no obvious trend in which of the precipitation levels were significant compared to others.

Discussion

2018

To address the question of enteric bacteria levels in three lakes in Northern Michigan we embarked on a multi-year, multi-lake, community-supported study on recreational lakes that each had over 75% of their shoreline developed. In 2018, we set out to investigate levels of qPCR detectable HF183 as a measure of human fecal contamination in the lake by collecting water samples every 153 metres around Glen Lake, Little Traverse Lake, and Lime Lake, each over the course of one to two days during the summer. This testing protocol models the "snapshot" approach, formulated by Grayson et al. (1997), in which numerous samples were taken over 4 days in a single catchment to perform a spatial analysis. We built on this concept by taking more samples (211) over two days on freshwater lakes instead of rivers. Like Grayson et al. (1997), we found the costs of such a large-scale sampling effort to be minimized by using local volunteers to accompany scientists, without which the cost of such a program may have been prohibitive. These water samples were tested via qPCR for the human enteric bacteria marker (HF183), and approximately 28% of the samples were shown to have detectable human waste.

Numerous studies have utilized the HF183 marker to assess human fecal pollution in a water source (Ahmed et al., 2014; Celico et al., 2004; Greaves et al., 2020; Jeanneau et al., 2012; Liang et al., 2012; Ragot & Villemur, 2022). However, the widespread spatial distribution of HF183 in

a freshwater lake is less frequently a study focus. Our "snapshot" data in 2018, proved informative to the prevalence of HF183 contamination on these lakes. These three lakes have an average of one septic system every 50 metres, along with a high percentage of developed shoreline (over 75%). These densely placed septic systems likely contribute to the human fecal contamination we observed. This corresponds to Verhougstraete et al. (2015) who found that more septic systems in a watershed correlated with increased human fecal contamination in Michigan rivers. Another study undertaken by Peed et al. (2011) suggests a correlation between septic system density and human MST markers in an Ohio stream system. When comparing septic system densities, the lakes included in this study fall into the upper half of density categories defined by Peed et al (2011). However, we use linear km of shoreline due to the abundance of human activity such as swimming and wading in these areas, whereas Peed et al. (2011) utilized total area. Because of this, we would categorize our three lakes as having high septic system density. Our study corroborates with the work of Verhougstraete et al. (2015) and Peed et al. (2011) by showing a high frequency of sites contaminated by human fecal material. Studies have also shown the persistence of HF183 in water and sediment to be lower than other FIB bacteria (Green et al., 2011; Jeanneau et al., 2012; Zimmer-Faust et al., 2017), thus reported HF183 values may underestimate fecal contamination in these lakes.

2019

In the subsequent year, we wanted to clarify the source of contamination and hypothesized that it may be entering the lake via inlets after rain events. Because Northern Michigan has primarily sandy soil, with a water table level very close to the surface, leaching from septic fields could contribute to the groundwater, which could concentrate to streams and lake inlets. This phenomenon is exacerbated by the sandy soil found in Northern Michigan. Extensive testing in urban environments has revealed increased FIB levels due to stormwater runoff (Galfi et al., 2016; Hathaway et al., 2010; Paule-Mercado et al., 2016; Reeves et al., 2004; Sidhu et al., 2012). However, studies have yet to be done on the impact of inlet specific runoff on rural freshwater lakes lacking large upstream municipalities, although Peed et al. (2011) showed that wet weather events correlated with human MST markers in a freshwater stream.

Thus, in 2019, we took water samples from inlets before and after a rain event to see if human bacteria was entering the water via inlets after rain events. We saw an increase in *Enterococcus*

bacteria after a rain event, and 38% of samples tested for human fecal contamination were positive. Analysis of these samples for HF183 markers revealed no difference in the number of in inlet sites positive for HF183 compared to other shoreline sites before or after rain events. Thus, the inlets are not the only contributing source of human fecal pollution. We concluded that although inlets did contribute significant amounts of *Enterococcus* to the lake after rain events, they did not contribute enough HF183 in our large recreational lakes to account for HF183 levels found in 2018 and decided to investigate alternative sources of human fecal contamination.

It should be noted that FIB are also shown to persist in the sediment at a higher rate than the water column (Ahmed et al., 2019; Eichmiller et al., 2013), thus a mixing of the sediment into the water on a windy day could increase FIB levels in the water. In our 2018 study there was no relationship between turbidity of water and HF183 signal, showing that these signals were not likely due to resuspension of DNA in the water column. Yet, some *Enterococcus* could have been resuspended during the rain event in 2019, increasing levels in our post-rain sampling.

2020-2022

Verhougstraete et al. (2015) have demonstrated that areas in Michigan with more septic systems display higher levels of human enteric bacteria markers in the water, while Peet et al. (2011) and Sowah et al. (2014) have shown a correlation between septic system density and fecal contamination. Verhougstraete et al. (2015) also provided evidence that may indicate a higher proportion of failing septic systems surrounding lakes than rivers. Thus, we dedicated the next three years of our study to testing well water and surface water for general and human fecal contamination via *Enterococcus* and HF183, respectively. Well water samples were taken to measure groundwater bacterial levels, while surface water samples were taken to see how many bacteria were directly in the lake. We hypothesized that improperly functioning septic systems would contribute fecal contamination to both the groundwater and surface water. Data was collected regarding the age, depth and location of the well and septic systems using county records, and a use log for each study participant was generated to quantify the number of people staying in each residence during the study period.

Data from these three years of study showed that another contributing factor to the fecal contamination in an area's groundwater may be septic system usage level. Our data showed that

those systems having greater total usage had a higher, though not statistically significant, level of positivity in their well water *Enterococcus* levels. This is logical, as even a properly functioning septic system may leach increased FIB DNA, detectable by qPCR, into the groundwater with increased usage. Conversely, a failing septic system which is rarely used may not contribute significantly to well water contamination. While there is currently no septic ordinance in the state of Michigan, this study may encourage lawmakers, either local or statewide, to consider septic inspection laws to preserve freshwater ecosystems. Further, lake associations may focus educational efforts on making sure riparians are not exceeding occupancy limits for their septic system.

Although there was no samples that were positive for HF183 in both well and surface waters simultaneously, the fact that the HF183 marker was detected in both well and surface water samples indicates that septic systems are likely the cause of human waste contamination in these lakes. Well water and surface water samples had 16 and 20 positive HF183 samples, respectively, which may indicate point source contamination of groundwater without surface water runoff impacting results. The 16 positive HF183 samples in well water represented 15 different residences, while the 20 positive HF183 samples represented 19 residences. There was one residence that had a positive sample for both well and surface water. Sunlight has been shown to increase decay of FIB signals in water, including HF183 (Greaves et al., 2020) and one study showed HF183 decayed faster than other FIB (*E. coli* and *Enterococcus*) (Dick et al., 2010), thus the DNA in well water may persist for longer periods of time due to a lack of direct exposure to sunlight. Some studies have shown cross-reactivity between the human-associated HF183 marker assay and dog fecal contamination (Ahmed et al., 2012; Kildare et al., 2007; McQuaig et al., 2009). Strong HF183 signals are less likely to be associated with dog fecal contamination since these signals are not as persistent in non-human feces as in human feces (Layton et al., 2013). Due to the high persistence of HF183 in our study and the high density of septic systems, it is likely that our results were due to human, not dog, fecal contamination.

