ENVIRONMENTAL FATE AND PERSISTENCE OF CYCLOSPORA CAYETANENSIS

BY

NORA HAZEL ONSTAD

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Masters of Science in Technical Systems Management
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

Master's Committee:

Assistant Professor Paul C. Davidson
Dr. Michelle L. Green, University of South Florida
Assistant Professor William H. Witola

ABSTRACT

Cyclospora cayetanensis is an emerging food-borne protozoan pathogen. Similar to other gastrointestinal pathogens, cyclosporiasis causes prolonged diarrhea. Unlike *Cryptosporidium*, *Cyclospora* oocysts are not infective when they are shed by infected individuals. Oocysts mature in the environment for several weeks before sporulating. Little is known about how *C. cayetanensis* is transported in the environment. The literature indicates that water and food, such as leafy greens and berries, are common sources of infection. Contact with soil has also been correlated with cyclosporiasis infection. In addition to acting as a vector to transport oocysts from the environment to the body, water and soil may be important reservoirs to not only allow *C. cayetanensis* to persist, but also transport the oocysts from one location to another. This research utilizes discrete sampling from an urban area where human waste entered the environment. Nested PCR and two rounds of non-nested PCR analyses identified possible *Cyclospora* DNA in the soil, water, and wildlife scat found in the area following Combined Sewage Outfall (CSO) events. This may indicate that *C. cayetanensis* is prevalent in the environment. In addition, an experimental investigation of oocyst adhesion to soil was completed, but was inconclusive.

ACKNOWLEDGEMENTS

I would not have been able to learn all that I did without the help of my advisor and committee members: Drs. Paul C. Davidson, Michelle L. Green, and William H. Witoloa.

I would also like to express my appreciation for all the help from the following people, who made all this research possible. Matthew Miller was a great lab partner who helped with lab work, field sampling, and edits. He also kept me excited about the research when it seemed to stall. Yi Fei (Monica) Millard worked hard to optimize the PCR primers and produce beautiful gels. Sharon Dubosky helped with sampling and lab work, and answered all my questions about molecular techniques. Jorge San Juan produced Figure 4 describing the environmental pathways that *Cyclospora* can take. All the WE CAN fellows (Alyson Cervantes, Alondra Estrada, Erin Holland, Patricia Leon, Naomi Waldon and Alisha Weatherspoon) asked questions and helped me to explain my work in different ways. Dr. Johnathan Beever guided me to develop a new set of primers and taught me everything I wanted to know about PCR and DNA.

All my friends from the Dog Training Club of Champaign-Urbana, listened to all my stories about research and kept me motivated outside university with dog sports.

Finally, thank you to my family, Dawn Dockter, David Onstad, and Emma Onstad, who listened to all my excitement and frustration about *Cyclospora* for 2 years, they are the best.

For my parents, who are the greatest teachers.

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CHAPTER 1: INTRODUCTION

Cyclospora cayetanensis is an emerging food-borne pathogen. *Cyclospora* is similar to *Cryptosporidium* in morphology and also in the symptoms of human infection. *Cyclospora* oocysts are not infective when they are shed by infected individuals. Oocysts mature in the environment for one or more weeks before sporulating. Little is known about how *C. cayetanensis* is transported in the environment and which factors inhibit or promote sporulation. Water and fresh produce, such as leafy greens and berries, are common sources of infection. Contact with soil has also been correlated with cyclosporiasis infection. In addition to acting as a vector to transport oocysts from the environment to the body, water and soil and even wildlife may be important reservoirs to not only allow *C. cayetanensis* to persist, but also transport the oocysts from one location to another.

This research adds to the limited knowledge about *Cyclospora cayetanensis* by considering the environmental life stage of *C. cayetanensis* as oocysts. To understand more about *C. cayetanensis* in the environment, we address several objectives:

1. Complete a literature review of the existing knowledge on environmental fate and transport of *C. cayetanensis*,
2. Determine whether *C. cayetanensis* is present in soil, water, or animal scat near Combined Sewer Outfalls (CSOs) and how samples from up- and downstream of the CSOs differ, and
3. Identify what portion of oocysts adhere to soil particles or remain accessible in different soil types to see how *C. cayetanensis* oocysts interact with the soil matrix.

