Risks associated with exposure to *Cryptosporidium* and *Giardia* parasites in North Seattle recreational waters

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Abstract

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Cryptosporidium and *Giardia* are diarrhea-causing microscopic parasites transmitted through ingestion of fecally-contaminated water. Both parasites can have a significant impact on the health and financial burden of communities. In King County, WA, annual reported cases of *Cryptosporidium* during 2012-15 ranged from 18-25 and reached 43 cases in 2016. Reported *Giardia* cases during 2012-15 ranged from 170-219 and in 2016 reached 253 cases. Seattle, the most populated city in King County, uses protected water sources and heavily treats drinking water before distribution, resulting in negligible parasite prevalence, suggesting sources other than drinking water may cause infection. To date, no studies have assessed risk associated with the levels present in natural recreational waters of Seattle. My research adapts a Bag-Mediated Filtration System (BMFS) to use Envirochek® HV filters which are processed using Environmental Protection Agency (EPA) 1623.1 methodology to detect and enumerate oocysts and cysts. Quantitative PCR (qPCR) was also used to identify the source of oocysts and cysts. Twelve-liter water samples from 6 recreational beaches in N. Seattle were taken from July 2017

through November 2018 and filtered on-site using the BMFS. Filters were eluted, eluates re-concentrated, purified and analyzed by immunofluorescent microscopy. The oocysts were scraped from the slide and underwent DNA extraction and qPCR for species-typing following established methods. Preliminary results show 2/6 locations and 14% of all samples were positive for *Cryptosporidium* during summer months, with levels ranging from 1-2 oocysts. *Giardia* was detected in 4/6 locations and 31% of all samples during summer, ranging from 1-5 cysts. Both fell to 0 oocysts and cysts in fall. Samples collected before and after a New Years "Polar Bear Plunge" resulted in an increase from 0 to 4 *Cryptosporidium* oocysts and 0 to 27 *Giardia* cysts. For all locations, avian feces and human feces were present in at least one of the water samples taken. There was some discordance between the two human assays; however, the majority of samples were concordant. The location with the lowest number of samples positive for avian feces was Matthews Beach (57% of samples) and for human feces was Magnuson Beach (43% of samples). Multiple locations had 100% of samples positive for avian and human feces. Dog feces were present in at least one sample at all locations, but all locations had at least one sample where there was discordance between the two assays. Magnuson Beach was the location with the least number of positive samples (14%) while Carkeek Park had the highest number of positive samples (50%). A Quantitative Microbial Risk Assessment characterizing the risk of infection based on the source of contamination showed that at all locations where organisms were present there was at least an 8.08 in 10,000,000 risk of infection. The scenario with the highest risk estimate was for children swimming after the Polar Bear Plunge, where 95% of those who swam had a probability of ≤9.07 infection per 10,000 primary contact children recreators. None of the scenarios fell above the EPA thresholds of 8/1000 illnesses for freshwater and 19/1000 illness for marine water.

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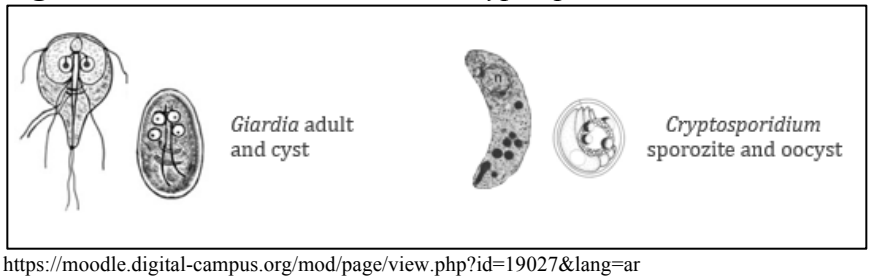
Chapter I: Introduction

1.1 *Cryptosporidium* spp. and *Giardia* spp.

According to the Centers for Disease Control and Prevention, diarrheal diseases are the second leading cause of death in children under the age of five globally. *Cryptosporidium* and *Giardia* are two widely distributed environmentally microscopic parasites that cause diarrhea and dehydration, infecting both humans and animals (Caccia et al., 2005; Hunter et al., 2005). In 2006 *Giardia* was stated to be the most frequently reported intestinal parasite worldwide, with a prevalence rate of 10–50% among children in developing countries (Savioli et al., 2006). The Global Enteric Multicenter Study (GEMS) - the largest study conducted on diarrheal disease to date - reported that *Cryptosporidium* was the second most common cause of diarrhea in infants, and was associated with an increased risk of death in the first 2 years of life (Murray et al., 2010). Given the large impact that diarrhea-causing illnesses have on the health of populations in developed and developing countries, it is important to study the prevalence and distribution of *Cryptosporidium* and *Giardia* (Kenzie et al., 1994).

The two microscopic parasites are spread by fecal-oral contact through the feces of animals and humans, and are highly associated with ingestion of fecally contaminated water (Leitch et al., 2011). Both parasites form protective thick-walled stages during their life cycle that are responsible for the spread of the organism, as the protective form can survive in the environment for long periods of time (Figure 1.1). For *Giardia*, these forms are called "cysts" and for *Cryptosporidium*, they are called "oocysts" (Ryan et al., 2014; CDC, 2015; CDC, 2017). The protective outer coating also make disinfection of the parasites relatively hard, with *Cryptosporidium* being extremely resistant to commonly used disinfection methods such as bleach (Fayer et al., 1997).

Figure 1.1 - Forms of *Giardia* and *Cryptosporidium*



https://moodle.digital-campus.org/mod/page/view.php?id=19027&lang=ar
https://www.slideshare.net/HamaNabaz/lab-10-cryptosporidiosis
https://www.jstor.org/stable/3281435

Children who live in resource-limited countries often experience multiple episodes of diarrheal illness during their first five years of life (Bern et al., 1994) The GEMS study identified that children with moderate-to-severe diarrhea grew significantly less in length in the two months following their episode compared to age- and gender-matched controls. For those who do not die, frequent diarrhea in the first 2 years of life is associated with lower brain development and a decrease in school performance (Bern et al., 2004; Fayer, 2004; Xiao, 2010; Murray et al., 2010; Eppig et al., 2010; Halliez et al., 2013; Abou-Shady et al., 2011; CDC, 2015). Since diarrhea is the second leading cause of death in children under the age of five globally, and *Giardia* and *Cryptosporidium* are major contributors to the burden of diarrheal disease, there is great significance in understanding the risks associated with exposure. Other highly susceptible populations to these parasites are the elderly, pregnant women, and those that are immuno-compromised; however, healthy adults can contract the diseases as well (Naumova et al., 2003).

Although the disease burden tends to be larger in developing countries, it is a problem in developed countries as well. For example, *Cryptosporidium* was the culprit behind the 1993 Milwaukee, USA outbreak where over 400,000 residents became ill and over 100 died, making it the largest waterborne outbreak in US history (Kenzie et al., 1994). *Giardia* can also be found within every region of the US, and in an analysis looking at 1,010 waterborne outbreaks in the United States from 1971 to 2000, it was reported that historically more waterborne disease outbreaks have been from *Giardia* than any other organism (CDC, 2015; Craun et al., 2003). In 2012, 44 states in the US voluntarily reported cases of gastrointestinal illness due to *Giardia* (Painter et al., 2015). In the late 1970s and early 1980s, *Giardia* was an important waterborne protozoan, and although *Giardia* continues to cause outbreaks, *Cryptosporidium* is one of the foremost causes of waterborne disease among humans in the United States (Pontius, 2003)

In the past, researchers thought that zoonotic transmission of *Cryptosporidium* from cattle was the main cause of cases of human infection (Xiao, 2010). However, new research suggests that most of the cases in developing countries are spread from human to human. The two most common subgroups of *Cryptosporidium* isolated from humans are: *C. parvum* (host-adapted to livestock) and *C. hominis* (host-adapted to humans) (Xiao et al., 2008). Ninety percent of *Cryptosporidium* infections in rural settings are attributable to *C. hominis* and *C. parvum,* and while *C. hominis* is spread between humans, *C. parvum* is the most often reported zoonotic species (spread between humans and animals). However, studies have shown that not

all transmission of *C. parvum* in humans is zoonotically spread (Xiao et al., 2008). Anthroponotic transmission of *C. parvum* in humans does occur. Subtype IIc has only been isolated in humans in resource-limited settings, but not in cattle, suggesting that there is the possibility that some types of *C. parvum* are human-adapted (Alves et al., 2006; Soba et al., 2008).

Unlike *Cryptosporidium*, *Giardia* species tend to be less specific to one host. The most common species of *Giardia*, *G. duodenalis,* has been found in a wide range of mammals, including humans (Hunter et al., 2005; Thompson et al., 2010). In fact, *G. duodenalis* is the only species of the six that causes human infection. The other five species were isolated from, birds (*G. ardeae* and *G. psittaci*), mice (*G. muris*), voles (*G. microti*) and amphibians (*G. agilis*). Due to this lack of specificity, it can make identifying the source of fecal contamination a challenge, as many mammals could be potentially infected with one species of *Giardia*.

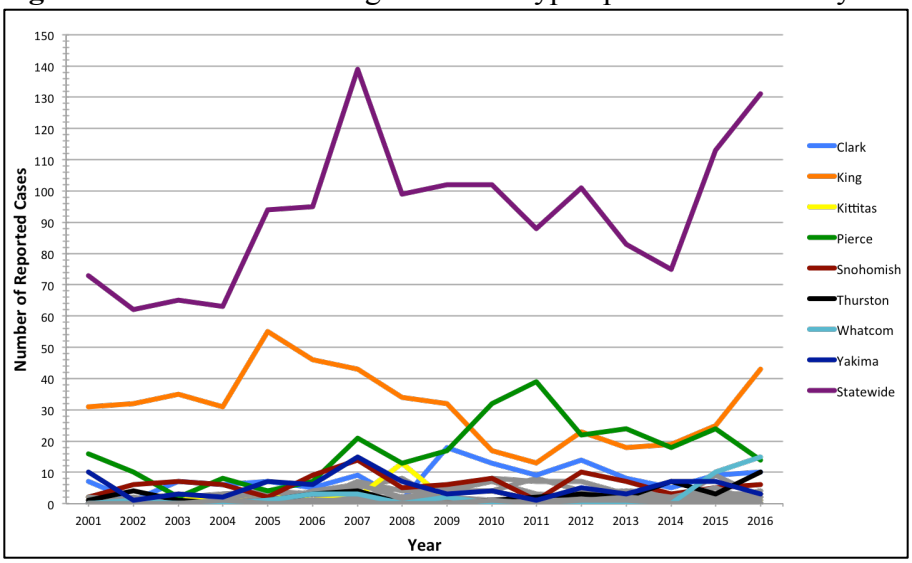
1.2 North Seattle Recreational Waters

While there are some limited data on the distribution of *Cryptosporidium* and *Giardia* subtypes and transmission geography, none are reported for the Seattle area. There are also no local estimates of risk for North Seattle recreational water sources. This does not mean that there are not cases of cryptosporidiosis and giardiasis occurring in the area. Cryptosporidiosis and giardiasis became reportable diseases in 2001 and since then the Washington State Department of Health has collected data on the number of reported cases by county per year (WSDOH, n.d.). In figures 1.2 and 1.3, only counties with 10 or more cases in at least one of the years between 2001-2016 are shown in the key. The counties with less than 10 cases are colored grey and excluded from the key. The highest purple line in both figures shows the overall number of cases per year with all counties combined, or "statewide", and the orange line represents King County, which for giardiasis has the highest number of cases of all counties for all years. King County has the highest number of reported cases of cryptosporidiosis for most years, but from 2010-2013, Pierce County had slightly higher case numbers.

An important consideration when looking at these graphs is also the fact that these are clinically reported cases. This means that someone with cryptosporidiosis or giardiasis went to the doctor/hospital with symptoms and was tested for one of the diseases. The figures do not include cases where patients were infected but asymptomatic, where illness was resolved at

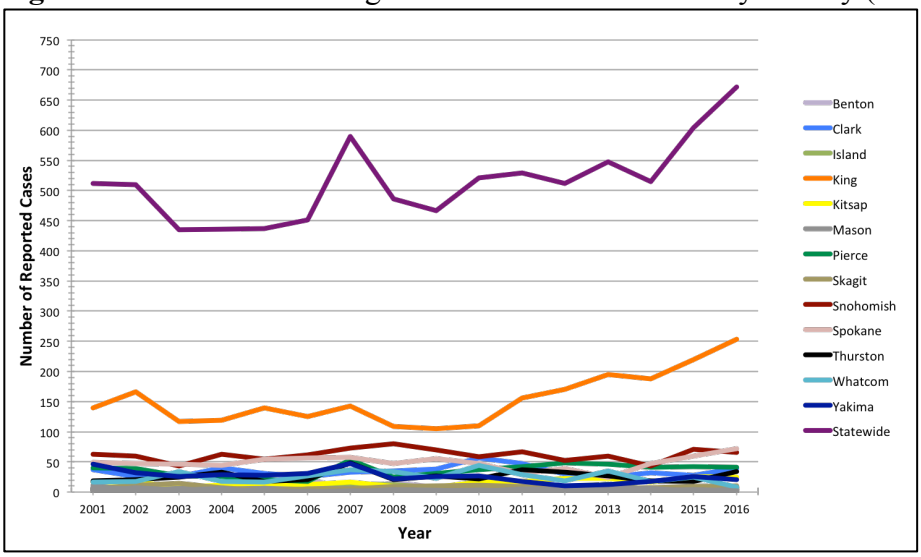
home, or where patients were treated for symptoms but were not tested for the organisms. This means that there are cases of infection that are not being reported to Washington State, and so it is expected that the actual number of cryptosporidiosis and giardiasis cases are greater than what is represented in the figures of reported cases below.

Figure 1.2 - Annual Washington State Cryptosporidiosis Cases by County (2001-2016)



Data obtained from the Washington State Department of Health - Annual Communicable Disease Report

Figure 1.3 - Annual Washington State Giardiasis Cases by County (2001-2016)



Data obtained from the Washington State Department of Health - Annual Communicable Disease Report

Given that King County generally has the highest number of cases of both diseases in all counties in Washington and because Seattle is the largest city in King County, the North Seattle area was chosen as the location from which beaches would be sampled. Another reason North

Seattle was chosen is due to its pristine drinking water. The city obtains its drinking water from the Tolt and Cedar River Watersheds, which get their water from protected mountain sources. The mountain water sources start off with a low or non-detectable concentration of *Cryptosporidium* and *Giardia* (SPU, 2016). The water is then heavily treated and undergoes ozonation and chlorination, and in the case of the Cedar River treatment plant also undergoes ultraviolet (UV) light treatment as an extra precaution against chlorine-resistant organisms such as *Cryptosporidium* (SPU, n.d.). The water that is distributed for drinking has negligible pathogen prevalence, and if the cases are not a result of drinking water exposure then recreational water may play a role as a source of infection.

As summer begins and more people use water recreationally, the risk of getting infected and spreading the parasites increase. Reports of cryptosporidiosis and giardiasis cases in the U.S. and Canada show that the number of infections increases during the summer months (Painter et al., 2015; Painter et al., 2015; Hlavsa et al., 2015). In 2011-2012, a 4.4-fold increase occurred in cryptosporidiosis symptom onset in late summer and a two-fold increase in giardiasis cases was reported in summer versus winter. These patterns are consistent with those previously observed in the United States and Canada (Painter et al., 2015). In order for implementation of infection prevention strategies, it is essential to understand the risk posed by current parasite levels in recreational water. This thesis seeks to identify and understand the risks associated with exposure to *Cryptosporidium* and *Giardia* in popular recreational water bodies frequented by humans in North Seattle, Washington, during the summer and fall months. Understanding how much and where these two pathogens are is critical for assessing an individuals exposure during their use of recreational water. As there are currently no local estimates of risk in the Seattle area, this study will contribute to the body of literature that assesses the risk posed by *Cryptosporidium* and *Giardia*, and is useful for future tracking of changes in parasite risk in North Seattle.

1.2.1 Environmental Protection Agency Method 1623.1

Subsequent to the Safe Drinking Water Act Amendment in 1996, the U.S. Environmental Protection Agency (EPA) is required to evaluate waterborne parasite risk to public health (U.S. EPA, 2012). Due to the amendment, the EPA developed a method that describes processes to collect, filter, elute, concentrate and purify a water sample, and then apply it to a slide to count the number of *Cryptosporidium* and *Giardia* using fluorescence microscopy. The first iteration,

validated in 1999, was a method specific for capture of only *Cryptosporidium* and was called "Method 1622". In 1998, a method for immunomagnetic separation (IMS) of *Giardia* was developed and so a revised method for capture of both *Cryptosporidium* and *Giardia* was validated in 1999 under the title "Method 1623" (U.S. EPA, 2005).

Since 1999, the method has been modified to incorporate changes and new technology that increase the recovery of the parasites. In 2012, the method was again revised and changed to "Method 1623.1", which included changes such as dispersant addition using sodium hexametaphosphate required for capsule filter elution, addition of bead pellet wash step during IMS procedure, and clarified text for adjustment of pellet volume and requirements for *Cryptosporidium* and *Giardia* characterizations. Many of the additions or changes in the methods were created to, "enhance program-wide data quality and consistency...updated method provides laboratories with the flexibility to select from options for various procedural components that do not require an alternate test procedure study " (U.S. EPA, 2012).

Although the general sample processing steps for the method must occur in a specific order and in a specified amount of time (Table 1.1), it does allow for some changes that the laboratory can make if the laboratory can demonstrate equivalent or superior performance when doing initial precision and recovery (IPR), and matrix spike (MS) tests prior to sampling (see sections 2.1.1 and 2.1.4). For example, even though the method was validated for filtration of bulk 10-L and 50-L water samples shipped from the field and filtered inside a laboratory, if a laboratory demonstrates equivalent recovery of a different volume of water using field-filtration, then that method can also be used.