Amount of precipitation from 24 hours prior to sampling was recorded during our study period and compared to *Enterococcus* levels grouped by amount of precipitation in both the well and surface water. Well water *Enterococcus* levels showed no association with precipitation; however, surface water *Enterococcus* levels did show a significant difference in *Enterococcus*

levels between different precipitation groups, although there was no clear trend as to which precipitation levels had a significant difference compared to the 0.0 cm precipitation group. Further analysis of this data may be needed since these data are not homoscedastic.

Because Michigan is the only state in the United States that does not have a statewide septic code (Michigan Legislature, n.d.), and has no laws regulating microbiological well water testing (Michigan Department of Health & Human Services, 2023), the issue of human-associated fecal pollution of lakes is a priority for many people living and owning properties in the state. There are an estimated 1.3 million septic systems in Michigan (Department of Environment, Great Lakes, and Energy, 2023), and many recreational lake properties rely on well water for drinking. The lakes in this study ranged from having 91% to 76% of their shoreline developed, thus there were many interested riparians who were eager to participate. These community partnerships proved essential in this study, as sampling and important usage data depended on reliable community participation. The evolutionary nature of this project encouraged continued participation over 5 years. Each year, we summarized data from the previous year and allowed it to drive the direction of next steps, continuously engaging partners in the study development. A long-term collaboration with lake associations and community partners has built trust and open communication, both of which were necessary for the completion of this project.

This project has already resulted in positive consequences on water quality for these lakes. One riparian had a positive sample for HF183 in 2018 in front of their property, followed by a positive *Enterococcus* sample in 2019. In 2020, their septic system was one of our volunteer sites and their well water came back positive for HF183, along with finding evidence of *Cladophora* spp. growing along their shoreline. These positive results stimulated conversations with the county, testing for coliform bacteria and, ultimately, the replacement of their septic system, as it was deemed ‘failing’. Also, our preliminary results contributed to Leelanau County passing a septic ordinance for point-of-sale septic testing in 2022 (Department of Environment, Great Lakes, and Energy, 2023).

This study showed that in three lakes in Northern Michigan, there was a high presence of human fecal contamination in surface waters, which could only partially be explained by water entering

the lakes via inlets after a rain event. Furthermore, *Enterococcus* presence in well water may be linked to high usage of septic systems.

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Table 3-1: Lakes in the study with their size (km^2), depth (m), shoreline kilometres, number of parcels, density of septic systems around each lake (septic systems per m) and percent of developed shoreline.

Lake Name	Area (km^2)	Max Depth (m)	Shoreline kilometres	Number of parcels	Average distance between septic systems (m)	Developed shoreline (%)
Glen Lake	25.56	39.62	27.36	732	37.49	90.85%
Little Traverse Lake	2.59	15.24	6.44	145	44.50	87.59%
Lime Lake	2.71	19.81	6.76	115	58.83	84.85%
South Lake Leelanau	21.73	18.90	42.16	818	51.51	75.98%
North Lake Leelanau	11.94	36.88	24.14	489	49.38	90.85%

Table 3-2: Primer and probe sequences for *Enterococcus* and HF183 assays in use during this study.

Assay	Forward primer (5'-3')	Reverse primer (5'-3')	TaqMan Probe (5'-3')	Reference
Enterococcus	GAGAAATTCCAAACGAACTTG	CAGTGCTCTACCTCCATCATT	TGGTTCTCTCCGAAATAGCTTTAGGGCTA	U.S. Environmental Protection Agency (2012a)
HF183	ATCATGAGTTCACATGTCCG	CGTAGGAGTTTGGACCGTGT	CTGAGAGGAAGGTCCCCCACATTGGA	Haugland et al. (2010)

Table 3-3: Results from Wilcoxon rank-sum test comparing variables based on HF183 results.

Water Source	Variable	HF 183	Mean	Median	Min	Max	WRS p-value
Surface	Well age (year)	Positive	1998.65	2000.00	1975	2017	
		Negative	1996.46	1997.00	1973	2017	0.41
	Septic age (year)	Positive	1996.88	1999.00	1978	2015	
		Negative	1995.07	1997.00	1968	2017	0.62
	Use Logs (people nights[1])	Positive	169.38	160.00	56	336	
		Negative	147.61	125.00	0	459	0.12
	Precipitation 24 hours before sampling (cm)	Positive	0.22	0.00	0	0.91	
		Negative	0.30	0.00	0	1.17	0.28
Well	Well age (year)	Positive	2002.75	2003.50	1980	2016	
		Negative	1996.28	1997.00	1973	2017	0.08
	Septic age (year)	Positive	2001.36	2003.50	1968	2016	
		Negative	1994.83	1997.00	1968	2016	0.04*
	Use Logs (people nights[1])	Positive	130.46	122.00	6	255	
		Negative	150.07	135.00	0	459	0.54
	Precipitation 24 hours before sampling (cm)	Positive	0.42	0.25	0	1.1684	
		Negative	0.29	0.00	0	1.1684	0.41
	Well depth (m)	Positive	16.69	16.15	12.80	22.86	
		Negative	16.85	15.24	9.14	59.74	0.41
	well distance from septic (m)	Positive	17.37	15.24	15.24	30.48	
		Negative	17.45	15.24	13.41	36.57	0.98

[1] People nights represent the number of people that spent the night at each residence throughout the months of June and July.
* Indicates significance at the 95% confidence level

Table 3-4: Spearman's rank correlation values and accompanying p-values comparing surface water samples and well water sample log transformed *Enterococcus* data with well age, septic system age, use log, well depth and well distance from septic.

Water Source	Variable	rho	p-value
Surface *Enterococcus* levels	Well age (year)	-0.04	0.52
	Septic age (year)	-0.09	0.13
	Use Logs (people nights[1])	0.01	0.83
Well *Enterococcus* levels	Well age (year)	0.00	0.96
	Septic age (year)	-0.01	0.88
	Use Logs (people nights[1])	0.06	0.29
	Well depth (m)	-0.02	0.73
	well distance from septic (m)	-0.05	0.46

[1] People nights represent the number of people that spent the night at each residence throughout the months of June and July.