CHAPTER 2: LITERATURE REVIEW

Cyclosporiasis, a human illness marked by diarrhea, has recently emerged as a food-borne illness of concern in North America (CDC, 2013). While other reviews have focused on the epidemiology of *Cyclospora cayetanensis* (Chacín-Bonilla, 2010; Fayer et al., 2000; Ortega & Sanchez, 2010; Shields & Olson, 2003), here we will focus on the environmental fate and transport of *C. cayetanensis*. Given the fact that cyclosporiasis is emerging in countries with advanced sanitation practices, the movement of oocysts through the environment is an important facet of the pathogen. There is relatively little known about *C. cayetanensis*, as it is difficult to source in developed countries, and there is no known method to culture the protozoan in the lab. This chapter will look at the characteristics of *Cyclospora* that allow it to survive in the environment and the effect of *C. cayetanensis* on humans. Additionally, techniques used to identify and distinguish *Cyclospora* from other organisms will be discussed.

2.1 Introduction

2.1.1 Taxonomy

Cyclospora spp. are members of the phylum Apicomplexa, along with *Cryptosporidium*, *Eimeria*, *Plasmodium*, and *Toxoplasma* (Cinar et al., 2015). When the organism was detected in humans in the 1970s, it was described as a large *Cryptosporidium* coccidian-like body (Ortega et al., 1994). For taxonomic classification, human *Cyclospora* was initially proposed as a new *Eimeria* species (Pieniazek & Herwaldt, 1997). Despite similarities to *Eimeria* and other encysted protozoa, the human pathogen was added to the genus *Cyclospora*, which includes similar organisms that infect a variety of hosts such as snakes and primates.

Phylogenetically, *Cyclospora* is closest to *Eimeria* given 94-96% similarity in the 18S rRNA sequence (Relman et al., 1996). Cinar et al. (2016) sequenced a variety of *C. cayetanensis* samples from around the world and found they largely overlapped. However, additional sequencing of various geographically and temporally distinct organisms is needed in order to confidently define the genus. (Eberhard et al., 1999) produced the phylogenetic tree found in Figure 1 based on the small subunit ribosomal RNA sequences.

Although genetically similar to *Eimeria*, *Cyclospora* is morphologically closer to *Cryptosporidium* (Fricker et al., 2002; Pieniazek & Herwaldt, 1997; Relman et al., 1996). Furthermore, both organisms exhibit similar life cycles, which include humans as a host (*Cyclospora cayetanensis* exclusively infects humans) and an environmental life stage (Eberhard et al., 2000; Fricker et al., 2002). Cryptosporidiosis and cyclosporiasis have similar symptoms in humans, such as diarrhea, intestinal discomfort and nausea (Huang et al., 1995).

While morphologically similar, several major differences distinguish *Cyclospora* and *Cryptosporidium* oocysts. *Cyclospora* is 8-10 µm compared to 4-6 µm for *Cryptosporidium* (Li et al., 2015; U.S. EPA Office of Water, 2005). *Cryptosporidium* is fully mature and infective when excreted from the host, while *Cyclospora* requires over a week to mature in the environment before becoming infective (Sathyanarayanan & Ortega, 2006). Additionally, *Cyclospora* contains two sporocysts with cell walls, whereas *Cryptosporidium* has four sporocysts with no additional protective layers inside the oocyst (Eberhard et al., 1999).

