2012

Use of In-Situ Biofilms to Monitor for Cryptosporidium in Eastern Pennsylavnia Watersheds

Robin Kelly Barnes-Pohjonen
Lehigh University

Use of *In-situ* Biofilms to Monitor for *Cryptosporidium* in Eastern Pennsylvania Watersheds

by

Robin Kelly Barnes-Pohjonen

A Thesis

Presented to the Graduate and Research Committee

Of Lehigh University

In Candidacy for the Degree of

Master of Science

In

Environmental Engineering

Lehigh University

June, 2012

Certificate of Approval

This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science degree.

_____

Date

_____

Thesis Advisor

_____

Chairperson of Department

Acknowledgements

I would like to acknowledge the advice and guidance of Dr. Kristen Jellison. I also thank lab members, Dr. Elizabeth Wolyniak-DiCesare and Julie Napotnik for their patience, guidance and suggestions. Their advice, encouragement, friendship and assistance helped make this project successful. I also thank Colin McLeod for his assistance. A special thanks to Beth Couillard for assistance with sample processing, as well as for being a great office mate! Without the knowledge and assistance of these few, this study would not have been successful.

I acknowledge the Philadelphia Water Department, Office of Watersheds, for their financial support for this project.

The major experiment materials for this study, two different prototype biofilm samplers, were constructed by Dan Zeroka of Lehigh University. His help in laboratory up-keep was invaluable as well. I appreciate his time, effort and support.

I would like to thank my husband, Kasperi Pohjonen, and my parents, Kitsy and Darrell Barnes, for supporting and encouraging me to pursue this degree.

Table of Contents

List of Tables

List of Figures

Abstract

Cryptosporidium is a waterborne pathogen that causes significant gastrointestinal infection in humans. Oocysts, commonly found in surface waters in low concentrations, are difficult to detect, and traditional water filtration sampling can underestimate concentrations. A new method uses natural biofilms grown over time on glass slides *in-situ* to monitor for *Cryptosporidium*. Slides were placed by the Philadelphia Water Department at the Queen Lane drinking water intake (Philadelphia, PA) and in Sandy Run (Abington, PA). The Queen Lane site, in the Schuylkill River, 0.5 miles downstream from the confluence with Wissahickon Creek, drains approximately 54 million L/day of treated wastewater from 5 municipalities. Sandy Run sites are upstream and downstream of a wastewater treatment outfall. Lehigh University sampled Saucon Creek (Hellertown, PA), and Monocacy Creek (Bethlehem, PA) as control, or non-wastewater impacted sites, and the Lehigh River, upstream and downstream of the Bethlehem wastewater treatment plant (BWWTP). Oocysts embedded within biofilm growth were recovered using immunomagnetic separation and processed by fluorescent *in-situ* hybridization (FISH) resulting in *Cryptosporidium* oocyst counts. Water filter samples were taken at Queen Lane and Monocacy Creek for comparison to biofilm samples. Queen Lane samples showed consistent detection of oocysts in biofilm samples while only sporadic occurrences were observed in water filter samples. Use of the biofilm method coupled with FISH processing resulted in the most sensitive oocyst detection when compared with water filtration processed by any method. The Sandy Run and BWWTP sites showed consistently higher oocyst counts in the downstream sites compared to upstream sites. Monocacy and Saucon Creeks, the control sites, exhibited consistent oocyst contamination thereby displaying the sensitivity of the

biofilm sampling method to disperse *Cryptosporidium* sources such as golf courses or agricultural runoff. Overall, biofilm slide sampling was highly sensitive to even low oocyst concentrations, and the biofilm method combined with FISH processing was most sensitive for detection. These results could be due to higher water volumes sampled, as the biofilm integrates particulates in the water column over time. Further investigation and validation of this method is needed, however current results support this method for identifying the presence of *Cryptosporidium* in watersheds and hotspots of contamination.

1. Introduction

Waterborne disease has been a concern in the United States since the early 1900s, and water treatment methods such as filtration and chlorination have long been considered necessary components in public drinking water systems. Even with these methods in place, waterborne diseases have continued to be problematic in the United States, with outbreaks of disinfectant-resistant microorganisms such as *Giardia*, rotavirus and Norwalk virus and *Cryptosporidium* plaguing many cities in the 1960s, 1970s and 1980s, respectively (Rose, 1997). *Cryptosporidium*, which is now found worldwide, during the past two decades has become recognized as one of the most common causes of waterborne disease in humans in the United States (Rose, 1997).

Cryptosporidium, a group of waterborne protozoan parasites, affects the intestines in mammals by causing an acute gastrointestinal infection, cryptosporidiosis (Smith and Rose, 1990). In the 1980s, soon after the first recorded case of human cryptosporidiosis was reported by Nime et al. (1976), cryptosporidiosis emerged as a common life-threatening disease for immunocompromised individuals such as the elderly or those with aquired immune deficiency syndrome (AIDS) (Smith, 1990). Recorded cryptosporidiosis cases in humans were up to the count of 58 by 1984, 40 of which (69%) were in immunocompromised patients and 33 of these 40 (83%) immunocompromised patients had AIDS (Guerrant, 1997). These 40 immunocompromised patients subsequently contracted severe, often irreversible, diarrhea, and in 65% of the 40 individuals these symptoms lasted longer than 4 months, and 55% died from the disease (Guerrant, 1997).

While infection in immunodeficient individuals is very common, immunocompetent individuals are also at risk. Guerrant (1997) reported that 17 to 32 % of immunocompetent

individuals in the states of Wisconsin, Virginia and Texas, along with Peace Corps volunteers before they had travelled overseas, showed seriological signs of infection by *Cryptosporidium* by young adulthood. Furthermore, around the world in industrialized and developing nations, *Cryptosporidium* infection was found in 2.1 and 6.1 %, respectively, of immunocompetent individuals. (Guerrant, 1997). While recent advances have now made medical treatment of cryptosporidiosis possible, these options are not entirely effective for those with compromised immune systems (Abuvakar et al., 2007).

Cryptosporidium has a life cycle that enhances its ability to infect many different mammals including domesticated animals, wildlife and humans, through waterborne transmission (Walker, Montemagno and Jenkins, 1998). Infection occurs upon the ingestion of disinfectant-resistant, thick-walled oocysts. Oocysts, which are the dormant form of the *Cryptosporidium* parasite, are about 4-6μm in diameter and the thick walls allow them to persist outside a host, and retain their infectivity, for long periods of time (Smith, 1990; Graczyk et al., 2000; Helmi et al. 2008). Once the oocysts reach the upper bowels in their hosts, excystation, or the opening of the thick shell wall, is triggered releasing four infectious sporozoites. The sporozoites tend to settle in the lower intestinal epithelial environment and proceed to reproduce either sexually or asexually (Walker, Montemagno and Jenkins, 1998). Through the sexual cycle, both thick and thin-walled infectious oocysts are created, the former for excretion into the outside environment through feces and the latter to continue infection within the same host (Guerrant, 1997). Given this autoinfectious cycle, as well as the excretion of large numbers of hardy, infectious oocysts into the environment, it is not surprising that as few as 30 oocysts can cause infection, and cases in which one oocyst has caused infection have been observed (Smith, 1990; Haas and Rose, 1994).

A variety of settings have been reported conducive to the spread of Cryptosporidium, from day care centers and hospitals via person-to-person transmission (Alpert et al., 1986), hospitals also spread person to person via personnel (Kock et al., 1985) to international travel via ingestion of unclean water (Jokipii, Pojola & Jokipii, 1985). The importance of waterborne transmission of *Cryptosporidium* has now been accepted worldwide as well as in the United States where the Centers for Disease Control report an increasing number of cases from 2003 (3505 cases) through 2008 (10,500 cases) often attributable to multiple large recreational water-associated outbreaks (Yoder and Beach, 2007; Yoder, Harral and Beach, 2010). In addition to recreational water-associated outbreaks, infection has been caused by drinking water contamination or treatment failure in many cases, the largest report to date being the Milwaukee outbreak in 1993, which was caused by the failure of one of the city's treatment plant filtration systems (MacKenzie et al., 1994). During this major outbreak, an estimated 403,000 people were infected with *Cryptosporidium* (Guerrant, 1997).

The ultimate source of *Cryptosporidium* oocysts in the hydrologic environment is now widely accepted to be via feces from infected hosts, whether they be wildlife, domesticated animals or humans. Surface waters used as public sources of drinking water or recreational waters, once contaminated with animal feces can cause cryptosporidiosis outbreaks (Howe et al., 2002) and the primary source of infection in humans is from the consumption of contaminated drinking water (Casemore, 1990). Surface waters used by humans can become contaminated in a variety of ways such as inadequate or transient failure of water treatment measures (MacKenzie et al., 1994), direct contamination via wildlife feces (Howe et al., 2002; Smith et al., 2006; Jellison, Lynch and Ziemann, 2009), non-point source contamination

from run-off associated with agricultural usage (Todd et al., 1991; Jellison, Hemond and Schauer, 2002), wastewater discharges into surface waters (Madore et al., 1987) or use of recreational waters by infected individuals (Walker, Montemagno and Jenkins, 1998; Smith et al., 2006). By one report, between 5.6% and 87.1% of drinking water samples tested that were not impacted by domestic and/or agricultural waste (i.e., surface, spring and groundwater) contained 0.003 to 4.74 *Cryptosporidium* oocysts/L (Lisle and Rose, 1995). In another study, cattle pasture run-off was associated with higher Cryptosporidium counts in rivers adjacent to pasture land when compared to protected watersheds in British Columbia (Ong *et al.,* 1996a and 1996b).

Oocysts can gain access to drinking water systems through a variety of routes which were discussed previously. Furthermore, once this contamination occurs, human infection has been reported after consumption of the chlorinated and treated water from the drinking water treatment systems, and this process of contamination and infection occurs in a matter of days (Smith, 1990; MacKenzie et al., 1994; Walker, Montemagno and Jenkins, 1998). These observations indicate that oocysts are small enough to effectively move through filtering systems and are not adversely affected by chlorination, which is the most utilized method of disinfection in water treatment systems (Smith, 1990). Therefore, environmental control and adequate testing of surface waters and drinking water sources for *Cryptosporidium* is paramount to public health and safety. The widespread prevalence and hardy nature of this parasite has led to a surge in research regarding *Cryptosporidium* over the last three decades. This research has in turn led to vast improvements in methods for detecting oocysts in natural and drinking water supplies.

Currently, the standard method for monitoring for *Cryptosporidium* in surface waters is EPA's Method 1622 in which a capsule water filter is utilized for collection of a minimum of 10 L of water for one filter sample (USEPA, 1999). Furthermore, in response to the major breakout of cryptosporidiosis in Milwaukee in 1993, the USEPA developed the Long Term 2 Enhanced Surface Water Treatment Rule (LT2) in 2003 that required water treatment providers to monitor for *Cryptosporidium* in source waters through water sampling (USEPA, 2007). However, although monitoring is now required by water treatment providers, literature suggests that oocyst recoveries from natural surface waters using EPA Method 1622 are lower and more variable when compared with spiked control samples using the same methods (Simmons et al., 2001; Francy et al., 2004). Often these lower recoveries of *Cryptosporidium* in natural waters are related to increased turbidity (DiGiorgio, Gonzales and Huitt, 2002; Francy et al., 2004), however the variability of the natural water environment has been shown to generally result in variable oocyst recoveries when using water filtration methods. Results of water filtration methods have sometimes shown no detection, even though oocysts were present, and a general underestimation of concentrations within water bodies tested (Smith and Rose, 1990; DiGiorgio, Gonzales and Huitt, 2002; Francy et al., 2004). Furthermore, monitoring for Cryptosporidium in surface waters can be extremely difficult, expensive and subsequently yield very few samples which may often underestimate oocyst concentrations (Graczyk et al., 2000).

Biofilms have recently become an important area of study with regards to pathogens and viruses in water environments (Flemming, Percival and Walker, 2002; Långmark et al., 2005; Filippini et al., 2006). Biofilms, which are complex aggregations of microorganisms that grow on solid substrates, such as rocks and pipes, are ubiquitous in water environments,

as well as in water distribution pipes (Långmark et al., 2005). Not only are these biofilm environments ubiquitous and available, but many types of particles such as viruses (Filippini et al., 2006; Helmi et al., 2008), pathogenic microorganisms (Långmark et al., 2005; Juhna et al., 2007) and pathogens (Wolyniak, Hargreaves and Jellison, 2009 & 2010) can become embedded for long periods within them. Helmi et al. (2008) observed that both viruses and pathogens (including *Cryptosporidium*) became embedded within a drinking water biofilm after 1 hour of inoculation and were still present within the biofilm after 34 days. Several other studies have also observed that human infectious species of oocysts are able to attach to biofilms very quickly (often within 24 hours of inoculation) and remain there for prolonged periods of time (Keevil, 2003; Searcy et al., 2006; Wolyniak, Hargreaves and Jellison, 2009 & 2010). This potential for adsorption of viable pathogens has become a major concern especially due to observations that often these concentrations of embedded organisms can occur at much higher concentrations than those in the surrounding water environment (Flood and Ashbolt, 2000). For example, Keevil (2003) observed attachment of *C. parvum* to a biofilm composed of a natural microbial assemblage on the order of 14,000 oocysts/cm^2 of biofilm area, and this concentration remained high for several months.

The importance of biofilm processes, such as attachment, penetration and detachment of particles, has been recognized and studied for these reasons. One key feature of biofilms is the ability of the integrated community to detach or slough off due to any variety of reasons, such as internal pressures within the biofilm, increased disinfectant concentrations in the environment, or sudden changes in the hydraulic environment that physically force the biofilms from their substrates (Angles et al., 2007). For example in the study by Helmi et al. (2008) described briefly above, once water flow velocity was increased

in the pipe where the biofilms were grown, the viable oocysts were readily transferred from the biofilm back to the water phase. Another study by Howe et al. (2002) investigated a cryptosporidiosis outbreak in Lancashire, England in 2000 which involved contamination of a single spring source by domesticated animal run-off from near-by grazing land. Continued study of the public's tap water showed a persistence of oocysts within the distribution system even though the drinking water source for the town was switched immediately after it was known to be contaminated and the whole system flushed for up to 23 days afterwards (Howe et al., 2002). Even though there was evidence that no one became infected after the source water was switched, oocysts still remained within the system suggesting that oocysts had become embedded within the biofilms of the distribution system and subsequently released from the biofilms only after a period of time. Results such as these imply that biofilms could potentially serve as contamination sources for drinking water supplies and subsequently pose an important human health risk.

Currently accepted monitoring methods use water filtration for monitoring of *Cryptosporidium* in water, however, as described above, biofilms have been recognized as an important possible factor controlling the migration and transport of this pathogen in natural water environments and water distribution systems. Furthermore, it has been suggested that biofilms have the potential to be helpful ecosystem monitors, especially regarding *Cryptosporidium*, due to their stability even in the presence of environmental variability and their apparent integrative nature over a specific incubation period (Snyder et al., 2005). This thesis study attempts to investigate the potential of using environmentally grown biofilms as a means to more efficiently and cheaply quantify *Cryptosporidium* contamination *in-situ* in natural environments. Similarly to results described above, previous work in our lab showed

that oocysts readily were embedded within laboratory grown biofilms and that oocysts remained within biofilms for up to 25 days after inoculation (Wolyniak, Hargreaves and Jellison, 2009 & 2010). This thesis study describes a novel method of sampling, by biofilm sampler, in which biofilms are grown *in-situ* in natural environments, thus resulting in detection of *Cryptosporidium* contamination over time rather than a "snap shot" view which results from a 10 L water filter sample. Ultimately, ramifications of these cumulative studies to the water industry could potentially be cheaper water sampling methods that utilize biofilms for *Cryptosporidium* monitoring.

1.1 Problem Statement

Water filtration can be extremely expensive, and with implementation of more stringent guidelines for water sampling required for Cryptosporidium monitoring, this can prove problematic especially for smaller scale companies within the water industry. The Pall Corporation offers the Envirocheck Sampling Capsules for sale for $110 each. This amounts to the price per sample, which can severely minimize the ability of the water industry to monitor for *Cryptosporidium* in surface waters.

Presently in our laboratory, once samples are collected, processing methods allow one of two methods for any given sample monitoring for *Cryptosporidium*: 1) fluorescent staining of the oocysts for enumeration purposes or 2) genetic analysis which results in a negative or positive identification in a sample and can ultimately give species specific information for that sample. Both methods result in valuable information necessary for better water management practices, however a combination, if possible, of these methods could potentially provide both pieces of information for all samples.

1.2 Objectives

Previous work in our lab resulted in the creation of a biofilm sampler which could be placed at environmental sites to generate biofilms reflective of those environments where placed. Evidence that the pathogen *Cryptosporidium* was thereafter embedded in the new biofilm growth was gathered from this previous work. The first objective of this thesis, therefore, was to optimize the biofilm *Cryptosporidium* sampling method. Ultimately, this thesis wanted to test the hypothesis that biofilms can be an effective and more affordable sampling method (when compared to water filtration methods) for *Cryptosporidium* contamination in surface waters.

Continued sampling during this study revealed that at sites not expected to be contaminated with *Cryptosporidium* did in fact have oocysts embedded within the biofilms much of the time. Even after more careful selection of 'non-impacted' sites, the definition of non-impacted being that the site has no wastewater treatment discharge flows upstream of sampling, *Cryptosporidium* was still observed at the new sites. The second objective, therefore, was to determine whether a baseline of *Cryptosporidium* contamination existed in non-impacted waters.

Current methods for processing samples potentially contaminated with *Cryptosporidium* utilize the immunomagnetic separation cleaning protocol followed by either fluorescent staining for enumeration of oocyst or polymerase chain reaction and sequencing of the DNA to identify specific species. Only recently has the option to combine protocols, in order to obtain the most information possible on *Cryptosporidium* within samples, become available (AWWA, 2010). Our laboratory, with help from George D. DiGiovanni (American Water Works Service Co., Inc., Belleville, Illinois), created one such method we called the

FISH-to-PCR method. The third objective of this research was therefore to optimize this FISH-to-PCR method.

2. Methods

2.1 Samples

Samples were collected in two watersheds, one nearer Philadelphia, the Wissahickon watershed, and the Delaware watershed nearer Lehigh University (LU). Two main sample types were collected during this thesis study, biofilm slides and water filters. Different sample types and processing combinations were used at various sites and are discussed in more detail below.

2.1.1. Sites

Sites have been divided into Philadelphia Water Department (PWD) sampled regions and LU sampled regions. Figure 1 displays the Wissahickon watershed in which Sandy Run/Abington WWTP and the Queen Lane WTP sampling sites are located. Sandy Run is a tributary on the eastern side of the Wissahickon River on which the Abinton WWTP is located at approximately the half-way point of the creek length as it flows east. The Queen Lane WTP is located just south of the Wissahickon watershed approximately two miles downstream of where the Wissahickon discharges into the Schuylkill River. These two sites were sampled exclusively by PWD personnel.

LU sampled areas were in the Lehigh River watershed, or on a larger scale, the Delaware River watershed. A map of a portion of the Lehigh watershed is depicted in Figure 2 along with specific site locations. The sites designated in Figure 2 in the Lehigh watershed were sampled exclusively by LU students.