Table 3-5. Comparison of *Enterococcus* spp. from well water to septic system use on linked properties on Lime Lake, Glen Lake, and Little Traverse Lake from 2020-2022.

Septic Use ≥ 300 people nights	*Enterococcus* detected # (%)*		*Enterococcus* $\log_{10}$ (GE/100 mL)			WRS Test p-value
	No	Yes	Mean	Median	Min/Max	
Yes	4 (4.35)	14 (10.07)	122.59	53.41	0/613.6	0.22
No	88 (95.65)	125 (89.93)	190.18	26.79	0/1194.8	
Total	92	129				

* Logistic regression: odds ratio 2.46 (p=0.12, 95% CI 0.78-7.74).
WRS – Wilcoxon rank sum test.

Table 3-6: Kruskal-Wallis with Dunn's multiple comparison test of *Enterococcus* levels grouped by amount of precipitation in the previous 24 hours.

		Dunn's Multiple Comparison Test			
Water Source	**Kruskal-Wallis results[1]**	**Precipitation (cm) grouping level based on previous 24 hours**	**Mean rank difference compared to 0.0 cm precipitation level**	**Z-value[2]**	**p-value**
Surface	34.06, p < 0.0001*	0.10	-6.13	0.37	>0.9999
		0.25	58.83	3.68	<0.001*
		0.48	64.31	2.87	0.03*
		0.66	19.65	1.16	>0.9999
		0.81	20.75	1.27	>0.9999
		0.91	-3.09	0.22	>0.9999
		1.17	79.29	3.73	<0.001*
Well	4.49, p = 0.72	0.10	-3.55	0.22	>0.9999
		0.25	3.90	0.25	>0.9999
		0.48	20.86	0.95	>0.9999
		0.66	-23.19	1.40	>0.9999
		0.81	-13.64	0.85	>0.9999
		0.91	5.52	0.39	>0.9999
		1.17	8.83	0.42	>0.9999

* Signifies significance at the 95% level
[1] Kruskal-Wallis value followed by p-value for significance
[2]Z-value is a comparison between the *Enterococcus* levels between that precipitation grouping level and the *Enterococcus* levels in the 0.0 cm precipitation group

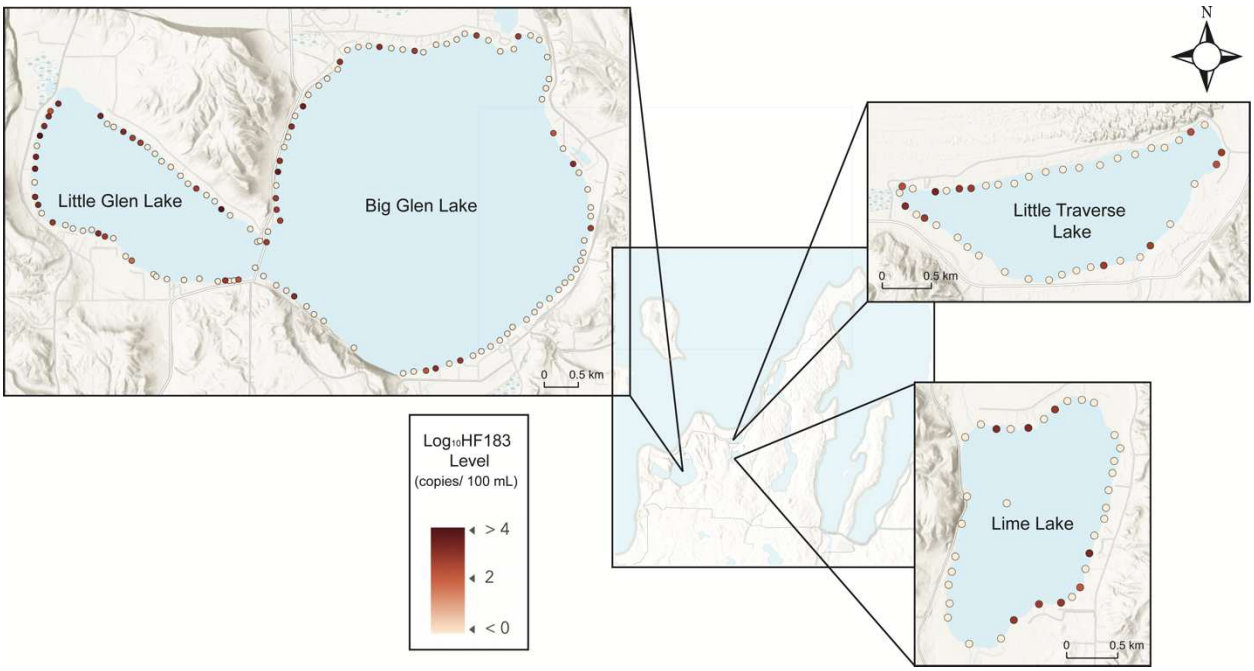
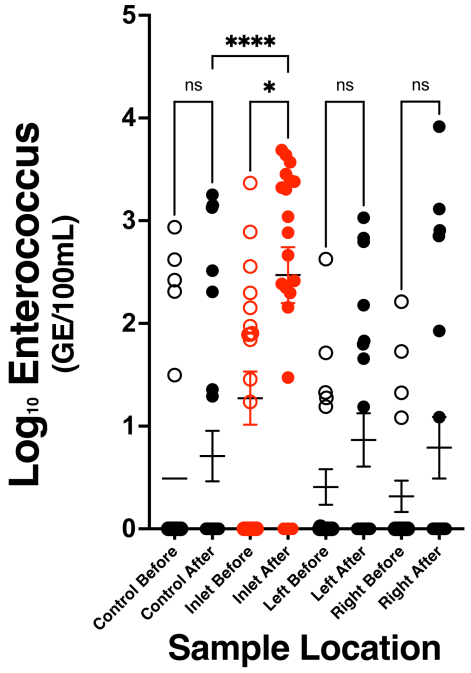
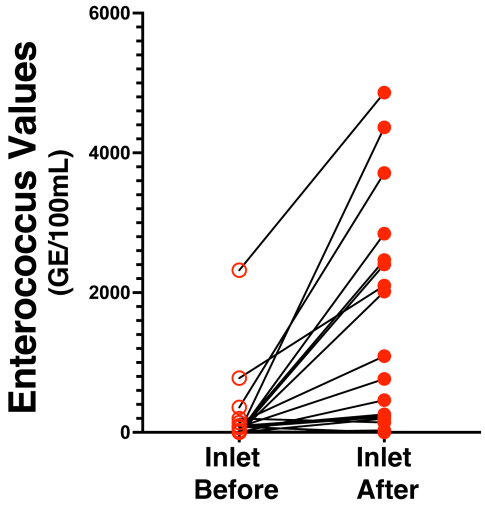


Figure 3-1: Spatial assessment of HF183 levels in Glen Lake, Little Traverse Lake, and Lime Lake taken on July 16 & 17, 2018, July 17, 2018, and July 9, 2018, respectively. HF183 values are reported as log_{10} copies/100 mL.