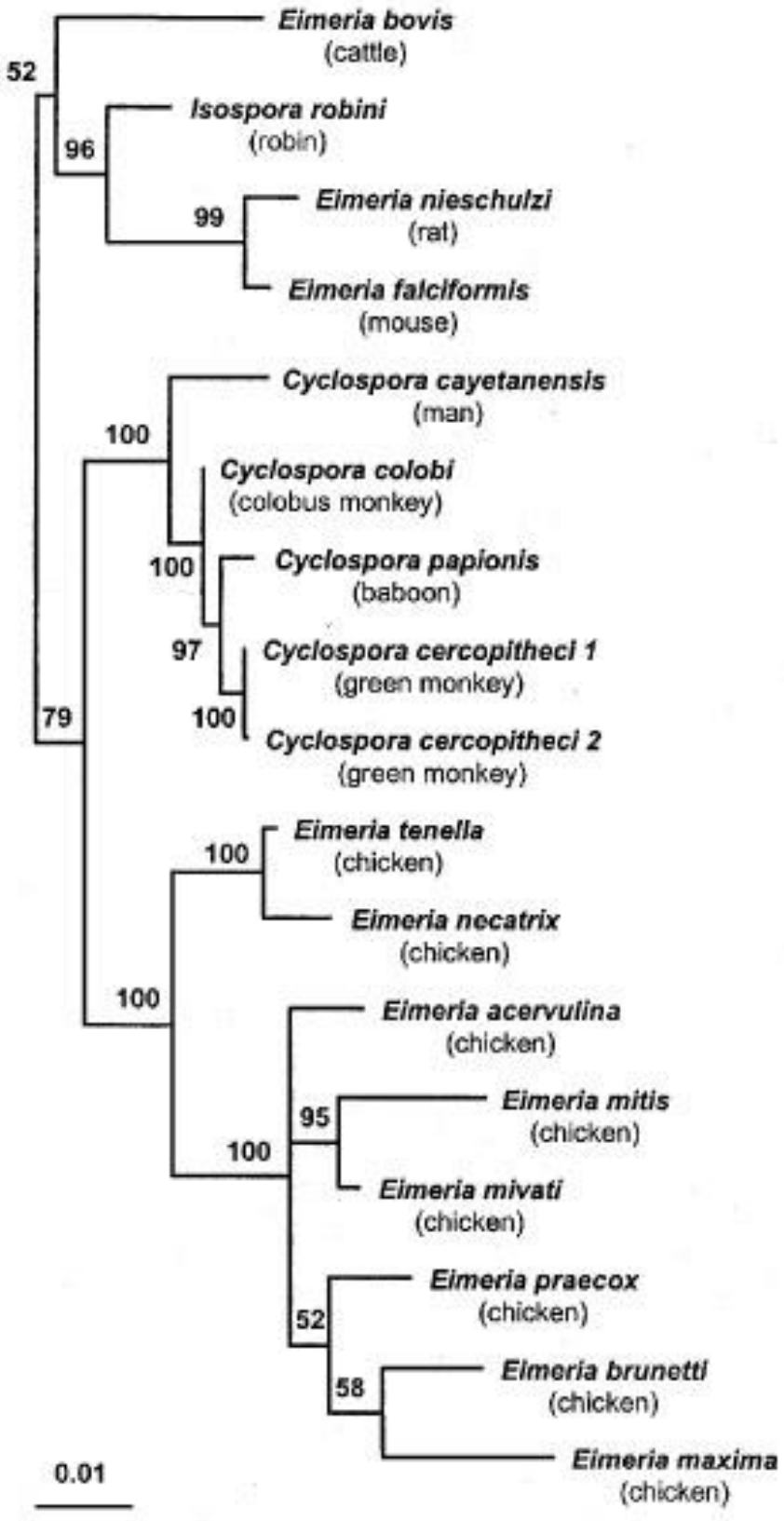

Figure 1. Phylogenetic tree of *C. cayetanensis* and related species from Eberhard et al., 1999.

2.1.2 *Cyclospora* spp.

Nineteen species of *Cyclospora* have been identified to date. They infect organisms from the Diplopoda, Reptilia and Insectivora classes as well as the Rodentia and Primate orders of Mammalia. Table 1 provides an overview of the species of *Cyclospora* that infect each host or hosts. These are considered the main hosts; however, there have been cases of DNA from one species of *Cyclospora* found in a non-traditional host; see the host specificity section below.

2.1.2.1 Host specificity

Several studies have found infrequent infections of *Cyclospora* spp. outside the main host species. *Cyclospora* spp. were detected in non-host primates in the wild and captivity (Li et al., 2015; Marangi et al., 2015) and in shellfish (Aksoy et al., 2014). However, the majority of infections have been detected within the primary host species. Humans have not been found to be infected with any other *Cyclospora* spp. and therefore this review will focus on *C. cayetanensis*.