Table 1.1 - Maximum Time Allowed Between Sample Processing Steps

Step	**Time between steps**
Collection/Filtration	4 days
Elution/Concentration/Purification	Same day
Application to slide	3 days
Staining slide	7 days
Examination of slide	

1.2.2 Bag-Mediated Filtration System (BMFS)

Developed by the Environmental and Occupational Health Microbiology Laboratory (EOHML) at the University of Washington, the Bag-Mediated Filtration System (BMFS) is a novel method used for concentrating large volumes of water in the field by gravity (Fagnant et al., 2018). Easy to use, cost-effective and practical in rural or urban settings, the BMFS does not require large volumes of water to be transported and kept on ice, but instead filters samples in field, resulting in a much easier method for filtration, as only the small filter needs to be kept on ice during transportation (Figure 1.4). The BMFS has been validated for use for environmental surveillance of Poliovirus, but the filter at the end of the system can be replaced depending on the target organism. In this study we replace with an Envirochek® HV Filter Capsule (Figure 2.3).

Figure 1.4 - Setup of Bag-Mediated Filtration System

Photo credit: Alex Kossik and Joanna Harrison

Data from a preliminary study completed by myself at the University of Washington during the summer of 2016 showed that the recovery rates of *Cryptosporidium* and *Giardia* using a combined BMFS-EPA 1623 method are consistent with recoveries of both pathogens seen using the published EPA 1623 10-liter grab method (Table 1.2). The BMFS was utilized for collection and filtration onto a 1.0um pore size Envirochek® HV filter, and all processes after filtration were done by EPA 1623 methods. Samples were taken from two locations: Magnuson Park and Squire's Landing. Two samples from each location were taken, with known amounts of cysts and oocysts spiked into one of the samples, and all samples were processed in the

laboratory. Recoveries were calculated by subtracting the spiked samples from the non-spiked, and total percent recovery was calculated by adding the recoveries together, dividing by sample size (2) and multiplying by 100. Based on these results, the BMFS method is useful for collection and filtration of water samples for both parasites in urban settings such as Seattle.

Table 1.2 - Recoveries for 2016 Study of *Cryptosporidium* and *Giardia* Capture using BMFS

	Magnuson Dog Park (n=2)	**Squire's Landing (n=2)**	**Total Percent Recovery**
Oocyst Recovery (% Recovery)	83%	70%	66%
Cyst Recovery (% Recovery)	79%	69%	74%

1.3 Species Typing and Microbial Source Tracking

Some *Cryptosporidium* species infect humans, some infect non-human mammals and others infect both humans and animals (Ryan et al., 2014). Due to this fact, by just assessing the number of oocysts in a water sample - as done in EPA method 1623.1 - scientists cannot tell if the species of oocyst they are looking at under the microscope is one that could infect a human. In order to assess the risk posed to humans from exposure to *Cryptosporidium*, it is important to know what species of *Cryptosporidium* they are exposed to. For example, if a human is exposed to *C. fragile*, which is specific to toads and has not been shown to cause human infection, than it is highly unlikely - but not impossible - that said human would become ill due to ingesting that oocyst. Using quantitative polymerase chain reaction (qPCR), the species of an organism can be determined.

Quantitative PCR is a molecular technique in which DNA molecules are amplified. The primers and probes that are used are specific to certain regions of the DNA template, resulting in amplification of only target genes, which are then detected by the qPCR machine which measures released fluorescence. There are two methods of qPCR used: non-specific fluorescence (most commonly SYBR® Green) and specific fluorescence (TaqMan®).

Non-specific fluorescence utilizes primers that are added into a master-mix, which also includes a fluorescent dye. When a DNA sample is added to the mix the dye binds to all nucleic acid on double-stranded DNA and releases fluorescence (Tajadini et al., 2014). The qPCR machine quantifies this fluorescence in real-time. As the machine goes through a denaturation

step, double-stranded DNA is separated. The machine then cools during an annealing step where the primers attach to the separated DNA strands. Once the primers are attached, the machine increases the temperature again in order for the DNA strands to elongate between where the primers are located, creating a new double-stranded DNA (Thermo Fischer Scientific, n.d.). This whole process is called a "cycle" and as the machine goes through multiple cycles, the targeted DNA replicates exponentially until there are no primers left in the reaction. As more DNA strands are created, there is increased fluorescence in the reaction as the dye binds to the new strands (Tajadini et al., 2014). Increased fluorescence correlates to increased DNA load and based on the intensity of the fluorescence the amount of product can be quantified. If there is DNA with a similar sequence from other organisms present in the sample, the primers will attach and replicate the sequence and give off fluorescence. To combat this non-specificity, after amplification each well undergoes a "melt curve analysis" in which the DNA is slowly heated and begins to dissociate. When 50% of the DNA has denatured, you get a "melt temperature" which is specific to an organism's DNA and is associated with the A-T and G-C amounts in the gene sequence (Ririe et al., 1997; Ahrberg et al., 2015). By comparing the melt temperature of your positive control with your sample, one can determine if the sample contains the target organism.

A much more specific but slightly more costly qPCR method is the specific fluorescence method, using TaqMan® probes. In this method, primers are used to attach to specific regions of the DNA in order to replicate the strands, but a probe is also used to attach to a sequence between the two primers on the DNA strand (Thermo Fischer Scientific, n.d.). There is no dye in the master-mix to attach to nucleic acid, so when DNA is added to the primer/probe/master-mix no fluorescence is detected. Instead, the fluorescent dye is attached to one end of the TaqMan® probe and a dye quencher is attached to the other side of the probe. The quencher does not allow for fluorescence to be released, even when the probe attaches to single-stranded DNA. Only when the probe is cleaved during the polymerase elongation step is fluorescence emitted, since the dye and quencher are no longer intact (Tajadini et al., 2014). The greater amount of organism in a sample, the more probes bind and release, and the greater the fluorescence. Since an additional sequence is needed for amplification and because the dye is not attached to all nucleic acid, the specific detection method is just that, more specific. A melt curve is also not necessary due to the increased specificity.

Both non-specific and specific fluorescence qPCR methods can be used in Simplex reactions, but also as Multiplex reactions where primer and probe sets of different organisms or species are included in the same mixture with the sample and analyzed jointly (Ahrberg et al., 2015). The Water Research Foundation (WRF) has produced a report on downstream genotyping of *Cryptosporidium* using qPCR after the application of oocysts to the slide from EPA method 1623. The report is titled "WRF 4099: *Cryptosporidium* Genotyping Method for Regulatory Microscope Slides". The WRF 4099 method utilizes non-specific detection of samples using two primer sets in a multiplex assay, and differentiates between human-pathogenic *Cryptosporidium* species and animal-associated species. The human-pathogenic primer set is specific to *Cryptosporidium* species that cause the majority of infection in humans: *C. hominis*, *C. parvum,* and *C. meleagridis* (McLauchlin et al. 2000; Leoni et al., 2006). Infection from species other than the previously mentioned is rare (Xiao et al., 2008). The other primer set used in the assay detects all species of *Cryptosporidium*. When primer sets are combined, the assay distinguishes human-pathogenic *Cryptosporidium* species from animal-associated species, but does not specify which human-associated are in the sample.

In line with species typing of *Cryptosporidium*, qPCR can also be used for Fecal Source Tracking, which informs researchers about potential sources of microbial water contamination. According to the USGS, fecal source tracking can determine the source of fecal contamination using different types of analytical protocols that, "use host-associated characteristics of various microorganisms present in feces; that is, physiological differences in hosts are expected to select for specific characteristics...in associated enteric microorganisms." (Stoeckel, 2005). One of the most common methods of fecal source tracking uses Bacteroides as a fecal source tracker (Valerie et al., 2014; Gómez‐Doñate et al., 2016; Kreader, 1995). Bacteroides is a ubiquitous bacterium that is a host-associated identifier, which is specific to the gut of different animals (Gómez‐Doñate et al., 2016). This means that the source of gut where the Bacteroides came from can be determined, versus if you are only looking for an organism like *E. coli* that is a general fecal indicator. *E. coli* will show that there is fecal contamination in the water, but it is not specific to where the feces might have originated (Ravaliya et al., 2014; U.S. EPA, 2005). Using Bacteroides, researchers can better assess what types of animals are defecating in the water, and therefore can better describe where a pathogenic organism might be originating from - and who can in turn get sick from that pathogen.

One does need to consider that the source of the pathogen can be different than the source of the microbial indicator - i.e. Bacteroides (Kabiri et al., 2016; Wexler, 2007). This means that while we can use Bacteroides to identify what sources of feces are present in the water, the *Cryptosporidium* and *Giardia* may not actually be coming from those sources. Another consideration is that the primer sets used for the detection of different Bacteroides species are not specific to only one animal. Some animals share similar microbiomes and result in cross-amplification in qPCR (McLain et al., 2009; Ryu et al, 2012). Studies have also shown that gut microbiome of humans and companion pets have similar microbes, and this needs to be taken into consideration when making claims about the source of a pathogen (Caugant et al., 1984; Song et al., 2013). Overall, however, fecal source tracking is very useful in suggesting what kinds of pathogen fecal sources may be present in water and can strengthen the conclusions made in studies assessing human risk based on exposure to human fecal pathogens.

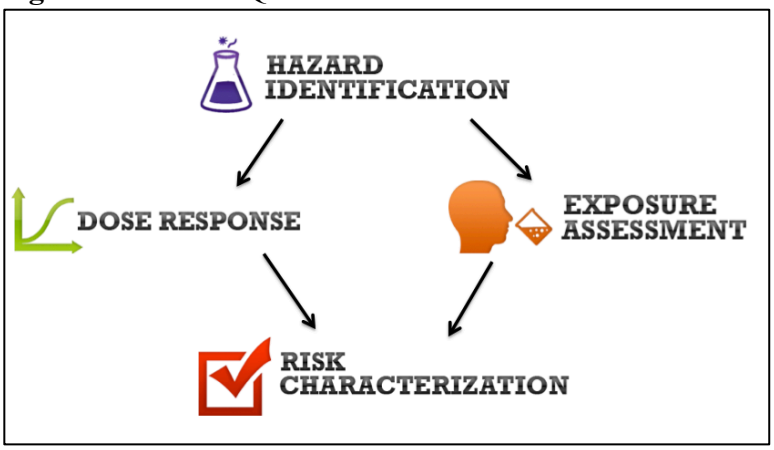
1.4 Quantitative Microbial Risk Assessment (QMRA)

Knowing the prevalence and distribution of pathogens in water sources is information that can then be used to understand how an organism may impact those exposed. A Quantitative Microbial Risk Assessment (QMRA) gives a numerical risk estimate of what a person's risk is based on multiple exposure parameters (Haas et al., 2014). For this reason, QMRA is useful to policy makers because certain parameters can be changed to see what risk to an individual or population is or what it could be, and based on those numbers set policies to reduce a person's or population's risk of infection, death, or other outcome (Stenström et al., 2007). Using QMRA to prioritize hazards will result in an objective, quantitative prioritization of the hazards, provided there is sufficient quantitative information available. For this reason, a QMRA will also help to identify what information is lacking in the literature.

A QMRA consists of primarily four parts (Figure 1.5), with a pre-step of formulating a question that you want answered (Haas et al., 2014). The first step of the framework is called Hazard Identification in which the organism(s) that are a problem are identified and specific characteristics of the microbial agent are classified - such as transmission routes, fatality ratios, previous outbreaks, strains that cause illness, etc. (QMRAwiki, 2015). Next, a dose-response or exposure assessment can be done - these can be done in parallel. A dose-response analysis is a quantitative relationship between the amount of organism present and the resulting adverse

affect. The QMRA Wikipedia, a community portal for current QMRA knowledge run by Michigan State University, has already reported many dose-response curves for different organisms. One dose-response equation has been reported for human infection from *Giardia*, and five dose-response equations for human infection from different *Cryptosporidium* species.

Figure 1.5 - Basic QMRA Framework



http://qmrawiki.canr.msu.edu/index.php/Quantitative_Microbial_Risk_Assessment

In addition to the dose-response analysis, an exposure assessment is needed. According to the QMRAwiki, an exposure assessment, "identifies affected populations, determines the exposure pathways and environmental fate and transport, calculates the amount, frequency, length of time of exposure, and estimates dose or distribution of doses for an exposure." In its essence, the exposure assessment takes in all the parameters that result in a dose, which is then input into the dose-response equation, resulting in a risk characterization. The risk characterization is the outcome of all parameters together, and will produce an estimate of how much risk, including variability and uncertainty, the hazard will create to a defined population. This quantifiable number can be used to set guidelines for reducing exposure to a hazard, incorporate strategies to reducing the hazard in the environment, and overall increase the health of those initially identified in your research question (Stenström et al., 2007).

Similar studies done on the risk of infection from *Cryptosporidium* and *Giardia* at recreational beaches have shown that there is measureable risk from *Giardia* and *Cryptosporidium* if their presence can be quantified (Adell et al., 2016; Mcbride et al., 2013; Ehsan et al., 2015). Ashbolt et al., 1997 showed that for those ingesting 100mL of water from select Australian beaches, there was a 5.4-19/100,000,000 risk of infection from

Cryptosporidium and a 1.45-5.35/10,000 risk of *Giardia* infection. A study done at beaches in Norway showed a greater than 0 risk for both adults and children in terms of getting *Cryptosporidium* and *Giardia* infection, while *Cryptosporidium* had a greater risk of infection (<1.8/10,000) than Giardia (<1.6/1000). For both organisms, the risk was greater for children then for adults (Eregno et al., 2016). A study done in a similar water temperature, the North Sea in the Netherlands, estimated that adult swimmers spending an average time of 45 minutes in the water had an average *Cryptosporidium* infection risk of 8.1/10,000 – 3.7/1000 and an average *Giardia* infection risk of 3.6/100,000-7.6/10,000 (Schets et al., 2011). Children in this same study had an increased risk of infection from both organisms, with an average *Cryptosporidium* infection risk of 9.0/10,000 – 4.9/1000 and an average *Giardia* infection risk of 7.6/100,000-1.0/1000. At this time, no studies have quantified the risk of infection from swimming at recreational waters in Seattle, Washington.

1.5 Specific Aims

Aim 1: To determine the prevalence and distribution of *Cryptosporidium* and *Giardia* in popular recreational water bodies frequented by humans and animals in North Seattle.

Based on preliminary data from 2016, my hypothesis is that *Cryptosporidium* and *Giardia* are present in North Seattle recreational waters (mean > 0). I believe *Giardia* prevalence is greater than *Cryptosporidium* prevalence and the concentration of both parasites will decrease after the rainy season starts, when compared to the concentration of parasites during the summer months.

Aim 2: To understand the source of the parasites (i.e. source tracking), by characterizing *Cryptosporidium* at the species-level and including Bacteroides as source tracking markers.

My hypothesis is that the majority of Cryptosporidium species are human-associated. I believe that the source-tracking marker Bacteroides will correlate with species of *Cryptosporidium* present in the sample.

Aim 3: To assess the risk associated with the identified levels of *Cryptosporidium* and *Giardia.*

Due to the low levels of *Cryptosporidium* that are expected, I hypothesize that the probability of harm (i.e. risk) from *Cryptosporidium* is at the benchmark risk of 8 cases per 1,000 persons per event in freshwater and risk of 19 cases per 1,000 persons per event in marine water. The risk of infection of *Giardia* will exceed the benchmark risk per event because the concentration of cysts in the water will be higher than the concentration of *Cryptosporidium* oocysts.

Chapter II: Methods

2.1 North Seattle Water Sampling

2.1.1 Initial Precision and Recovery & Ongoing Precision and Recovery

Following EPA Method 1623.1, before water samples could be tested for *Cryptosporidium* and *Giardia*, initial precision and recovery (IPR) testing was conducted to ensure that oocyst and cyst recovery would be in the EPA's acceptable range. For *Giardia* cysts the acceptable IPR percent recovery is from 27-100% and for *Cryptosporidium* oocysts it is 38-100% (U.S. EPA, 2012). The IPR is a quality assurance procedure that ensures that personnel have control of the analytical system and that laboratory equipment/reagents are working properly. Four consecutive IPR samples must meet acceptance criteria before being able to sample sites. If there is a failure to meet the acceptance criteria, then it indicates laboratory problems that must be addressed before processing field samples. Along with IPR, three ongoing precision and recovery (OPR) tests were performed throughout the sampling period. OPR tests are conducted the same as the IPR tests and are also used to assure that the laboratory is in an acceptable recovery range. For *Giardia* cysts the acceptable OPR percent recovery is from 22-100% and for *Cryptosporidium* oocysts it is 33-100% (U.S. EPA, 2012). For both IPR and OPR, precision is calculated as maximum relative standard deviation. *Cryptosporidium* acceptable IPR/OPR precision is 37% and *Giardia* IPR/OPR acceptable precision is 39%.

All procedures for IPR and OPR were completed inside the laboratory following EPA Method 1623.1. A sterile carboy was filled with 12-liters of cold tap water and left at room temperature while the spiking solution was prepared. The spiking solution used was BTF EasySeed™ (BTF, North Ryde, Australia). Every tube of EasySeed™ contains approximately 100 gamma-irradiated *Cryptosporidium parvum* oocysts and 100 gamma-irradiated *Giardia lamblia* cysts. One tube of EasySeed™ was used per IPR test and tubes were prepared and inoculated into the 12-liter carboy according to manufacturer's instructions (Appendix B). The carboy was shaken for 2 minutes to mix the cysts and oocysts, then poured into a BMFS bag to be filtered through an Envirochek® HV Sampling Capsule by gravity. This 1.0um pore size filter is specific for capture of *Cryptosporidium* and *Giardia* (Figure 2.3). When filtration was done, the plastic caps at the end of the capsule were replaced and the filter was stored inside a 4˚C

fridge until it was eluted. Processing after this point was done via *Section 2.2 Analysis of Water Samples,* below**.** Percent recovery of IPR and OPR tests were calculated as follows:

$$\textbf{\textit{Cryptosporidium Recovery}}\ (\%) = \left(\frac{Cryptosporidium\ oocysts\ detected}{Number\ of\ Cryptosporidium\ in\ EasySeed^{TM}}\right) 100$$

$$\textbf{\textit{Giardia Recovery}}\ (\%) = \left(\frac{Giardia\ cysts\ detected}{Number\ of\ Giardia\ in\ EasySeed^{TM}}\right) 100$$

2.1.2 Matrix Spikes

Another quality assurance procedure conducted was matrix spiking. A matrix spike is a separate sample aliquot from the same source as your sample, and is used to determine the effect that everything in the water (the matrix) has on oocyst and cyst recovery. A matrix spike accompanied the first samples taken from each location. After the sample water was collected for filtration using the BMFS in the field, a sterilized carboy was filled with 12-liters of water from the same locations that the sample was taken. Within 3 hours the carboy was returned to the laboratory and spiked with EasySeed™ on the same day, in the same manner as the IPR/OPR tests and filtered through an Envirochek® HV Sampling Capsule with the BMFS in the laboratory. If the carboy could not be spiked immediately, it was placed in a 4˚C fridge until it was spiked less than 22 hours after being put in the fridge. Processing after this point was done via *Section 2.2 Analysis of Water Samples,* below. Acceptable matrix spike mean recovery percent for *Giardia* cysts is from 8-100% and for *Cryptosporidium* oocysts is 32-100%. Due to time constraints, matrix spikes duplicates were not performed. Mean percent recoveries were calculated as follows:

$$\textbf{\textit{Cryptosporidium Recovery}}\ (\%) = \left(\frac{Number\ of\ oocysts\ counted\ in\ spiked\ sample - number\ of\ oocysts\ counted\ in\ unspiked\ sample}{Number\ of\ oocysts\ in\ EasySeed}\right) 100$$

$$\textbf{\textit{Giardia Recovery}}\ (\%) = \left(\frac{Number\ of\ cysts\ counted\ in\ spiked\ sample - number\ of\ cysts\ counted\ in\ unspiked\ sample}{Number\ of\ cysts\ in\ EasySeed}\right) 100$$

2.1.3 Recreational Beach Sites

In order to assess the health risks that *Cryptosporidium* and *Giardia* pose to those who swim in surface water at recreational sites in North Seattle, six popular recreational beaches were

chosen in the North Seattle area based on expected moderate to high levels of activity during summer and fall months. Half of the sites were fresh water - two sites on Lake Washington - and the other half marine water from the Puget Sound (Table 2.1). Surface water samples were obtained from July through November, with an additional sample taken on January 1st from Matthews Beach (see Chapter 3). All beaches were sampled at least 5 times over the course of the sampling period, with Carkeek having an additional 6th sample, and Matthews Beach and Magnuson Park having two additional samples for a total of 7 samples. Due to low matrix spike recoveries and time consideration, Greenlake, Golden Gardens and Richmond Beach were not further sampled after November 1st.