A.



B.

Figure 3-2A: Log$_{10}$ *Enterococcus* values (GE/100 mL) based on location, before (7/10/19) and after (7/16/19) the 3.5 cm rain event. Significant relationships according to the Kruskal-Wallis test are shown with *. Red color represents the inlet sites before and after the rain event. Left and right water samples were taken 10 m to the respective side of the inlet. Control sites were located around each lake, far enough away from the inlets to not be directly impacted by flowing water. B: *Enterococcus* values (GE/100 mL) paired by inlet location before (7/10/19) and after (7/16/19) the 3.5 cm rain event.

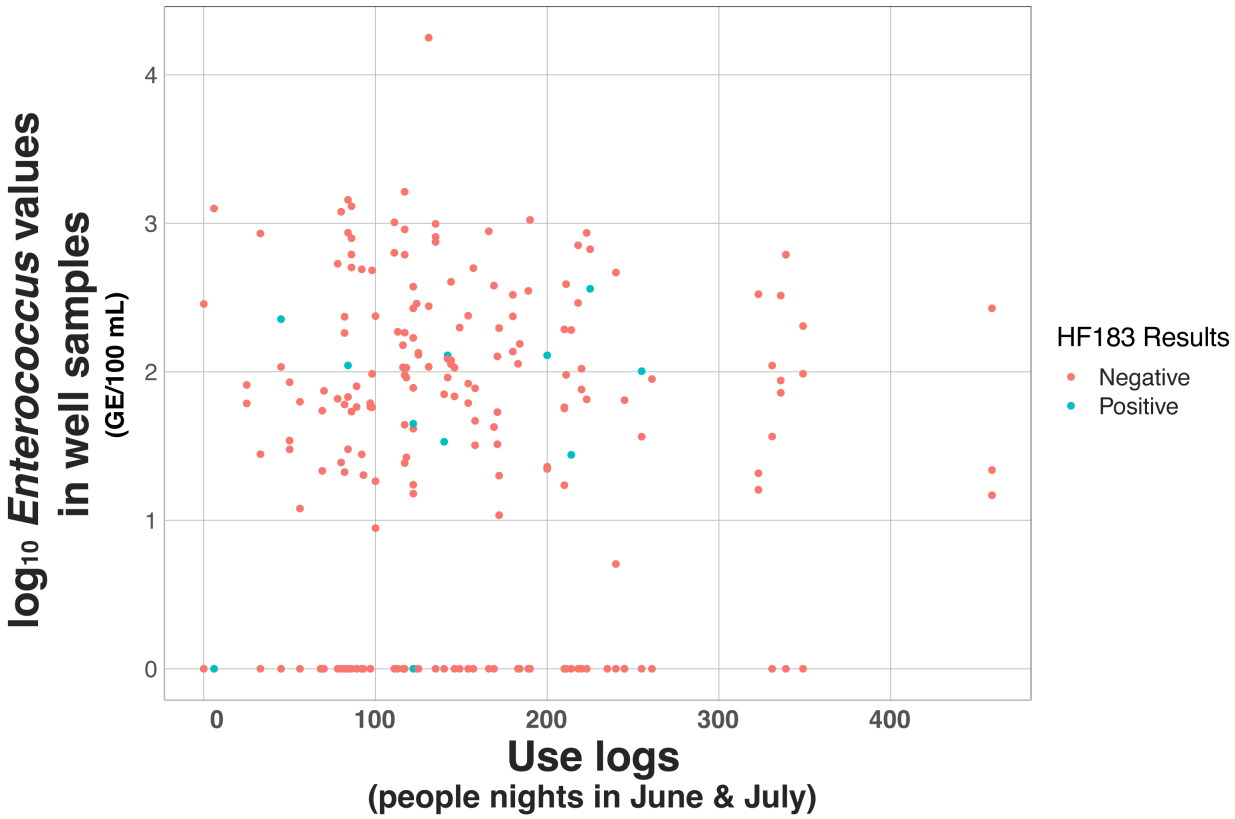


Figure 3-3: *Enterococcus* values (GE/100 mL) of well water samples collected from 2020-2022 compared to usage logs. People nights are equal to the sum of the number of individuals at a residence each night in June and July. People nights were calculated for each residence individually. HF183 results are shown as positive or negative based on color of dot.