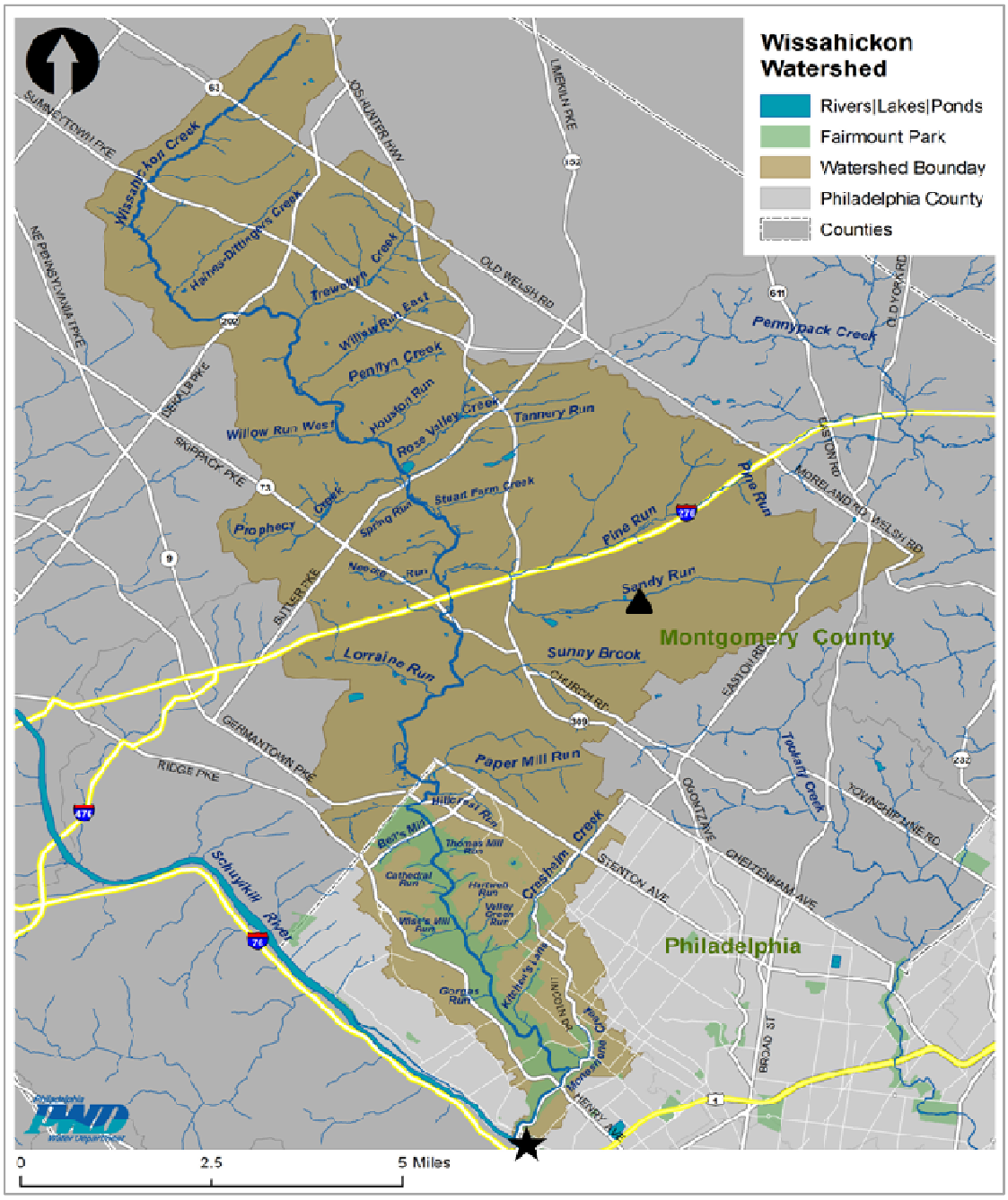

Figure 1. Map of the Wissahickon watershed designating PWD sampled sites
Sandy Run/Abington WWTP is designated by the black triangle at the top and the Queen Lane WTP is designated by the black star at the bottom below where the Wissahickon enters the Schuylkill River. Queen Lane and Sandy Run were sampled as part of Result Set 1 and 2, respectively. Map courtesy of the Philadelphia Water Department from http://www.phillywatersheds.org

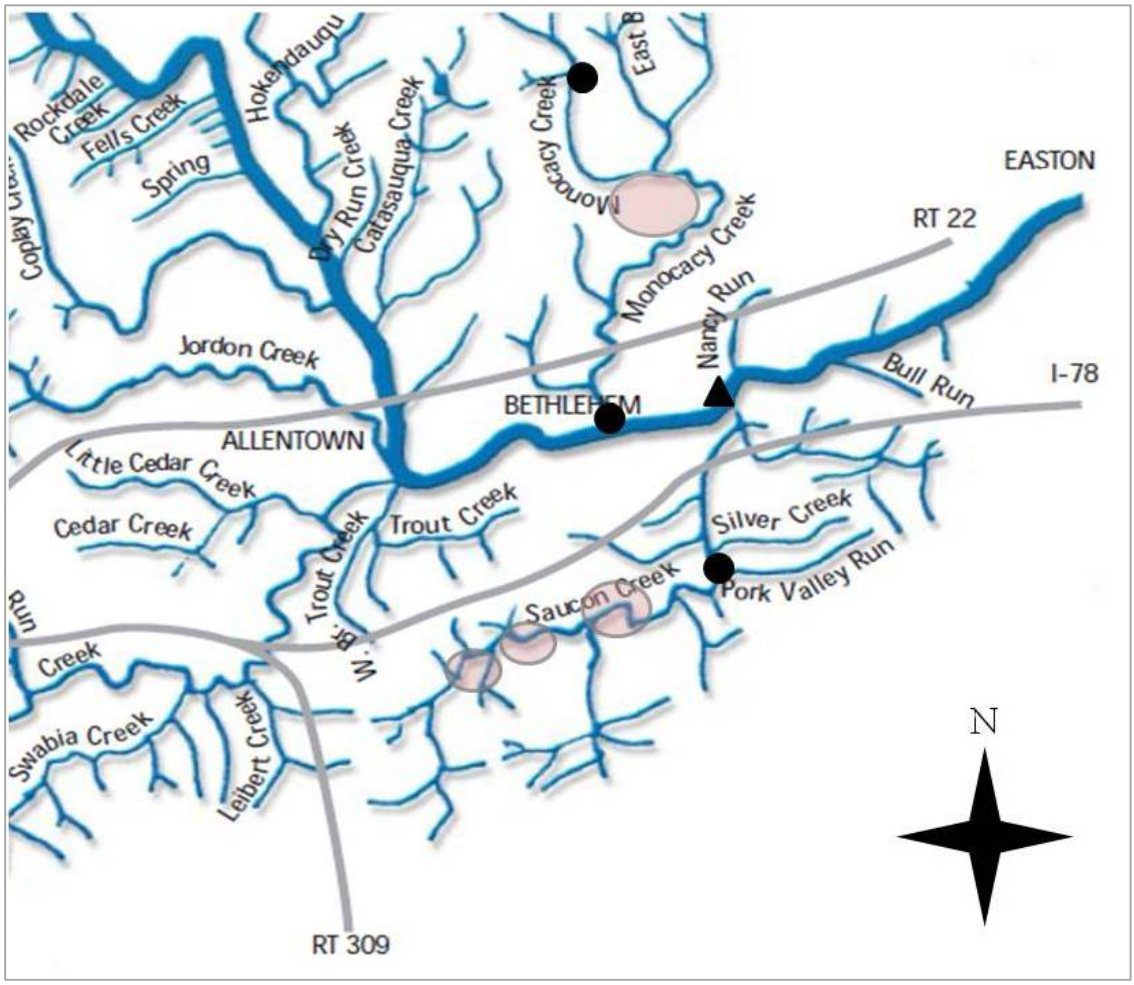

Figure 2. Map of a portion of the Lehigh River watershed designating LU sampled sites
Major city names are displayed as well as major roads, Route 22, Interstate 78 and Route 309, designated by gray lines. The black cirles, respectively from top to bottom, designate sampling locations for Monocacy Creek (New) as part of Result Set 3, Monocacy Creek (the original site) sampled as part of Result Set 2, and Saucon Creek, sampled as part of Result Set 3. The triangle designated the Bethlehem WWTP sampling location, sampled as part of Result Set 3. Shaded circles designate golf course locations, which are potential oocyst sources for sites. Map courtesy of Lehigh Valley Water Suppliers, Inc. at http://www.lvwater.org

2.1.2 Biofilm Slides

Biofilms were sampled in natural stream environments using a biofilm sampler. For the majority of the study, an 'old' prototype biofilm sampler was used, however, a 'new' prototype sampler was constructed later in the thesis study, and the efficacy was studied by using side-by-side oocyst count comparisons in early 2012, at the end of the thesis study

period (Figure B). The old prototype is composed of a plastic box frame, dimensions 8in x 2in x 4in (l x w x h), with an open front and back. Within the frame are six slots, each slot

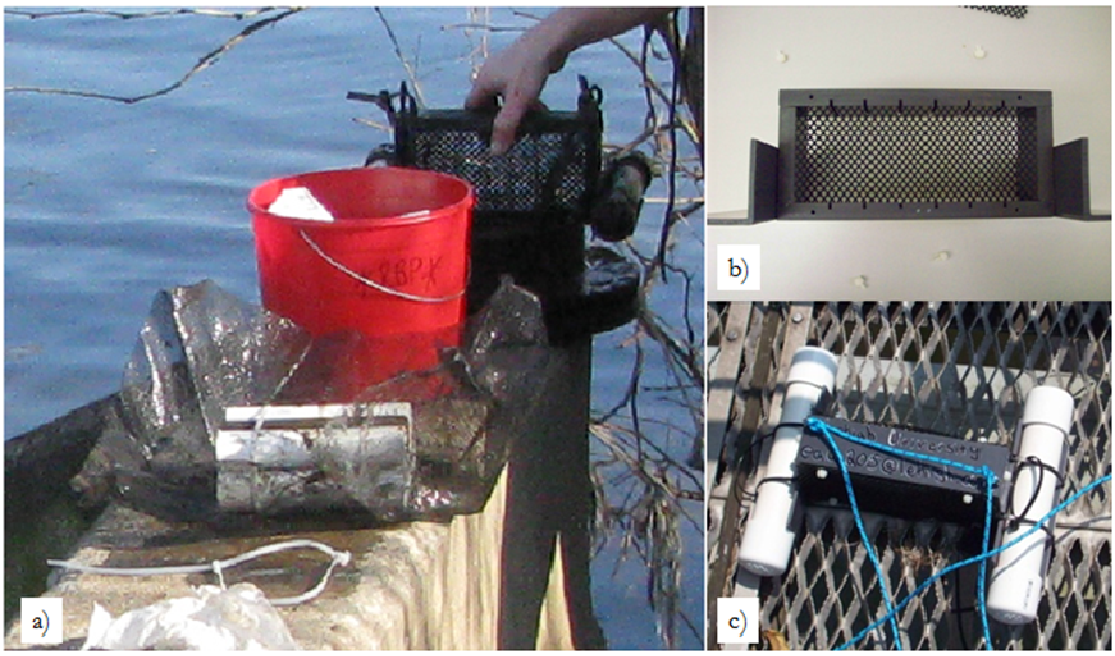

Figure 3. Photographs of the old and new prototype biofilm samplers
a) depicts both samler prototypes: in the background is the old prototype biofilm sampler attached to a heavy weight, while in the foreground is the new prototype biofilm sampler. b) shows the side view of the 'old' prototype biofilm sampler, while c) shows the same sampler from the top with attached side weights and screens.

about one inch apart from the other, in which two microscope slides are fit into each slot, holding a total of twelve slides. The front and back of the frame are protected by a wide mesh plastic screen (~0.4cm opening), which is secured with plastic screws, to prevent macroinvertebrates from grazing on the slide biofilms or debris from impacting the slides. On each side of the box frame, 6 inch segments of 1 inch diameter PVC piping, enclosed and filled with sand, act as weights to ensure minimal sampler movement within the stream bed. In cases of high stream flow, extra weight was added to the bottom of the sampler.

The new prototype biofilm sampler is composed of a steel 1 inch diameter pipe weight with one-inch diameter PVC piping tubes, approximately 6-7 inches in length, on

either side of the pipe weight and four below it secured together with zip ties. All the 6-7 inch tubes are open on both ends, and two glass slides fit securely within each. A square piece of mesh screen was then flattened against both ends of the sampler tube openings (so only one layer of mesh screen was in front and back of the tube openings), wrapped like a present, and secured with more zip ties. Both samplers provided a screen-enclosed area to protect the biofilms, with weights to maintain a position in the stream bed, in which the slides were perpendicular to stream flow. Both sampler prototypes held 12 glass slides on which biofilm would grow during their duration of submersion in the streams, which ranged from 1 to 3 weeks (a few exceptions include a few 28 day periods and one maximum of 36 days), but was usually approximately 2 weeks time. After this submersion period, the 12 slides from each sampler would be carefully removed and placed two slides per 50 mL centrifuge tube. Approximately 10-20 mL of clean creek water would be added to each 50 mL centrifuge tube to prevent desiccation of the biofilm during transport.

Biofilm slides were transported quickly to the lab in 50 mL centrifuge tubes, retrieved from the tubes with forceps, scraped with a clean cell scraper, and rinsed with Millipore water (Milli-Q System; Millipore Corp., Bedford, Mass.) until the slides appeared clear of any particles or debris. The scrapings/rinse water for one sample location were combined and centrifuged at 1100 g for 15 minutes. The supernatant was reduced to 2.5 mL, vortexed, and transferred to an Immunomagnetic Separation (IMS) tube ensuring that no more than 0.5 mL of solids went into any one IMS tube. The centrifuge tube was rinsed with 2.5 mL Millipore water, and the rinse water was transferred to the same IMS tube. The combined 5 mL of scrapings/rinse water was then processed by IMS and Fluorescent *In-Situ* Hybridization (FISH).

2.1.3 Rock Scrapings

To sample rock biofilms, approximately two medium-sized rocks (each nicely fitting in the palm of a hand) and 500 mL of creek water were placed in a bucket. A soft brush was used to gently remove the biofilm from the surface of the rocks. Rocks were brushed until they appeared clean and were thoroughly rinsed with the creek water in the bucket before being returned to the creek. The mixture of scraped biofilm and rinse water was stirred in the bucket to resuspend all particles and carefully poured into 500 mL centrifuge tubes. The samples were transported to the lab and centrifuged at 1100 g for 15 minutes. The supernatant was reduced to 2.5 mL, vortexed, and transferred to an IMS tube. The centrifuge tube was rinsed with 2.5 mL Millipore water, and the rinse water was transferred to the same IMS tube. The combined 5 mL of scrapings/rinse water was then processed by IMS and FISH.

2.1.4 Water Filters

At the Queen Lane WTP and Monocacy Creek sites only, water filter samples were taken in conjunction with biofilm slide samples. From the Queen Lane WTP site, the PWD would collect water filters and ship them in insulated containers with freezer packs to LU via overnight delivery. Water filters were stored at 4°C until processing could commence.

LU site water samples were obtained by filtering surfacewater through Gelman Envirochek™ Sampling Capsules (Pall Gelman Sciences, Inc., Ann Arbor, Michigan) at approximately 1 L per minute, according to manufacturer's recommendations. Filtration continued for about 30 minutes or until the filter clogged precluding more filtration.

Typically, approximately 10 L of water were filtered. Filters were transported to the laboratory, where they were stored at 4°C until processing could commence.

All water filters were eluted according to manufacturer's recommendations within 24 hours of sample collection. Eluted solids were resuspended in 5 mL of Millipore water for each 0.5 mL of solids, stored at 4°C, and processed within 24 hours by IMS and FISH (Jellison, Hemond & Schauer, 2002).

2.2 Processing

Methods of processing included IMS; FISH; genomic DNA extraction, polymerase chain reaction (PCR), cloning and sequencing; and FISH to PCR combination. IMS is a tool that allows us to isolate oocysts from biofilm/sediment slurries and clean up the samples before further processing. All sample types were first processed using IMS. IMS products can then either go through the FISH process, the FISH to PCR process or directly to the DNA extraction and PCR process.

Once the samples are clean, they can proceed to the FISH process, which is a cytogenetic technique in which fluorescent probes bind only the the parts of the chromosome desired (in our case, the probe only bionds with that part of the oocyst RNA that designates it as potentially human infectious). FISH allows us to enumerate oocysts within our samples as well as give us an idea as to percentage of viability within that number.

Any samples in which species data is desired could next go through DNA extraction in which the DNA of any oocysts on the slides or in IMS products are collected through DNA extraction techniques. This small amount of DNA collected would then continue on to PCR, a technique which allows us to multiply a single or few copies of a portion of DNA by several orders of magnitude finally making sequencing possible. Cloning and sequencing

allows us to figure out which specific species of oocysts were encountered within the samples.

2.2.1 Immunomagnetic Separation

Oocysts were purified from water pellets and biofilm slurries by immunomagnetic separation (IMS) with the Virusys IMS kit (ImmTech, Inc., New Windsor, MD) according to the manufacturer's recommendations. If the total volume of water or biofilm pellet exceeded 0.5 mL, the sample was split into multiple IMS tubes (with no more than 0.5 mL of pellet per IMS tube) to ensure that the entire sample was analyzed properly. Oocysts were dissociated from the magnetic beads using 0.05 M HCl; if multiple IMS tests were performed for a given sample, the samples were recombined after the oocyst dissociation step just before subsequent processing. Oocyst suspensions were neutralized with 0.5 M NaOH, centrifuged for 3 minutes at 13,000 g, the supernatant removed and the pellet resuspended in 100 µL of Millipore water. IMS products were stored at 4 °C until subsequent processing, which commenced within 48 hours after completion of IMS. Positive IMS controls consisted of 5 mL of Millipore water spiked with 100 µL of a 1.25 x 10^6 oocyst per mL stock suspension. IMS controls were processed approximately once per month alongside samples to monitor IMS operator efficacy, as well as reduce material waste.

2.2.2 Fluorescent In-Situ Hybridization

Once IMS processing was complete, oocysts were identified from the water filter and biofilm slide pellets using a combined Fluorescent *In-Situ* Hybridization (FISH) and immunofluorescent antibody (IFA) method outlined by Graczyk et al. (2003). The FISH method employs fluorescently-labeled oligonucleotide probes targeted to species-specific sequences of 18S rRNA. The FISH method allows for the numeration of viable and non-

viable (or 'other' as they are named throughout the remaining thesis) oocysts, since viable oocysts only will have enough rRNA, due to its short half-life, to be targeted by the fluorescently-labeled FISH probe. This FISH probe, a *C. parvum/C. hominis* specific oligonucleotide probe, i.e., Cry 1, (5' CGG TTA TCC ATG TAA GTA AAG 3'), targets the positions between 138 and 160 on the *C. parvum/C. hominis* 18S rRNA. The Cry1 probe was synthesized by Sigma Aldrich (St. Louis, Mo.) in 0.05 μm scale, purified by high-performance liquid chromatography, and labeled with hexachlorinated 6-carboxyfluorescein. The concentration of the oligonucleotide probe used in processing was 5 or 1 mmol. The IFA method targets the cell wall antigens of *Cryptosporidium* and *Giardia* using a FITC-conjugated combination of mAb. The IFA method is achieved using a MER*IFLUOR*™ *Cryptosporidium/Giardia* test kit (Meridian Diagnostic, Inc., Cincinnati, OH). FISH and IFA processes were performed on all biofilm and water samples (sometimes only half of the water filter samples when they were split for both FISH and PCR processing) retrieved in the studies of this thesis. The concentration of the mAb used in IFA processing was 1:1 diluted. Positive IFA/FISH controls consisted of 100 μL of Millipore water spiked with 10 μL of 1.25 x 10^6 oocysts/mL stock suspension. Those controls were performed approximately one time per month alongside other samples.

Upon completion of IMS, the pellet was rinsed with Phosphate Buffer Solution (PBS) and oocysts walls were permeabilized in acetone for 15 minutes at room temperature. The permeabilized oocysts were rinsed again with PBS, resuspended in PBS with the Cry1 probe (Graczyk et al., 2003; Vesey et al., 1998), and incubated for one hour at 48 °C. Following incubation, the solution was centrifuged, rinsed with PBS and stained by using the IFA method via the MER*IFLUOR*™ kit. Following 30 minutes incubation in the dark with

the FITC-conjugated combination of mAb's as per the MER*IFLUOR*™ kit instructions, the sample pellets were rinsed with Millipore water and centrifuged twice at 4 °C at 8,000 g for 4 minutes, and then resuspended in approximately 30 µl of Millipore water. Each 30 µl sample was transferred onto a separate MER*IFLUOR*™ slide well and dried in an incubator at 46 °C. When using the FISH method for most samples (all samples collected prior to 1/2012), up to all three wells on a slide were often utilized and then covered with one, long cover slip which was adhered with nail polish to ensure it was locked in place. For later samples, processed in 1/2012 or after, the 30 µL pellets were placed at opposite-end wells of the MER*IFLUOR*™ slides, leaving the central well empty, and each sample was covered with separate, smaller cover slips, which were adhered with nail polish (Figure C). Wells were then examined and oocysts were counted using a Nikon epifluorescence microscope (Nikon, Inc.).

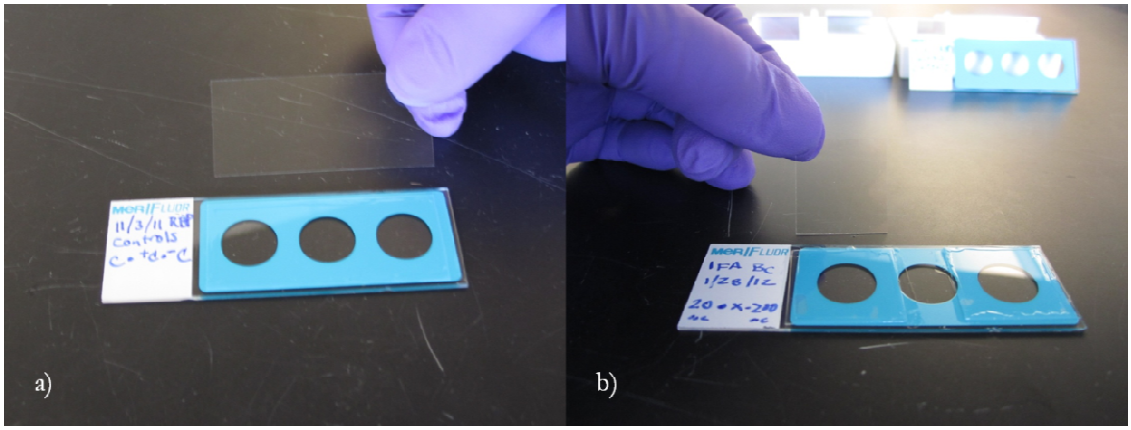

Figure 4. Pictures depicting both slip cover methods used in the FISH protocol
a) shows the older method using the longer, rectangular slip cover that covers all three wells of the slide. b) depicts the use of two smaller, square slip covers to cover a single well on either end of the slide. The second method reduces possible contamination between well samples which is important for the FISH to PCR assay.