Figure 2.1 - Locations of Beaches Sampled in North Seattle

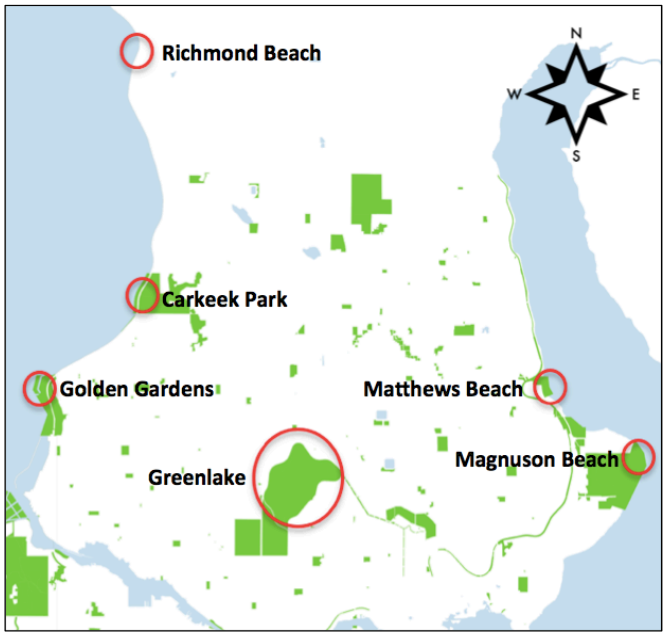


Table 2.1 - Location of Sampling Sites

Location	Area Sampled	Estimated Human Activity Level	Type of Water	Total # of Samples
Matthews Beach	Along swimming area	High	Fresh	7
Magnuson Park	Along swimming area	Moderate	Fresh	7
Greenlake	West and East beaches	High	Fresh	5
Carkeek Park	Along beach	Moderate	Marine	6
Golden Gardens	Along beach	High	Marine	5
Richmond Beach	Along beach	Moderate	Marine	5

2.1.4 Collection and Filtration

Between the hours of 9am-2pm, 12-liter grab samples of surface water were taken and filtered at each beach. The depth of the marine water samples varied because of the tide and were between 1-2.5 feet deep, but samples from fresh water beaches were consistently ~2 feet deep. To collect samples, waders were worn and the sampler walked out into the water to be sampled. For all locations except Greenlake, the length of the beach was divided into three sections, and 4-liters of surface water from each section was collected into the BMFS bag using a 1-liter sterilized bottle. Figure 2.2 shows how beaches were split into the three sections to get a representative water sample from the beach; red stars are locations where samples were taken. At Greenlake where there are two swimming beaches, 6-liter grab samples were taken from each beach; 3-liters from one half of the beach and 3-liters from the other half, for a total of 12-liters from both beaches combined. The sampler collected water using a down-and-up scooping motion to ensure that water from different depths was obtained - maximum depth was 2.5 feet down.

Once the BMFS bag was filled with 4 liters of water from one section of a beach, or 3-liters for Greenlake beaches, the bag was returned to the BMFS stand to be filtered by gravity through an Envirochek® HV Sampling Capsule (Figure 2.3). After the 3 or 4-liters of water was filtered, the caps at the ends of the capsule were replaced to seal the filter and avoid contamination, and all equipment was moved to the next section of the beach to continue collection and filtration. One filter was used for each 12-liter sample taken. During filtration, data was recorded on the number of humans and animals present, the activities they were doing, air temperature and weather, and qualitative observations of water quality. When all 12-liters were filtered, the ends of the capsule was sealed with the plastic caps, the filter was placed inside the plastic bag it came in, and the capsule was put inside an insulated backpack or lunch-bag with multiple icepacks to keep it cool. The filter was transported back to the laboratory within 4 hours and put inside a 4˚C fridge until it was eluted. All reusable materials were autoclaved, washed and then autoclaved again before re-use.

Figure 2.2 - Sampling Scheme of Locations Sampled for Matthews Beach

Figure 2.3 - Envirochek® HV Filter Capsule

Photo Credit: Joanna Harrison

2.2 Analysis of Water Samples

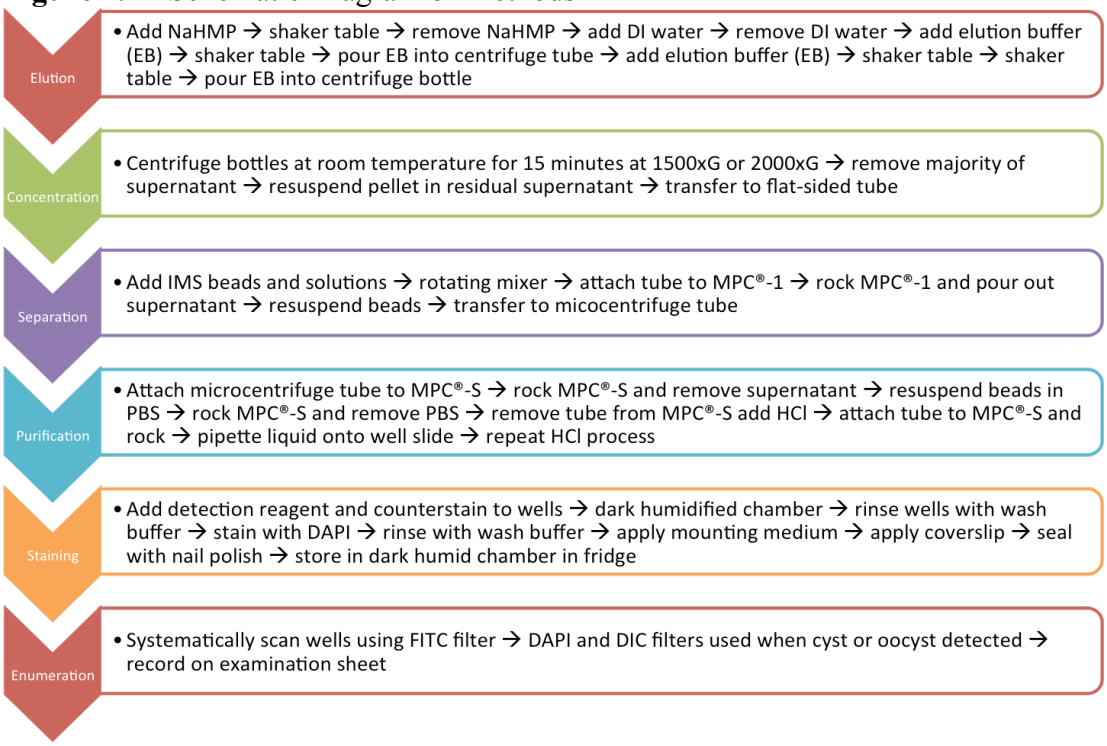
2.2.1 Elution & Concentration

The Envirochek® HV sampling capsules were eluted within 4 days (96 hours) of filtration following the EPA 1623.1 protocol. Elution, separation and purification were all performed on the same day. Five percent (5%) sodium hexametaphosphate (NaHMP) was

prepared according to elution protocol in Appendix B, poured into the inlet end of capsule, and the end cap was replaced. NaHMP was added before the elution buffer in order to pre-treat the filter and decrease the impact of turbidity caused by inorganic constituents on the recovery of the oocysts and cysts. The entire capsule was wrapped in plastic wrap and placed horizontally on a Barnstead Lab-Line shaker table (Model SHKA2000), secured with metal bungees and rubber bands, and shaken for 5 minutes at maximum speed. The capsule was removed from the shaker and tubing was attached to the effluent end of the capsule, through a Masterflex L/S peristaltic pump (Model 07557-04), and into a waste container. As much NaHMP as possible was pumped out of the filter. The capsule was filled with distilled water in the same manner as NaHMP was added, and pulled through the filter with the peristaltic pump - without being agitated beforehand.

After the pre-treatment, elution buffer was poured into the capsule. The buffer was prepared weekly. The end cap was replaced, the capsule covered in plastic wrap and shaken horizontally on the shaker table for 5 minutes. The eluent was then poured out of the inlet end and into a 175mL sterile centrifuge bottle. The process was repeated, but with two 5-minute shakes on the shaker table after re-aligning the bleed valve to a different position. The eluent of the 2nd elution was poured into a different 175mL sterile centrifuge bottle. The filter was then swung through an arc of 18˚ to remove more eluent from the filter. To concentrate the filter eluate, the two tubes were spun in a centrifuge at 1500xG or 2000xG - room temperature - for 15 minutes. Spinning the tubes at a higher speed increases the recovery of organisms; however, if the sample contains sand or other gritty material spinning at a higher speed will degrade the cysts and oocysts. For this reason Matthews Beach and Magnuson Beach were spun at 2000xG and all other locations were spun at 1500xG.

Figure 2.4 - Schematic Diagram of Methods



2.2.2 Separation & Purification

Centrifugation resulted in a pellet being formed at the bottom of the tube. If the pellets were ≤0.5mL, then all but 5mL of the supernatant was removed with a Pasteur pipet, leaving 5mL of supernatant total for both of the centrifuge tubes combined. If the pellet was >0.5mL, then volume of supernatant left had to be calculated using the following equation:

$$\boldsymbol{Total\ volume\ required\ (mL)} = \left(\frac{Pellet\ volume\ (mL)}{0.5mL}\right) 5mL$$

In most cases, the pellet volume was below 0.5mL. The tubes were then vortexed until pellet and supernatant were completely homogenized, and the mix was pipetted into one of the centrifuge tubes. The empty centrifuge tube was rinsed with 1/2 of the volume needed to bring the total volume of sample to 10mL. For example:

$$\boldsymbol{Amount\ of\ rinse\ (mL)} = \frac{10mL - (Pellet + Supernatant\ volume)}{2}$$

The rinse was pipetted into flat-sided tube to be used for Immunomagnetic Separation (IMS) and the centrifuge tube checked to ensure all sample was removed. The concentrate from the other centrifuge tube was also added to the flat-sided tube, and that centrifuge tube was

rinsed using the same calculation above, to reach a total volume of 10mL in the flat-sided tube. For samples that had over 5mL of supernatant due to larger pellet size, a total of 10mL of the pellet/supernatant were pipetted into the flat-sided tube, and later the percent of the sample that was examined was calculated using the following equation:

$$\textbf{Percent examined} = \left(\frac{total\ volume\ of\ resuspended\ concentrate\ transferred\ to\ flatsided\ tube}{total\ volume\ of\ resuspended\ concentrate\ in\ both\ centrifuge\ tubes}\right) 100\%$$

To separate out the oocysts and cysts from the extra material in the mix, Dynabeads™ DC-Combo magnetic beads from the Applied Biosystems™ IMS kit (Cat# 73012) were added to the water sample concentrate. The flat-sided tube was attached to a Lab-Line rotating mixer (Model 4631) and rotated for 1 hour at speed 8 (equivalent to 18 rpm). During the rotation, the antibodies on the beads attach to the antigens on the oocysts and cysts and are removed via a magnetic particle concentrator (MPC). After the tube was removed from the rotator, the flat side was attached to a MPC®-1 (Thermo Fischer, Cat#12001D) and rocked by hand so that the magnetic beads affixed to the side of the tube on the magnet. The supernatant was poured out, leaving just the magnetic beads with the cysts and oocysts attached. The beads were resuspended in 1X SL-buffer-A from the IMS kit and transferred to a 1.5mL microcentrifuge tube, followed by two rinses with 1X SL-buffer-A.

The microcentrifuge tube was inserted into a second magnetic particle concentration, MPC®-S (Thermo Fischer, Cat#A13346), and rocked by hand, forming a brown dot on the back of the microcentrifuge tube. After the supernatant was removed, the beads were rinsed with 1X PBS, rocked again and the 1X PBS was removed with a micropipette, leaving only the beads behind. To dissociate the cysts and oocysts from the magnetic beads, the microcentrifuge tube was removed from the MPC®-S and 0.1 N hydrochloric acid (HCl) was added. The tube was vortexed at the highest speed for 50 seconds, and then left to sit for 10 minutes, then vortexed again for 30 seconds. The microcentrifuge tube was replaced into the MPC®-S, forming the black dot of magnetic beads at the back of the tube. At this point, the oocysts and cysts had dissociated from the magnetic beads due to the HCl, so while the beads were attached to the back-magnetized side of the tube, the oocysts and cysts were in the HCl. The sample was transferred using a micropipette to a labeled well slide that contained 5 µL of 1.0 N sodium hydroxide (NaOH). The acid dissociation process was repeated once more and placed on a different well, but on the same slide. The slide was placed inside a non-airtight box, and into a

dark cabinet to air dry. See Appendix B for full separation and purification procedure. None of the samples were complex, so additional IMS techniques were not needed.

2.2.3 Staining & Enumeration

Within 72 hours of application of the sample to the slide, slides were stained using a MeriFluor® *Cryptosporidium/Giardia* staining kit (Meridian Bioscience, Memphis, Tennessee) following EPA instructions (see Appendix B). The MeriFluor® kit simultaneously detects *Cryptosporidium* oocysts and *Giardia* cysts utilizing FITC labeled anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies. The detection reagent used contains these monoclonal antibodies, which attach to antigens on the outer wall of oocysts and cysts. When excited under FITC fluorescent microscopy, the antibodies result in a bright green color, making the oocysts and cysts quantifiable.

Control slides were prepared using the positive and negative control solution supplied by MeriFluor®. Following manufacturer's instructions, detection reagent and counterstain were added to all wells and slides were placed in a dark humidified chamber - a tightly sealed plastic container containing damp paper towels and covered in foil - for 30 minutes. The slides were removed from the chamber and gently rinsed with wash buffer, allowing for the sample to stay secured to the well. DAPI staining solution was made daily, and was applied to each well for 5-9 minutes. The DAPI stain penetrates through the outer wall of the cysts and oocysts, staining the nucleic acid inside, resulting in nuclei appearing blue under DAPI fluorescent microscopy. After gently rinsing again with wash buffer, mounting medium was added to the well. Because qPCR would be later completed on these samples, the mounting medium used was not from MeriFluor®. Instead, 2% DABCO/glycerol mounting medium was used, and a glass coverslip was applied over top. The coverslip was sealed with nail polish and the slide was placed inside a different dark humidified chamber in a 4°C fridge until examined.

Within 7 days (168 hours) of staining, the slides were examined using immunofluorescence (IMF) microscopy. Each well was scanned in a systematic side-to-side pattern and the numbers of cysts and oocysts were recorded on a data sheet. The well was first scanned at 400X under FITC, which fluoresces the cysts and oocysts a bright green, making them identifiable against the dark background. Once a possible oocyst or cyst was spotted, the magnification was changed to 1000X (oil emersion lens) and the UV filter was changed to DAPI,

then DIC to determine if the organism was truly *Cryptosporidium* or *Giardia*, or instead another organism that fluoresced. Organism identification was based on the internal staining under DAPI and the external/internal characteristics under DIC. After examination, slides were placed back into the dark humid chamber in the fridge until removal of the cysts from the slides.

2.3 Quantitative Polymerase Chain Reaction (qPCR)

2.3.1 DNA extraction from slides

Quantitative PCR was used to determine the *Cryptosporidium* species present on the slides. Following WRF 4099, oocysts were removed from the slides (WRF, 2010). Molecular biological grade water (MBGW) was used to resuspend the organisms, and a piece of closed cell foam (2 x 3 x 3 mm square) was used to scrape the well. One piece of closed cell foam was used on both of the wells of one sample slide and then placed inside a microcentrifuge tube. Residual MGBW was aspirated and placed in the same microcentrifuge tube. After adding a 1:1 combination of Chelex/MBGW, the oocysts were freeze-thaw lysed using liquid nitrogen and a hot plate. Everything in the microcentrifuge tube was transferred to a spin column, centrifuged, eluted, and the resulting 40uL DNA was immediately stored at -80°C until use.

2.3.2 Controls

Controls for the *Cryptosporidium* qPCR were obtained from the Oregon Veterinary Diagnostic Laboratory at Oregon State University. Sixteen bovine fecal specimens from different cows confirmed positive with an unknown *Cryptosporidium* species were shipped on dry ice to the University of Washington Environmental and Occupational Health Laboratory and immediately stored in a 4°C fridge upon arrival. Within 24 hours, DNA from six cow specimens with the highest fecal *Cryptosporidium* load were extracted using the Qiagen QIAamp® Fast DNA Stool Mini Kit (Cat# 51604). DNA samples were immediately stored at -80°C until use.