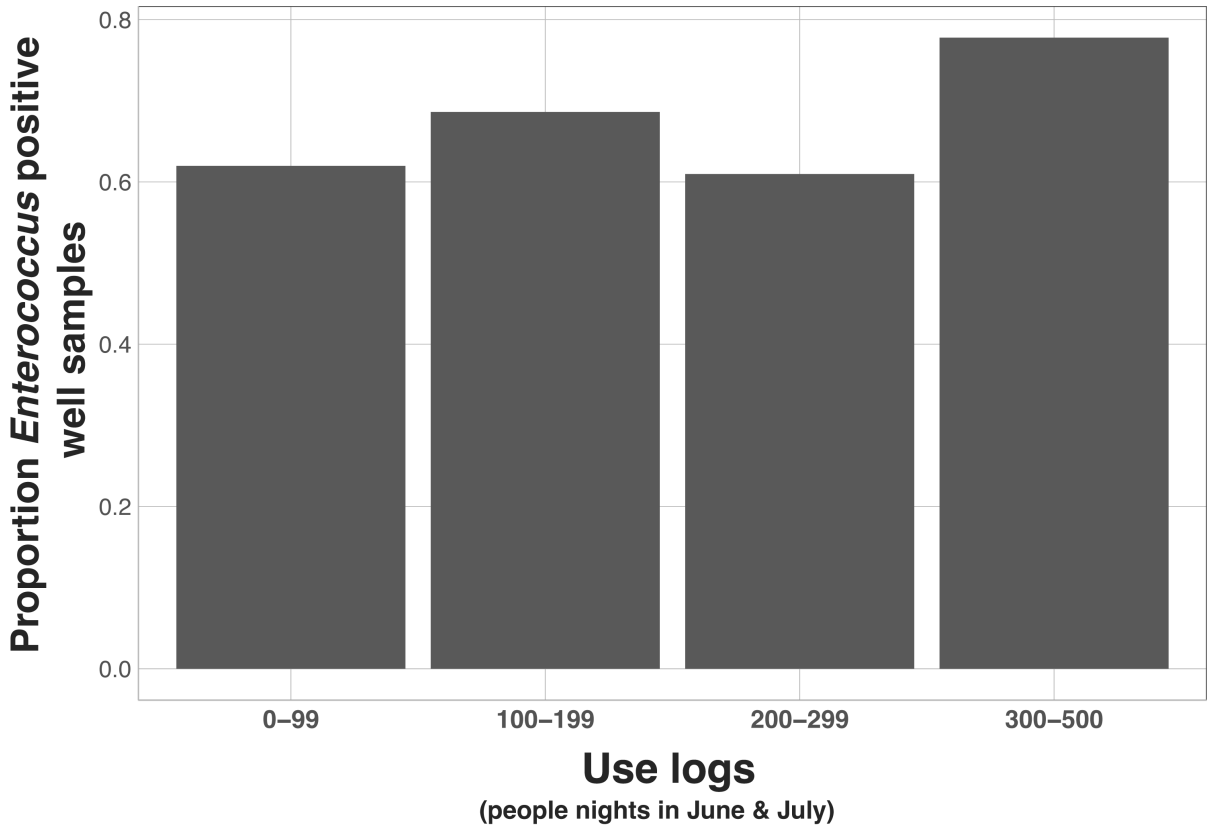


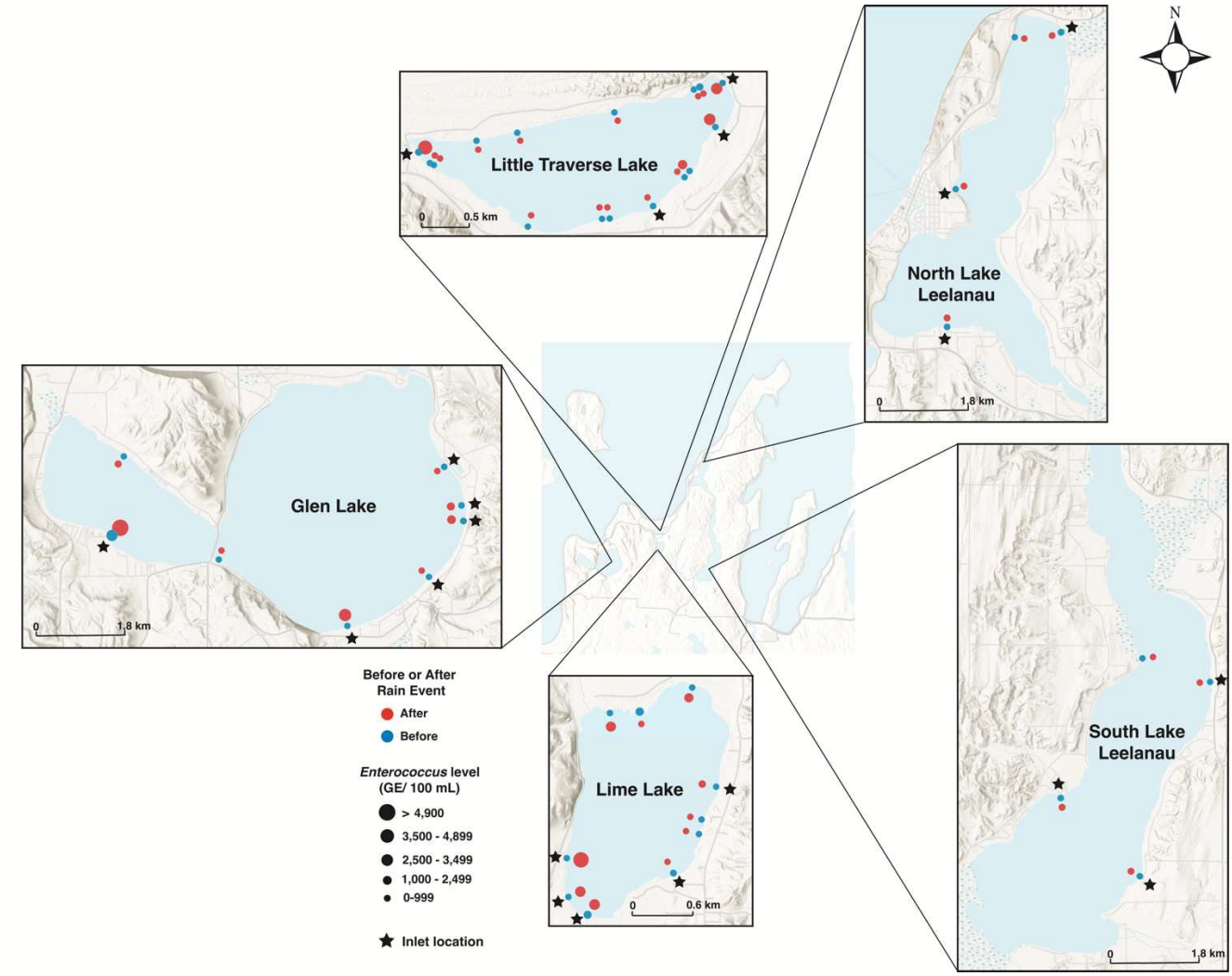
Figure 3-4: Proportion of well water samples that were positive compared to a range of people nights from usage logs. People nights were calculated based on how many individuals stayed in a residence each night for the months of June and July. People nights were calculated for each residence individually.

Supplementary Table 3-1: Pre- and post-rain event levels of *Enterococcus* (GE/100 mL). Data is grouped based on the site of the inlet. Left and right water samples were taken 10 m to the respective side of each inlet. Control sites were located around each lake, far enough away from the inlets to not be directly impacted by flowing water. Water samples that had levels of *Enterococcus* above 1,280 GE/100 mL (colored red) have HF183 source tracking results (log_{10} copies/100 mL). ND represents samples that were labelled 'not detected' for HF183 source tracking.

Lake	Site	Location	Pre-rain *Enterococcus* (GE/100mL)	Post-rain *Enterococcus* (GE/100mL)	HF183 (Log_{10} copies/ 100 mL)
Glen Lake	1	Inlet	79.23	0.00	
Glen Lake	1	Left	0.00	61.57	
Glen Lake	1	Right	0.00	708.58	
Glen Lake	2	Inlet	76.92	765.69	
Glen Lake	2	Left	0.00	0.00	
Glen Lake	2	Right	52.31	0.00	
Glen Lake	3	Inlet	140.77	1,091.65	
Glen Lake	3	Left	0.00	0.00	
Glen Lake	3	Right	0.00	0.00	
Glen Lake	4	Inlet	0.00	0.00	
Glen Lake	4	Left	0.00	1,068.87	
Glen Lake	4	Right	161.54	1,299.90	ND
Glen Lake	5	Inlet	0.00	2,842.85	ND
Glen Lake	5	Left	0.00	149.75	
Glen Lake	5	Right	0.00	803.80	
Glen Lake	6	Inlet	2,320.45	4,862.33	2.72 / 3.07
Glen Lake	6	Left	0.00	677.28	
Glen Lake	6	Right	0.00	0.00	
Glen Lake	Control A	Control A	0.00	0.00	
Glen Lake	Control B	Control B	0.00	325.75	
Lake Leelanau	1	Inlet	0.00	196.91	
Lake Leelanau	1	Left	0.00	44.44	
Lake Leelanau	1	Right	0.00	0.00	
Lake Leelanau	2	Inlet	196.92	142.84	