2.2.2.1 Fish Counts

FISH slides were viewed under an epifluorescence microscope to gain counts of oocysts, both viable and other, within each sample. At 40X magnification, the FISH slides

were initially inspected; when a potential oocyst was found at 40X, oil was placed atop the adhered slide cover and viewed with the 100X oil objective. Prior to the use of the 100X objective, my predecessor used only the 40X objective for oocyst enumeration.

To perform counts, the FITC examination occurs first, which generally identifies the outer shell of *Cryptosporidium* oocysts. When brilliant, apple-green fluorescing ovoid or sphilical objects were found, and were within the proper diameter range for oocysts (4-6 μm), the magnification would be increased to 100X. The potential oocyst would be carefully examined (USEPA, 1999). Potential oocysts would be examined for proper color (the apple green color should be brightest on the outer shell, with perhaps a very light inner green fluorescence. If this description fit the potential oocyst, then the UV filter block for the Cry1 probe stain would be put in place. Viewed through this filter, if there was only light red staining within the shell of the oocyst, it was considered 'other'. If intense internal red staining was observed, the oocyst was considered 'viable'. Most times that intense internal staining was observed, the distinct sporozoites were not clearly seen. Figure 5 depicts examples of often-observed 'viable' oocysts in environmental samples, compared with control viable and other oocysts from stock concentrations.

Our laboratory does not have the magnification capabilities required by EPA Method 1622/23 to perform an internal morphological exam on oocysts (requires 1000X objective), however, the four stained sporozoites, representative of viable oocysts, sometimes were detected in control samples, but seldom in environmental ones. Figure 6 depicts an example of a viable oocyst with the four clearly detected sporozoites within.

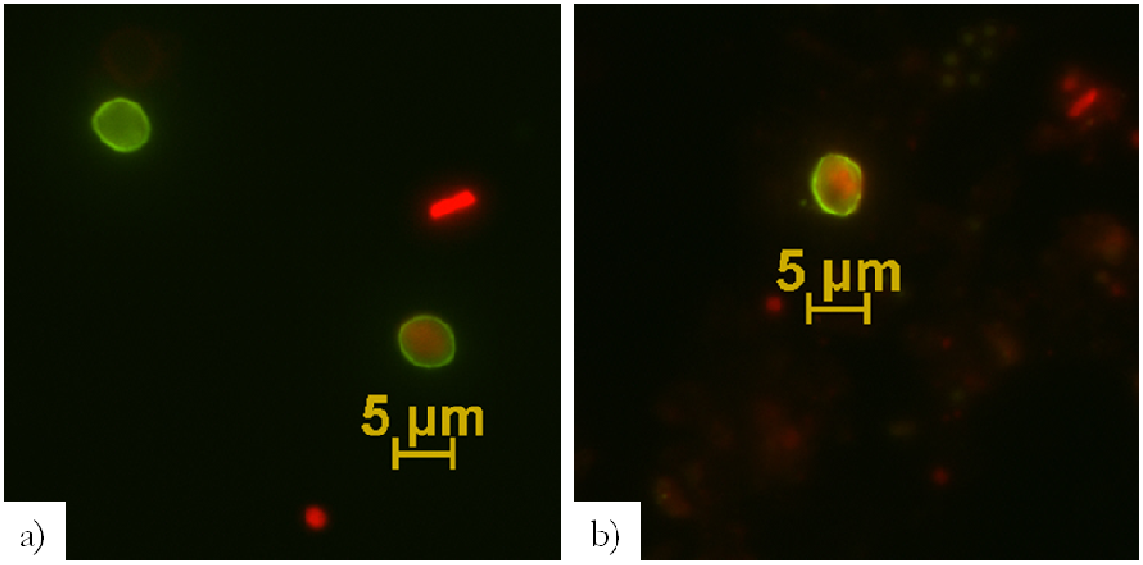

Figure 5. Common example of a viable environmental oocyst compared with laboratory controls
a) depicts two oocysts, the upper left defined as other and the lower right as viable (internal red staining). b) depicts an environmental oocyst, obtained from a rock scraping sample, with internal red staining and comparable characteristics to the viable control.

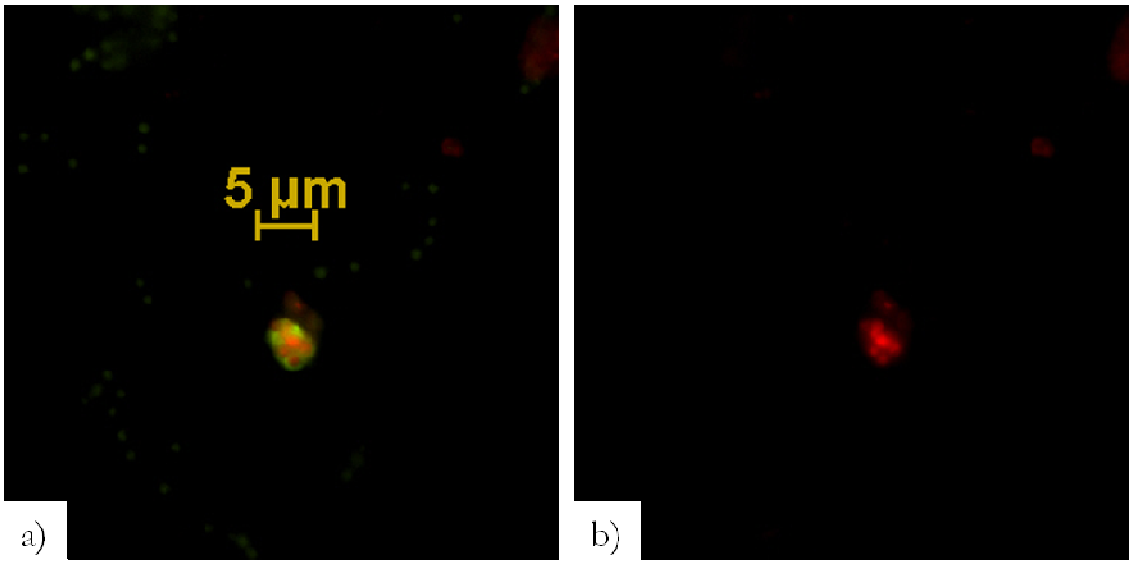

Figure 6. A photograph of an environmental sample viable oocyst with a clear view of the sporozoites within
a) depicts a viable oocyst from an environmental sample where both the FITC and Cry1 probe stains are visible; the sporozoites within are fairly clear. b) depicts only the Cry1 probe stain of the picture viewed in a) so that the sporozoites are more clearly viewed.

2.2.3 Genomic DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing & Cloning

Environmental samples collected from Queen Lane only, early on in the time period of this thesis work, were split following the IMS protocol allowing half of the IMS pellet to be used for both FISH and DNA extraction followed by PCR (See Table A). For all other samples, the processing described in this section was performed on all samples only after they were first processed by the FISH protocol. Following the methods outlined in a grant report written by Jellison (2011), oocysts from these early samples were lysed by adding the IMS product to 450 µl of Tris-EDTA (TE) buffer containing 0.2 g proteinase K per L and 0.4% sodium dodecyl sulfate and incubating the mixture overnight at 45 °C (for samples that went through IMS, to FISH and then to PCR, please see Section 2.2.4. FISH to PCR). Positive DNA extraction controls (consisting of 25 µL of a suspension of 104 oocysts per mL in 475 µL of TE buffer) and negative DNA extraction controls (consisting of 500 µL of TE buffer) were performed for each set of environmental samples. Using phenol-chloroform, DNA was extracted several times, followed by precipitation with 0.2M NaCl and two volumes of absolute ethanol, and resuspension in 30 µL of TE buffer. Nested PCR amplification of the hypervariable region of the 18S rRNA gene and cloning and sequencing of the secondary PCR products positive for *Cryptosporidium* was performed as previously described in a report to the PWD (Jellison, 2011). These methods were not performed by the author but by other LU laboratory operators over the duration of the thesis work period.

2.2.4 FISH to PCR

In an effort to gain information from both FISH and PCR protocols on a whole environmental sample, efforts to combine the FISH and PCR/genotyping protocols were

made early on and environmental samples were soon run using this combined method (all samples collected after 2/2011 used this processing method). Samples undergoing PCR from FISH slides necessitated using a special non-formalin mounting medium (No-Fade Mounting Medium, M101NF, Waterborne, Inc., New Orleans, LA) that would not interfere with genotyping protocols as would the mounting medium provided in the MER*IFLUOR*™ Kit (Personal Communication, G. D. DiGiovanni, American Water Works Service Co., Inc., Belleville, Illinois). Following the FISH protocol with the above provision, slides were wiped with a 6% bleach solution followed by isopropyl alcohol. Non-acetone nail polish remover on a swab was used coupled with a sterile scalpel to carefully remove cover slips, which were then inverted and set aside. Millipore water was added to the slide well, to remove residual mounting medium, aspirated and discarded. Following the Millipore water wash, 15 µL of Millipore water was added to the center of the slide well, and the surface of the slide well was thoroughly scraped with a cell scraper. Upon rotating the slide 90°, this action was repeated, and the Millipore water was aspirated from the slide well and transferred to a microcentrifuge tube. The cover slip was then treated and scraped in the same manner and the 15 µL of Millipore wash water was transferred to the slide well. The slide was rotated 90°, scraped again, and the wash water, originally from the cover slip, was aspirated and transferred to the same microcentrifuge tube. The contents of the microcentrifuge tube were centrifuged briefly before proceeding to the protocols for genomic DNA extraction, PCR, cloning and sequencing, for phylogenetic analysis.

2.3 Sampling Regimes

Biofilm and water filter sampling regimes are summarized in Table 1. Upon collection or receipt of biofilm slides and rock scrapings, the samples were treated and processed as outlined in Sections 2.1.2 Biofilm Slides, 2.1.3. Rock Scrapings, and 2.2.1 IMS unless described otherwise for specific samples; water filters were treated and processed as outlined in Sections 2.1.4 Water Filters and 2.2.1 IMS.

Table 1. Biofilm slide/rock scraping and water filter sampling regimes for the duration of the thesis period
WTP designates a water treatment plant, WWTP designates a wastewater treatment plant and * designates sites where a different operator took over after February 2012.

Site	Biofilms/Rock Scrapings	Water Filters
Queen Lane (WTP)	April 2010 – May 2011 ↓ ~2 X / month FISH (4/10 - 2/11) FISH to PCR (3/11 - 4/11)	April 2010 – April 2011 ↓ ~2 X / month Samples split ½ FISH; ½ PCR (4/10 – 8/10) FISH to PCR (3/11 - 4/11) PCR Only (8/24/10 - 2/11)

Site	Biofilms/Rock Scrapings	Water Filters
Sandy Run (WWTP)	September 2010 – May 2011 ↓ ~2 X / month ↓ FISH (9/10 - 2/11) FISH to PCR (3/11 - 4/11)	Not sampled
Monocacy Creek	July 2010 – April 2011 ↓ ~2 X / month ↓ FISH (7/10 - 2/11) FISH to PCR (3/11 - 4/11)	September 2010 – April 2011 ↓ ~2 X / month ↓ FISH (9/10 - 2/11) FISH to PCR (3/11 - 4/11)
Monocacy Creek (New)	June 2011 – July 2011 ↓ 2 X / month ↓ FISH to PCR	Not sampled.

Site	Biofilms/Rock Scrapings	Water Filters
Saucon Creek*	June 2011 – February 2012 ↓ 2 X / month ↓ FISH to PCR	Not sampled.
Bethlehem (WWTP)*	June 2011 – February 2012 ↓ 2 X / month ↓ FISH to PCR	Not sampled.

2.3.1 Queen Lane Water Treatment Plant (WTP)

Glass slides were deployed using old prototype biofilm samplers at the Queen Lane WTP intake and looked after by the PWD (Figure 1). Slides were collected after varying durations of deployment from April 2010 to September 2010, after which slides were routinely sampled twice per month until May 2011 when sampling halted (Note: a separate operator led the processing from April 2010 through June 2010; the thesis author was in training and FISH counts were double-checked by the lead processor for this period). Biofilm slides collected from April 2010 through February 2011 were processed via FISH only. The FISH to PCR protocol was up and running by March 2011, and samples from March 2011 through April 2011 were processed using this method (Table 1).

Water filter samples were taken in conjunction with biofilms during most of the time biofilm collection was occurring, from April 2010 through April 2011. Water filters were collected twice per month and the samples were split and half processed separately by FISH and PCR from April 2010 through August 10, 2010. From August 24, 2010 through February 2011, water samples were not split but processed via PCR and sequencing only. From March 2011 through April 2011, water samples were processed via the FISH to PCR protocol (Table 1).

2.3.2 Sandy Run/Abington Wastewater Treatment Plant

Sandy Run, into which the Abington WWTP discharge flows, was the location of a quick rock scraping experiment before determining its usefulness as a site in this study (Figure 1). The PWD personnel gently brushed the biofilm off of six rocks into a bucket, mixed the slurry and collected the slurry in 4-50mL centrifuge tubes (Note, this rock scraping protocol differs slightly from that used by LU; See Section XX. Rock Scraping). This was performed both in the upstream and downstream areas of the discharge pipe flow into the creek. These one-time rock scraping samples were shipped on ice to LU for processing. Upon determination the site would yield useful results, it was afterwards sampled using only biofilm samplers in both the upstream and downstream areas of the discharge pipe, from September 2010 through May 2011. These samplers were looked after by the PWD, and biofilm slides were collected approximately twice per month. Biofilm slides collected from September 2010 through February 2011 were processed via FISH only, while samples collected from March 2011 through May 2011 were processed using the FISH

to PCR method (Table 1). Due to steep grades at this location, no water filters could be collected.

2.3.3 Monocacy Creek

Monocacy Creek, monitored by LU students, was sampled approximately twice per month for biofilm slides and natural rock scrapings from July 2010 through April 2011 (Figure 2). Water filter samples were also collected at this site approximately twice per month from September 2010 through April 2011. Water filters were collected by a different operator, which usually resulted in different sampling days for biofilms and water filters. All sample types, biofilm slides, rock scrapings and water filters, were processed only by FISH from the beginning of collection through February 2011, after which processing was performed using the FISH to PCR protocol (Table 1).

2.3.4 Monocacy Creek (New), Saucon Creek and Lehigh River/Bethlehem WWTP

In April 2011, new sites were chosen for only biofilm slide sampling: Monocacy Creek (New) (upstream of the previous location on the same creek), Saucon Creek and the Bethlehem WWTP, both upstream and downstream of the discharge pipe into the Lehigh River (Figure 2). Monocacy Creek (New) biofilm slides were only collected twice per month by LU from June 2011 through July 2011 due to high creek flow and loss of biofilm samplers. Both Saucon Creek and the Bethlehem WWTP (upstream and downstream) biofilm slides were sampled approximately twice per month by LU from June 2011 through February 2012, after which sampling and processing was taken over by another operator.

Only biofilm slides were collected from these three sites and were processed via the FISH to PCR method; no water filters were collected at any time (Table 1).

2.3.4.1 New Biofilm Sampler Prototype

Two short-term experiments used the Bethlehem WWTP upstream and downstream sites, as well as the Saucon Creek site. One experiment tested the efficacy of a new prototype of biofilm sampler that was constructed during 12/2011. The new prototype biofilm sampler (See Section 2.1.2 Biofilm Slides for a description of the new prototype sampler) was used in conjunction with the old prototype to acertain whether the two samplers yielded comparable results. Only four dates using both sampler prototypes were sampled by the author, from 1/11/12 through 2/21/12.

2.3.4.2 Biofilm Thickness

A second experiment run from 1/24/12 through 2/28/12, using the same two sites as described for the new biofilm sampler prototype, focused on biofilm thickness over time. Table 2 depicts the sampling regime set up for the biofilm thickness experiment. Glass slides were placed in old prototype biofilm samplers at each of the sites, allowed to sit for

Table 2. Sampling regime for the biofilm thickness experiment
'ns' designates a day no samples were taken.

	Slides placed at site	Week 1 picked up	Week 2 picked up	Week 3 picked up
Date:				
1/24/12	BWWTP	--	--	--
1/31/12	--	ns	--	--
2/7/12	SC	--	BWWTP	--
2/14/12	--	SC	--	BWWTP
2/21/12	--	--	SC	--
2/28/12	--	--	--	SC

1, 2 or 3 weeks, collected after each exposure duration and carefully brought back to the lab. The samples sat for no more than 48 hours before analysis under a laser scanning confocal microscope. Fifty thickness measurements were taken on one slide per site and collection date. The measurements were taken down the center transect of the biofilm slide using the ZEN 2008 software at 10X magnification resulting in a μm thickness at each of the 50 points across the slide. These measurements were then averaged to yield the mean biofilm thickness per slide.

3. Results

Data was worked up in several rounds, or 'result sets', using data from certain sites to compare to other sites where comparable samples were collected over the same periods. The data has been grouped and analyzed based on sampling location and time and are reported as 'result sets'. Figure 7 depicts the sampling performed at each site including sample type and duration for each result set, i.e. sets 1, 2 and 3 (Figure 7).

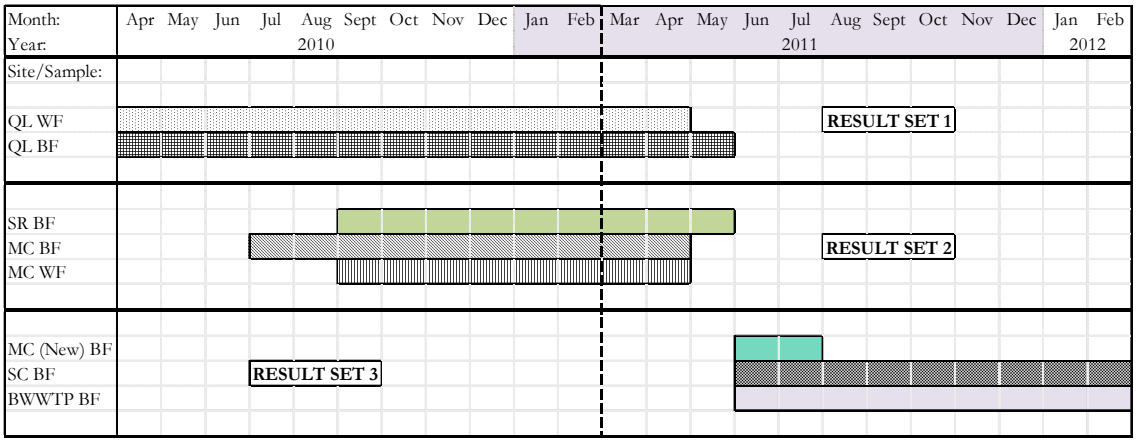

Figure 7. Site and sample types taken and the duration of sampling for each
Groupings of site and sample types are separated into the three result sets in which they will be discussed and compared within this thesis. The bold dotted line represents when FISH to PCR processing was begun for all samples. Site names have been abbreviated as the following: QL-Queen Lane; SR-Sandy Run; MC-Monocacy Creek; SC-Saucon Creek and BWWTP-Bethlehem WWTP. Sample types have been abbreviated as the following: WF-water filter; BF-biofilm slides.

3.1 Result Set 1

Data set 1 provides a direct comparison between the two different sample collection methods, specifically water filters and biofilm slides, from the same location, the Queen Lane WTP intake. To evaluate the sensitivity, or performance, of various sampling and processing method combinations, the Queen Lane samples were processed by FISH only, PCR only, and a combination of FISH to PCR. Thus over the sampling duration at Queen Lane for Result Set 1, the data is grouped into three sampling periods for four different sampling type/processing method combinations: 1) biofilm slides/FISH, 2) water filters/FISH, 3) water filters/PCR , and 4) biofilm slides/FISH/PCR (using the FISH to PCR protocol described in Section 2.2.4).