2.3.3 Species Typing

Previously described primers reported in the WRF 4099 report that target the *Cryptosporidium* genes for 18S ribosomal RNA (18S) or heat shock protein 70 (hsp70) were used (LeChevallier et al., 2003; Aboytes et al., 2004; Di Giovanni et al., 2005; Johnson et al., 1995). 18S genes target all species of *Cryptosporidium*, while hsp70 genes target human-

pathogenic species of *Cryptosporidium*. These primers were run as a single-round Multiplex assay using SYBR® Green and a high resolution melt (HRM) analysis. The WRF report utilizes EvaGreen® as the binding dye, but after testing controls with SYBR® Green (Bio-Rad, Hercules, California) prior to running samples and having all positive bovine fecal samples amplify, a SYBR® Green assay was used for this study. Table 2.2 below gives information on the primers used. Ten randomly selected slide DNA samples were also tested on an Invitrogen™ Qubit™ 3.0 fluorometer (Model Q33216) to determine the load of overall DNA present in the sample.

Table 2.2 - Primer Sequences for *Cryptosporidium* qPCR

Primer	Sequence (5→3)	Orientation
Human-Pathogenic Species (hsp70)		
CPHSPT2F	TCCTCTGCCGTACAGGATCTCTTA	Forward
CPHSPT2R	TGCTGCTCTTACCAGTACTCTTATCA	Reverse
All Cryptosporidium Species (18S)		
CPB DIAG PCR F	AAGCTCGTAGTTGGATTTCTG	Forward
CPB DIAG PCR R	TAAGGTGCTGAAGGAGTAAGG	Reverse

2.4 Fecal Source Tracking

2.4.1 Water Sample Collection

An additional one-liter grab sample of water was collected during the filtration of samples at each location to later do Bacteroides typing. A sterile bottle was filled with ~333mL of water from each of the 3 locations where water was sampled at a beach, resulting in a total of ~1-liter composite sample. The bottle of water was stored in an insulated backpack or lunch-bag with multiple icepacks to keep it cool. The pack was transported back to the laboratory within 4 hours and the bottle was put inside a 4˚C fridge until DNA extraction.

2.4.2 DNA extraction

DNA extraction of the water sample occurred the same day that the sample was taken. The water was agitated and 500mL was membrane filtered through a 0.45um Millipore membrane (Cat# F4SA32924). The membrane was cut into strips, and following Qiagen DNeasy® PowerSoil Kit® (Cat# 12888-50) procedure, the strips were placed into PowerBead® tubes and the nucleic acid was extracted. DNA samples were immediately stored at -80°C until use.

2.4.3 Quantitative Polymerase Chain Reaction (qPCR)

The samples were processed for detection of two human, two dog and one avian marker. TaqMan® probe qPCR was performed using previously described primer and probe sequences (Table 2.3). Quantitative PCR analysis was completed in duplicate. Ten randomly selected DNA samples were also tested on the Qubit™ fluorometer to determine the load of overall DNA present in the sample. Both human markers utilize the well-described HF183 forward primer located on the 16S rRNA gene (Ahmed et al., 2016). The human reverse primer and probe sequences are different however, with Human 1 assay described by Haugland et al., 2012 and Human 2 assay by Shanks et al., 2016. These assays are quite specific to human feces, although there have been studies that show minor cross-amplification with fish, dog and cat feces (Green et al., 2014; Haugland et al., 2012). The two dog markers are also quite specific to canine feces and in studies showed no cross-amplification from other animals. The gene target for Dog 1 assay is the 16S rRNA (Dick et al., 2005; Sinigalliano et al., 2010) while for Dog 2 it is a non-16S rRNA gene (Green et al., 2014). The avian marker, or "Gull2" marker utilizes primers and a probe described by Sinigalliano et al., 2010 and although it is under the Bacteroides qPCR category of the paper, is really an assay targeting the 16S rRNA gene of the bacteria, *Catellicoccus marimammalium.* Although one study showed the Gull2 assay was specific for seagull feces (Lu et al., 2008), another showed that while it does not readily amplify non-avian DNA it does amplify DNA from other avian sources, such as poultry and waterfowl (Ryu et al., 2012). Due to this fact and because there were multiple types of birds present at all sampling locations (seagulls, ducks, geese, crows, etc.) the Gull2 assay will be considered an “avian” fecal marker for this thesis.

Table 2.3 - Primer and Probe Sequences for Bacteroides qPCR

Primer	Sequence (5→3)	Orientation
	Human 1 (16S)	
HF183	ATCATGAGTTCACATGTCCG	Forward
BFDrev	CGTAGGAGTTTGGACCGTGT	Reverse
BFDFAM	CTGAGAGGAAGGTCCCCCACATTGGA	Probe
	Human 2 (16S)	
HF183	ATCATGAGTTCACATGTCCG	Forward
BacR287	CTTCCTCTCAGAACCCCTATCC	Reverse
BacP234MGB	6FAM-CTAATGGAACGCATCCC-MGB	Probe
	Dog 1 (16S)	
DF475F	CGCTTGTATGTACCGGTACG	Forward
Bac708R	CAATCGGAGTTCTTCGTG	Reverse
DogBac	6FAM-ATTCGTGGTGTAGCGGTGAAATGCTTAG-BHQ1	Probe
	Dog 2 (non-16S)	
DG3	TTTTCAGCCCCGTTGTTTCG	Forward
	TGAGCGGGCATGGTCATATT	Reverse
	[FAM] AGTCTACGCGGGCGTACT [MGB]	Probe
	Avian (16S)	
Gull2 TaqMan®	TGCATCGACCTAAAGTTTTGAG	Forward
	GTCAAAGAGCGAGCAGTTACTA	Reverse
	6FAM-CTGAGAGGGTGATCGGCCACATTGGGACT-BHQ1	Probe

2.4.4 Controls

Controls for qPCR were obtained using various methods. Three unique human fecal samples were collected by putting a sterile plastic bucket underneath a toilet seat during excretion and mixed together in even amounts by weight. Dog feces were collected by dog owners – differing in breed and age - and brought to the laboratory in sterile conicals. Three dog samples were mixed together in even amounts by weight. Seagull feces were collected at Golden Gardens Park in Seattle. A group of seagulls was found at the park, and their feces were scraped into a conical using a sterile tongue depressor. Four seagull fecal samples were collected and mixed together in even amounts by weight. The DNA was extracted from each composite sample using the Qiagen QIAamp® Fast DNA Stool Mini Kit (Cat# 51604). Controls were tested and confirmed positive using the qPCR primers before running samples.

2.5 Polar Bear Plunge 2018

2.5.1 Sample Collection

In addition to the 7 samples taken at Matthews Beach, one twelve liter water sample was collected and filtered 30 minutes before and 30 minutes after the 12:00PM Polar Beach Plunge at Matthews Beach on January 1st, 2018. During the Polar Bear Plunge, participants enter a body of water all at the same time despite the low water temperature. Participants generally stay in the body of water about 2-6 minutes after submerging their head. For a video of the 2018 Polar Bear Plunge, use the following URL: http://www.seattlechannel.org/videos?videoid=x86962. All processes from collection to enumeration were performed in the same manner as previous samples (see Section 2.2). After microscopic enumeration, the before and after-Plunge samples were prepared for *Cryptosporidium* species typing (see Section 2.3). One liter of water was collected before and after as well for Bacteroides source tracking (Section 2.4) and an additional liter of water was collected for fecal coliform, *E. coli* and Enterococcus testing.

2.5.2 Fecal Coliform, E. coli and Enterococcus

In order to assess a change in bacterial water quality, fecal coliform and *E. coli* numbers were quantified using the IDEXX Colilert-18® and Quanti-Tray/2000® system. This method is easy, fast and is widely used for detection of fecal coliform and *E. coli* and estimates the Most Probable Number (MPN) of coliform in the water. For quantification of Enterococcus, the IDEXX Enterolert® and Quanti-Tray/2000® system was also used, which is similar to the Colilert-18® but instead gives MPN for Enterococcus. The use of IDEXX for quantification of coliform and Enterococcus organisms in the water has been validated for testing water samples, and for this reason and the ease of its use, these methods were chosen for quantification of fecal coliform, *E. coli* and Enterococcus in the water (Schang et al., 2016; Eckner 1998; Fricker et al., 1997; Palmer et al., 1993).

Chapter III: Results

3.1 IPR, OPR and Matrix Spike Recoveries

All IPR recoveries meet the EPA 1623.1 acceptance criteria of 38-100% recovery for *Cryptosporidium* and 22-100% recovery for *Giardia* (Table 3.1). OPR recoveries were also in the range of EPA acceptance criteria (33-100% for *Cryptosporidium* and 22-100% for *Giardia*). For both IPR and OPR, the precision - described as maximum relative standard deviation of all samples - was well above the EPA's 37% value, falling between 69.09% and 88.24%. For raw data values, see Appendix A.

Matrix spike recoveries for *Cryptosporidium* were in the EPA acceptable range for the Lake Washington samples (32-100%), but fell below 32% for Greenlake and all marine beaches (Table 3.2). Samples that did not meet the criteria are in bold text. *Giardia* matrix spike recoveries were in the 8-100% acceptable range for all locations (Table 3.3). Matrix spike recoveries were not continued for Greenlake, Golden Gardens or Richmond Beach due to time constraint, but were redone for sample 6 of Magnuson Beach, Matthews Beach and Carkeek Park (sampled in November) due to a notification from IDEXX regarding a possible compromised lot of Dynabeads™ IMS kits that was used for all previous samples. Since there was little change in the recovery of cysts and oocysts after using the new Dynabeads™ kit compared to the first kit, it was concluded that there was little to no loss of additional cysts and oocysts when using the first Dynabeads™ kit.

Table 3.1 - Recoveries for IPR and OPR

	IPR (n=4)		OPR (n=3)	
	Recovery (%)	**Precision (%)**	**Recovery (%)**	**Precision (%)**
Cryptosporidium	48.25	79.88	48.67	87.96
Giardia	51.25	69.09	51.00	88.24

Table 3.2 - *Cryptosporidium* Oocyst Matrix Spike Recovery

Sample #	**Matthews Beach (%)**	**Magnuson Beach (%)**	**Greenlake (%)**	**Carkeek Park (%)**	**Golden Gardens (%)**	**Richmond Beach (%)**
1	47	64	**5**	**2**	**22**	**7**
2	NA	NA	**10**	**10**	**10**	**6**
6	43	54	NA	**16**	NA	NA
Average	45	59	7.5	9.33	16	6.5

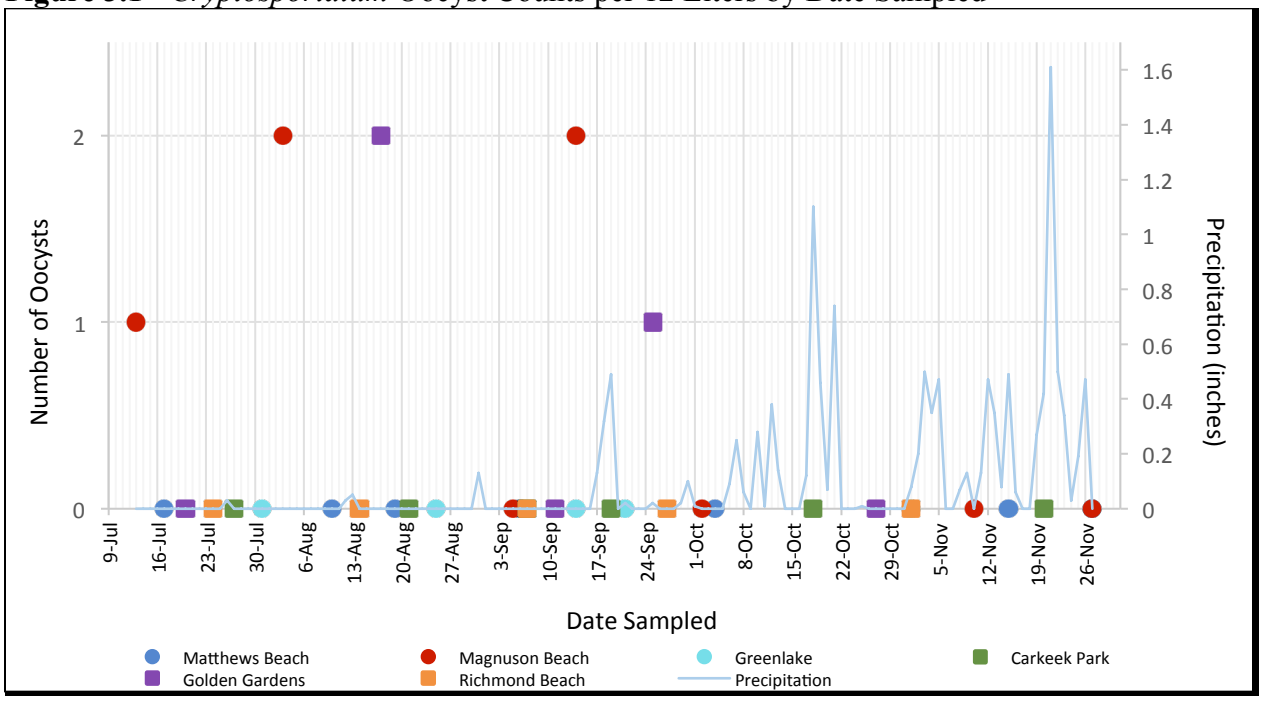
Table 3.3 - *Giardia* Cyst Matrix Spike Recovery

Sample #	Matthews Beach (%)	Magnuson Beach (%)	Greenlake (%)	Carkeek Park (%)	Golden Gardens (%)	Richmond Beach (%)
1	64	54	15	30	26	20
2	NA	NA	50	40	56	42
6	64	57	NA	52	NA	NA
Average	64	55.5	32.5	40.67	41	31

3.2 Oocyst and Cyst Counts

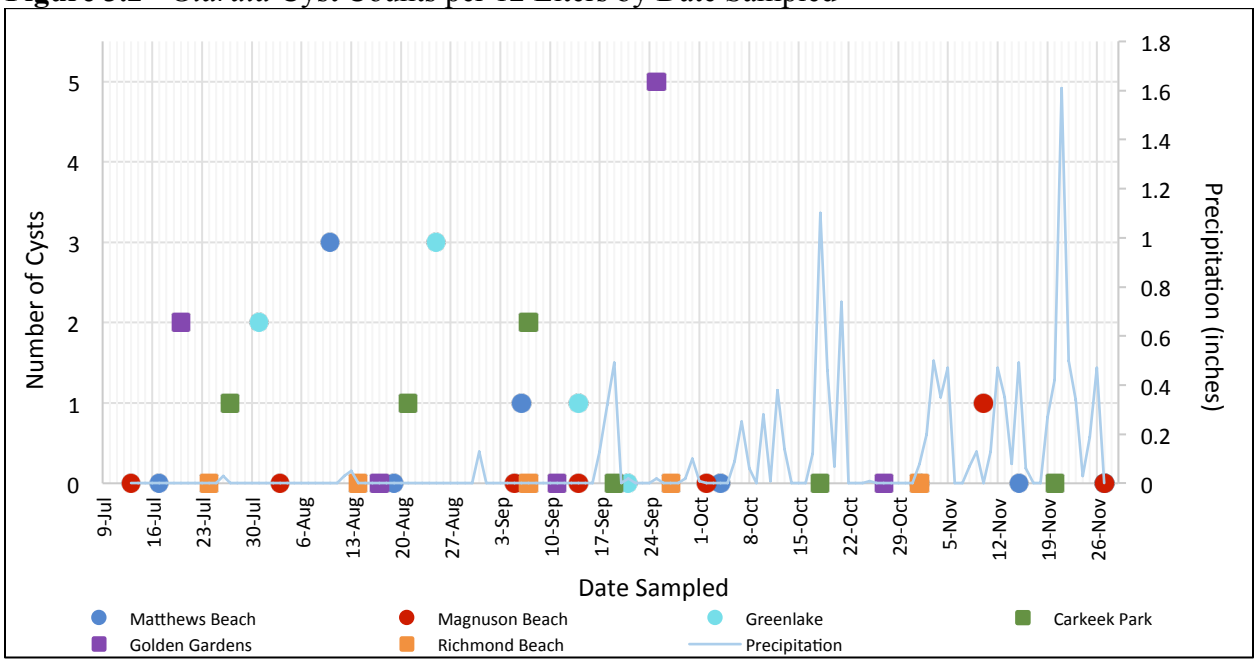
Magnuson Beach and Golden Gardens were the only locations sampled that resulted in measurable *Cryptosporidium* counts (Figure 3.1). All other locations had either zero or non-detectable levels of *Cryptosporidium*. Magnuson Beach had a maximum of 2 oocysts counted in one sample and also had the highest total number of oocysts counted for all samples combined (5 oocysts). The highest number of oocysts at Golden Gardens was 2 oocysts, and all samples combined had a total of 3 oocysts. Precipitation data from NOAA shows that the first rains occurred on September 19th, after which a sample from Golden Gardens resulted in one oocyst (NOAA, 2018). Oocyst numbers for all locations fell to zero after precipitation increased in early October.

Figure 3.1 - *Cryptosporidium* Oocyst Counts per 12 Liters by Date Sampled



All locations except Richmond Beach had one or more samples with non-zero *Giardia* cyst counts (Figure 3.2). Golden Gardens had the highest number of cysts counted on one day (5 cysts), as well as the highest total number of cysts when all samples were combined (7 cysts). Greenlake and Carkeek Park had three consecutive positive samples. Precipitation data shows that after the first rain on September 19th, one sample from Golden Gardens resulted in five cysts, the highest number recorded for one sample from all locations. Cyst numbers for all locations fell to zero after precipitation increased in early October, except for one sample at Magnuson Beach in November.

Figure 3.2 - *Giardia* Cyst Counts per 12 Liters by Date Sampled



3.3 Quantitative PCR

Although DNA extracted directly from *Cryptosporidium*-positive cattle feces did amplify, results for *Cryptosporidium* species typing of oocysts scraped from the slides came back negative; none of the samples amplified with qPCR. The ten randomly selected samples of extracted DNA from the slides that were analyzed using the Qubit™ fluorometer all showed very low concentrations of DNA that were below the detection limit, resulting in an "Out of Range" response. This could mean that *Cryptosporidium* species could not be detected due to low DNA content in the samples.