Lake Leelanau	2	Left	20.28	0.00	
Lake Leelanau	2	Right	0.00	0.00	
Lake Leelanau	3	Inlet	92.82	241.78	
Lake Leelanau	3	Left	0.00	0.00	
Lake Leelanau	3	Right	11.08	83.43	
Lake Leelanau	4	Inlet	76.76	258.95	
Lake Leelanau	4	Left	0.05	0.00	
Lake Leelanau	4	Right	0.00	0.00	
Lake Leelanau	5	Inlet	68.44	222.18	
Lake Leelanau	5	Left	0.00	14.45	
Lake Leelanau	5	Right	0.00	11.25	
Lake Leelanau	6	Inlet	0.00	28.72	
Lake Leelanau	6	Left	0.00	66.55	
Lake Leelanau	6	Right	0.00	0.00	
Lake Leelanau	Control A	Control	0.00	18.55	
Lake Leelanau	Control B	Control	0.00	21.78	
Lime Lake	1	Inlet	0.00	460.79	
Lime Lake	1	Left	0.00	0.00	
Lime Lake	1	Right	0.00	0.00	
Lime Lake	2	Inlet	776.74	2,101.37	2.79
Lime Lake	2	Left	0.00	0.00	
Lime Lake	2	Right	0.00	0.00	
Lime Lake	3	Inlet	0.00	2,014.31	ND
Lime Lake	3	Left	0.00	N/A	
Lime Lake	3	Right	0.00	0.00	
Lime Lake	4	Inlet	0.00	4,365.48	ND
Lime Lake	4	Left	0.00	0.00	
Lime Lake	4	Right	0.00	0.00	
Lime Lake	Control A	Control A	0.00	0.00	
Lime Lake	Control B	Control B	0.00	0.00	
Lime Lake	Control C	Control C	263.30	202.19	
Lime Lake	Control D	Control D	203.46	0.00	

Lime Lake	Control E	Control E	0.00	0.00	
Lime Lake	Control F	Control F	0.00	1,785.85	ND
Lime Lake	Control G	Control G	863.43	0.00	
Lime Lake	Control H	Control H	0.00	1,339.64	2.71
Little Traverse Lake	1	Inlet	27.54	2,465.98	ND
Little Traverse Lake	1	Left	50.62	0.00	
Little Traverse Lake	1	Right	20.15	0.00	
Little Traverse Lake	2	Inlet	16.24	0.00	
Little Traverse Lake	2	Left	14.48	0.00	
Little Traverse Lake	2	Right	0.00	0.00	
Little Traverse Lake	3	Inlet	0.00	2,402.90	ND
Little Traverse Lake	3	Left	17.84	623.56	
Little Traverse Lake	3	Right	0.00	8,244.61	3.02
Little Traverse Lake	4	Inlet	357.02	3,712.40	ND
Little Traverse Lake	4	Left	421.80	0.00	
Little Traverse Lake	4	Right	0.00	0.00	
Little Traverse Lake	Control A	Control	0.00	0.00	
Little Traverse Lake	Control B	Control	30.35	0.00	
Little Traverse Lake	Control C	Control	0.00	0.00	
Little Traverse Lake	Control D	Control	0.00	0.00	
Little Traverse Lake	Control E	Control	0.00	0.00	
Little Traverse Lake	Control F	Control	0.00	0.00	
Little Traverse Lake	Control G	Control	0.00	1,410.58	2.91
Little Traverse Lake	Control H	Control	416.14	0.00	
Little Traverse Lake	Control I	Control	0.00	0.00	
Little Traverse Lake	Control J	Control	0.00	0.00	
Little Traverse Lake	Control K	Control	0.00	0.00	
Little Traverse Lake	Control L	Control	0.00	0.00	



Supplementary Figure 3-1: All sites sampled in 2019 pre- and post-rain event. Blue circles represent *Enterococcus* values (GE/100 mL) before the rain event and red circles represent values after the rain event. Samples pre- and post-rain were taken at identical locations but are offset on the map to show change in *Enterococcus* values. Stars represent the inlet location. While sampling was completed to the left and right of the inlet, only the inlet and control data are presented here due to space limitations in the graph. Points without a star served as control sites.

Chapter 4: General Discussion & Conclusion

This thesis contributes to the scientific understanding of community-based monitoring (CBM) practices and their impact on freshwater systems, particularly in relation to stormwater and high usage septic systems. CBM has proven to be an integral part of this project, allowing us to collect many samples through our collaboration with trained community partners. Using these samples, we have demonstrated the influence of high-density septic systems and stormwater discharge on freshwater bacterial levels. Moreover, we have identified a positive trend between high use septic systems and stormwater outflows and enteric bacterial levels in the water.

In the work presented in this thesis, projects were designed in a CBM context. University of Alberta was the core laboratory that was supporting the work in Michigan. Beginning in 2016, Freshwater Solutions, LLC (FWS) became a community partner as part of work done by Rudko to expand the use of qPCR in satellite laboratories (Rudko, 2020). Since then, FWS has become the satellite laboratory that other community partners in Northern Michigan work with. Because of this unique relationship, we not only collected a wealth of water samples but have accumulated the infrastructure required to analyze data and communicate appropriately with partners (Figure 4-1).