3.1.1 Sampling Period 1

The first sampling period of data set 1 was from 4/10/2010 to 8/10/2010 and consisted of nine sampling dates with the objective of evaluating the biofilm slides/FISH

method against the current water filter/PCR method. Samples were obtained using both collection methods; the biofilm slides were then processed by FISH whereas the water filter samples were split (following IMS), with half processed by FISH and the other half by PCR. This sampling regime resulted in the comparison of three different sampling type/processing method combinations: 1) biofilm slides/FISH, 2) water filters/FISH and 3) water filters/PCR. For comparison purposes, a positive sample processed via FISH corresponds to an oocyst count greater than zero. For the biofilm/FISH, water/FISH, and water/PCR combinations, positive results were observed 100, 55 and 0 percent of the days sampled, respectively (Figure 8a, Tables 3 & 10). Split water filter samples analyzed separately by FISH and PCR were in agreement 33 percent of the days sampled (3 out of 9 sampling dates), however, these results correspond to negative results, i.e., a negative (0 oocysts) FISH result and corresponding negative PCR result. The 55 percent (5 out of 9 sampling dates) of positive water filter samples were the result of occyst counts ranging from 2 to 13; however, all of the corresponding samples processed via PCR were negative. The lack in correlation of the processing methods could be attributed to the relatively low number of oocysts combined with the fact that the water filter samples were split after IMS for three of the nine sampling days in this period and thus were discrete and not duplicate samples. However, this is unlikely the case since 15 filter samples (2 filters * 6 sampling dates, and 1 filter * 3 sampling dates) were all negative via PCR.

Table 3. Sampling date, sample type, processing methods and results for data from sampling period 1 within result set 1.

Sampling Date	Sample Type	Days Slides in Stream	FISH Count (Total)	PCR Results	Sequencing Results
4/13/2010	Filters (2)	--	0	Negative	--
	Slides	36	18		--
4/27/2010	Filters (2)	--	6	Negative	--
	Slides	15	9		--
5/11/2010	Filters (2)	--	0	Negative	--
	Slides	15	2		--
5/25/2010	Filters (2)	--	2	Negative	--
	Slides	8	5		--
6/8/2010	Filters (2)	--	0	Negative	--
	Slides	8	3		--
6/29/2010	Filters (2)	--	13	Negative	--
	Slides	20	6		--
7/20/2010	Filter	--	2	Negative	--
	Slides	22	3		--
7/28/2010	Filter	--	2	Negative	--
	Slides	8	5	--	--
8/10/2010	Filter	--	1	Inconclusive	--
	Slides	15	1	--	--

3.1.2 Sampling Period 2

The second sampling period at the Queen Lane WTP was from 8/24/2010 to 2/23/2011 and consisted of 11 and 13 sampling dates for biofilm slides and water filters, respectively. Biofilm slides and water filters were processed separately, the slides via FISH, and the water samples by PCR. The objective of this sampling regime was to compare the two different sampling type/processing method combinations, specifically: 1) biofilm slides/FISH, and 2) water filters/PCR, to investigate the sensitivity of each combination at the same location. As shown in Figure 8b, positive results for the biofilm slides/FISH and water filter/PCR combinations were observed 64 and 38 percent of the days sampled, respectively (Tables 4 &

10). Based on these results, the biofilm slides/FISH method is the more sensitive assay, with an increased likelihood of detecting a low number of oocysts in a natural surface water environment, as compared to the current method (water filter/PCR).

Table 4. Sampling date, sample type, processing methods and results for data from sampling period 2 within result set 1.

Sampling Date	Sample Type	Days Slides in Stream	FISH Count (Total)	PCR Results	Sequencing Results
8/24/2010	Filter	--	--	Negative	--
	Slides	14	2	--	--
9/21/2010	Filters (2)	--	--	Negative	--
	Slides	28	5	--	--
9/28/2010	Filters (2)	--	--	Positive	*C. Parvum*
	Slides	7	0		--
10/12/2010	Filters (2)	--	--	Negative	--
10/26/2010	Filters (2)	--	--	Negative	--
11/9/2010	Filters (2)	--	--	Negative	--
	Slides	14	1	--	--
11/23/2010	Filters (2)	--	--	Positive	*C. Parvum*
	Slides	14	2	--	--
12/7/2010	Filters (2)	--	--	Negative	--
	Slides	14	2	--	--
12/21/2010	Filters (2)	--	--	Negative	--
	Slides	14	5	--	--
1/11/2011	Filters (2)	--	--	Positive	*C. Parvum*
	Slides	21	0	--	--
1/25/2011	Filters (2)	--	--	Positive	*C. Parvum-like*
	Slides	14	0	--	--
2/8/2011	Filters (2)	--	--	Negative	--
	Slides	14	1	--	--
2/23/2011	Filters (2)	--	--	Positive	*C. Parvum*
	Slides	15	0	--	--

3.1.3 Sampling Period 3

For the third sampling period, from 3/8/2011 to 5/10/2011, biofilm slides were collected and processed via the FISH process and then directly through the PCR process using the FISH to PCR protocol (See Section 2.2.4). The water filters were collected and processed only by PCR. Based on the results of the previous sampling periods, the FISH processing method appears to offer greater sensitivity in the detection of low numbers of oocysts. However, as previously mentioned, PCR processing and subsequent genotyping allows for the specific speciation of all oocysts in the sample. The objective of this sampling period was to try to combine the FISH and PCR processing methods to yield a high sensitivity for detection and enumeration of oocysts while maintaining the ability to obtain specific genotype information. This sampling period consisted of five and four sampling days for biofilm slides and water filters, respectively, and resulted in the comparison of two different sampling type/processing method combinations: 1) biofilm slides/FISH to PCR processing, and 2) water filters/PCR only (Table 5). Figure 8c) displays the percent positive observations for the two sampling/processing combinations. The biofilm samples yielded an 80 percent positive rate after FISH with the subsequent PCR processing of these samples yielding a 40 percent positive rate, resulting in agreement between the FISH and PCR processing from the biofilm slides in 60 percent of the samples. Water filters that were processed via PCR only had zero positives. These results continue to support the hypothesis that the biofilm slide/FISH processing method offers greater sensitivity in the detection of oocysts. In addition, the combination of performing FISH and PCR in series, while only successful on half of the positive samples for this data set, offers proof that this method has potential and should be further investigated and optimized.

Table 5. Sampling date, sample type, processing methods and results for data from sampling period 3 within result set 1.

Sampling Date	Sample Type	Days Slides in Stream	FISH Count (Total)	PCR Results	Sequencing Results
3/8/2011	Filters (2)	--	--	Negative	--
	Slides	13	8	Positive	*C. Parvum*
3/22/2011	Filters (2)	--	--	Negative	--
	Slides	14	5	Negative	--
4/12/2011	Slides	21	3	Negative	--
4/19/2011	Filters (2)	--	--	Negative	--
4/26/2011	Filters (2)	--	--	Negative	--
	Slides	14	2	Positive	*C. Parvum*
5/10/2011	Slides	14	0	Negative	--

3.2 Result Set 2

This data set consists of Sandy Run WWTP biofilm slides and rock scrapings collected by PWD) from 9/21/10 through 5/10/11, Monocacy Creek biofilm slides (collected by LU) from 7/19/10 to 4/13/11, and Monocacy Creek water filters (collected by LU) from 9/21/10 to 4/28/11. Data summaries for the Sandy Run and Monocacy Creek sites are displayed in Tables 6 and 7, respectively. It should be noted that the samples collected at both Sandy Run and Monocacy Creek were initially processed via FISH only until the FISH to PCR protocol was initiated in March 2011.

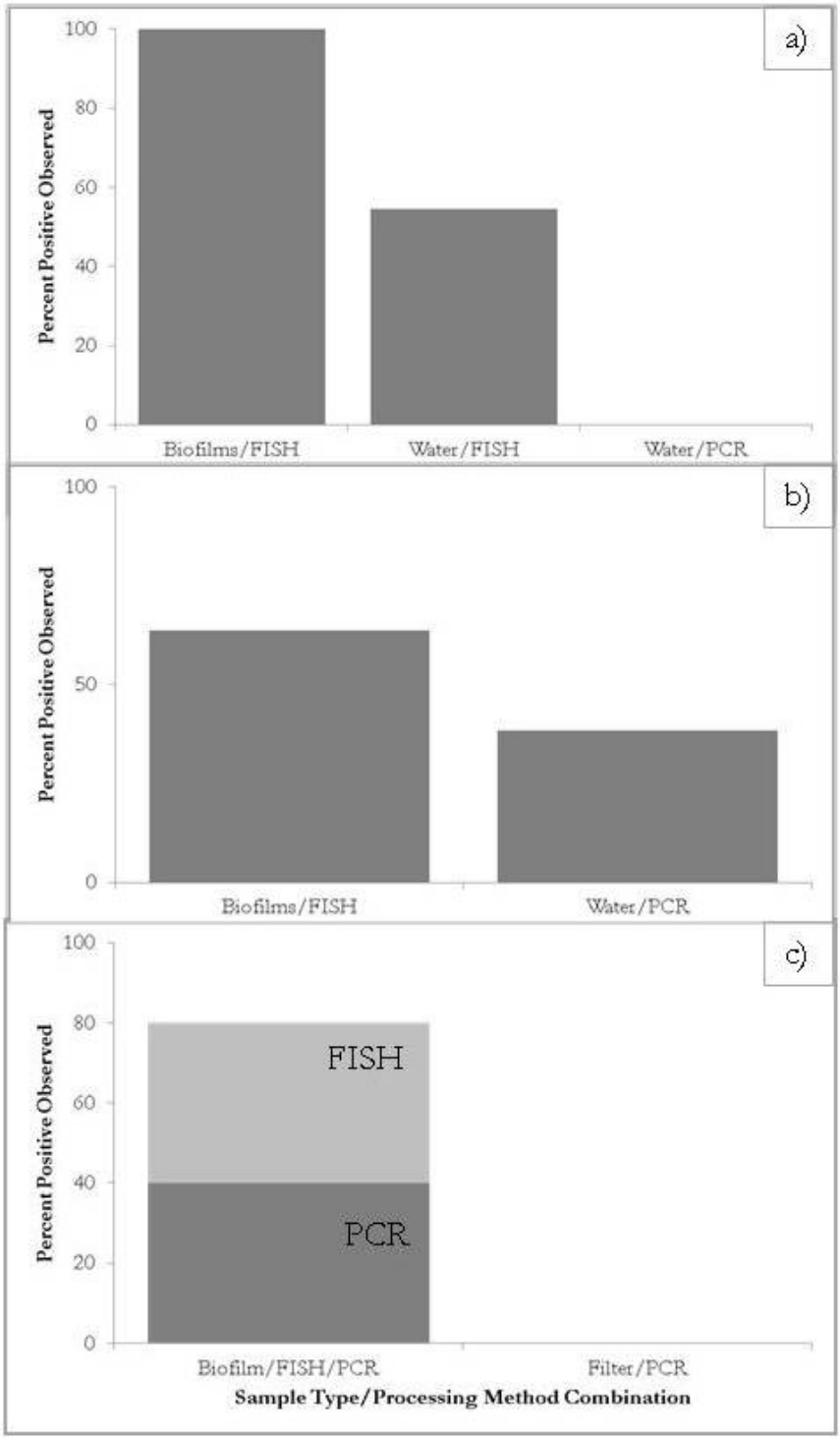

Figure 8. Comparison of percent positive observations versus sample type/processing method combinations for result set 1

a) Depicts sampling period 1, from 4/13/10 through 8/10/10, b) depicts sampling period 2, from 8/24/10 through 2/23/11 and c) depicts sampling period 3, from 3/8/11 through 5/10/11.

Table 6. Summary of sampling, sampling type, processing methods and results for Sandy Run WWTP samples from result set 2

The lightly shaded region designates the only time rock scrapings were obtained, and 'ns' means that particular item was 'not sampled'. The astericks (*) designates the day samples could not be obtained at the upstream site because it was covered with ice.

		Upstream			Downstream		
Sampling Date	Days Slides in Stream	FISH Count (Total)	PCR Results	Sequencing Results	FISH Count (Total)	PCR Results	Sequencing Results
9/8/10	--	5	ns	--	19	ns	--
9/21/10	14	1	ns	--	4	ns	--
9/28/10	7	0	ns	--	2	ns	--
10/12/10	14	1	ns	--	1	ns	--
10/26/10	14	1	ns	--	2	ns	--
11/9/10	14	1	ns	--	1	ns	--
11/23/10	14	0	ns	--	3	ns	--
12/7/10	14	1	ns	--	4	ns	--
12/21/10	14	0	ns	--	4	ns	--
1/11/11	21	0	ns	--	1	ns	--
1/25/11	14	0	ns	--	0	ns	--
2/8/11	14	ns*	ns	--	0	ns	--
2/23/11	15	0	ns	--	1	ns	--
3/8/11	13	0	Negative	--	1	Negative	--
3/22/11	14	1	Negative	--	1	Positive	*C. Parvum*
4/12/11	21	0	Negative	--	1	Negative	--
4/26/11	14	0	Positive	*C. Parvum*	0	Negative	--
5/10/11	14	0	Negative	--	0	Negative	--

Table 7. Summary of sampling, sampling type, processing methods and results for Monocacy Creek samples from result set 2

Positive counts are all bolded, and 'ns' means that particular item was not analyzed. The '**a**' designates that sample in which counts could not be obtained due to well contamination. The '**b**' designates that sample in which a different analyst performed FISH improperly, and the slide was unreadable.

Sampling Date	Days Slides in Stream	Sample Type	Viable:	Other:	FISH Count (Total)	PCR Results
7/19/10	7	Slides	0	0	0	ns
	--	RS	0	0	0	ns
7/26/10	7	Slides	--	--	**a**	ns
	--	RS	0	**1**	**1**	ns
8/2/10	7	Slides	0	0	0	ns
	--	RS	0	0	0	ns
8/9/10	7	Slides	0	0	0	ns
	--	RS	0	0	0	ns
8/16/10	7	Slides	**1**	0	**1**	ns
	--	RS	0	0	0	ns
8/23/10	7	Slides	**1**	0	0	ns
	--	RS	0	0	0	ns
9/7/10	15	Slides	0	**1**	0	ns
	--	RS 1	0	**1**	0	ns
	--	RS 2	0	0	0	ns
9/21/10	--	RS	0	**1**	**1**	ns
	--	Filter	0	0	0	ns
10/20/10	--	Filter	--	--	**b**	ns
11/8/10	--	Filter	0	0	0	ns
11/16/10	25	Slides	**1**	0	**1**	ns
	--	RS	0	0	0	ns
11/23/10	--	Filter	0	0	0	ns
12/3/10	17	Slides	**1**	0	**1**	ns
	--	RS	**1**	0	**1**	ns
12/8/10	--	Filter	0	0	0	ns
12/15/10	12	Slides	0	0	0	ns
	--	RS	0	**1**	**1**	ns
12/22/10	7	Slides	**1**	**1**	**2**	ns
	--	RS	0	0	0	ns
	--	Filter	0	0	0	ns
1/7/11	16	Slides	0	0	0	ns
	--	RS	0	0	0	ns
	--	Filter	0	0	0	ns
1/25/11	--	Filter	0	0	0	ns

Sampling Date	Days Slides in Stream	Sample Type	Viable:	Other:	FISH Count (Total)	PCR Results
1/31/11	24	Slides	0	0	0	ns
	--	RS	0	0	0	ns
2/9/11	--	Filter	0	0	0	ns
2/15/11	15	Slides	0	0	0	ns
	--	RS	0	0	0	ns
2/24/11	--	Filter	0	0	0	ns
3/2/11	15	Slides	0	0	0	ns
	--	RS	0	0	0	ns
3/9/11	--	Filter	0	0	0	Negative
3/23/11	21	Slides	0	**1**	**1**	Negative
4/13/11	21	Slides	**1**	0	**1**	Negative
	--	RS	**1**	0	**1**	Negative
4/20/11	--	Filter	**1**	0	**1**	Negative
4/28/11	--	Filter	0	0	0	Negative

It was hypothesized that natural biofilms in the streambed would provide an indication of background oocyst contamination and could be utilized to identify potential sites of interest for sampling and monitoring within a watershed. This hypothesis was tested at the Sandy Run site, where natural biofilms were obtained from rock scrapings, both upstream and downstream of the WWTP discharge, and then processed via FISH. The initial rock scrapings, obtained by PWD on 9/8/10, showed a higher oocyst count of 19 at the downstream site, which is much higher than the 5 oocyst count observed at the upstream site. Of the 19 and 5 oocysts counted in these samples, 13 (67%) and 4 (80%) were designated as viable by using the FISH protocol, respectively (Figure 9).

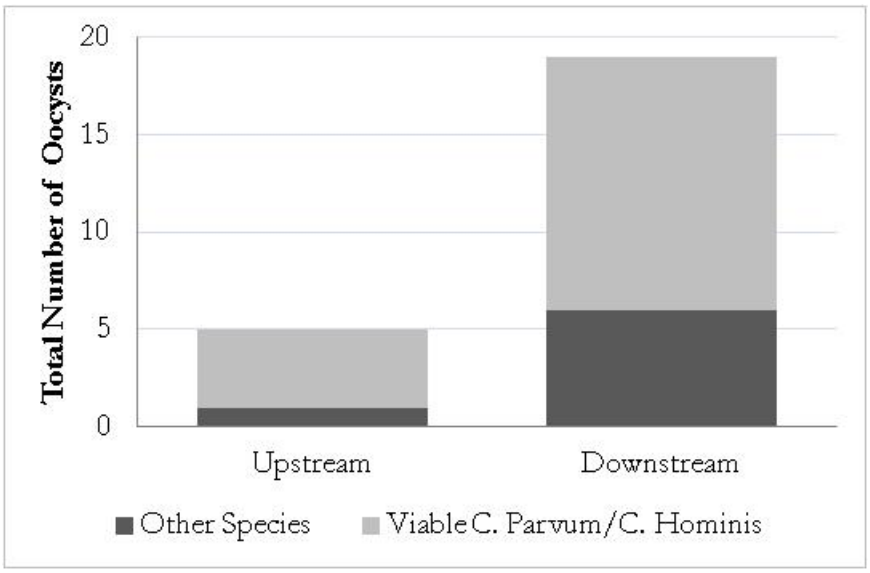

Figure 9. Total number of oocysts detected by FISH in initial rock scrapings up and downstream of the Abington WWTP
Light gray bars indicate the total number of viable *C. parvum* and *C. hominis* oocysts detected; dark gray bars indicate the total number of other not viable *C. parvum* and *C. hominis* oocysts and other species of *Cryptosporidium* (both viable and not viable) detected.

Biofilm slides at Sandy Run continued to show the same trend shown initially with rock scrapings. Generally, downstream oocyst counts were higher (63%, n=10/16, excluding the one sampling day the upstream samples were not retrieved) or equal to upstream oocyst counts (38%, n=6/16). At no time were upstream counts observed to be higher than downstream counts (Table 4, Figure 10).

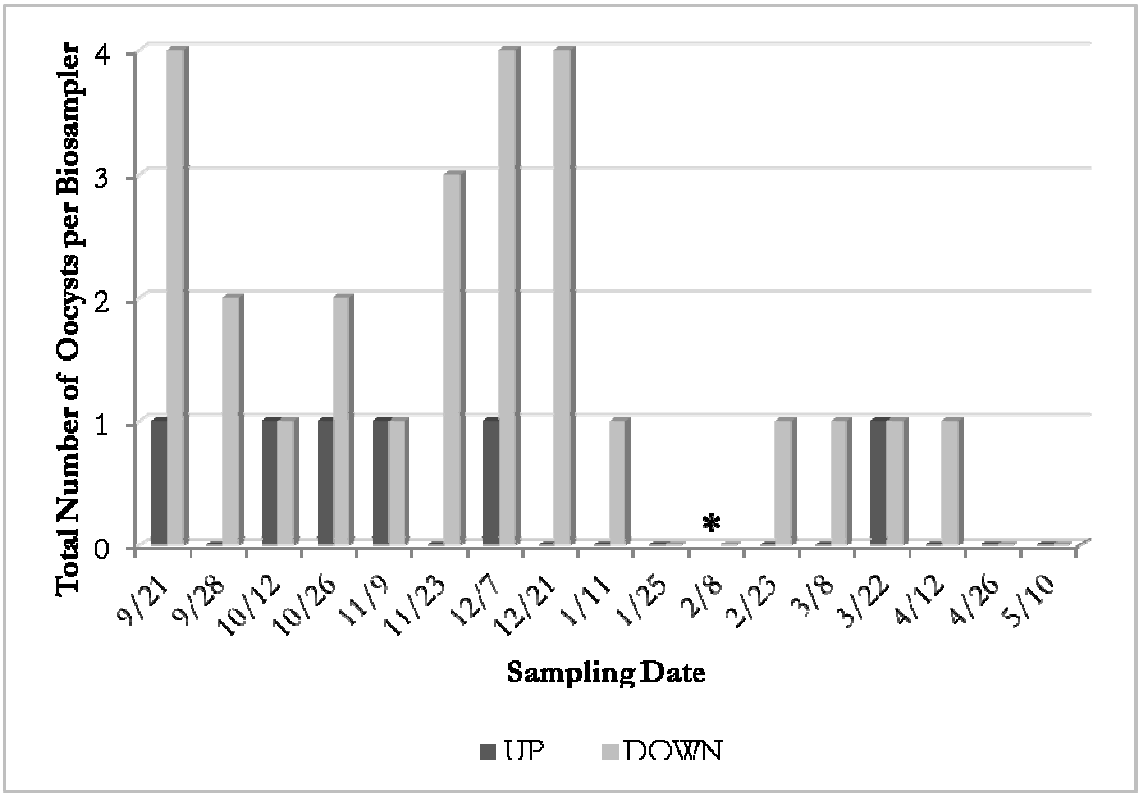

Figure 10. Total number of oocysts detected by FISH in biofilm slides located upstream and downstream of the Abington WWTP discharge on Sandy Run versus sampling date
Dark gray and light gray bars designate counts from the upstream ('UP') and downstream ('DOWN') locations, respectively. The astricks (*) indicates the one sampling day that the upstream location was covered with ice and precluded retrieving samples.