The results of Bacteroides qPCR show detectable levels of avian feces in the majority of samples taken from all beaches (57-100% of samples from each location). Table 3.4 shows that both human-associated assays detected measureable levels of human feces in all samples from the saltwater beaches and ≤60% of samples in the freshwater beaches. Magnuson beach resulted in both assays with less than 50% of samples having detectable levels of human feces, Matthews Beach assays both resulted in 57% of samples with detectable levels and one assay in Greenlake did not amplify any human feces while the other assay showed that 60% of samples had detectable levels. The dog-associated fecal assays were quite different, in that there was a lot of discordance present between the two assays. One assay amplified for at least one sample at all locations, while the other did not amplify any samples at the majority of locations. The location with the highest number of samples that amplified dog feces was Carkeek Park, at 50%, while the lowest was Magnuson that had only 14% sample amplification.

Table 3.4 - Fecal Source Tracking qPCR Results

	Avian Assay	**Human Assays**		**Dog Assays**	
	Positive	**Positive**	**Discordance between assays**	**Positive**	**Discordance between assays**
Magnuson Beach	7/7 (100%)	3/7 (43%)	2/7	1/7 (14%)	1/7
Matthews Beach	4/7 (57%)	4/7 (57%)	0/7	3/7 (43%)	1/7
Greenlake	3/5 (60%)	3/5 (60%)	3/5	2/5 (40%)	2/5
Carkeek Park	6/6 (100%)	6/6 (100%)	0/6	3/6 (50%)	3/6
Golden Gardens	5/5 (100%)	5/5 (100%)	0/5	1/5 (20%)	1/5
Richmond Beach	4/5 (80%)	5/5 (100%)	0/5	2/5 (40%)	2/5

Positive = number of samples positive for at least one assay/total number of samples

3.4 Polar Bear Plunge 2018

There was a notable increase in *Cryptosporidium* and *Giardia* after the PBP, compared to the non-detectable levels before. Before the PBP, *Cryptosporidium* count was zero (0) oocysts, and afterwards increased to 4 oocysts. *Giardia* count before was also zero (0) but rose to 27 after. A qualitative assessment of the water turbidity also showed an increase in turbidity after the PBP (Figure 3.3), which is supported by the total coliform, *E. coli* and Enterococcus results

(Table 3.5). The numbers for all three of the previously mentioned were lower before the PBP than after the PBP; there was an increase of >3.84% MPN/100mL of total coliform, 45.3% MPN/100mL of *E. coli* and 4.93% MPN/100mL of Enterococcus. Bacteroides results show that avian, human and dog feces were present in samples before and after the PBP, although one dog assay only detected dog feces after the PBP (Table 3.6).

Figure 3.3 - Filters from Sampling Before (left) and After (right) the Polar Bear Plunge

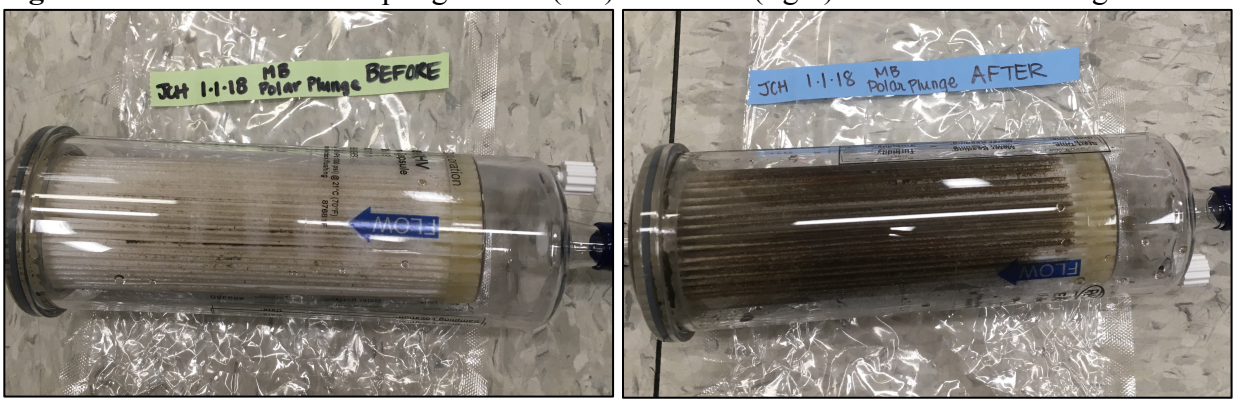


Photo Credit: Joanna Harrison

Table 3.5 - IDEXX results for Total Coliform, *E. coli* and Enterococcus

	Total Coliform (MPN/100mL)	***E. coli* (MPN/100mL)**	**Enterococcus (MPN/100mL)**
Before	629.4	2.0	6.1
After	>2419.6	90.6	30.1
Percent Increase	>3.84%	45.3%	4.93%

Table 3.6 - Fecal Source Tracking qPCR Results for Polar Bear Plunge

	Avian Assay	**Human Assays**		**Dog Assays**	
	Positive	**Positive**	**Discordance between assays**	**Positive**	**Discordance between assays**
Polar Bear Plunge	2/2 (100%)	2/2 (100%)	0/2	2/2 (100%)	1/2

Positive = number of samples positive for at least one assay/total number of samples

Chapter IV: Quantitative Microbial Risk Assessment (QMRA)

4.1 Problem Formulation

As stated previously in Chapter 1, diarrheal diseases are a major contributor to human morbidity and mortality across the globe. Not only do they impact the health of children and populations with weaker immune systems – or YOPIs (Young, Old, Pregnant and Immunocompromised) - but they also lead to disease in healthy adults (CDC, 2015; CDC, 2017; Naumova et al., 2003). While the majority of diarrheal disease burden occurs in developing regions of the world, there are cases that occur in developed regions. For example, in 1994 *Cryptosporidium* caused a massive diarrheal outbreak in Milwaukee, Wisconsin, leading to over 400,000 cases of diarrhea. There were also some deaths associated with the outbreak and the economic cost was estimated to be about $96.2 million – this includes medical cost and productivity loss cost (Corso et al., 2005). Diarrheal diseases can have a large impact on the health and financial burden of communities in any country, so it is important to understand the risks associated with identified levels.

There are both cryptosporidiosis and giardiasis cases reported every year in King County, Washington – of which Seattle is the biggest, most densely populated city (Tables 1.2 and 1.3). Due to the advanced drinking water treatment of the Tolt and Cedar River Watersheds, negligible cyst and oocyst numbers are released when water is distributed to Seattle residents (SPU, 2016). This leads to the question, if the reported cases are not coming from the pristine drinking water, then where are people being exposed to the two waterborne protozoa? One route may be through the ingestion of untreated recreational water. For Seattle, Washington, recreational beach water does not get tested for *Cryptosporidium* and *Giardia*. Since the beach water is not tested, there have been no reported estimates of risk due to swimming in the water. To address this lack of information, this risk assessment will estimate the levels of risk from ingesting the two parasite's cysts and oocysts during the sampling period of June through November 2017, based on the number of cyst and oocyst that were quantified during that period. The results of this assessment will inform to what extent children and adults should reduce their exposure to untreated recreational waters in Seattle.

4.2 Hazard ID

Cryptosporidium and *Giardia* are two protozoan parasites that can be transmitted between humans or zoonotically (Caccia et al., 2005; Hunter et al., 2005). Although there are multiple species of *Cryptosporidium*, two are the most medically important for humans: *C. parvum* and *C. hominis* (Xiao, 2008). While *C. parvum* has broad host range – it can infect cattle and dogs – *C. hominis* is almost only transmitted between humans. *Giardia* on the other hand, has only one species that infects humans – *G. duodenalis* – which also infects a broad range of animal host (Hunter et al., 2005; Thompson et al., 2011). These protozoa are most commonly spread through the fecal-oral route and are highly associated with ingestion of contaminated water – either intentionally via drinking water or accidentally via ingestion of recreational water (CDC, 2015; CDC, 2017). The parasites create protective barriers called cysts and oocysts that are the infectious forms of the organism. When a cyst or oocyst is ingested, it goes through the life cycle inside the body, and oocysts and cysts are excreted in feces. These hearty cysts have a long persistence time in the environment and are also resistant to many common disinfection methods such as chlorine (Ryan et al., 2014).

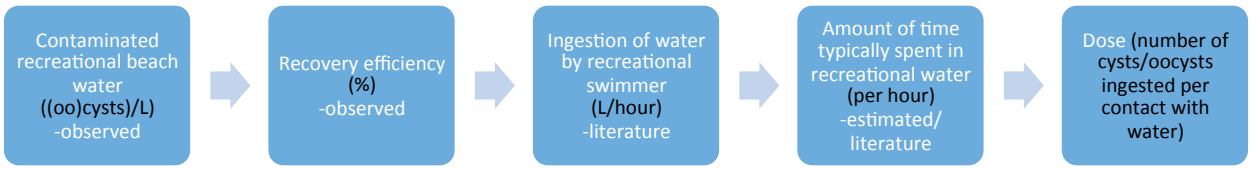
The infectious dose of the parasites plays a large role in their spread. Research has shown that as low as 1 cyst or oocyst can cause an infection in humans, although normally about 10-30 cysts or oocysts is the low estimate that can lead to an infection (Ryan et al., 2014; Okhuysen et al. 1999; Chappell et al. 2006; DuPont et al., 1995). For these parasites, infection does not mean that symptoms will appear. Many infections from *Giardia* and *Cryptosporidium* are asymptomatic, although the infected person continues to shed the cysts and oocysts in their feces, spreading the disease (Certad et al., 2017). While watery diarrhea is the most common symptom of infection, cramps, vomiting and dehydration may also occur, and without replacing lost fluids, infection can sometimes lead to death (CDC, 2015; CDC, 2017). Symptoms usually last about 1-2 weeks, but both organisms have been shown to cause symptoms for longer. For severe cases, there are anti-parasitic or anti-diarrheal medications that patients can take, but generally patients are treated by fluid replacement as bodily fluids are lost (CDC, 2015; CDC, 2017).

4.3 Exposure Assessment

In this QMRA scenario, both adults and children in the community are using recreational beach water for swimming during July through November in 2017. While swimming,

recreational water that is contaminated with the measured levels of cyst and oocyst is unintentionally ingested. Other routes of ingestion are not considered. In order to get to the dose, or the number of cysts or oocysts ingested per contact with water, the number of cysts or oocysts present, the recovery efficiency, the ingestion rate and the amount of time spent in the water were multiplied together (Figure 4.1).

Figure 4.1 – QMRA Exposure Assessment Model



In this scenario, it is assumed that there will be greater ingestion of fresh water locations than marine water locations due to increased amount of time spent in the water and because we assume that swimmers are more likely to spit out marine water than fresh water. For fresh water, it is also assumed that children ingest more water compared to adults as children tend to spend 1.5x more time in the water than adults and because their ingestion rate of water (L/hour) is greater than adults (DeFlorio-Barker et al., 2018; Schets et al., 2011). The estimates for ingestion rate of fresh water were based on those reported by the World Health Organization of 20-50mL/hour and divided in half to get the estimate for marine water (WHO, 2003). The estimates for amount of time spent in water were based on what was seen in the literature. Due to the temperature of the Puget Sound being colder than the locations that were in the literature, the numbers were adjusted in order to account for the reduced amount of time that people spend in our colder marine water (DeFlorio-Barker et al., 2018; Schets et al., 2011). Tables 4.2 and 4.3 show the ingestion rate and time parameters used for adults and children. Cyst and oocyst input into the model were done using lognormal distributions and the average and standard deviation of cysts and oocysts were calculated using measured data from July through November (see Appendix A for raw data values). The matrix spike recovery used a uniform distribution. No parameters were point data, all were distributions in Oracle® Crystal Ball (Table 4.1).

Table 4.1 - Exposure Assessment Model Parameters

Parameter	Units	Distribution	Reference
Oocysts in water	Cyst or oocyst / L	Lognormal	Personal data
Recovery efficiency	Unitless	Uniform	Personal data
Ingestion of water	L / hour	Uniform	WHO and DeFlorio-Barker et al., 2017
Time spent in water	Hours	Triangular	Assumption based on location

Table 4.2 - Adult Parameters Used for QMRA

Parameter	Units	Distribution	Min	Likeliest	Max
Ingestion of fresh water	L / hour	Uniform	0.02	NA	0.05
Time spent in fresh water	Hours	Triangular	0.017	0.13	0.5
Ingestion of marine water	L / hour	Uniform	0.01	NA	0.025
Time spent in marine water	Hours	Triangular	0.017	0.067	0.25

Table 4.3 - Child Parameters Used for QMRA

Parameter	Units	Distribution	Min	Average	Max
Ingestion of fresh water	L / hour	Uniform	0.02	NA	0.05
Time spent in fresh water	Hours	Triangular	0.025	0.2	0.75
Ingestion of marine water	L / hour	Uniform	0.01	NA	0.025
Time spent in marine water	Hours	Triangular	0.017	0.067	0.25

4.4 Dose-Response

Two types of dose-response models were used to estimate the probability of infection: Exponential (1) and Beta-Poisson (2).

$$P(response) = 1 - \exp(-k \times dose) \qquad (1)$$

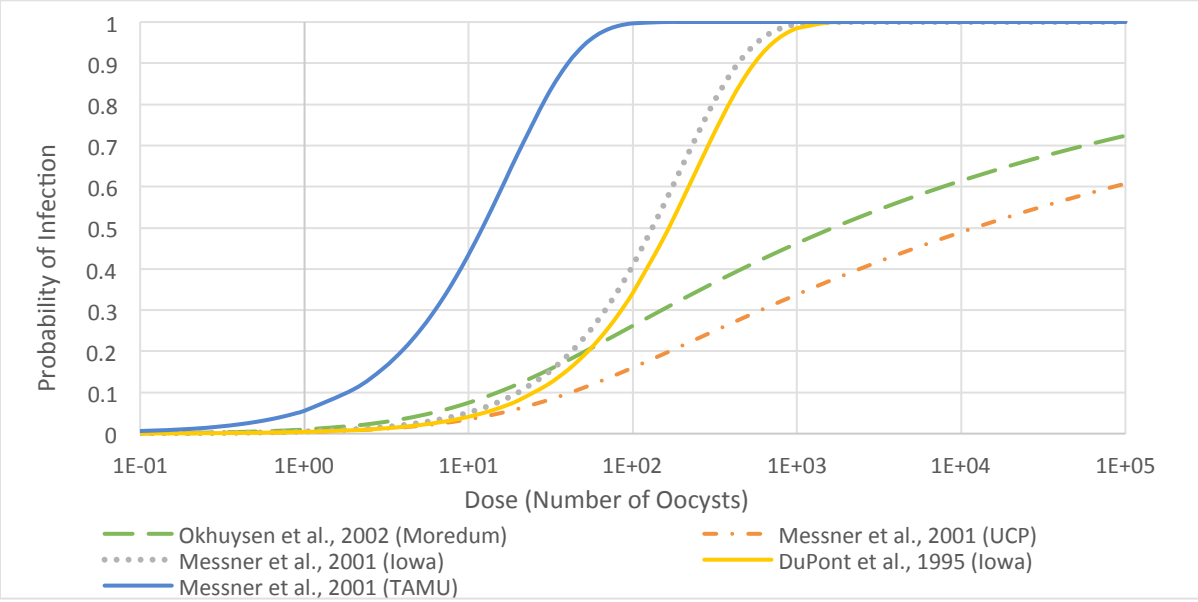
$$P(response) = 1 - \left[1 + dose\frac{\left(2^{\frac{1}{\alpha}} - 1\right)}{N_{50}}\right]^{-\alpha} \qquad (2)$$

Due to the fact that *C. parvum* and *C. hominis* are the predominant species of *Cryptosporidium* that cause infection in humans, and because qPCR was unable to determine what species were present in the samples, the *Cryptosporidium* dose-response model toggled between published exponential and beta-Poisson models that have a response of infection (QMRAwiki, 2015). Dose-response model parameters are provided in Table 4.4; model curves are depicted in Figure 4.2. The first model comes from a study done by Okhuysen et al. (2002) where healthy individuals were given single doses of different numbers of *C. parvum* Moredun isolate oocysts. Oocyst counts in participant's feces and resulting symptomology were recorded and analyzed for median infectious dose (ID_{50}) using a simple cumulative percent endpoint method, resulting in a beta-Poisson model. A similar study to this, but instead looking at *C. parvum* Iowa strain was done by DuPont et al. (1995). Again, healthy individuals were given different doses of oocysts and their responses were recorded. The same methodology was used to create a model, this time resulting in an exponential model. The last three models came from a study done by Messner et al. (2001), where data obtained from three dose-response studies of different *C. parvum* strains were assessed using maximum likelihood dose-response parameter estimates, instead of the a simple cumulative percent endpoint method (DuPont et al., 1995; Chappell et al., 1999; Okhuysen et al., 1999). The methods of the three studies Messner et al. used were the same as Okhuysen et al. (2002) and DuPont et al. (1995), where healthy individuals were given single doses of different numbers of oocysts. The different isolates given were UCP, Iowa and TAMU. Using both the oocysts in stool and clinical diarrhea as their response, Messner et al. generated two exponential models and one beta-Poisson model.