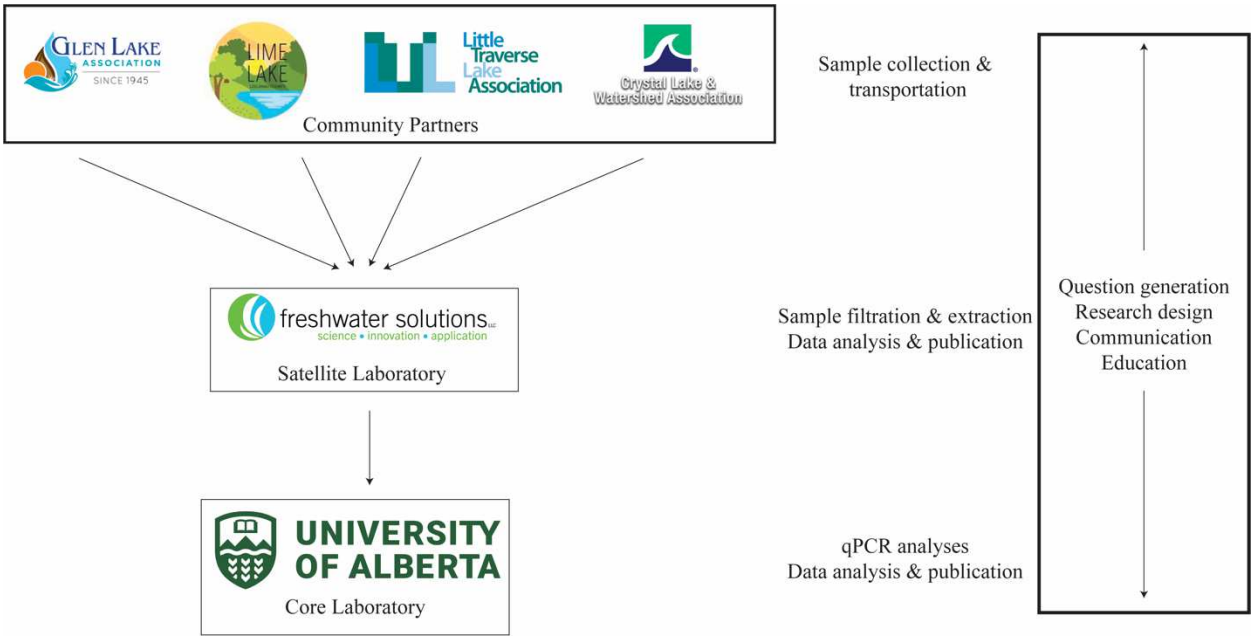


Figure 4-1: Demonstration of workflow with community partners.

Our main community partners were lake associations motivated by generating data to assess local water quality concerns, a common motivating factor (Carlson & Cohen, 2018). They also were clearly interested in preserving and improving their environment, helping the community, and contributing to scientific knowledge (Alender, 2016). Thus, all question generation and research design was completed with input from all members of the CBM project. Community members were mainly concerned about their water quality and the lack of a septic system ordinance in the state of Michigan. They wanted to learn more about their lake health and sources of fecal contamination.

Sample collection and transportation was the piece of each project that was conducted purely by community partners, which allowed for a much greater number of samples to be collected than would otherwise be possible. The satellite lab at FWS solely performed the DNA extractions for this program and all samples were shipped to UA to run qPCR tests for *Enterococcus* and MST markers. To ensure continued engagement in this research from all partners, it was essential that partners participated in question generation and research design so that the needs of the community partners were adequately represented and pursued. Communication and education needed to be a part of each level to ensure data was analyzed, communicated, and used appropriately. Lake associations had the main responsibility of educating their members on how to use/interpret results from these studies to preserve lake health, although FWS played a large role in this knowledge translation process as well. When required, UA partners presented technical information.

Although many CBM projects aim to connect partners with government agencies, there is often a disconnect between the objectives of each partner. Thus, long term planning and communication is recommended (Conrad & Daoust, 2008). In the CBM framework proposed by Conrad and Daoust (2008), they presented four steps to a successful CBM project, which we followed to completion. The first step is to "identify stakeholders", which occurred naturally in this project, due to long-term relationships with lake associations in the area of study. Stakeholders approached FWS and UA with environmental concerns and questions that they wished answered. This was a strength of the work in these projects because we could be confident that the results from our studies would be used by community members quickly.

The second step was to “identify skills and resources”, and this also naturally fell into place for our projects. Within this step, our ‘champion identification’ was inherent, as there were key members of lake associations with whom we had built relationships with and often a lake biologist from the lake to work with who would lead the initiative among the lake association. We spent time recruiting volunteers in this step to make sure we had adequate participation, along with committed partners.

The third step was to “create a communication plan”, and this step was especially important in the work presented here. With our stakeholders, it was imperative to be clear about who the work would be done in collaboration with and what organization would receive preliminary results, prior to publication. We needed to be clear about the timeline involved in this work, as our goals were not to monitor water for recreational use, but to address larger, more complex questions. We were communicating results with lake association members, which needed to be done in a way that was understandable to general members of the public. A key area of communication that we needed to focus on was relaying the message of what we could and could not conclude from the data we provided our partners. This education was critical as community members were quick to draw conclusions that our data could not support. Care needed to be taken when communicating complex data to the public, to avoid misinterpretation.

The fourth step was to “create a monitoring plan”, whereby we focused on who our volunteer water collectors would be, when sampling would take place and what the water sampling protocols were. Many of our water samplers were trained in lake biology and all were competent at following protocols and providing requested data. Thanks to extensive planning, we had all samples collected on the appropriate days and times. Preliminary results were shared with stakeholders during this project, with further clarification when studies were published. We found this framework to be realistic and a natural progression through our CBM studies.

As we worked to decentralize DNA-based water monitoring using the CBM framework, there were challenges at each step. The first step of water monitoring, collecting the samples, needed to be consistent between all samplers. This has been shown to be possible, as we have trained over 10 volunteers to collect water samples for qPCR analysis of swimmer’s itch-causing parasites (Rudko et al., 2022). While enteric bacteria targets require different sampling

techniques, the methodology and training of volunteers was comparatively simpler for the projects presented here.

The transport and timing of sample processing were other concerns since we had multiple individuals collecting samples for qPCR analysis. When we were sampling for enteric bacteria, all samples were kept on ice and, according to US EPA Method 1611, all filtering was started within six hours of water collection (U.S. Environmental Protection Agency, 2012a). If samples need to be transported, this could constrain the ability to sample in areas of interest or could limit the number of samples taken. We found that with proper water sampling techniques in place and sampling sites predetermined, our volunteers could collect samples and deliver them to the FWS laboratory within the given time frame.

We have found that CBM projects present a unique opportunity to environmental scientists. Geographic location is often prohibitive to timely water samples being collected and processed for DNA-based analyses. However, if multiple volunteers are working during the same time period, many samples can be taken simultaneously, eliminating temporal differences between samples. While extending CBM programming to technically challenging laboratory work may be difficult, simple laboratory tasks, such as water filtration, could be implemented by trained volunteers with the proper equipment. Using community volunteers in these ways broadens the scope of work and the type of questions that can be answered in a scientific study and may be very appealing for water monitoring efforts.

Governmental agencies could even expand their recreational water monitoring efforts by enlisting the help of community partners. In the most recent report by the Michigan Department of Environment, Great Lakes, and Energy, 448 beaches had water quality monitored at some point over the summer, while there were a total of over 1,300 identified public beaches in Michigan (Armstrong et al., 2019). Given these realities, assuming a government agency has the resources for comprehensive water monitoring programs is unrealistic. Thus, many decisions must be made to prioritize public swimming areas. With the help of volunteer water collectors, the number of monitored areas could greatly increase.