The Monocacy Creek site was chosen as a control site and was expected to be free of oocysts since there is no WWTP discharge upstream, however the samples collected showed an unexpected number of positive oocyst observations. For biofilm slides, rock scrapings and water filters, positive observations were made 35, 35 and 8 percent of the time, respectively, when processed via the FISH protocol only (sampling dates 7/19/10 through 3/2/11). All the positive observations in this set were 1-oocyst counts with the exception of a 2-oocyst count from biofilm slides on 2/22/10 (Table 5). After 3/8/11, all samples were

processed using the FISH to PCR protocol, however, from those five sampling days, no PCR positive observations were observed for any sample type. Of the five sampling days, positive observations from FISH counts were observed for biofilm slides, rock scrapings and water filters 100 (n=2), 100 (n=1) and 33 (n=3) percent of the time, respectively (Table 5). Further investigation of the land use for the watershed area of the sampling location at Monocacy Creek identified a golf course upstream of the site. Previous work (Jellison, Lynch and Ziemann, 2009) has identified that geese are a potential vector for *Cryptosporidium* in eastern Pennsylvania watersheds and golf courses are a flocking site for geese. This is one potential explanation for the presence of *Cryptosporidium* at this particular site.

3.3 Result Set 3

This data set includes biofilm slide sampling of the 'New' Monocacy Creek site (upstream of the previous location) from 6/20/11 to 7/27/11, the Saucon Creek site from 6/20/11 through 2/21/12, and the upstream and downstream locations of the Bethlehem WWTP discharge into the Lehigh River from 6/22/11 to 2/21/12, all collected approximately every two weeks by LU students. The New Monocacy Creek site and Saucon Creek were studied to gather additional information on different types of control, or non-wastewater impacted, sites. By selecting a site upstream of the golf course on Mononcacy Creek, at which there were no known potential upstream sources of any type, the potential *Cryptosporidium* oocyst baseline, if present, could be evaluated. In addition, Saucon Creek offered another site that was non-impacted by wastewater but that had three golf courses and multiple agricultural land sites upstream of the sampling site as potential non-point sources. The third site, at the Bethlehem WWTP discharge, was chosen to investigate

whether the trend identified at Sandy Run, specifically that greater numbers of oocysts are detected downstream as compared to upstream of a WWTP discharge, could be confirmed.

The new upstream sampling location on Monocacy Creek was chosen as the new control site because it was upstream of the golf course, thus it was un-impacted by both wastewater and other potential non-point sources (e.g., golf course) and was sampled on four days. All *Cryptosporidium* counts were zero, with the exception of a 1-oocyst count on the last sampling day of 7/27/11 (Table 8). This data suggests that there is a possibility that *Cryptosporidium* could be more ubiquitous in the natural environment than previously thought. It also suggests that biofims, both natural and those collected on slides, offer a greater sensitivity for detecting the potential of low occurrences of oocysts in these environments.

Table 8. Summary of viable, other and total oocysts detected by FISH in biofilm slides from the Monocacy Creek (New) site

Sampling Date	Days Slides in Stream	Viable	Other	FISH Count (Total)	PCR Results
6/20/11	19	0	0	0	Negative
6/29/11	9	0	0	0	Negative
7/13/11	14	0	0	0	Negative
7/27/11	14	0	1	1	Negative

Saucon Creek was sampled from 6/20/11 through 2/21/12 via biofilm slides. A summary of these data are displayed in Table 9. Positive observations, which were either one or two oocyst counts at this site, were observed 38% (n=6/16) of the time, while zero counts were observed 63% (n=10/16) of days sampled. These results are in agreement with the previous sampling set at Monocacy Creek and further support the idea that a waterway

does not have to be impacted by wastewater to have either a potential background/baseline level and/or non-point source for *Cryptosporidium*.

Table 9. Summary of viable, other and total oocysts detected by FISH in biofilm slides, as well as PCR results for those slides, for samples from Saucon Creek
'ns' depicts samples not taken or analyzed.

Sampling Date	Days Slides in Stream	Viable	Other	FISH Count (Total)	PCR Results
6/20/11	19	0	2	2	Negative
6/29/11	9	1	0	1	Negative
7/13/11	14	1	1	2	Negative
7/27/11	--	--	--	ns	ns
8/10/11	12	0	0	0	Negative
8/24/11	14	0	0	0	Positive
8/31/11	7	1	1	2	Negative
9/21/11	21	0	1	1	Negative
10/12/11	21	0	0	0	Negative
10/24/11	14	0	0	0	Negative
11/16/11	23	0	0	0	Negative
12/14/11	28	0	0	0	Negative
12/29/11	15	0	1	1	Negative
1/11/12	14	0	0	0	Negative
1/24/12	13	0	0	0	Negative
2/7/12	14	0	0	0	Negative
2/21/12	14	0	0	0	Negative

Further examination of the Sacucon Creek results identified a potential trend in the data with regards to time of year and temperature. In general, the warmer months (June through September) yielded more positive observations (71%, n=5/7) than colder months (October through February) with only 11% positive observations (n=1/9), which can be easily seen in Figure 11.

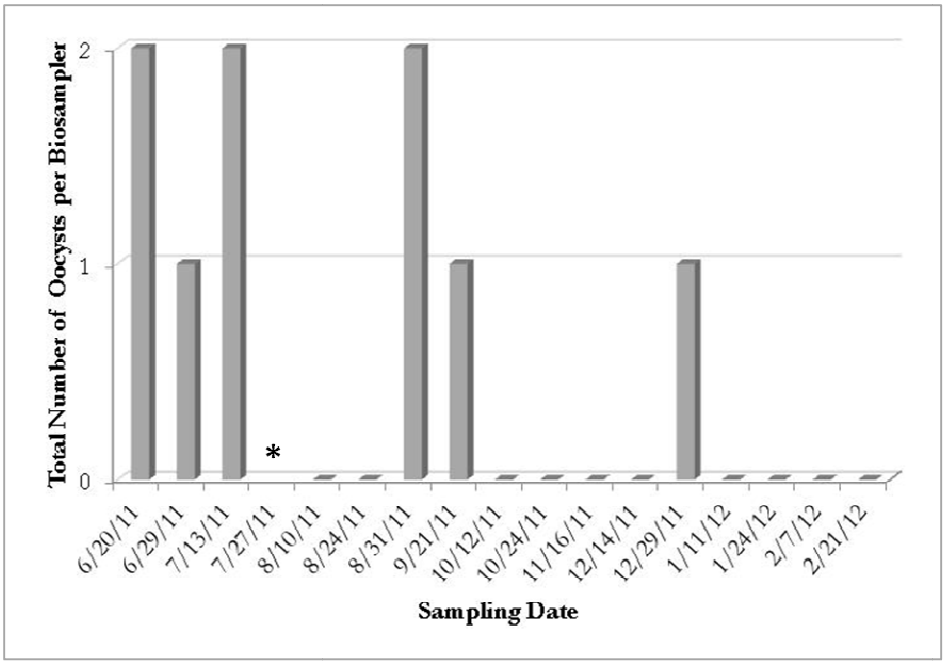

Figure 11. Total number of oocysts detected by FISH in biofilm slides located at Saucon Creek versus sampling date
The astricks (*) indicates the one day the sampler was missing which precluded sample retrieval.

The Bethlehem WWTP was chosen as a site at which we would expect to see similar results to the Sandy Run data from result set 2. A summary of these data are shown in Table 10. Samplers located at the upstream location were missing on 8/3/11, downstream samplers were missing on 7/6/11 and 8/17/11, and these unsampled dates are shaded in Table 8. Figure 12 shows these data in graphical form, which displays a similar trend to the Sandy Run data, in which higher oocyst counts were observed in the downstream samples compared with the upstream site. These data show more variation between the upstream and downstream positive counts than did Sandy Run data. Counts downstream were higher than at the upstream location 58% of the time (n=7/12, excluding the three dates in which one location was not sampled), counts at the two locations were equal 25% of sampling

dates (n=3/12) and upstream counts were higher than downstream counts on 17% of sampling days (n=2/12) (Figure 12).

Table 10. Summary of total oocysts detected by FISH in biofilm slides, as well as PCR and sequencing results for those samples upstream and downstream of the Bethlehem WWTP
Light shading (or 'ns' designation) depicts those dates when samples were not obtained.

		Upstream			Downstream		
Sampling Date	Days Slides in Stream	FISH Count (Total)	PCR Results	Sequencing Results	FISH Count (Total)	PCR Results	Sequencing Results
6/22/2011	14	0	Negative	--	1	Negative	--
7/6/2011	14	2	Negative	--	ns	ns	ns
7/20/2011	14	0	Negative	--	3	Negative	--
8/3/2011	12	ns	ns	ns	3	Negative	--
8/17/2011	12	1	Negative	--	ns	ns	ns
9/21/2011	42	1	Negative	--	4	Negative	--
10/12/2011	21	1	Negative	--	1	Negative	--
10/24/2011	14	0	Negative	--	3	Negative	--
11/16/2011	23	1	Negative	--	0	Negative	--
12/14/2011	28	1	Negative	--	3	Negative	--
12/29/2011	15	0	Negative	--	0	Negative	--
1/11/2012	14	0	Negative	--	1	Positive	not run yet
1/24/2012	13	0	Negative	--	0	Negative	--
2/7/2012	14	0	Negative	--	1	Negative	--
2/21/2012	14	1	Negative	--	0	Negative	--

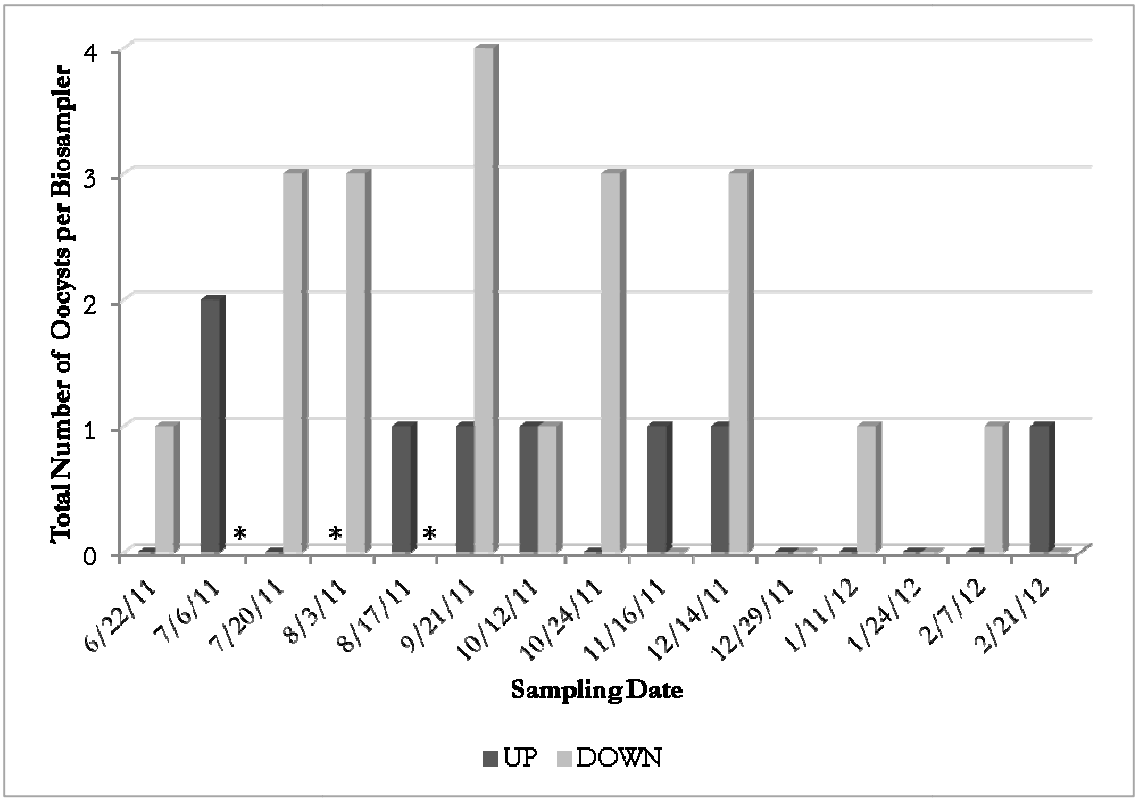

Figure 12. Total number of oocysts detected by FISH in biofilm slides located upstream and downstream of the Bethlehem WWTP discharge on the Lehigh River versus sampling date
Dark gray and light gray bars designate counts from the upstream ('UP') and downstream ('DOWN') locations, respectively. The astricks (*) indicate sampling days that samplers were missing and could not be collected.

3.3.1 New Biofilm Sampler Prototype & Biofilm Thickness

Separate data gathered from sites included in result set 3 were separate oocyst counts obtained from slides placed in 'new' prototype biofilm samplers (See section 2.1.2 Biofilm Slides). Only four sample dates were processed and analyzed by the author, from 1/11/12 through 2/21/12, and can be viewed in comparison to their 'old' prototype biofilm sampler counterparts in Table 11. Counts for the Bethlehem WWTP upstream site, downstream site and Saucon Creek site were in agreement between prototypes 67%, 75% and 100% of the time, respectively.

Table 11. Comparison of total oocysts detected by FISH in biofilm slides collected from old and new prototype biofilm samplers
Only four samples were taken from 1/11/12 through 2/21/12. 'ns' depicts those times when samples were not taken. Light shading designates those dates for each site that showed matching results.

	BWWTP Up		BWWTP Down		Saucon Creek	
Sampler Prototype	Old	New	Old	New	Old	New
Sampling Date						
1/11/2012	0	ns	1	1	0	0
1/24/2012	0	0	0	0	0	0
2/7/2012	0	1	1	1	0	0
2/21/2012	1	1	0	1	0	0

Biofilm thickness measurements were also collected from all three sites assessed in result set 3 (Table 12). All three sites depict an increasing trend in mean biofilm thickness over the course of two or three weeks, which was expected. Saucon Creek displayed the quickest growth of biofilms when compared to both the Bethlehem WWTP sites and maintained the thicker biofilm into three weeks. This trend of increasing biofilm growth over time was often observed by the author although no other thickness measurements were made throughout the rest of the study.

Table 12. Biofilm thickness measurements in μm from biofilm slides collected from sites grouped in result set 3
'ns' depicts those times when samples were not taken.

Site	BWWTP up	BWWTP down	Saucon Creek
Week			
1	ns	ns	17.4
2	19.8	24.6	30
3	39.3	33.5	43.5

4. Discussion

Result set 1 is composed of two long-term data sets obtained from samples taken at the Queen Lane WTP site (Figure 1) to ultimately gain a better understanding of sampling methods (water filtration versus biofilm slides) and the efficacy and potential of each to monitor for *Cryptosporidium* in surface waters. In an attempt to elucidate the performance of the methods, several variations were employed and thus complicates comparison of the data (Table 1). To provide an accurate comparison, data in result set 1 were combined by sample type and processing methods. As can more easily be seen in Table 11, the sampling type and processing method combination of biofilm slides/FISH (designated in the table as BF/FISH) yielded the best results for all three sampling periods in that this combination yielded the highest percent of positive observations.

In the first sampling period of data set 1, 100% of samples included in the biofilm/FISH combination set (n=11) yielded positive results, while the combination of water filter/FISH yielded positive observations 55% of the time, and the water filter/PCR combination showed the worst results at 0% positive observations (Figure 8a). This data directly supports the hypothesis that the combination of sample collection via biofilm slides and FISH processing is the most sensitive combination when sampling surface waters for *Cryptosporidium* oocysts (Table 13).

Table 13. Percent positive observations for each sampling type/processing method combination for each of the three sampling periods in result set 1
'ns' depicts when the combination type was not sampled. The percentages are on the left in bold and the number of dates that combination was positive per total dates sampled are in parentheses on the right.

Sampling Type/ Processing Method Combination	Sampling Period 1	Sampling Period 2	Sampling Period 3
BF/FISH	**100** (11/11)	**64** (7/11)	**80** (4/5)
BF/FISH/PCR	ns	ns	**40** (2/5)
Water/FISH	**55** (6/11)	ns	ns
Water/PCR	**0** (0/11)	**38** (5/13)	**0** (0/4)

In the second sampling period, out of the two sample type/processing method combinations, the biofilm/FISH combination again yielded the best results. For this sample set, the biofilm slides/FISH combination resulted in 64% positive observations, while the water filter/PCR combination showed 38% positive results, however the biofilm/FISH combination results were lower than expected (Figure 8b). The biofilm slides/FISH positive observations at 100, 64, and 80 percent for three sampling periods, respectively, support the hypothesis that this method offers greater sensitivity than the current water filter/PCR method that only yielded positive results 0 , 38, and 0 percent of the time for the same sampling periods.

Sampling period 2 had a lower percent positive observation of oocysts, as compared to the other two sampling periods, 64 compared to 100 and 80, respectively. One explanation deals with the fact that for 3 of the 4 zero oocyst counts that were observed for the BF/FISH combination during sampling period 2, occurred during winter months (Table 3). The previous observation that time of year and temperature influence the biofilm growth and subsequent capture capability of oocysts could be the cause. In the second sampling

period, out of the two sample type/processing method combinations, the biofilm/FISH combination again yielded the best results. The biofilm slides/FISH combination, however, only resulted in 64% positive observations, while the water filter/PCR combination showed 38% positive results, the former being lower than expected (Figure 8b). One explanation deals with the fact that for 3 of the 4 zero oocyst counts that were observed for the BF/FISH combination during the 11 days of biofilm sampling during this period, occurred during winter months (Table 3). The trend that biofilms were thinner during colder months and thicker during warmer months was generally observed by the author over the duration of this thesis. This trend has been recognized by others in the literature as well (Wolyniak, Hargreaves and Jellison, 2009; Melo and Bott, 1997). Wolyniak, Hargreaves and Jellison (2009) observed specifically that summer biofilms, at an average thickness of 42.1 μm, were thicker than winter biofilms, at an average thickness of 37.0 μm, and these differences were statistically different (P of 0.03 by independent *t* test). The importance of the biofilm thickness becomes apparent when considering that the biofilm sampler method depends heavily on a thicker, healthy biofilm in order to take in *Cryptosporidium* oocysts. Thinner biofilms are probably less likely to embed any oocysts present in surface waters, and since during colder months of the year biofilms tend to be thinner, this may result in lower counts and subsequent *Cryptosporidium* estimations in the winter using this method.

Although Wolyniak, Hargreaves and Jellison (2009) reported that biofilm thickness did not appear to be related to the number of oocysts attached to any given biofilm grown in laboratory experiments, biofilm roughness was shown to be positively correlated. The standard deviation of the biofilm thickness measurements correlates positively with biofilm roughness. In the short-term thickness experiment conducted during this thesis study,

results of which are displayed in Table 10, standard deviations of thickness measurements at each site increased over time (there was a positive correlation between thickness measurements and their standard deviation for thickness). The increase in biofilm surface roughness, which tentatively increases with thickness at these sites (more study is needed to ascertain this certainly) may potentially cause the increase in oocyst counts during warmer periods, observed in this study, when thicker biofilms were present.