Table 4.4 – *C. parvum* Dose-Response Model Parameters

Study	Strain	Model	α	N50
Messner et al., 2001	UCP isolate	Beta-Poisson	1.14E-01	4.55E+02
Okhuysen et al., 2002	Moredun isolate	Beta-Poisson	1.45E-01	1.79E+02
Study	**Strain**	**Model**	**k**	
Messner et al., 2001	TAMU isolate	Exponential	5.72E-02	
DuPont et al., 1995	Iowa strain	Exponential	4.19E-03	
Messner et al., 2001	Iowa strain	Exponential	5.26E-03	

Figure 4.2 – *C. parvum* Dose-Response Model Curves

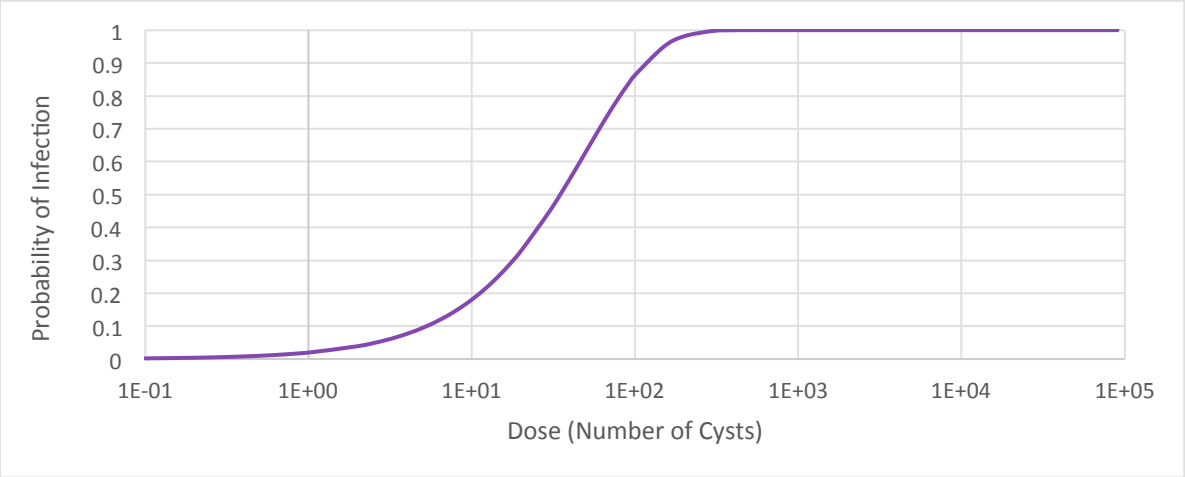


The QMRAwiki reports only one model for *Giardia* infection, meaning that there is no need for toggling. Dose-response model parameters are provided in Table 4.5; model curve is depicted in Figure 4.3. The model comes from a study conducted by Rendtorff et al. (1952) where 40 healthy men were given single doses of different numbers of *G. duodenalis* cysts. The symptoms of the participants was recorded and resulted in the exponential model below.

Table 4.5 – *G. duodenalis* Dose-Response Model Parameters

Study	Model	k
Rendtorff et al., 1954	Exponential	1.99E-02

Figure 4.3 – *G. duodenalis* Dose-Response Model Curve



4.5 Risk Characterization

Using the primary data collected on the number of cysts and oocysts present in the North Seattle beach water, risks associated with the levels present by means of probabilistic QMRA were assessed. A 2-D Monte Carlo methods simulation was performed for 1001 x 10,001 trials (uncertainty x variability) in Oracle® Crystal Ball, which generated median estimates of risk and >95% confidence around the estimates (QMRAwiki, 2015). Figures 4.4 - 4.7 show the S-curves created in Crystal Ball for Golden Gardens; all other S-curves are located in Appendix A. Tables 4.6 and 4.7 show the results from the median S-curve for adults and children.

Figure 4.4 – Probability of Infection from *Cryptosporidium* for Adults at Golden Gardens

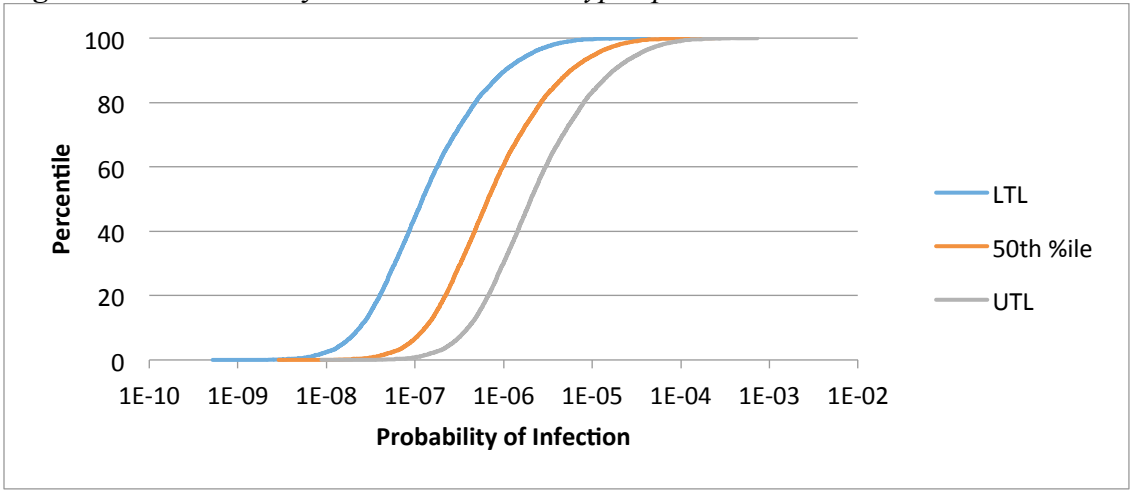


Figure 4.5 – Probability of Infection from *Giardia* for Adults at Golden Gardens

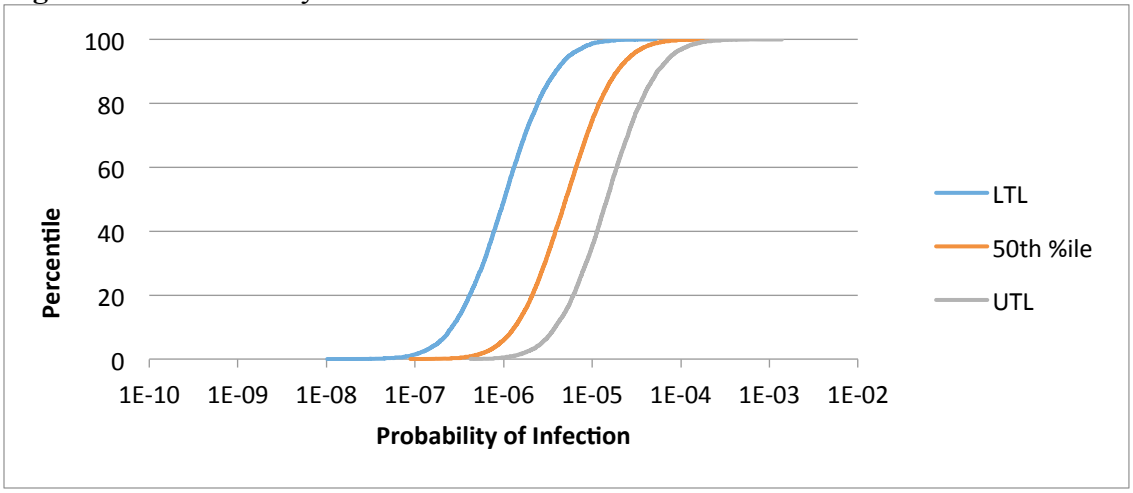


Table 4.6 - Median Results for Adult Swimming Exposure

	Cryptosporidium **(Probability of Infection)**		***Giardia*** **(Probability of Infection)**	
	Median	**95%**	**Median**	**95%**
Magnuson Beach	2.67E-06	4.01E-05	8.08E-07	9.29E-06
Matthews Beach	NA	NA	4.10E-06	3.20E-05
Greenlake	NA	NA	1.88E-05	7.19E-05
Carkeek Park	NA	NA	5.19E-06	2.39E-05
Golden Gardens	6.67E-07	1.09E-05	5.02E-06	2.78E-05
Richmond Beach	NA	NA	NA	NA
After Polar Bear Plunge	2.40E-05	2.98E-04	4.42E-04	6.12E-04

All locations fall under the EPA marine and freshwater illness thresholds of 19/1000 and 8/1000, respectively, during the July through November sampling period. The location and organism resulting in the highest probability of infection during that period for adults is getting *Giardia* at Greenlake, where 95% of the population has a probability of infection of ≤7.19/100,000. The lowest probability of infection during the summer and fall months is from *Giardia* at Magnuson beach, where 50% of the population has a ≤8.08/10,000,000 probability of infection. All other scenarios fall between the high and low estimates. Looking at the risk of

infection for 95% of adults if they swam in Matthews Beach after the Polar Bear Plunge, all levels are below the thresholds, but are generally greater than the July through November sampling locations.

Figure 4.4 – Probability of Infection from *Cryptosporidium* for Children at Golden Gardens

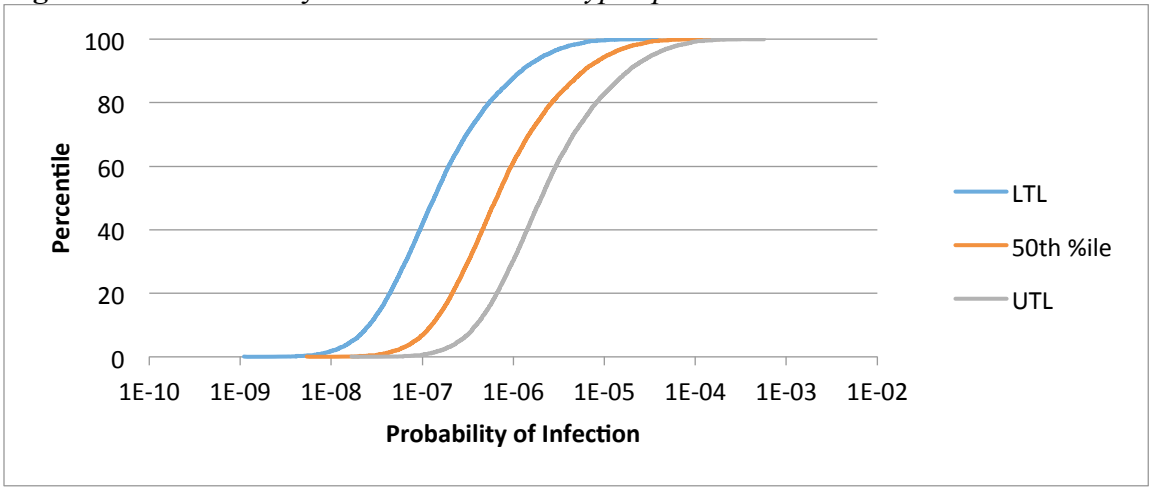


Figure 4.4 – Probability of Infection from *Giardia* for Children at Golden Gardens

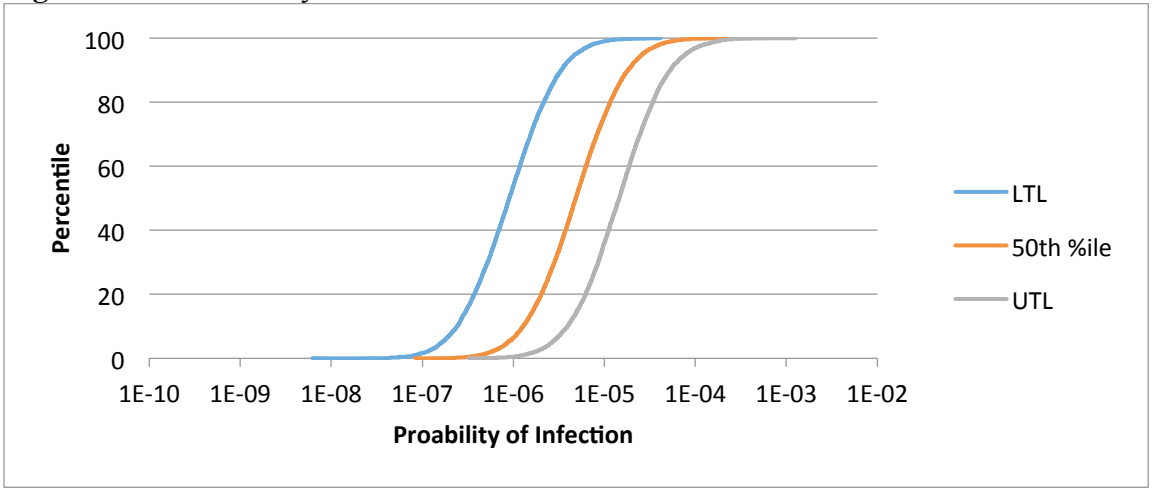


Table 4.7 - Median Results for Child Swimming Exposure

	Cryptosporidium (Probability of Infection)		*Giardia* (Probability of Infection)	
	Median	**95%**	**Median**	**95%**
Magnuson Beach	3.88E-06	5.88E-05	4.17E-06	3.94E-05
Matthews Beach	NA	NA	2.43E-06	1.12E-05
Greenlake	NA	NA	8.02E-05	3.52E-04
Carkeek Park	NA	NA	5.74E-06	2.65E-05
Golden Gardens	6.56E-07	1.10E-05	9.98E-06	6.41E-05
Richmond Beach	NA	NA	NA	NA
After Polar Bear Plunge	4.83E-05	4.11E-04	6.53E-04	9.07E-04

The location and organism resulting in the highest probability of infection fur July through November for children is getting *Giardia* at Greenlake, where 95% of the population has a probability of infection of ≤3.52/10,000. The lowest probability of infection during this time is from *Cryptosporidium* at Golden Gardens, where 50% of the population has a ≤6.56/10,000,000 probability of infection. All locations fall under the EPA marine and freshwater illness thresholds of 19/1000 and 8/1000, respectively, during the July through November sampling period. Looking at the risk of infection for 95% of children if they swam in Matthews Beach after the Polar Bear Plunge, all levels are above the EPA thresholds.

A tornado analysis showed that the first driving factor in the risk estimate was the toggling of the different risk models for *Cryptosporidium*. The second driving factor was the number of oocysts present in the water, and the third and fourth were ingestion rate of water and time spent in water, respectively. The *Giardia* tornado analysis came up with similar results. Since the simulation did not toggle between models, the first driving factor was the number of cysts in the water, with ingestion rate and time spent in water having much less effect on the risk estimates.

Chapter V: Discussion

5.1 Prevalence and Distribution

The *Cryptosporidium* oocyst recovery - which is the infective form of the protozoan - had quantifiable numbers at Magnsuon Beach and Richmond Beach during the sampling period of July through November 2017. Though numbers range between only 1-2 oocysts, research shows that ingestion of just 1 oocyst can result in infection (Ryan et al., 2014). The other four locations resulted in zero oocyst recovery throughout the sampling period, which could mean that the concentration of oocyst is actually zero or instead, below the limit of detection. Because the number is low enough that it can't be quantified, it means that the risk of infection would be very low. While it can't be said there is no risk, since there may be little exposure to the organism, there would be low risk. It is important to note however, that the recovery of oocysts may be low due to the methods used. For the fresh water locations other than Greenlake, matrix spike recovery was acceptable (between 43-64%), so numbers for Magnuson Beach and Matthews Beach can be trusted. Matrix spike recoveries were below the EPA acceptable recovery percentage for Greenlake and all saltwater beaches. This does not mean that oocysts could not be recovered, but that the recovery was below 32%. For these locations, matrix spike recovery ranged from 2-22%.

Giardia cyst recovery had quantifiable numbers for at least one sample in all locations during the sampling period, except Richmond Beach. The number of *Giardia* cysts recovered did vary by location (1-5 cysts) but no consistency was identified between the location and number of cysts counted. Due to acceptable matrix spike recoveries, these numbers hold less uncertainty than the *Cryptosporidium* oocyst recoveries. The better recovery of *Giardia* could be due to the salt water having less of an effect on the organisms; or if the turbidity of the water plays a factor in recovery, the cysts are larger than the oocysts, 5-18um versus 4-6um, and the vibrant green of *Giardia* is easier to see under fluorescent microscopy (EPA Method 1623.1).

Based on the low matrix spike recoveries seen for marine water locations, it seems very likely that saltwater decreases recovery. One study looking into the recovery of *Cryptosporidium* and *Giardia* using the EPA method showed that recovery efficiencies varied substantially using Envirochek® filtration in marine waters (Betancourt et al., 2014). Another possibility is that the increased turbidity of the water reduced oocyst recovery. If the water is too turbid, it is harder to

purify the sample. When the sample is stained on a slide, leftover debris can cover up the oocyst, resulting in the technician being unable to see the fluorescent green color. Water turbidity varied between field sample sites, with marine water locations having greater qualitative turbidity.

A trend that can be seen in both the *Cryptosporidium* and *Giardia* data is that after the rains became more consistent in October, the levels of both organisms drop to zero, resulting in no detectable cysts or oocysts (Tables 3.1 and 3.2). As the dry season ends and it starts to rain more consistently, pathogen concentrations can be diluted in the water to undetectable levels (Stenstrom et al., 2005). Atherbolt et al. (1998) showed that there was a positive correlation between initial rainfall and increased Giardia and *Cryptosporidium* concentrations in the Delaware river; while Ajeagah et al. (2009) showed a decrease in cyst and oocyst load during the rainy season versus the long dry season in Cameroon. This is hypothesized as one of the reasons why there was a drop to zero cysts and oocysts in all samples after October (except one sample from Magnuson Beach positive for *Giardia* in November). This concept is supported by numerous studies looking at pathogenic load after more frequent rainfall (Daniels et al., 2016; Aguire et al., 2016; Rose et al., 2002).

Another reason for the higher counts in the summer and lower in the fall may be because less people are using recreational beaches as the weather gets colder. When less people use beaches, there is less incidence of contaminated fecal content shedding off of those persons into the water. This will result in a lower number of organisms present. A study by Gerba (2000) estimated that recreational beach users shed about 0.14 grams of feces. When you take into account that feces of infected humans generally contain 10^5 to 10^7 cysts or oocysts per gram, it results in a large concentration of parasites being shed into the water when more people are swimming (Gerba, 2000). Yet another hypothesis for the shift between seasons is that with less people using the beaches, there is less stirring of sediment since people are not entering the water. A study by Graczyk et al. (2010) showed that waterborne parasite levels increased when there was re-suspension of bottom sediment by marine water users. If cysts and oocysts are settled in the sediment when sampled, it will result in a non-detection of the organisms.

The Polar Bear Plunge (PBP) results were supportive of the hypothesis that oocysts and cysts either come off the body of humans when they enter the water, or that oocysts and cysts settle in the sediment and when sediments are disturbed they resuspended in the water and are able to be detected and possibly ingested. With an increase from 0 to 4 oocysts and 0 to 27 cysts

thirty minutes after the PBP ended, one of these two hypothesized ways are a likely cause of the increased number. The bacterial data before and after the PBP show that there is also a bacterial load increase after people went into the water; fecal coliform, *E. coli* and Enterococcus levels all rose by at least 3.84%. While this bacterial data does not specifically mean that pathogens are present in the water, studies have shown a positive relationship between coliform and pathogen levels (Ferguson et al., 2012; Graczyk et al., 2010; Wu et al., 2011).