Using this CBM framework, we were able to gain valuable information about sources of enteric bacteria in a freshwater environment. We found that on three freshwater lakes in Leelanau

County, Michigan with a high density of septic systems (approximately 1 septic system every 50 meters), there was a high level of HF183, the human fecal contamination marker from *Bacteroides* spp. When samples were taken every 152 meters around these lakes, over 28% of samples contained HF183 signals. While we cannot rule out cross-reactivity with dog fecal material, the high persistence of HF183 leads us to conclude that human fecal material is getting into these lakes. This coincides with work done by Verhougstraete et al. (2015), in which they tested river systems in Michigan to compare general enteric and human fecal bacteria markers to amounts of septic systems in a given area. They found areas with more septic systems to have higher levels of human enteric bacteria markers. Similarly, Peed et al. (2011) found a significant positive correlation ($r = +0.75$, $p = 0.021$) between septic system density and HF183 values in Ohio stream systems after a wet weather event.

For four of the lakes in our study, inlets after a rain event were contributors of fecal contamination, as *Enterococcus* values were significantly higher after rain events, but it was concluded that these inlets would not likely be the cause of the widespread contamination of human fecal bacteria that we saw the previous year. On a lake not far from Leelanau County, Crystal Lake, city stormwater outflows correlated with higher *Enterococcus* and HF183 levels than other sampling sites. These two studies combined show that precipitation runoff and stormwater are major sources of fecal contamination in the studied freshwater systems, sometimes contributing human fecal material.

The contribution of fecal contamination due to stormwater that we saw in our studies correlates well with previous research. Past studies have focused mainly on urban environments, in which stormwater runoff has been shown to increase FIB in freshwater systems (Galfi et al., 2016; Hathaway et al., 2010; Paule-Mercado et al., 2016; Reeves et al., 2004; Sidhu et al., 2012). Little work has been done studying the effect of stormwater on rural environments (Stea et al., 2015). Thus, our work provides further evidence of the increase in FIB, specifically of human and dog origin, in a rural freshwater systems from stormwater outflows.

Septic systems were also studied to look for a correlation between a variety of criteria and enteric bacteria levels. While enteric bacteria levels did not show a statistically significant association with the age of septic system, well age, well distance from septic field or depth of well, septic

system use corresponded to a higher positivity rate of *Enterococcus* tested well water samples. Septic systems that are old and not functioning properly may not contribute fecal contamination to groundwater if they are not often used. Conversely, even well-functioning septic systems may fail if they are overused. This conclusion is important to lakes that frequently observe seasonal use, as they often sit vacant for long periods of time and then are overused during the summer months. These studies demonstrate the importance of properly maintaining a septic system that has high usage capacity.

The knowledge gained in this study has had numerous impacts on the communities in focus. The main reason that lake associations were interested in participating in these studies was to get information about inputs of contamination to their lakes and streams. This project uniquely fit the needs of both lake associations and researchers as we were able to provide lake associations with this information, while furthering our scientific understanding about fecal contributions to rural, freshwater lakes. In one instance, a riparian had their septic system entirely replaced after positive samples were found in three years of our study and further testing was done. Also, the county where most of our work took place, Leelanau County, passed a septic ordinance for point-of-sale septic testing during our study period, in part because of our preliminary results (Brandt Burgess, 2022). The tie between building scientific understanding about a freshwater system and helping communities work toward more ecologically friendly infrastructure is a unique and impactful benefit of this CBM program.

The future of microbial nucleic acid water testing

While qPCR is an adequate testing mechanism for the MST of a small number of species at a time, traditional qPCR methods are often limited in the quantity of targets tested for. Multiplexing samples is a commonly used technique to run qPCR on up to four different targets at the same time, using different colored probes. This reduces the time needed for analysis and the volume of the sample used. In freshwater testing, multiplexing has been used to detect mussel species at risk of extinction (Redden et al., 2023; Rodgers et al., 2020), cyanobacteria levels (Al-Tebrineh et al., 2012; Ngwa et al., 2014; Zuo et al., 2018), fish species (Hulley et al., 2019), and viruses found in wastewater (Fumian et al., 2010; Wolf et al., 2010).

Digital PCR (dPCR) is another quickly evolving technology that takes a small volume sample and disperses it to a high number of separate PCR reactions. The number of reactions that occur is quantified and theoretically correlates to the original number of template molecules present in the sample (Zhong et al., 2011). The sensitivity of a reaction is only limited by the number of droplets analyzed (Pekin et al., 2011), and it alleviates the need for quantitative standards. It reduces the chances of multiplexing interfering with chemical dynamics amongst the primers and probes of the multiple targets (Zhong et al., 2011) and inhibitions from water samples (Te et al., 2015). It has been established as a better option for testing water samples with a low volume of DNA in them (Doi et al., 2015; Mauvisseau et al., 2019a) thanks to its heightened sensitivity (Jiang et al., 2023). dPCR has already been applied to the water monitoring setting for *Enterococcus* and HF183 markers with excellent results in regards to precision, sensitivity and specificity compared to qPCR (Cao et al., 2015). Digital PCR has also been used to study many endangered species in freshwater that may not be found in high abundances (Mauvisseau et al., 2019b). Although the CBM framework can be utilized for tasks like water sample collection, filtration, and extraction in the context of dPCR testing, the actual dPCR testing itself presents challenges for community partners due to the high equipment costs and limited options for decentralized testing. These factors should be considered when determining the appropriate water testing methods to integrate into a CBM project.

Furthermore, using newer technology to test environmental DNA (eDNA) along with metabarcoding has shown great promise for the future of microbial and invasive species water testing (Garlapati et al., 2021; Nevers et al., 2018; Wu et al., 2022). In a study done in South Africa, authors measured temporally changing microbial community composition in a watering hole and hypothesize that changes were due to the variety of vertebrate hosts visiting throughout the sampling period (Farrell et al., 2019). With further microbial community assessment, concatenated communities could be used to clarify animal populations that are present, but not often seen in an ecosystem. Although current eDNA with metabarcoding strategies do not quantify organisms in a water sample, future work may be done to overcome this downfall. qPCR or dPCR are still the desired technology when targeting a specific list of species (Wood et al., 2019), however eDNA with metabarcoding would be advantageous to get a snapshot of the biodiversity in an area (Roy et al., 2018). eDNA with metabarcoding may show microbes in an

ecosystem that have not been tested for before and could change the target species that are used to signify fecal contamination in freshwater systems.

This thesis demonstrates the advantages of employing a CBM framework to address scientific research questions that offer benefits to both the local community and the scientific community at large. The methodology presented in this work and that which has been developed in the time of this project creates an exciting future for the field of water monitoring. The ever-expanding wealth of information accessible from water samples is poised to accelerate progress in addressing critical ecological queries, safeguarding endangered species, and effectively identifying and responding to invasive species.

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