The third sampling period of result set 1 showed again that the biofilm/FISH combination yielded the highest number of positive observations at 80% (n=5), followed by the biofilm/FISH/PCR (processed first by FISH and then through PCR using the FISH to PCR protocol; See Section XX FISH to PCR) combination at 40% and last by the water/PCR combination at 0% (n=4) (Figure 8c). Since the FISH to PCR method combined the two analytical methods in this third sampling period, the first two combinations in this instance really reflect the efficacy of this combined analytical method. It appears in this sampling period, half the observations for positive FISH results also tested positive in the PCR and DNA analysis. This suggests that of the two analytical methods, FISH would appear to be the most sensitive. This conclusion is also supported by data from sampling period one, where water filters were split and processed half by FISH and half by PCR, in which FISH again seemed to be more sensitive a processing method for oocyst detection. All three sampling periods in result set 1 seem to support the idea that the biofilm/FISH combination is the most sensitive for perceiving whether *Cryptosporidium* contamination exists within a specific surface water location. These results led our group to consider the next series of experiments, which involved sampling at the upstream and downstream locations of a waste water treatment plant outfall pipe, (Sandy Run/Abington

WWTP) as well as at what we considered a wastewater discharge control location (Monocacy Creek) (Figure 2).

The initial trial for Sandy Run/Abington WWTP, which was comprised of sampling natural biofilms or rock scrapings at the sites (Figure 9), was a complete success. Expectations were met regarding results, with downstream counts being much higher (19) than upstream counts (5). This suggests that biofilms do indeed embed oocysts and that they are detectable using the processing methods described in this study. A second interesting observation in the initial trial was the fact that a higher percentage of viable oocysts compared to other oocysts was observed at both the up and downstream locations (downstream: 67% viable, n=13/19; upstream: 80% viable, n=4/5) (Figure 9). The largest, most important source of potentially human infectious *Cryptosporidium* species (i.e. viable oocysts) in this location is undoubtedly the discharge pipe from the WWTP. If water flow occurred always only in one direction, and the outfall pipe was the only source of oocysts, the expectation would be to find viable oocysts only in the downstream location. However, flow does not always simply move smoothly in one direction; eddies in water flow are not uncommon in creeks and rivers, and they have often been observed by the author even in the larger Lehigh River (Figure 13), at the site of the second WWTP sampled in this study. These variations in water flow could be responsible for viable oocyst distributions upstream.

Other possible sources of viable to either site are random and intermittent human/animal waste inputs into surface waters. Jellison (2010) and Jellison, Lynch, and Ziemann (2009) identified deer and geese as sources of human infectious species *Cryptosporidium* to studied watersheds. Graczyk et al. (2000) and Howe et al., (2002)

identified cattle/domestic animals as sources of *Cryptposporidium*, while Ruecker et al. (2007) stated that in addition to cattle, deer, muskrats, voles, birds, and other wildlife species, as well as sewage (human or agricultural), may also potentially have impacted water quality in the study watershed. While human inputs are typically related to 'viable' oocyst inputs and animal sources are usually responsible for 'other' oocyst inputs, geese and deer are known vectors of human infectious oocyst species to watersheds. Any of these sources listed could account for the variability of oocyst species at the sites.

Another possible source of oocysts to sites could be sloughing of creekbed biofilms, their resuspension into the water column and finally their subsequent incorporation into biofilms grown on slides placed in those creeks. Wolyniak, Hargreaves and Jellison (2009) observed that oocysts would in fact become embedded within environmental biofilms grown in the laboratory, but only on the surface of the biofilms. Furthermore, Wolyniak, Hargreaves and Jellison (2010) observed sloughing off of biofilms in similarly grown laboratory biofilms during flow experiments. Others have observed increased sloughing with an increase in biofilm thickness associated with increased shear stress (Morgenroth and Wilderer, 2000). These cumulative observations make it feasible that oocysts within grown biofilms in the study may potentially be from sloughed biofilms from the streambed in which the oocysts had been embedded previously.

In the long-term study of Sandy Run in which biofilm samplers were used to collect biofilm slides, the counts were not as high in either location as in the rock scraping study. These results support the idea that rock scrapings are older, thicker and more descriptive of the historical distribution of *Cryptosporidium* over a longer period of time compared to the slides. The same trend observed for the rock scraping samples in Figure 9 (Table 4) was

observed in the longer term study as well (Figure 10), with generally higher counts downstream of the outfall (1-4 oocyst counts) than upstream (0-1 oocyst counts). Downstream counts were higher than upstream counts 63% (n=10/16) of the time and equal to upstream counts 38% (n=6/16) of the days sampled. These counts, both for downstream and upstream locations, are lower than those for the rock scrapings probably due to the relatively young biofilms collected (two weeks old) when compared with the older, historical biofilms present on the rocks at the location. Numbers of viable and other oocysts were varied at both locations with no apparent pattern as observed in the initial rock scraping study, however, 67% of positive observations from both the up and downstream sites consisted of at least one viable oocyst (Appendix A). As discussed previously, there are a multitude of possible reasons for this observation and potential sources which could have contributed to the variability in oocyst species found at both locations.

Monocacy Creek served as a wastewater discharge control site (a control site in which there was no wastewater input) with which to compare results from the Sandy Run WWTP site samples. Interestingly, as is shown in Table 5, approximately 35% (n=6/17) of both the biofilm slides and rock scrapings collected resulted in positive observations for *Cryptosporidium*, as well as 8% of water filters (n=1/13). These results show that the biofilm samplers could be extremely sensitive in detecting oocysts from even far-away, disperse sources such as golf courses upstream of the sampling location (Figure 2). The Bethlehem golf course, approximately 3000 m upstream of the sampling location, could be a source of oocysts since these locations are a common meeting place for geese that are sources of both types of oocysts. In order to test whether this possible source had contributed to the positive *Cryptosporidium* results observed and whether there could be a baseline of

contamination at sites with no known point or non-point sources, e.g., WWTP and golf course, new sites were chosen to be examined in result set 3.

Upon discussing the results from result set 2, the advisors for the study were interested in establishing potential 'baseline' data for what we now termed 'non-impacted' sites. Monocacy Creek results were interesting in the fact that they had any *Cryptosporidium* contamination at all, and we wondered if this may be true for other sites with long-distance, disperse sources as well as sites with no known upstream sources. In the last set of experiments, grouped in result set 3, a new location upstream of the previous Monocacy Creek site (but still in that creek), labeled Monocacy Creek (New), along with a location on Saucon Creek, a site with no wastewater inputs but where potential upstream *Cryptosporidium* sources exist (i.e. golf courses, agricultural land), were chosen as two more 'control' sites for assessment. Unfortunately, logistics of site location soon precluded sampling at the Monocacy Creek (New) site, but Saucon Creek continued to be sampled to the end of the thesis study. The few results from the Monocacy Creek (New) site showed positive observations for 25% (n=1/4, Table 6) of sampling days, which is interesting with respect to the fact that there are no known upstream sources of *Cryptosporidium* at that location. This could support the idea that there is in fact a baseline of *Cryptosporidium* in supposed 'non-impacted' surface waters. The Saucon Creek site displayed 38% positive results (n=6/16, Table 7, Figure 11) of all sampled days. The golf courses impacting this site are located approximately 3400 m upstream of the sampling location, and agricultural land is ubiquitous upstream of the golf courses. The golf courses and agricultural land serve as potential sources of oocysts at this location, although other non-point sources exist as described earlier. The fact that oocysts were detected at this site proves the sensitivity of this biofilm

monitoring method, since these results show that they have the capability of detecting even low concentration, disperse, non-point sources. Furthermore, as observed at other sites, of the positive results, 50% of both Monocacy Creek (New) and Saucon Creek samples were comprised of at least one viable oocyst (Appendix A). As mentioned previously, there are a multitude of sources for both oocyst types, and this result is not surprising.

Similarly to Queen Lane data from result set 1, sampling period 2, a higher number of positive observations occurred in warmer months (71%, n=5/7, June - September) than colder months (11%, n=1/9, October - February) in Saucon Creek (Table 7, Figure 11). This observation is interesting when compared with other sources who observed the opposite effect. Jellison, Hemond, and Schauer (2002) observed that water filter samples positive for oocysts were limited to late fall, winter and early spring and that no oocysts in water filter samples were observed between the much warmer months of April through October. This again supports the idea that the biofilm thickness or surface roughness could be having an effect on oocyst counts collected using the biofilm method, since biofilms are typically thinner (with lower surface roughness, at least in the samples collected in this study) in cooler weather when compared to warmer weather biofilms, which is probably due to the sensitivity of the microbial community growth rates to temperature (Melo and Bott, 1997).

A second site chosen for analysis in result set 3 was the Bethlehem WWTP as a follow-up site for comparison to the Abington WWTP analyzed previously. The expectation was to observe similar trends when collecting biofilms from upstream and downstream locations of the outfall pipe. This expectation was generally met as can be observed in Figure 12 (Table 8) however with more variation between the up and downstream counts than observed at the Abington WWTP sites. Downstream counts were higher than or equal

to those from the upstream locations 58% and 25% of sampling days (n=12), respectively, (compared with 63% and 38% (n=16) for Abington WWTP results) while upstream counts were higher than downstream counts on 17% of sampling days (compared with 0% for Abington WWTP) (Table 8, Figure 12). The higher variability at this site may possibly be due to changes in processing methods between the Abington and Bethlehem WWTP samples. For the Abington WWTP the biofilm samples were all processed via FISH, while the Bethlehem WWTP samples were processed via FISH to PCR. The fluorescence of oocysts viewed when using the alternate mounting medium necessary for the FISH to PCR process is extremely faint when compared with those incubated with the intended mounting medium. While I am absolutely sure that positive FISH counts were correct, this lower fluorescence may have resulted in lower counts of oocysts than were actually present. This difference in processing between WWTP sites could have resulted in the variability in the data between them. Although more variable than expected after studying the Abington WWTP, these results continue to support the efficacy of the biofilm sampling method in order to monitor for *Cryptosporidium* in surface waters.

As discussed with respect to Sandy Run results, the viability of the oocysts observed in upstream and downstream locations of the Bethlehem WWTP sites were also variably distributed (Appendix A). Of the positive observations for Cryptosporidium in the upstream and downstream sites, 43% and 67% of the positive samples, respectively, were comprised of at least one viable oocyst. This seems more in line with expectations regarding the location where we would think to find higher numbers of viable oocysts, but again it is my conclusion that flow pattern or alternative sources are the reasons behind this variability. As shown in Figure 2, the Saucon Creek flows into the Lehigh River adjacent to the

discharge pipe from the BWWTP, and just downstream from the sampling sites by about 50 feet. During some flow periods for the Lehigh River, the Saucon Creek flowing into the Lehigh River resulted in large, back-flowing eddies, and a sort of skirting of the discharge outflow in the upstream direction of the Lehigh River, as seen in the bottom photograph of Figure 13. In this picture, the bubbles on the water surface, which designate the discharge outflow from the WWTP, can be observed even reaching all the way back to the upstream sampling location. This Saucon Creek flow inevitably pulls the WWTP discharge water with it when these eddies are present, probably increasing the likelihood of viable oocysts becoming embedded even in short-term biofims like the ones growing on the glass slides of this study.

Figure 13. Photographs depicting variations in stream flow at the Bethlehem WWTP
The top photograph, from summer 2011, shows the clear outline of discharge flow by way of white bubbles or froth on the surface. The discharge, most times, flowed out in a circle from the opening and then continued downstream as shown in the top picture. The bottom photograph shows the surface froth actually flowing upstream and skirting upstream along the edge of the concrete just past the upstream sampling location.

Another observation by the author is that, surprisingly, the Bethlehem WWTP outfall pipe area is frequented often in warmer months by many (i.e. fisherman, kids, domestic animals, etc.), and often for several hours while fishing or visiting. These visitors could pose another source of viable oocysts to either sampling location, especially since there is no restroom available in this area. The variability observed in the oocyst viability data at the Bethlehem WWTP could stem from either of these sources, the literature and likelihood of both possibilities having been discussed earlier.

The biofilm samplers were all made in-house at LU, however, the labor and supplies necessary to build these samplers can still be high (approximately $35 to $50 per sampler depending on labor, personal correspondence with Dan Zeroka, Engineering Technician, LU). With the addition of more sites and the employment of more samplers at one time, the necessity arose to attempt to build a cheaper prototype that still yielded the same results as the old. A new prototype was designed and deployed alongside the old prototype to collect data as to the efficacy of the new prototype, however this experiment was only begun towards the end of this thesis period (1/11/12 to 2/21/12). The results shown in Table 8 show fairly good comparison between the new and old prototypes with agreement between the Bethlehem WWTP upstream, downstream and Saucon Creek sites 67% (n=2/3), 75% (n=3/4) and 100% (n=4/4) of the time, respectively. A word of caution however, is that only one and zero counts were observed during all dates of observation between prototypes; furthermore, of the times the data were in agreement between the two prototypes, 50% (n=1/2), 33% (n=1/3) and 100% (n=4/4) of the agreements resulted from both prototypes having zero counts. More study is necessary to ascertain whether these results are reliable.

Tentative logistical analysis and experience using the new prototype tend to suggest that the old prototype is preferable. The new prototype uses a mesh screen with 0.10 inch x 0.16 inch openings which seems to slow the water flow through the pipes in which the slides are held resulting in an unwanted build-up of dirt and other sediment. This build-up minimizes the already small area for biofilm growth on the glass slides placed in the sampler, and this build-up does not seem to be alleviated by elevating the sampler in the water column (i.e. by placing atop rocks in the stream bed). Furthermore, the weight (necessary for all samplers to prevent too much movement within the streambed) for the new prototype is a 1" steel rod-shaped weight which rusted quite a bit even after short periods of time in the water, possibly resulting in contamination of the biofilms (rust was observed on all glass slides on all dates the biofilms were collected).

Results from Queen Lane and Saucon Creek supported the need for another study in which biofilm thickness was assessed throughout the year to compare to oocyst counts during those same time periods. A study as such was attempted later in the thesis study, the results of which can be viewed in Table 9. Another analyst observed winter month biofilm thicknesses from Monocacy Creek (probably most similar to Saucon Creek of the three sites in which thicknesses were measured in the current study) over a two week period in 2008 which resulted in average biofilm thicknesses of 27.87, 26.62, 28.00 and 26.51 μm (Personal Communication, Dr. Elizabeth Wolyniak-DiCesare). These correlate extremely well with the winter month measurements taken in this study (Table 9). The same analyst took measurements in the fall (36.47 and 37.36 μm), spring (44.25 and 42.98 μm), and summer months (38.18, 37.89, 33.57 and 32.64 μm) at the same site showing that at a site used in this study (Monocacy Creek), colder months did foster thinner biofilms and it is likely lower

oocyst counts as well (Personal Communication, Dr. Elizabeth Wolyniak-DiCesare). Unfortunately, this experiment was only begun at the end of this thesis study yielding few results with which to work, however these data will continue to be observed by another analyst. More study of these trends is necessary to better understand the efficacy and realistic potential of using the biofilm sampling method to analyze surface waters for *Cryptosporidium*.

4.1 Conclusions

All the studies described above support the idea that biofilms can in fact be used to assess surface water bodies for *Cryptosporidium* contamination. Expected trends up and downstream of two WWTP outfalls were observed, with higher counts of oocysts found downstream when compared to upstream at both sites. Even at non-wastewater impacted control sites, as well as at sites where there are no known upstream influences or sources of *Cryptosporidium* contamination, the presence of oocysts were observed displaying the high sensitivity to pathogen detection using this biofilm sampling method.

The biofilm slide and FISH processing combination seemed to yield more positive results regarding oocyst numbers than any other combination analyzed here. The water filter and PCR combination, currently accepted monitoring method by the EPA, yielded the worst results in every comparison. These results suggest that the biofilm/FISH method is more sensitive with perhaps a lower detection limit for oocysts when compared to the other combination.

The studies described here certainly prove the biofilm method to be a useful tool to monitor for *Cryptosporidium* in watersheds, however, it is the view of the author that perhaps

biofilm thickness or surface roughness plays a role in oocyst embedment within biofilms. At a couple of sites during the study, lower or zero oocyst counts were obtained during cooler winter months. Perhaps microbial growth rates, which slow at lower temperatures, were responsible for generally thinner biofilms during winter months, and possibly led to these low or zero counts.

The presence of viable and other oocysts at both up and downstream locations of a WWTP outfall, as well as at all sampled control sites, suggests that there are other potential sources. One possible source is variation in flow patterns within the streams, which was observed at one of the WWTPs studied here. In this instance, eddies moved the discharge outflow farther upstream than the upstream sample site location. Another possible source is intermittent human, animal and domestic animal contamination. At the same WWTP where eddies were observed, humans and animals were observed to occupy the location for significant amounts of time potentially introducing a source. Furthermore, golf courses and agricultural land may have impacted the oocyst data at several of these sites.

4.2 Future Directions/Recommendations

As discussed previously (See Section XX FISH and XX FISH Counts) a different mounting media than that recommended by the manufacturers of the MERIFluor Kit is used in the FISH to PCR protocol. This is to prevent the formalin in the Kit mounting media from adversely affecting the PCR protocol and inhibiting the achievement of results from the combination of methods. It is the recommendation of the author that perhaps another PCR/DNA method (such as one more similar to those used by D. G. DiGiovanni first seen in the publication AWWA, 2010). This recommendation is made due to the poor

fluorescing results observed in the FISH method when using this alternate mounting media. Although difficult to see via photograph, a comparison of two control oocyst pictures can be viewed in Figure 14, where the use of the intended mounting media with the Kit is on the left, and the use of the alternate mounting media is on the right. When attempting to combine the two processing methods of FISH and PCR in our laboratory, no changes were made to the methods used for performing the protocols separately except for the change in mounting medium. The fluorescence of oocysts viewed when using the alternate mounting medium is extremely faint when compared with those incubated with the intended mounting medium, which results in very high uncertainty in the analysts regarding their FISH counts of controls and probably in inaccurate counts of oocysts on the control FISH slides.

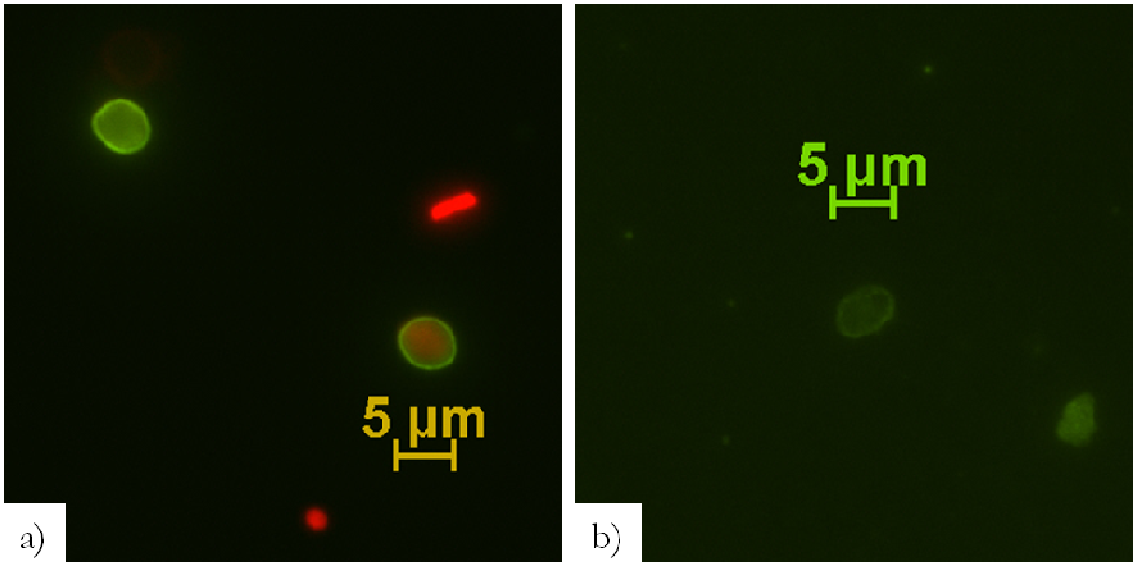

Figure 14. Photographs of control oocysts using mounting media from the MER*IFLOUR*™ Kit compared with the alternate non-formalin medium
Control oocysts were processed using the FISH protocol with a) using the Kit provided mounting medium and b) using the non-formalin based mounting medium

One issue with this method is the fact that it is very difficult to calculate just how much water each sample is exposed to before collection. Some way to obtain a calculation

of water exposure should be analyzed in future research. When using water filters, an exact amount of water filtered is known and thus the oocyst concentration per volume of water can be calculated. Having no real measure of volume of water each sampler is exposed to makes it difficult with respect to watershed management because there is no real way besides simple total oocyst counts to explain *Cryptosporidium* contamination; this count gives no idea as to the potential volume of water contaminated making watershed management difficult. If this volume of water exposure could somehow be calculated, it would make a better management tool and provide more information with a concentration of oocysts obtained from the samplers rather than just a count.