5.2 Fecal Source Tracking

Results from the Bacteroides qPCR suggest that there are feces from birds and humans present at all sampling locations for at least one point in time during the sampling period. There was no discernable pattern of feces number in the water during the summer versus the fall/early winter months although it would be expected that the level of human fecal content would decrease as people stopped using the water recreationally. The results for the dog feces were a bit more variable, but suggest that there are dog feces present in all locations sampled as well. All locations had some discordance between the dog assays, ranging from 14% discordance to 50% discordance (Table 3.4).

The reason for the discordance may be due to the Dog 2 assay being less sensitive than the Dog 1 assay. A reduction in the assays ability to detect low levels of DNA could be the reason why Dog 1 amplified samples that Dog 2 did not (Le Fichoux et al., 1999; Bustin et al., 2017). It is also normal that some sample replicates don't amplify since the DNA is not always evenly distributed in the matrix. To try and parse out which samples did not amplify because of no/low DNA or because of uneven distribution, samples that only amplified one of the two replicates were run again. One final reason that there might have been no amplification is because DNA samples were not aliquoted into smaller volumes and so the extracted nucleic acid went through multiple freeze-thaw cycles as assays were run on different days. Shao et al. (2012) showed that there was progressive DNA degradation of samples with increasing freeze-thaw cycles. In future studies, aliquoting the nucleic acid so that they are going through less freeze-thaw cycles would reduce the DNA degradation of the samples.

The results of the species typing of *Cryptosporidium* were inconclusive due to inability of qPCR to amplify the DNA. This does not mean that there was no *Cryptosporidium* present in the samples, but could be due to the low level of DNA present in the sample, a hypothesis supported

by the Qubit fluorometer results being "Out of Range". Similar to the Bacteroides hypotheses, the lack of amplification may also be due to DNA degradation, most likely while the DNA is on the slide in the dark humid chamber in the fridge. Since the DNA was not extracted 24 hours after the oocysts were placed on the slide - there was a 7-day period between staining and examination, and additional days in the fridge after examination and prior to extraction - the oocyst and it's DNA may have degraded even before extraction.. It's a possibility that the environmental matrix that the oocyst was in could also have interfered with the qPCR process in some way. A paper by Haugland (2012) supports this hypothesis, stating that there are two classes of qPCR interference: inhibition and DNA recovery. Haugland et al. explains that water constituents can reduce qPCR amplification, so this may be a reason why the *Cryptosporidium* DNA did not amplify.

5.3 Quantitative Microbial Risk Assessment

The findings of this study suggest that there is measureable risk to human health from surface water exposure to *Cryptosporidium* and *Giardia* at some of the recreational beaches in North Seattle, Washington, but that risk is not above regulatory threshold. Based on the results of these simulations, there is measureable risk at all locations where *Cryptosporidium* and *Giardia* were found over the period of July through November, 2017. This risk changed based on location and which group was being looked at (adult versus children), but all locations had at least an 8.08 in 10,000,000 risk of getting infected.

The highest risk group was children, who generally had a slightly higher risk of infection at the same location as adults. This is likely due to the increased ingestion rate and greater amount of time spent in the water than the adults. Based on the literature, it was assumed that at fresh water locations, children spent 1.5x more time in the water (DeFlorio-Barker et al., 2018; Schets et al., 2011). Children swimming at Greenlake had the highest risk of infection when comparing children and adults as well. A child's risk of infection from *Giardia* at Greenlake was at least 3.52 /10,000 when looking at the 95%. This is likely due to the increased ingestion of fresh water for children, as well as the high number of cysts observed in Greenlake versus other locations. The lowest risk for adults was getting *Cryptosporidium* at Golden Gardens. This makes sense as there were less total number of oocysts present at Golden Gardens versus Magnuson beach, and because the assumption was that less marine water would be ingested

versus fresh water. A tornado analysis also showed that the first driving factor in the risk estimate was the toggling of the different risk models for *Cryptosporidium*, but the second was the number of cysts and oocysts present in the water. This means that since Golden Gardens had less number of oocysts than Magnuson Beach, the number of oocysts is probably what drove the risk down below the other locations.

The high risk estimates for swimming after the Polar Bear Plunge were above the EPA's acceptable risk in fresh water for children, but not for adults. According to the organizers of the event, there were an estimated number of 1,900 participants at the 2018 PBP at Matthews Beach. For both children and adults, the risk of infection for *Giardia* for both the median and 95th percentile were in the range of 4.42/1000-9.07/1000, but in both percentiles the children had a higher risk of infection (Tables 4.6 and 4.7). The risk of infection from *Cryptosporidium* was lower, likely due to less oocysts than cysts present in the water afterword. Each adult who participated and dunked their head under the water had a 95th percentile risk of 2.98/1000 from *Cryptosporidium* infection, while for children the 95th percentile risk was 4.11/1000. The median risk estimates were below the EPA threshold. It is important to consider though that the persons who were at the front of the crowd and entered the water first likely had a lower risk than what is reported above. This reasoning comes from the idea that there was less water movement and sediment disturbance before those people entered than when the people at the back of the crowd entered the already-turbid waters.

While the parameters in my study used different distributions for ingestion rate and time spent in water, the risk estimates from my simulations fall in line with published estimates from ingesting *Cryptosporidium* and *Giardia* in recreational water. A study by Coupe et al. (2006) estimated the average risk of infection to individuals exposed to lake and river water in Paris to be 1.5/1000 - 46.9/1000 for *Cryptosporidium* and 1.7/1000 - 56.2/1000 for *Giardia*. Their recorded number of Giardia was substantially higher during some sampling days then our studies cyst count. In the North Sea, Schets et al. (2011) showed that adults had an average *Cryptosporidium* infection risk of 8.1/10,000 – 3.7/1000 and an average *Giardia* infection risk of 3.6/100,000-7.6/10,000, while children had an average *Cryptosporidium* infection risk of 9.0/10,000 – 4.9/1000 and an average *Giardia* infection risk of 7.6/100,000-1.0/1000. In this study, researchers assumed that the time spent in the water was an average of 45 minutes, which is above the amount of time that we assumed swimmers spent in Seattle marine waters.

One other interesting observation was that the risk of infection was similar for marine water and fresh water. I had originally hypothesized that the risk of infectious would be greater at fresh water locations because I had made the assumptions that both children and adults ingest more water when they're swimming in fresh water. I believe the similarity is due to the fact that the driving factor that mainly determines the risk is not the ingestion rate or time spent in water, but instead the number of cysts and oocysts in the water; a hypothesis supported by the tornado plot**.**

5.4 Limitations and Directions for Future Research

A limitation of the prevalence and distribution portion of this study was that quantitative turbidity calculations were not taken, so being able to do a comparative analysis of turbidity versus recovery is not available. However, I think that the assumption that greater turbidity resulted in lower recovery is justifiable based on the qualitative turbidity data taken during each sample. When sampling the water, there was a cognitive decision to reduce the amount of sediment that was resuspended to aid in sample filtration. This decision however, could result in less cysts and oocysts being quantified, when in reality swimmers may be consistently resuspending sediment while they move around in the water. A limitation to this study is that we are unsure if the cysts and oocysts are coming off the swimmers body, or if they are being resuspended during sediment agitation. Future studies should look into the levels of *Cryptosporidium* and *Giardia* before and after the agitation of sediment, to help determine if it is the source of the cysts and oocysts.

Another limitation in assessing low recovery is that due to the fact that both the *Cryptosporidium* oocysts and *Giardia* cysts are stained a fluorescent apple green color for microscopy, it is sometimes difficult to distinguish between a cyst and an oocyst. Misidentification of a cyst or oocyst will lead to misclassification, and will result in a higher or lower prevalence of one organism. To combat this, we recorded the dimensions of each parasite identified and also compared these results to the positive controls used. Limiting factors to the recovery of cysts and oocysts also include the smaller volume of water taken for each sample. The Envirochek® HV filter is validated for filtration of 10L and 50L of water, depending on the water turbidity. In environments where there is low parasite prevalence, taking a sample volume on the low end may result in no detection of organisms just because they weren't picked up in the

small sample. By increasing sample size, it increases the likelihood that the organism will be captured. These factors in low organism recovery can then affect the risk estimates, reducing the amount of estimated risk to swimmers. Researchers should look into developing better methods for capture of cysts and oocysts in marine waters, since many untreated recreational water exposures around the world happen in saltwater.

As with most QMRA studies, a limitation of the model is the absence of concrete numbers for parameters. Assumptions about the ingestion rate and time spent in water had to be estimated based on the literature, but were not from observed data at the beaches. Another limitation was that my parameters did not have both variability and uncertainty; they were one or the other. It is important to have both variability and uncertainty because if not, the resulting risk estimates could be skewed. Not being able to state which species of *Cryptosporidium* were present in the water was another limitation. If the species of *Cryptosporidium* were those that do not results in human infection, then the risk would be negligible. Since this data was unavailable, assuming that all cysts and oocysts counted were infectious could lead to a larger estimate of risk. Future studies should look at developing other qPCR approaches to differentiate *Cryptosporidium* species when the number of oocysts is low.

Finally, what is being reported is the risk of infection per swim event. A more applicable number would be a cumulative risk over the number of times that people use these recreational water sources over the summer. The limitation in this approach however, is obtaining data on exposure factors. Studies that have estimated the number of times people use recreational beaches or lakes took the approach of a questionnaire (DeFlorio-Barker et al., 2018; Schets et al., 2011). This brings up the possibility of recall-bias. In theory, doing a cumulative risk would increase the probability of infection if people were using these sources more than once over the summer.

5.5 Conclusions

The data obtained in this study show that there are *Cryptosporidium* oocysts and *Giardia* cysts present in recreational water bodies in North Seattle. There is a seasonality associated with their presence however, with levels dropping below the limit of detection during early fall. Based on the results of the risk estimates, if there are cysts or oocysts present in the waters of North Seattle, there is some measurable risk to both children and adults when swimming in the water.

This measurable risk; however, is below the EPA recreational thresholds of 19/1000 for marine water and 8/1000 for freshwater. It is important that those who choose to swim in untreated recreational water understand the risk associated with their exposures, in order to reduce the likelihood of getting an infection.

Using the information from this study, possible transmission routes can be modeled in future research and populations with the greatest risk of infection from the two parasites can be identified. The research also shows that the BMFS can be combined with the EPA 1623.1 method to give recoveries for fresh water consistent when only using the EPA method. While this will be useful to water quality researchers working in rural or remote field sites, or areas where climate conditions make sample storage challenging, it is important that future researchers optimize recovery for marine water using EPA 1623.1 methods so that future risk estimates are more accurate.

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Appendix A: Miscellaneous Data

A.1 - Percent recovery of *Cryptosporidium* and *Giardia* (IPR and OPR)

	IPR #1	IPR #2	IPR#3	IPR#4	OPR#1	OPR#2	OPR#3
Date Run	July 6	July 7	July 10	July 11	Aug 7	Oct 15	Nov 8
***Crypto* (%)**	35	56	55	47	51	42	53
***Giardia* (%)**	31	69	49	56	57	45	51

A.2 - *Cryptosporidium* Oocysts Counts from Un-spiked Samples

Sample #	Matthews Beach	Magnuson Beach	Green-lake	Carkeek Park	Golden Gardens	Richmond Beach
1	0	1	0	0	0	0
2	0	2	0	0	2	0
3	0	0	0	0	0	0
4	0	2	0	0	1	0
5	0	0	0	0	0	0
6	0	0	NA	0	NA	NA
7	0	0	NA	NA	NA	NA

A.3 - *Giardia* Cyst Counts from Un-spiked Samples

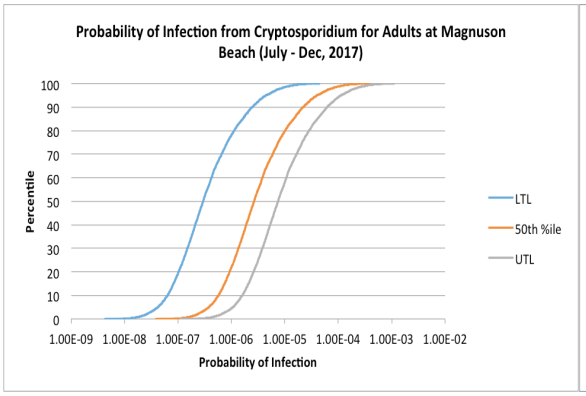
Sample #	Matthews Beach	Magnuson Beach	Green-lake	Carkeek Park	Golden Gardens	Richmond Beach
1	0	0	2	2	4	0
2	3	0	3	2	0	0
3	0	0	2	4	0	0
4	1	0	0	0	5	0
5	0	0	0	0	0	0
6	0	1	NA	0	NA	NA
7	0	0	NA	NA	NA	NA

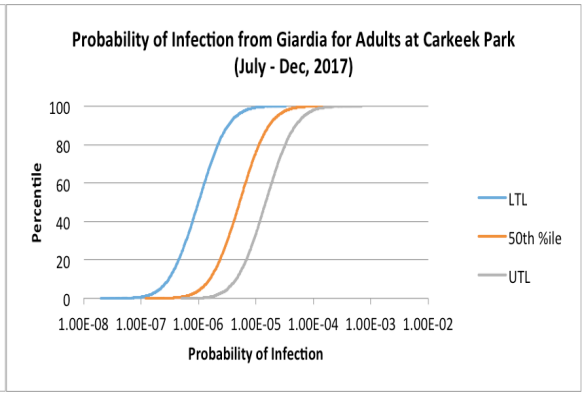
A.4 - Raw Bacteroides Data

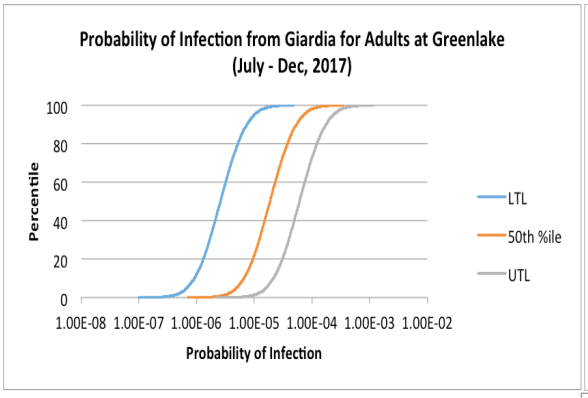
	Avian	Human 1	Human 2	Dog 1	Dog 2
	+/-	+/-	+/-	+/-	+/-
Matthews 1	-	-	-	-	-
Matthews 2	-	+	+	+	-
Matthews 3	+	+	+	-	-
Matthews 4	-	-	-	-	-
Matthews 5	+	-	-	-	-

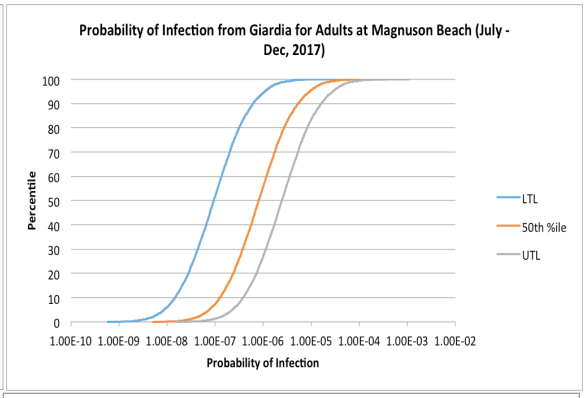
Matthews 6	+	+	+	+	+
Matthews 7	+	+	+	+	+
Magnuson 1	+	-	+	-	-
Magnuson 2	+	-	-	-	-
Magnuson 3	+	-	-	-	-
Magnuson 4	+	-	-	-	-
Magnuson 5	+	+	+	-	-
Magnuson 6	+	-	-	-	-
Magnuson 7	+	-	+	+	-
Richmond Beach 1	-	+	+	+	-
Richmond Beach 2	+	+	+	+	-
Richmond Beach 3	+	+	+	-	-
Richmond Beach 4	+	+	+	-	-
Richmond Beach 5	+	+	+	-	-
Golden Gardens 1	+	+	+	-	-
Golden Gardens 2	+	+	+	-	-
Golden Gardens 3	+	+	+	-	-
Golden Gardens 4	+	+	+	+	-
Golden Gardens 5	+	+	+	-	-
Greenlake 1	-	-	+	-	-
Greenlake 2	-	-	+	-	-
Greenlake 3	+	-	-	-	-
Greenlake 4	+	-	+	+	-
Greenlake 5	+	-	-	+	-
Carkeek 1	+	+	+	-	-
Carkeek 2	+	+	+	-	-
Carkeek 3	+	+	+	+	-
Carkeek 4	+	+	+	-	-
Carkeek 5	+	+	+	+	-
Carkeek 6	+	+	+	+	-
Pol Plunge Bef	+	+	+	+	-
Pol Plunge After	+	+	+	+	+

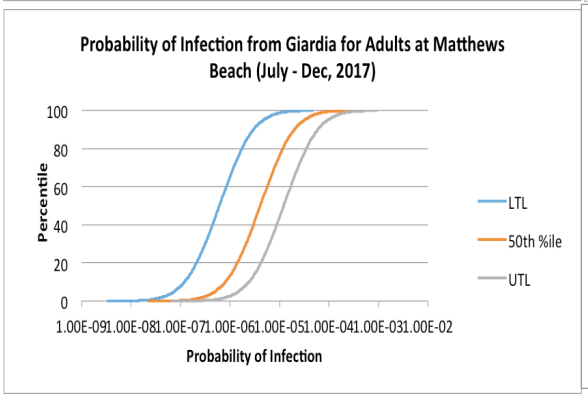
A.5 - S-curve Graphs for Probability of Infection in Adults