The oocyst counts themselves still hold uncertainty with respect to the low counts even at sites that we would expect the numbers to be higher. Most of the sites, even at WWTPs where we would expect higher numbers of oocysts in the downstream location, showed multiple one-oocyst counts. The fact that often these low oocyst counts were not detected when put through the PCR and sequencing processes shows that it is likely the detection limit is higher with PCR than one or two oocysts.

A recommendation in the paper is to collect more thickness measurements versus season at all the sites. These data seem to reflect that biofilm thickness is dependent on seasonal temperatures and oocyst counts, are in turn, affected by the thickness of the biofilms. Thickness measurements compared with oocyst counts at the same sites and seasons would provide invaluable data as to whether this method is highly dependent on biofilm thickness. Evaluation of the biofilm method, and its efficiency for monitoring for

Cryptosporidium contamination in watersheds, makes it necessary to understand if biofilm thickness prohibits oocyst embedment within the biofilms during certain seasons.

Also recommended is more analysis and comparison of biofilm slides and water filter samples. Collection and analysis of these sample types for comparison to each other will provide invaluable data as to the efficacy of the biofilm method for *Cryptosporidium* monitoring in watersheds. However, the old prototype should be used in future experiments and the new prototype should be no longer used. Too many logistical issues exist to make processing more slides from the new prototype sampler worthwhile.

5. List of References

Abuvakar, I., Aliyu, S. H., Arumugam, C., Usman, N. K., and Hunter, P. R. (2007). Treatment of cryptosporidiosis in immunocompromised individuals: systematic review and meta-analysis. *British Journal of Clinical Pharmacology 63*: 387–393.

Alpert, G., Bell, L. M., Kirkpatrick, C. E., Budnick, L. D., Campos, J. M., Friedman, H. M., and Plotkin, S. A. (1986). Outbreak of cryptosporidiosis in a day-care center. *Pediatrics, 77*: 152-157.

American Water Works Association (AWWA). (2010). New molecular method IDs pathogenic Crypto. Streamlines, 2(18). Retrieved from http://www.awwa.org/index.cfm

Angles, M. L., Chandy, J. P., Cox, P. T., Fisher, I. H., and Warnecke, M. R. (2007). Implications of biofilm-associated waterborne *Cryptosporidium* oocysts for the water industry. *Trends in Parasitology 23*: 352-356.

Casemore, D. (1990). Epidemiological aspects of human cryptosporidiosis. *Epidemiology and Infection, 104*: 1-28.

Flemming, H. C., S. L. Percival, and J. T. Walker. 2002. Contamination potential of biofilms in water distribution systems. *Water Science and Technology, 2*: 271–280.

Flood, J. A., and N. J. Ashbolt. 2000. Virus-sized particles can be entrapped and concentrated one hundred fold within wetland biofilms. *Advances in Environmental Research, 3*: 403–411.

Graczyk, T. K., Evans, B. M., Shiff, C. J., Karreman, H. J., and Patz, J. A. (2000). Environmental and geographical factors contributing to watershed contamination with *Cryptosporidium parvum* oocysts. *Environmental Research Section, 82 (A)*: 263-271. doi:10.1006/enrs.1999.4022

Graczyk, T. K., Grimes, B. H., Knight, R., DaSilva, A. J., Pieniazek. N. J., and Veal, D. A. (2003). Detection of *Cryptosporidium parvum* and *Giardia lamblia* carried by synanthropic flies by combined fluorescent in situ hybridization and a monoclonal antibody. *American Journal of Tropical Medicine and Hygiene, 68,* 228-232.

Guerrant, R.L. (1997). Cryptosporidiosis: An emerging, highly infectious threat. *Emerging Infectious Diseases, 3(1)*: 51-57.

Haas, C. N., and Rose, J. B. (1994). Reconciliation of microbial risk models and outbreak epidemiology: the case of the Milwaukee outbreak. Proceedings of the American Water Works Association, 1994: 517-23.

Helmi, K., Skraber, S., Gantzer, C., Willame, R., Hoffmann, L., and Cauchie, H.-M. (2008). Interactions of *Cryptosporidium parvum*, *Giardia lamblia*, Vaccinal Poliovirus Type 1, and bacteriophages φX174 and MS2 with a drinking water biofilm and a wastewater biofilm. *Applied and Environmental Microbiology, 74 (7)*: 2079-2088. doi:10.1128/AEM.02495-07

Howe, A. D., Forster, S., Morton, S., Marshall, R., Osborn, K. S., Wright, P., and Hunter, P. R. (2002). *Cryptosporidium* oocysts in a water supply associated with a cryptosporidiosis outbreak. *Emerging Infectious Diseases, 8*: 619-624.

Jellison, K. L., Hemond, H. F. & Schauer, D. B. (2002). Sources and species of *Cryptosporidium o*ocysts in the Wachusett Reservoir Watershed. *Applied and Environmental Microbiology*, 569–575. doi: 10.1128/AEM.68.2.569–575.2002

Jellison, K. L., Lynch, A. E., and Ziemann, J. M. (2009). Source tracking identifies deer and geese as vectors of human-infectious *Cryptosporidium* genotypes in an urban/suburban watershed. *Environmental Science and Technology, 43(12)*: 4267-4272.

Jellison, K. (2010). Canada geese: tranquil symbol of the wild or pathogen carrier? *Opflow, August 2010*: 16-18.

Jellison, K. L. (Lehigh University, Bethlehem, PA). *Detection and Genotyping of Cryptosporidium spp. Oocysts in Water and Geese Feces in the Wissahickon Watershed; September, 2008-June, 2011: A comprehensive final report to PWD*. Philadelphia (PA): Philadelphia Water Department. 2011 June.

Jokipii, L., Pohjola, S., and Jokipii A. M. (1985). Cryptosporidiosis associated with traveling and giardiasis. *Gastroenterology, 89*: 838-842.

Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Shapiro, A., Azevedo, N. F., Ménard-Szczebara, F., Castagnet, S., Féliers, C., and Keevil, C. W. (2007). Detection of *Escherichia coli* in biofilms from pipe samples and coupons in drinking water distribution networks. *Applied and Environmental Microbiology, 73*: 7456-7464.

Keevil, C. W. 2003. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. *Water Science and Technology, 47*: 105–116.

Kock, K. L., Phillips, D. J., Aber, R. C., and Current, W. L. (1985). Cryptosoridiosis in hospital personnel: evidence for person-to-person transmission. *Annals of Internal Medicine, 102*: 593-596.

Lisle, J. and Rose, J. (1995). *Cryptosporidium* contamination of water in the USA and UK: a mini-review. *Journal of Water Supply: Research and Technology-Aqua, 44(3)*: 103-117.

MacKenzie, W. R., Hoxie, N. J., Proctor, M. E., Gradus, M. S., Blair, K. A., Peterson, D. E., Kazmierczak, J. J., Addiss, D. G., Fox, K. R., Rose, J. B., and Davis, J. P. (1994). A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *New England Journal of Medicine, 331*: 161-167.

Madore, M., Rose, J., Gerba, C., Arrowood, M., and Sterling, C. (1987). Occurrence of Cryptosporidium oocysts in sewage effluents and selected surface waters. *Journal of Parasitology, 73(4)*: 702-705.

Melo, L. F. and Bott, T. R. (1997). Biofouling in water systems. *Experimental Thermal and Fluid Science, 14*: 375-381.

Morgenroth, E., and Wilderer, P. A. (2000). Influence of detachment mechanisms on competition in biofilms. *Water Resources, 34*: 417–426.

Nime, F. A., Burek, J. D., Page, D. L., Holscher, M. A., and Yardley, J. H. (1976). Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. *Gastroenterology, 70*: 592-598.

Ong, D., Moorehead, W., Ross, A., and Isaac-Renton, J. L. (1996a). *Giardia* spp. and *Cryptosporidium* spp. in British Columbia watersheds. *Journal of Eukaryotic Microbiology, 43(5)*: 65S.

Ong, C., Moorehead, W., Ross, A., and Isaac-Renton, J. L. (1996b). Studies of *Giardia* spp. and *Cryptosporidium* spp. in two adjacent watersheds. *Applied and Environmental Microbiology, 62(8)*: 2798-2805.

Rose, J. B. (1997). Environmental ecology of *Cryptosporidium* and public health implications. *Annual Review of Public Health, 18*: 135-161. doi: 10.1146/annurev.publhealth.18.1.135

Ruecker, N. J., Braithwaite, S. L., Topp, E., Edge, T., Lapen, D. R., Wilkes, G., Robertson, W., Medeiros, D., Sensen, C. W., and Neumann, N. F. (2007). Tracking host sources of Cryptosporidium spp. in raw water for improved health rish assessment. *Applied and Environmental Microbiology, 73(12)*: 3945-3957.

Searcy, K. E., Packman, A. I., Atwill, E. R., and Harter, T. (2006). Capture and retention of *Cryptosporidium parvum* oocysts by *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology, 72(9)*: 6242-6247.

Simmons, O. D., M. D. Sobsey, C. D. Heaney, F. W. Schaefer, and D. S. Francy. 2001. Concentration and detection of *Cryptosporidium* oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration. *Applied and Environmental Microbiology, 67*: 1123–1127.

Smith, A., Reacher, M., Smerdon, W., Adak, G. K., Nichols, G., and Chalmers, R. M. (2006). Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. *Epidemiology and Infection 134(6)*: 1141-1149. Doi: 10.1017/S0950268806006406

Smith, H. V. (1990). Environmental aspects of *Cryptosporidium* species: a review. *Journal of the Royal Society of Medicine, 83*: 629-631.

Smith, H. V. and Rose, J. B. (1990). Waterborne cryptosporidiosis. *Parasitology Today, 6*: 8-12.

Smith, H. V. and Rose, J. B. (1998). Waterborne cryptosporidiosis: current status. *Parasitology Today, 14(1)*: 14-22.

Snyder, R. A., Lewis, M. A., Nocker, A., Lepo, J. E. (2005). Microbial biofilms as integrative sensors of environmental quality. In: Bortone SA, ed. Estuarine Indicators. New York: CRC Press. p. 111-126.

Todd, S. C., Phillips, M. S., Marchin, G. L., and Upton, S. J. (1991). *Cryptosporidium* and *Giardia* in surface waters in and around Manhattan, Kansas. *Transactions of the Kansas Academy of Science, 94(3/4)*: 101-110.

United States Environmental Protection Agency (USEPA). (1999). Method 1622: *Cryptosporidium* in water by filtration/IMS/FA. United States Environmental Protection Agency, Office of Water. EPA-821-R-99-001.

United States Environmental Protection Agency (USEPA). (2007). Long Term 2 Enhanced Surface Water Treatment Rule (LT2). http://www.epa.gov/safewater/disinfection/lt2/index.html

Vesey, G., Ashbolt, N., Fricker, E. J., Deere, D., Williams, K. L., Veal, D. A., and Dorsch, M. (1998). The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labeling of viable *Cryptosporidium parvum* oocysts. *Journal of Applied Microbiology, 85*: 429-440.

Walker, M. J., Montemagno, C. D., and Jenkins, M. B. (1998). Source water assessment and nonpoint sources of acutely toxic contaminants: a review of research related to survival and transport of *Cryptosporidium parvum. Water Resources Research, 34*: 3383-3392.

Wingender, J., and Flemming, H.-C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health, 214 (6)*: 417-423. doi.org/10.1016/j.ijheh.2011.05.009

Wolyniak, E. A., Jellison, K. L., and Hargreaves, B. R. (2009). Retention and release of *Cryptosporidium parvum* oocysts by experimental biofilms composed of a natural stream microbial community. *Applied and Environmental Microbiology, 75*: 4624-4626.

Wolyniak, E. A., Jellison, K. L., and Hargreaves, B. R. (2010). Seasonal retention and release of *Cryptosporidium parvum* oocysts by environmental biofilms in the laboratory. *Applied and Environmental Microbiology, 76*: 1021-1027.

Yoder, J. S., and Beach, M. J., Centers for Disease Control and Prevention (CDC). (2007). Cryptosporidiosis surveillance – United States, 2003–2005. *MMWR Surveillance Summaries, 56(7)*: 1–10.

Yoder, J. S., Harral, C., and Beach, M. J., Centers for Disease Control and Prevention (CDC). (2010). Cryptosporidiosis surveillance – United States, 2006-2008. *MMWR Surveillance Summaries, 59(6)*: 1-14.

6. **Appendix A.** Complete data set information.

Date Collected	Type of Sample	# of days slides in creek	Sampling Location	Total # oos (Merifluor)	# viable *C. hominus* or *C. parvum* (FISH probe)	oos besides *C. hominus/C. parvum* or non-viable human	Tech	Date IMS performed	Date FISH performed	Date FISH slides counted
4/13/2010	Filter		Queen Lane A	0	0	0	EAW			
4/13/2010	Filter		Queen Lane B	0	0	0	EAW			
4/27/2010	Filter		Queen Lane A	2	1	1	EAW			
4/27/2010	Filter		Queen Lane B	4	4	0	EAW			
5/11/2010	Filter		Queen Lane A	0	0	0	EAW			
5/11/2010	Filter		Queen Lane B	0	0	0	EAW			
5/25/2010	Filter		Queen Lane A	1	1	0	EAW/RBP			
5/25/2010	Filter		Queen Lane B	1	1	0	EAW/RBP			
6/8/2010	Filter		Queen Lane A	0	0	0	EAW/RBP			
6/8/2010	Filter		Queen Lane B	0	0	0	EAW/RBP			
6/29/2010	Filter		Queen Lane A	13	11	2	EAW/RBP	7/21/10	7/22/10	7/22/10
6/29/2010	Filter		Queen Lane B	0	0	0	EAW/RBP	7/21/10	7/22/10	7/22/10
7/19/2010	Slides	7	Monocacy Creek	0	0	0	RBP	7/19/10	7/20/10	7/20/10
7/19/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	7/19/10	7/20/10	7/20/10
7/20/2010	slides	7	Queen Lane	2	1	1	EAW/RBP	7/21/10	7/22/10	7/22/10
7/26/2010	slides	7	Monocacy Creek	16	3	13	EAW/RBP	7/26/10	7/27/10	7/28/10
7/26/2010	Rock Scraping		Monocacy Creek	1	0	1	RBP	7/26/10	7/27/10	7/28/10
7/28/2010	Filter		Queen Lane	2	1	1	RBP	7/29/10	7/29/10	7/29/10
7/28/2010	slides	7	Queen Lane	7	6	1	RBP	7/28/10	7/29/10	7/29/10
8/2/2010	slides	7	Monocacy Creek	6	3	3	RBP	8/2/10	8/2/10	8/3/10
8/2/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	8/2/10	8/2/10	8/3/10
8/9/2010	Slides	7	Monocacy Creek	0	0	0	RBP	8/9/10	8/10/10	8/12/10
8/9/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	8/9/10	8/10/10	8/12/10
8/10/2010	slides	14	Queen Lane	1	0	1	RBP	8/11/10	8/11/10	8/12/10
8/10/2010	Filter		Queen Lane	1	0	1	RBP	8/11/10	8/11/10	8/12/10
8/16/2010	Slides	7	Monocacy Creek	1	1	0	RBP	8/16/10	8/16/10	8/16/10
8/16/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	8/16/10	8/16/10	8/16/10
8/23/2010	Slides	7	Monocacy Creek	1	1	0	RBP	8/23/10	8/24/10	8/24/10
8/23/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	8/23/10	8/24/10	8/24/10
8/24/2010	Slides	14	Queen Lane	2	0	2	RBP	8/25/10	8/26/10	8/26/10
8/24/2010	Filter		Queen Lane							
9/7/2010	Slides	15	Monocacy Creek	1	0	1	RBP	9/7/10	9/9/10	9/10/10
9/7/2010	Rock Scraping		Monocacy Creek	1	0	1	RBP	9/7/10	9/9/10	9/10/10
9/7/2010	Rock Scraping		Sandy Run up	5	4	1	RBP	9/8/10	9/9/10	9/10/10
9/7/2010	Rock Scraping		Sandy Run down	19	13	6	RBP	9/8/10	9/9/10	9/10/10
9/21/2010	Rock Scraping		Monocacy Creek	1	0	1	RBP/CMM	9/22/10	9/23/10	9/24/10
9/21/2010	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM	9/22/10	ns	ns
9/21/2010	Slides	28	5902 - Queen Lane	5	3	2	RBP/CMM	9/22/10	9/23/10	9/24/10
9/21/2010	Filter		SR1 - Sandy Run UP							
9/21/2010	Slides	14	SR1 - Sandy Run UP	1	1	0	RBP/CMM	9/22/10	9/23/10	9/24/10
9/21/2010	Filter		SR2 - Sandy Run DOWN	3	2	1	RBP/CMM	9/22/10	9/23/10	9/24/10
9/21/2010	Slides	14	SR2 - Sandy Run DOWN	4	4	0	RBP/CMM	9/22/10	9/23/10	9/24/10
9/21/2010	Filter		Monocacy Creek	0	0	0	RBP/CMM	9/22/10	9/23/10	9/24/10
9/28/2010	slides	7	Queen Lane	0	0	0	RBP/CMM	9/29/10	9/30/10	10/1/10
9/28/2010	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM			
9/28/2010	Slides	7	SR1 - Sandy Run UP	0	0	0	RBP/CMM	9/29/10	9/30/10	10/1/10
9/28/2010	Slides	7	SR2 - Sandy Run DOWN	2	0	2	RBP/CMM	9/29/10	9/30/10	10/1/10
10/12/2010	Filters (2)		Queen Lane	ns	ns	ns				
10/12/2010	Slides	14	SR1 - Sandy Run UP	1	1	0	RBP/CMM	10/13/10	10/13/10	10/15/10
10/12/2010	Slides	14	SR2 - Sandy Run DOWN	1	0	1	RBP/CMM	10/13/10	10/13/10	10/15/10
10/20/2010	Filter		Monocacy Creek	na	na	na	RBP/CMM	10/21/10	10/21/10	10/21/10
10/26/2010	Filters (2)		Queen Lane	ns	ns	ns				
10/26/2010	Slides	14	SR1 - Sandy Run UP	1	1	0	RBP	10/27/10	10/29/10	10/29/10
10/26/2010	Slides	14	SR2 - Sandy Run DOWN	2	1	1	RBP	10/27/10	10/29/10	10/29/10
11/8/2010	Filter		Monocacy Creek	0	0	0	CMM	na	na	na

Date Collected	Type of Sample	# of days slides in creek	Sampling Location	Total # oos (Merifluor)	# viable *C. hominus* or *C. parvum* (FISH probe)	oos besides *C. hominus/C. parvum* or non-viable human	Tech	Date IMS performed	Date FISH performed	Date FISH slides counted
11/9/2010	Filters (2)		Queen Lane	ns	ns	ns				
11/9/2010	Slides	14	Queen Lane	1	0	1	RBP/CMM	11/10/10	11/10/10	11/15/10
11/9/2010	Slides	14	SR1 - Sandy Run UP	1	0	1	RBP/CMM	11/10/10	11/10/10	11/15/10
11/9/2010	Slides	14	SR2 - Sandy Run DOWN	1	1	0	RBP/CMM	11/10/10	11/10/10	11/15/10
11/16/2010	Slides	25	Monocacy Creek	1	1	0	RBP	11/19/10	11/19/10	11/29/10
11/16/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	11/19/10	11/19/10	11/29/10
11/23/2010	Filter		Monocacy Creek	0	0	0	RBP/CMM	11/23/10	11/23/10	11/24/10
11/23/2010	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM	11/24/10	11/24/10	11/29/10
11/23/2010	Slides	14	Queen Lane	2	2	0	RBP	11/24/10	11/29/10	11/29/10
11/23/2010	Slides	14	SR1 - Sandy Run UP	0	0	0	RBP	11/24/10	11/29/10	11/29/10
11/23/2010	Slides	14	SR2 - Sandy Run DOWN	4	2	2	RBP	11/24/10	11/29/10	11/29/10
12/3/2010	Slides	17	Monocacy Creek	1	1	0	RBP	12/3/10	12/6/10	12/6/10
12/3/2010	Rock Scraping		Monocacy Creek	1	1	0	RBP	12/3/10	12/6/10	12/6/10
12/7/2010	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM			
12/7/2010	Slides	14	Queen Lane	2	2	0	RBP	12/8/10	12/10/10	12/10/10
12/7/2010	Slides	14	SR1 - Sandy Run UP	1	1	0	RBP	12/8/10	12/10/10	12/10/10
12/7/2010	Slides	14	SR2 - Sandy Run DOWN	4	2	2	RBP	12/8/10	12/10/10	12/10/10
12/8/2010	Filter		Monocacy Creek	0	0	0	CMM/RBP	12/8/10	12/9/10	12/10/10
12/15/2010	slides	12	Monocacy Creek	0	0	0	RBP	12/16/10	12/17/10	12/17/10
12/15/2010	Rock Scraping		Monocacy Creek	1	0	1	RBP	12/16/10	12/17/10	12/17/10
12/21/2010	Filters (2)		Queen Lane				RBP/CMM			
12/21/2010	Slides	14	Queen Lane	5	3	2	RBP	12/22/10	12/22/10	12/22/10
12/21/2010	Slides	14	SR1 - Sandy Run UP	0	0	0	RBP	12/22/10	12/22/10	12/22/10
12/21/2010	Slides	14	SR2 - Sandy Run DOWN	4	3	1	RBP	12/22/10	12/22/10	12/22/10
12/22/2010	slides	7	Monocacy Creek	2	1	1	RBP	12/22/10	12/22/10	12/22/10
12/22/2010	Rock Scraping		Monocacy Creek	0	0	0	RBP	12/22/10	12/22/10	12/22/10
12/22/2010	Filter		Monocacy Creek	0	0	0	RBP/CMM	12/22/10	12/22/10	12/22/10
1/7/2011	Rock Scraping		Monocacy Creek	0	0	0	RBP	1/14/11	1/17/11	1/21/11
1/7/2011	Slides	16	Monocacy Creek	ns	ns	ns	RBP	1/14/11	1/17/11	1/21/11
1/7/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	1/14/11	1/17/11	1/21/11
1/11/2011	Filters (2)		Queen Lane	ns	ns	ns				
1/11/2011	Slides	21	Queen Lane	0	0	0	RBP	1/14/11	1/17/11	1/21/11
1/11/2011	Slides	21	SR1 - Sandy Run UP	0	0	0	RBP	1/14/11	1/17/11	1/21/11
1/11/2011	Slides	21	SR2 - Sandy Run DOWN	1	0	1	RBP	1/14/11	1/17/11	1/21/11
1/25/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	1/26/11	1/27/11	1/28/11
1/25/2011	Filters (2)		Queen Lane	ns	ns	ns				
1/25/2011	Slides	14	Queen Lane	0	0	0	RBP	1/26/11	1/27/11	1/28/11
1/25/2011	Slides	14	SR1 - Sandy Run UP	0	0	0	RBP	1/26/11	1/27/11	1/28/11
1/25/2011	Slides	14	SR2 - Sandy Run DOWN	0	0	0	RBP	1/26/11	1/27/11	1/28/11
1/31/2011	slides	24	Monocacy Creek	0	0	0	RBP	2/10/11	2/11/11	2/11/11
1/31/2011	Rock Scraping		Monocacy Creek	0	0	0	RBP	2/10/11	2/11/11	2/11/11
2/8/2011	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM			
2/8/2011	Slides	14	Queen Lane	1	0	1	RBP	2/10/11	2/11/11	2/11/11
2/8/2011	Slides	14	SR2 - Sandy Run DOWN	0	0	0	RBP	2/10/11	2/11/11	2/11/11
2/9/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	na	na	2/26/11
2/15/2011	slides	15	Monocacy Creek	0	0	0	RBP	2/24/11	2/26/11	2/26/11
2/15/2011	Rock Scraping		Monocacy Creek	0	0	0	RBP	2/24/11	2/26/11	2/26/11
2/23/2011	Filters (2)		Queen Lane	ns	ns	ns	RBP/CMM			
2/23/2011	Slides	15	Queen Lane	0	0	0	RBP	2/24/11	2/26/11	2/26/11
2/23/2011	Slides	15	SR1 - Sandy Run UP	0	0	0	RBP	2/24/11	2/26/11	2/26/11
2/23/2011	Slides	15	SR2 - Sandy Run DOWN	1	1	0	RBP	2/24/11	2/26/11	2/26/11
2/24/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	3/9/11	3/10/11	3/18/11
3/2/2011	slides	15	Monocacy Creek	0	0	0	RBP	3/9/11	3/10/11	3/18/11
3/2/2011	Rock Scraping		Monocacy Creek	0	0	0	RBP	3/9/11	3/10/11	3/18/11
3/8/2011	Filters (2)		Queen Lane	ns	ns	ns				
3/8/2011	Slides	13	Queen Lane	8	4	4	RBP	3/9/11	3/10/11	3/18/11
3/8/2011	Slides	13	SR1 - Sandy Run UP	0	0	0	RBP	3/9/11	3/10/11	3/18/11
3/8/2011	Slides	13	SR2 - Sandy Run DOWN	1	0	1	RBP	3/9/11	3/10/11	3/18/11
3/9/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	na	na	3/18/11
3/22/2011	Filters (2)		Queen Lane	ns	ns	ns				
3/22/2011	Slides	14	Queen Lane	5	2	3	RBP	3/23/11	3/24/11	4/13/11
3/22/2011	Slides	14	SR1 - Sandy Run UP	1	0	1	RBP	3/23/11	3/24/11	4/13/11
3/22/2011	Slides	14	SR2 - Sandy Run DOWN	1	0	1	RBP	3/23/11	3/24/11	4/13/11
3/23/2011	slides	21	Monocacy Creek	1	0	1	RBP	3/23/11	3/24/11	4/13/11
3/23/2011	Filter		Monocacy Creek	ns	ns	ns	RBP	3/23/11	3/24/11	4/13/11
4/12/2011	Slides	21	Queen Lane	3	1	2	RBP	4/15/11	4/15/11	4/15/11
4/12/2011	Slides	21	SR1 - Sandy Run UP	0	0	0	RBP	4/15/11	4/15/11	4/15/11
4/12/2011	Slides	21	SR2 - Sandy Run DOWN	1	1	0	RBP	4/15/11	4/15/11	4/15/11
4/13/2011	slides	21	Monocacy Creek	1	1	0	RBP	4/15/11	4/15/11	4/15/11
4/13/2011	Rock Scraping		Monocacy Creek	1	1	0	RBP	4/15/11	4/15/11	4/15/11
4/19/2011	Filters (2)		Queen Lane	ns	ns	ns				
4/20/2011	Filter		Monocacy Creek	1	1	0	RBP/CMM	na	na	na

Date Collected	Type of Sample	# of days slides in creek	Sampling Location	Total # oos (Merifluor)	# viable *C. hominus* or *C. parvum* (FISH probe)	oos besides *C. hominus/C. parvum* or non-viable human	Tech	Date IMS performed	Date FISH performed	Date FISH slides counted
4/26/2011	Filters (2)		Queen Lane	ns	ns	ns				
4/26/2011	Slides	14	Queen Lane	2	1	1	RBP	4/30/11	5/1/11	5/1/11
4/26/2011	Slides	14	SR1 - Sandy Run UP	0	0	0	RBP	4/30/11	5/1/11	5/1/11
4/26/2011	Slides	14	SR2 - Sandy Run DOWN	0	0	0	RBP	4/30/11	5/1/11	5/1/11
4/28/2011	Filter		Monocacy Creek	0	0	0	RBP/CMM	5/13/11	5/20/11	5/26/11
5/10/2011	Slides	14	Queen Lane	0	0	0	RBP	5/13/11	5/20/11	5/26/11
5/10/2011	Slides	14	SR1 - Sandy Run UP	0	0	0	RBP	5/13/11	5/20/11	5/26/11
5/10/2011	Slides	14	SR2 - Sandy Run DOWN	0	0	0	RBP	5/13/11	5/20/11	5/26/11
NEW SITES:										
6/20/2011	Slides	19	Monocacy (upstream)	0	0	0	RBP	6/21/11	6/21/11	6/24/11
		19	Saucon Creek	2	0	2	RBP	6/21/11	6/21/11	6/24/11
6/22/2011	Slides	14	Bethlehem WWTP UP	0	0	0	RBP	6/23/11	6/24/11	6/24/11
		14	Bethlehem WWTP DOWN	1	0	1	RBP	6/23/11	6/24/11	6/24/11
6/29/2011	Slides	9	Monocacy (upstream)	0	0	0	RBP	6/30/11	7/6/11	7/11/11
		9	Saucon Creek	1	1	0	RBP	6/30/11	7/6/11	7/11/11
7/6/2011	Slides	14	Bethlehem WWTP UP	2	0	2	RBP	7/7/11	7/8/11	7/11/11
7/13/2011	Slides	14	Monocacy (upstream)	0	0	0	RBP	7/13/11	7/15/11	7/18/11
		14	Saucon Creek	2	1	1	RBP	7/13/11	7/15/11	7/18/11
7/20/2011	Slides	14	Bethlehem WWTP UP	0	0	0	RBP	7/20/11	7/21/11	7/21/11
	Slides	28	Bethlehem WWTP DOWN	3	1	2	RBP	7/20/11	7/21/11	7/21/11
7/27/2011	Slides	14	Monocacy (upstream)	1	0	1	RBP	7/27/11	7/28/11	7/29/11
8/3/2011	Slides	14	BWWTP Down	3	2	1	RBP	8/4/11	8/5/11	8/10/11
8/10/2011	Slides	12	Saucon Creek	0	0	0	RBP	8/10/11	8/10/11	8/10/11
8/17/2011	Slides	12	BWWTP Up	1	0	1	RBP	8/22/11	8/22/11	8/29/11
8/24/2011	Slides	14	Saucon Creek	0	0	0	RBP	8/25/11	8/26/11	8/29/11
8/31/2011	Slides	7	Saucon Creek	2	1	1	RBP	9/22/11	9/26/11	10/3/11
9/21/2011	Slides	35	BWWTP Down	4	2	2	RBP	9/22/11	9/26/11	10/3/11
9/21/2011	Slides	42	BWWTP Up	1	1	0	RBP	9/22/11	9/26/11	10/3/11
9/21/2011	Slides	21	Saucon Creek	1	0	1	RBP	9/22/11	9/26/11	10/3/11
10/12/2011	Slides	21	BWWTP Down	1	0	1	RBP	10/13/11	10/14/11	10/17/11
10/12/2011	Slides	21	BWWTP Up	1	1	0	RBP	10/13/11	10/14/11	10/17/11
10/12/2011	Slides	21	Saucon Creek	0	0	0	RBP	10/13/11	10/14/11	10/17/11
10/24/2011	Slides	14	BWWTP Down	3	2	1	RBP	10/25/11	11/18/11	11/21/11
10/24/2011	Slides	14	BWWTP Up	0	0	0	RBP	10/25/11	11/18/11	11/21/11
10/24/2011	Slides	14	Saucon Creek	0	0	0	RBP	10/25/11	11/18/11	11/21/11
11/16/2011	Slides	23	BWWTP Down	0	0	0	RBP	11/17/11	11/18/11	11/21/11
11/16/2011	Slides	23	BWWTP Up	1	1	0	RBP	11/17/11	11/18/11	11/21/11
11/16/2011	Slides	23	Saucon Creek	0	0	0	RBP	11/17/11	11/18/11	11/21/11
12/14/2011	Slides	28	BWWTP Down	3	3	0	RBP	12/15/11	12/19/11	12/20/11
12/14/2011	Slides	28	BWWTP Up	1	0	1	RBP	12/15/11	12/19/11	12/20/11
12/14/2011	Slides	28	Saucon Creek	0	0	0	RBP	12/15/11	12/19/11	12/20/11
12/29/2011	Slides	15	BWWTP Down	0	0	0	RBP	1/5/12	1/6/12	1/10/12
12/29/2011	Slides	15	BWWTP Up	0	0	0	RBP	1/5/12	1/6/12	1/10/12
12/29/2011	Slides	15	Saucon Creek	1	0	1	RBP	1/5/12	1/6/12	1/10/12
1/11/2012	Slides	14	BWWTP Down	1	1	0	RBP	1/12/12	1/16/12	1/17/12
1/11/2012	Slides	14	BWWTP Down NEW	1	0	1	RBP	1/12/12	1/16/12	1/17/12
1/11/2012	Slides	14	BWWTP Up	0	0	0	RBP	1/12/12	1/16/12	1/17/12
1/11/2012	Slides	14	Saucon Creek	0	0	0	RBP	1/12/12	1/16/12	1/17/12
1/11/2012	Slides	14	Saucon Creek NEW	0	0	0	RBP	1/12/12	1/16/12	1/17/12
1/24/2012	Slides	13	BWWTP Down	0	0	0	RBP	1/25/12	1/26/12	1/27/12
1/24/2012	Slides	13	BWWTP Down NEW	0	0	0	RBP	1/25/12	1/26/12	1/27/12
1/24/2012	Slides	13	BWWTP Up	0	0	0	RBP	1/25/12	1/26/12	1/27/12
1/24/2012	Slides	13	BWWTP Up NEW	0	0	0	RBP	1/25/12	1/26/12	1/27/12
1/24/2012	Slides	13	Saucon Creek	0	0	0	RBP	1/25/12	1/26/12	1/27/12
1/24/2012	Slides	13	Saucon Creek NEW	0	0	0	RBP	1/25/12	1/26/12	1/27/12
2/7/2012	Slides	14	BWWTP Down	1	0	1	RBP	2/8/12	2/8/12	2/9/12
2/7/2012	Slides	14	BWWTP Down NEW	1	0	1	RBP	2/8/12	2/8/12	2/9/12
2/7/2012	Slides	14	BWWTP Up	0	0	0	RBP	2/8/12	2/8/12	2/9/12
2/7/2012	Slides	14	BWWTP Up NEW	1	1	0	RBP	2/8/12	2/8/12	2/8/12
2/7/2012	Slides	14	Saucon Creek	0	0	0	RBP	2/8/12	2/8/12	2/9/12
2/7/2012	Slides	14	Saucon Creek NEW	0	0	0	RBP	2/8/12	2/8/12	2/9/12
2/21/2012	Slides	14	BWWTP Down	0	0	0	RBP	2/22/12	2/22/12	2/23/12
2/21/2012	Slides	14	BWWTP Down NEW	1	0	1	RBP	2/22/12	2/22/12	2/23/12
2/21/2012	Slides	14	BWWTP Up	1	0	1	RBP	2/22/12	2/22/12	2/23/12
2/21/2012	Slides	14	BWWTP Up NEW	1	0	1	RBP	2/22/12	2/22/12	2/23/12
2/21/2012	Slides	14	Saucon Creek	0	0	0	RBP	2/22/12	2/22/12	2/23/12
2/21/2012	Slides	14	Saucon Creek NEW	0	0	0	RBP	2/22/12	2/22/12	2/23/12

7. Vita

Robin K. Barnes-Pohjonen

EDUCATION & CERTIFICATION:

Engineer in Training Certification: Expected 10/2012.

M.S. Environmental Engineering. Lehigh University, Bethlehem, PA. Expected 5/2012. Advisor: Dr. Kristen Jellison.

M.S. Marine and Atmospheric Sciences. Stony Brook University, Stony Brook, NY. 8/2005. Advisor: Dr. Anne E. McElroy.

B.S. Oceanography (Chemistry Minor). Humboldt State University, Arcata, CA. 12/2002. Advisor: Dr. Marie de Angelis.

PROFESSIONAL EXPERIENCE:

2010-Present—Research Assistant, Environmental Engineering Laboratory

Lehigh University, Bethlehem, PA

Investigated *Cryptosporidium* contamination in surface water and the potential of a new sampling procedure to compliment EPA sampling method 1622/23 for detecting pathogens in drinking water supplies.

- Coordinated sampling with various organizations across several PA. watersheds (i.e. Philadelphia Water Department (PWD), Bethlehem Department of Water & Sewer Resources, Berks County Conservancy, Allentown Waste Water Treatment)
- Primary analyst for PWD samples for LT2 requirement (on-going research collaboration)—all samples analyzed via immunofluorescent (**IFA**) and fluorescent *in-situ* hybridization (**FISH**) assays for identifying presence of *Cryptosporidium* while also characterizing human infectious species using **infectivity assays**
- Conducted **water quality testing** (i.e. turbidity, pH, DO, nutrients, bacteria, pathogens)
- Assisted in general laboratory management (e.g., ordering supplies, maintaining and calibrating equipment, training new lab personnel, etc.)

2008-2010—National Marine Fisheries Service, North Pacific Groundfish Observer

NOAA: NMFS; Alaskan Observers, Inc., Seattle, WA/Dutch Harbor, AK

Observed and recorded commercial fisheries activities in the Bering Sea of Alaska (stationed in Dutch Harbor, AK) while conducting daily sampling and ensuring that vessels followed pertinent fishing laws/regulations (**> 5,000 hours of cruise experience**). Improved our ability to maintain the sustainability of the Alaskan fisheries by increasing our understanding of fishing activities on fish populations.

- Monitored and recorded catch data (up to 120,000 tons per tow), including species composition, weights, biological information as well as documented seabird sightings and marine mammal interactions
- Conducted fish dissection, organ and otolith sampling for NMFS scientific studies

2006-2007—Staff Aquatic Scientist
The Elizabeth River Project, Portsmouth, VA

Assisted multiple non-profit project managers with environmental projects including wetland restoration, best management practices, low impact development options and multi-million dollar sediment remediation.

- Facilitated the completion of multi-project technical and engineering documents (i.e. grant proposals, requests for proposals, etc.)
- Coordinated meetings between technical advisors from multiple agencies (i.e. EPA, DEQ, VMRC, consultants, etc.) to evaluate data for making restoration decisions
- Educated and involved the local community in environmentally friendly behavior and projects
- Managed a project regarding a contest for community members to win a 'backyard makeover' to make their environment beautiful and sustainable

2003-2005—Research Assistant, Aquatic Toxicology Laboratory

Stony Brook University, Stony Brook, NY

Conducted research on marine populations and deleterious effects caused by pesticide sprays targeting West Nile Virus/mosquito populations in Long Island, NY marshes. Responsible for leading the field investigation with caged animals (i.e. wetland ecosystem analysis) and laboratory chemical toxicity testing.

- Created sampling plans for both field and laboratory
- Coordinated sampling efforts with a diverse, multi-institutional team (i.e. USGS, Suffolk County Department of Health Office of Ecology, Suffolk County Department of Public Works, RTP Environmental Corporation, Cashin Associates, as well as Southampton College)
- Organized and managed the caged animal, toxicity and salt marsh sampling and analyzed data essential for the completion of the 'Vector Control & Wetlands Management Long Term Plan for Suffolk County'

MANUSCRIPTS/PUBLICATIONS:

Barnes, R.K. and A.E. McElroy. 2011. Relative acute toxicity of Anvil, Scourge, Altosid and their active ingredients to the grass shrimp *Palaemonetes pugio*. **Manuscript in Preparation**.

Barnes, R.K. and A.E. McElroy. 2011. Assessment of toxicity associated with aerial application of Resmethrin and Altosid on salt marsh shrimp and fish. **Manuscript in Preparation**.

Barnes, R. K. and C. L. Gallegos. 2005. Influence of particle size on specific-absorption and -scattering coefficients of inorganic and non-algal particulate matter and implications for submerged aquatic vegetation. MARSci. *In press*.

Barnes, R. K. 2005. Master's Thesis Stony Brook University. Pesticides used to control West Nile Virus: Toxicity to the estuarine grass shrimp, *Palaemonetes pugio*.

McElroy, A. E., Turner, R. and C. Gobler. 2005. Assessment of the potential effects of mosquito spraying on local organisms: caging study, saltwater assessment, Cashin Associates Technical Report. (Aided by performing field and laboratory assessments, creating tables and graphs and editing drafts).

Brownawell, B. J., Terracciano, S. A., Ruggieri, J. P., Barnes, R. K. & A. E. McElroy. 2005. Detection and persistence of pesticides used to control West Nile virus in salt marsh waters and sediments after aerial application. Report to Cashin Associates as part of the Vector Control & Wetlands Management Long Term Plan for Suffolk County.

PRESENTATIONS & AWARDS:

Pennsylvania Water Environment Association 2011 Student Research Poster Award: Barnes-Pohjonen, R., McLeod, C., Wolyniak DiCesare, E., Napotnik, J. and K.L. Jellison. Comparing Two Methods of *Cryptosporidium* Detection in Eastern Pennsylvania Watersheds. Pennsylvania Water Environment Association Annual Technical Conference. Lancaster, PA, June 5-8, 2011.

Lehigh Valley Ecology & Evolution Symposium Best Graduate Student Presentation 2011: Barnes-Pohjonen, R., Wolyniak, E., McLeod, C., Napotnik, J. and K. Jellison. Use of *In-Situ* Biofilms to Monitor for *Cryptosporidium* at a Drinking Water Intake and Wastewater Treatment Outfall in Southeastern Pa. Lehigh Valley Ecology & Evolution Symposium. Muhlenberg College, PA, April 16, 2011.

Barnes, R. K., A. E. McElroy, B. J. Brownawell, J. P. Ruggieri, R. Cerrato, and S. A. Terracciano. 2005. A Field and laboratory assessment of lethal and non-lethal effects of pesticides used in mosquito control in Suffolk County on estuarine shrimp. Benthic Ecology Meeting. Williamsburg, VA, April 7-11, 2005.