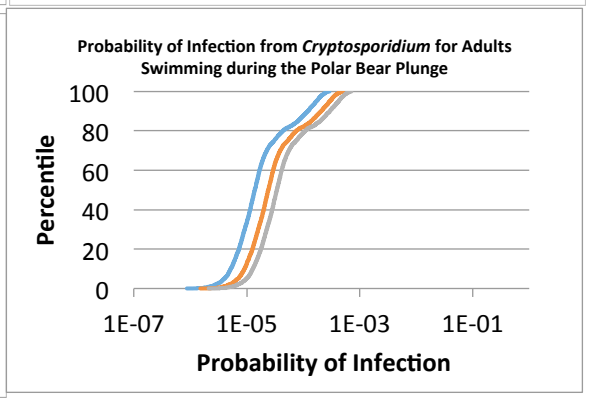




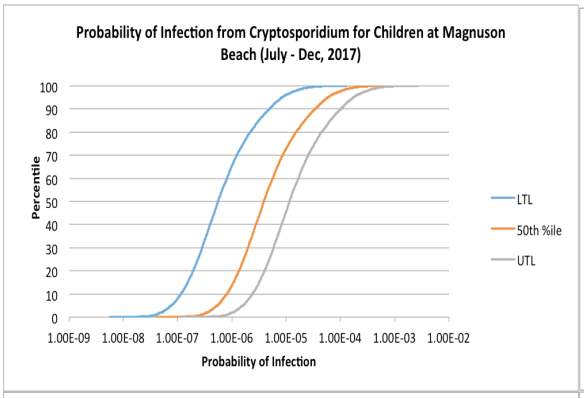


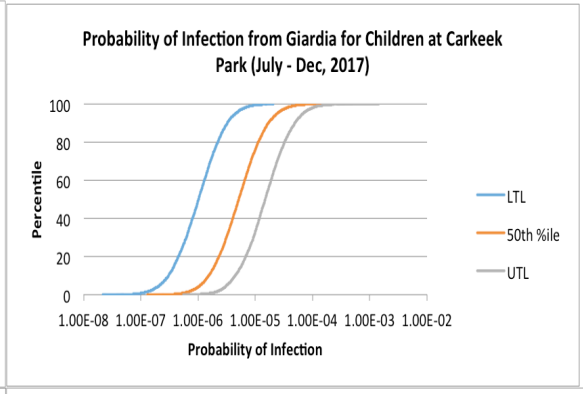


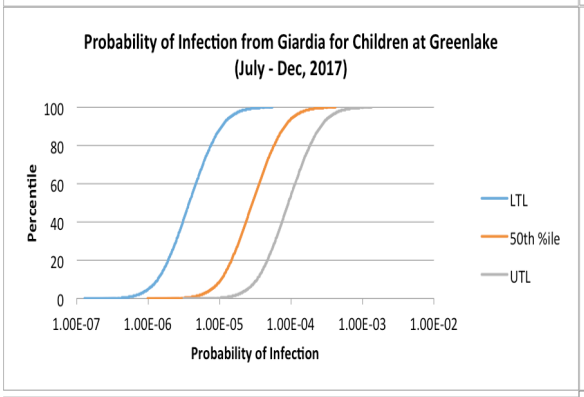


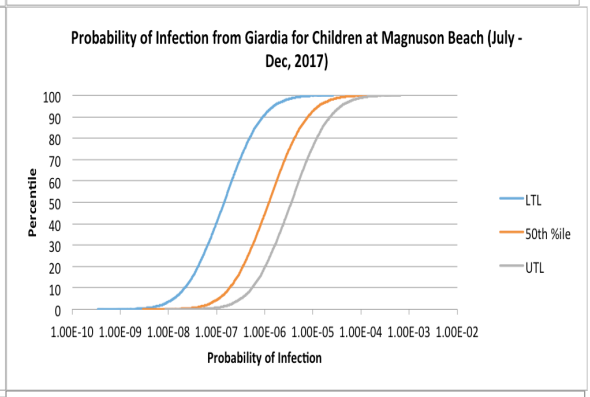


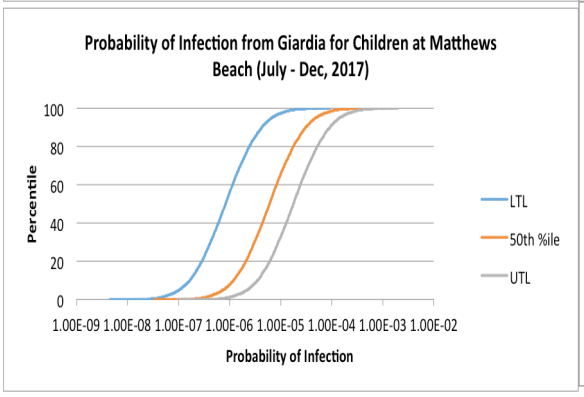
A.6 - S-curve Graphs for Probability of Infection in Children

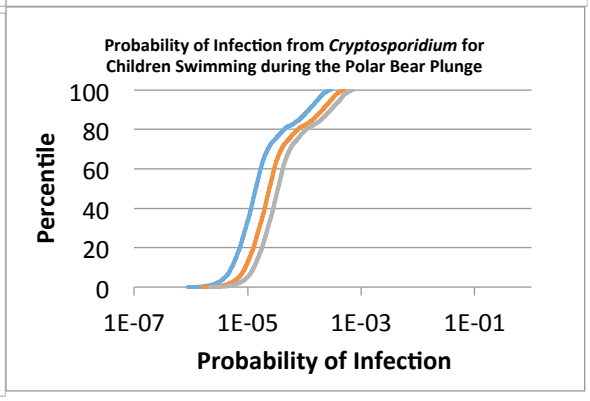












Appendix B: EPA 1623.1 Protocols

B. 1 - EasySeed Spiking Protocol

1. Remove and keep the tube cap.
2. Add 2mL of 0.05% (v/v) Tween 20 to the tube.
3. Replace cap and vortex for 20 seconds.
4. Remove and keep cap and pour tube contents into sample.
5. Add 3mL of reagent grade water to the empty tube.
6. Replace cap and vortex for 20 seconds.
7. Remove and keep cap and pour tube contents into sample.
8. Repeat steps 5, 6 and 7 once more.
9. Analyze the sample as per the laboratory Standard Operating Procedure.
10. Record the number of fluorescent Cryptosporidium and Giardia detected.
11. Calculate the Cryptosporidium and Giardia recovery

B.2 - Elution

Equipment:

Laboratory shaker
3 bench papers (5 if using bench)
2- 175mL conical centrifuge tubes
Conical centrifuge tube bottoms (blue and white things)
Parafilm
Rubber bands
Timer
3- 100mL graduated cylinder
Plastic wrap
Ruler
Pump
1 tube and tube adaptor
1 stub piece of tubing (for adaptor)
Waste container
5mL pipet

Reagent Preparation:

Elution buffer (made weekly)
NaHMP (made when runs out)
DI water

Procedure:

1. Prep hood and table
2. Make elution buffer or NaHMP if needed
3. Remove IMS supplies from fridge
4. Record elution date and time
5. Record anything that may be relevant during elution process (i.e. spills, deviation from protocol, etc.)
6. Hold filter in vertical position with inlet end up
7. Make sure that capsule is not clogged - record
8. Remove inlet cap

9. Swirl NaHMP
10. Using graduated cylinder pour ~125mL NaHMP solution through inlet
11. Allow liquid level to stabilize
12. Add sufficient NaHMP buffer to cover pleated white membrane & plastic
13. Record amount of NaHMP used & height above membrane (cm)
14. Replace cap
15. Cover both cap ends with parafilm and plastic wrap
16. Securely clamp filter on shaker (bleed valve up: 12 o'clock)
17. Use rubber bands and metal springs to secure
18. Set shaker speed to maximum (record speed)
19. Agitate for 5 minutes
20. Remove filter from shaker
21. Prepare pump, tubing and waste container
22. Remove filter outlet cap
23. Attach outlet to tubing & run through a pump
24. Tape tube to waste container
25. Hold filter upright, remove inlet cap
26. Turn on pump, allow to pull all NaHMP out
27. Do not allow filter pleats to collapse
28. Turn off pump
29. Fill capsule with reagent water
30. Add sufficient water to cover pleated white membrane & plastic
31. Allow liquid level to stabilize
32. Turn on pump, allow to pull all water out
33. Turn off pump
34. Replace inlet cap
35. Disconnect tubing and replace outlet cap
36. Record time
37. Hold filter in vertical position with inlet end up
38. Remove inlet cap
39. Using graduated cylinder pour ~125mL elution buffer through inlet
40. Allow liquid level to stabilize
41. Sufficient elution buffer to cover pleated white membrane & plastic
42. Record amount of buffer used and height above membrane (cm)
43. Replace cap
44. Cover both cap ends with parafilm and plastic wrap
45. Securely clamp filter on shaker (bleed valve up: 12 o'clock)
46. Use rubber bands and metal springs
47. Set shaker speed to maximum (record speed)
48. Agitate for 5 minutes
49. Remove filter from shaker
50. Remove inlet cap
51. Pour contents into first 175 mL centrifuge tube
52. Hold filter in vertical position with inlet end up
53. Remove inlet cap
54. Using graduated cylinder pour ~125mL elution buffer through inlet

55. Allow liquid level to stabilize
56. Sufficient elution buffer to cover pleated white membrane & plastic
57. Record amount of buffer used and height above membrane (cm)
58. Replace cap
59. Cover both cap ends with parafilm and plastic wrap
60. Securely clamp filter on shaker (bleed valve angled: 4 o'clock)
61. Set speed to maximum (record speed)
62. Agitate for 5 minutes
63. Securely clamp filter on shaker (bleed valve angled: 8 o'clock)
64. Set speed to maximum (record speed)
65. Agitate for 5 minutes
66. Remove filter from shaker
67. Remove inlet cap
68. Pour contents of capsule into second 175 mL centrifuge tube
69. Rinse filter with 5mL reagent water using pipet
70. Make sure as much of eluent as possible has been transferred
71. Replace the inlet cap
72. Manually swing the filter capsule through an arc of ~180 degrees to retrieve more eluate
73. Be careful not to spill when pouring into centrifuge tube
74. Record volume of elution buffer that came out of centrifuge tube 1
75. Record volume of elution buffer that came out of centrifuge tube 2 (after rinse)
76. Record number of 180 degree whips
77. Keep waste container for concentration/purification

B.3 - Concentration

Equipment:

Centrifuge
Conical centrifuge tubes with sample

Setup:

Run takes 40min from start

1. Set centrifuge to 2000xG for MatB and MB; use 1500xG for all others
2. Record speed
3. Deceleration: OFF
4. Time: 15 minutes
5. Rotor: JS-5.3
6. Temperature: 20 degrees Celsius
7. Weigh counterweights of the centrifuge tubes - use DI water
8. Make sure there is conical holder on bottom
9. Words facing out, tighten middle, try to lift
10. Listen after turning on
11. Record info in notebook on table
12. Prepare waste container and 25mL pipet while centrifuging
13. After cycle: return stop time and turn off centrifuge
14. DO NOT WAIT LONG AFTER REMOVING FROM CENTRIFUGE TO START PURIFICATION

B.4 - Purification

Equipment:

25mL pipet
2- 5mL pipets
4- 1mL pipets
Flat-sided tube
2- 1.5mL micro centrifuge tube
Waste container
MPC-1
MPC-M
Vortex
Slow shaker
Timer
2 well slides
NaOH
HCl
Dynabeads kit
Elution buffer
Kimwipe

Reagent prep:

1.6 mL of 1x SL-Buffer A dilution = 1.44 mL DI water + 160 uL 10x SL-Buffer A
1x PBS

Setup:

If both pellets add to <1mL (generally MatB and MB) do the following, if not see bottom of protocol:

1. Record estimated pellet volume
2. Using 25ml pipet pipette, carefully aspirate ALL supernatant in tube 1
3. DO NOT aspirate oocysts and cysts
4. Using same 25ml pipet, carefully aspirate supernatant in tube 2 to 5mL above pellet
5. Pre-rinse a 5ml pipet with elution buffer
6. Vortex tube 1 until re-suspended
7. Using pre-rinsed pipet transfer tube 1 in tube 2
8. Do not remove pipet tip from pipetor
9. CALCULATE tap water rinse: ((10mL – tube 2)/2). Record rinse amount.
10. Rinse tube 1 with calculated DI water, swish around tube sides
11. Pipet rinse into tube 2 – do not throw away pipet tip
12. Visually inspect tube 1 to ensure no concentrate remains
13. Swirl to reduce foaming
14. Add 1mL 10x SL-Buffer A (non-dilution) to flat sided tube
15. Add 1mL 10x SL-Buffer B to same flat sided tube
16. Vortex tube 2 10-15 seconds until re-suspended
17. Using previous pre-rinsed pipet tip, transfer sample concentrate to flat sided tube
18. Record volume transferred to IMS via pipet tip
19. CALCULATE tap water rinse: (10mL – volume). Record rinse amount.
20. Rinse tube 2 with calculated tap water, swish around tube sides
21. Pipet into flat tube with previous pre-rinsed pipet tip

22. Visually inspect centrifuge tube to ensure no concentrate remains
23. Vortex Dynabeads Crypto for 10 sec
24. Ensure beads are fully re-suspended: invert sample tube, no residual
25. Add 100uL of Dynabeads Crypto to flat tube touching side of tube
26. Vortex Dynabeads Giardia for 10 sec
27. Ensure beads are fully re-suspended: invert sample tube, no residual
28. Add 100uL of Dynabeads Giardia to flat tube
29. Affix tubes to rotating mixer flat side down (molecular lab)
30. Rotate at speed 8 for 1 hr at room temp
31. Record rotating speed
32. Make 1.6mL 1x SL Buffer A (recipe above)
33. Put IMS back into fridge
34. Remove from mixer
35. Prepare a 1mL Pasteur pipet and waste container
36. Place snugly in MPC-1, flat side toward magnet
37. Tape MPC-1 to magnet, do not remove tube from magnet
38. Place magnet side downwards, so tube is horizontal, flat side down
39. Gently rock tube by hand end-to-end through ~90˚, tilt cap end and base end up and down in turn
40. Tilt 2 minutes, 1 tilt/sec
41. If sample is allowed to stand motionless for >10sec, remove from MPC-1 shake tube and repeat 18-22
42. Return MPC-1 to upright position, cap at top
43. Immediately remove cap
44. Keeping flat side of tube on top, pour supernatant into waste container
45. Do not shake tube or remove from MPC-1
46. Allow more supernatant to settle
47. Aspirate with pipette
48. Use kimwipe to blot end of flat-sided tube and cap
49. Remove from MPC-1
50. Prepare a 1mL Pasteur pipet
51. Add 0.5mL 1x SL-Buffer A dilution to flat side of tube
52. Do not touch the round side
53. Mix gently using 1.0mL pipet to resuspend
54. Release liquid down flat side of tube
55. Do not vortex
56. Place microcentrifuge tube in MPC-M with magnetic strip
57. Pipette sample into labeled 1.5mL microcentrifuge tube
58. Rinse with 0.5mL 1x SL-Buffer A dilution
59. Mix gently using 1.0mL pipet to resuspend
60. Release liquid down flat side of tube
61. Pipette sample into labeled 1.5mL microcentrifuge tube
62. Repeat steps 58-60
63. Allow flat sided tube to sit for 5 min
64. Pipette sample into labeled 1.5mL microcentrifuge tube
65. Ensure all liquid and beads are transferred

66. Do not remove tube
67. Gently rock/roll tube through 180˚ by hand
68. Do it 1 min, 180˚ roll/sec
69. Beads should produce a distinct brown dot on back of tube
70. Hold tube against MPC-M and open carefully
71. Using 1mL micropipette, immediately aspirate supernatant from tube and cap - BE CAREFUL
72. Do not shake the tube
73. Gently add 1mL of 1X PBS to tube - Do not disturb pellet on tube wall
74. Remove magnetic strip from MPC-M
75. Gently rock sample 10 times at 180˚ until beads resuspended
76. Replace magnetic strip in the MPC-M
77. Repeat steps 66-72
78. Allow tube to sit for 1 min
79. Using 20uL pipet, aspirate residual liquid
80. Remove magnetic strip from MPC-M
81. Add 50uL of 0.1 N HCl
82. Briefly spin down tube
83. Vortex at highest speed for 50 sec
84. Place tube in MPC-M (no magnet)
85. Allow to stand in vertical position for 15min at room temp
86. Get well slide from fridge & label
87. Vortex for 30 sec
88. Very briefly spin down tube – if a pellet forms briefly spin down
89. Ensure all sample is at base of the tube
90. Place in MPC-M WITH magnetic strip
91. Allow to stand in vertical position for 15sec
92. Add 5 uL of 1N NaOH to well 1
93. Without removing microcentrifuge tube, pipette all sample into well - DO NOT disturb beads
94. Record time sample was applied
95. Repeat 80-94 with other well
    If beads get on outside of pipet tip, gently wipe off with kimwipe
96. Air dry well slides in pipet tip box in drawer – careful not to spill

If both pellets are above 1mL do the following:

- (3) Leave 10mL supernatant above pellet
- (8) CALCULATE tap water rinse: ((20mL – tube 2)/2).
- (17) Transfer 10mL of sample to flat sided tube. Use pipet to measure amount of sample left in conical. Record amount. Excess sample into waste container.
- (19) No rinse of tube 2

B.5 - Staining

Equipment:

Clear nail polish
Merifluor Staining kit

Sample well slide
Control well slide
Humidity chamber: foil & wet paper towels

Solutions:

Merifluor Solutions
DI water
DAPI staining solution

Setup:

1. Prepare humid chamber
2. Bring entire kit to room temperature
3. UV the hood
4. Put bench paper in hood
5. Prepare DAPI staining solution
6. Mix reagents thoroughly before use
7. Label control well slide
8. Use transfer loop to transfer drop of Positive control on well slide
9. Spread over entire well - DO NOT scratch
10. Use new transfer loop to transfer drop of Negative control on well slide
11. Spread over entire well - DO NOT scratch
12. Allow slides to dry (about 30 minutes)
13. One drop of Detection Reagent in each well
14. One drop of Counterstain in each well
15. Mix reagents with applicator stick, spread over entire well - DO NOT scratch
16. Incubate slides in humidified chamber - 30min at room temp - NO LIGHT
17. Allow condensate to evaporate if present
18. Apply 1 drop of 1X wash buffer to each well
19. No not submerge the slides in wash buffer
20. Tilt slide long edge of slide on clean paper towel
21. Remove excess buffer - aspirate with pipet or absorb with paper towel
22. DO NOT disturb sample, DO NOT allow slides to dry
23. Apply 50uL DAPI staining solution to each well
24. Let stand at room temp - 5min
25. One drop of 1X wash buffer to each well
26. Tilt slide long edge of slide on clean paper towel
27. Remove excess buffer - aspirate with pipet or absorb with paper towel
28. DO NOT disturb sample, DO NOT allow slides to dry
29. One drop of Mounting Medium to each well
30. Apply coverslip
31. Use tissue to remove excess mounting fluid on edges
32. Seal edges using nail polish
33. Record date and time
34. If slides not read immediately, store in humid chamber in the dark between 1C and 10C