Table 1. *Cyclospora* species and their host organisms.

***Cyclospora* species**	**Hosts**		**Study**
C. anglomurinensis	*Chaetodipus hispidus*	Hispid pocket mouse	Ford et al., 1990
C. ashtabulensis	*Parascalops breweri*	Hairy-tailed mole	Ford & Duszynski, 1989
C. babaulti	*Vipera berus*	Common European viper	Phisalix, 1924a
C. caryolytica	*Talpa europaea, Mogera wogura coreana*	European mole, Japanese mole	Schaudinn, 1902
C. cayetanensis	*Homo sapiens*	Human	Ortega et al., 1994
C. cercopitheci	*Cercopithecus aethiops*	Grivet	Eberhard et al., 1999
C. colobi	*Colobus guereza*	Mantled guereza	Eberhard et al., 1999
C. glomericola	*Glomeris* spp.	Pill millipede	Schneider, 1881
C. macacae	*Macaca mulatta*	Rhesus macaque	Li et al., 2015
C. megacephali	*Scalopus aquaticus*	Eastern mole	Ford & Duszynski, 1988
C. niniae	*Ninia sebae sebae*	Redback coffee snake	Lainson, 1965
C. parascalopi	*Parascalops breweri*	Hairy-tailed mole	Ford & Duszynski, 1989
C. papionis	*Papio anubis*	Olive baboon	Eberhard et al., 1999
C. scinci	*Scincus officinalis*	Sandfish skink	Phisalix, 1924b
C. schneideri	*Anilius scytale scytale*	American pipe snake	Lainson, 2005
C. talpae	*Talpa europaea*	European mole	Pellérdy & Tanyi, 1968
C. tropidonoti	*Natrix natrix, Natrix stolata*	Grass snake, Striped keelback snake	Phisalix, 1924c
C. viperae	*Coluber scalaris, Coronella austriaca, Natrix viperinus, Vipera aspis*	Ladder snake, Colubrid snake, Viperine snake, Asp viper	Phisalix, 1923
C. zamenis	*Coluber viridiflavus viridiflavus*	Green whip snake	Phisalix, 1924d

2.1.2.2 Variability within *C. cayetanensis*

The internal transcribed tracer (ITS) genes have some conserved and non-conserved regions. Olivier et al. (2001) evaluated geographically variable *C. cayetanensis* samples and identified a portion of the gene that could be targeted with polymerase chain reaction (PCR) to distinguish *C. cayetanensis* from other *Cyclospora* spp. The portions of ITS-1 that varied were not correlated with geography. Olivier et al. (2001) proposed that the observed variations could lead to polyparasitism, allowing multiple strains of *C. cayetanensis* to co-infect a host.

The 18S rRNA and heat shock protein (HSP70) were extremely similar across geographically distant *C. cayetanensis* isolates from Peru, Mexico and Nepal (Sulaiman et al., 2014), suggesting that the sampled populations have not significantly diverged into discrete populations. The 18S rRNA gene in *C. cayetanensis* is very similar to that of *Eimeria* spp. Primers used to target the 18S rRNA gene (Relman et al., 1996) also detect *Eimeria* spp. so care must be taken to distinguish the organisms after PCR or with another method.. Further analysis of the detected DNA, through sequencing or single-nucleotide polymorphisms PCR, is required to definitively identify *Cyclospora*.

Although the 18S rRNA gene has been studied more than other regions as a target for PCR, it is not as specific as the other regions. The ATCC *Cyclospora cayetanensis* standard also contains the genes for ITS-1 and ITS-2. The two regions are located near the 18S rRNA and provide another location for molecular detection of *C. cayetanensis* (Olivier et al., 2001).

The heat shock protein (HSP70) gene is one of the more recent genes to be identified and sequenced to the level required for PCR. Sulaiman *et al.* (1996) noted that HSP70 was the same across the 16 isolates used in the study. It was also found in other apicomplexan species, but with enough genetic variation to differentiate *C. cayetantenis*.

2.1.3 Life cycle

C. cayetanensis requires time in the environment to mature before it becomes infective. While other coccidian parasites are infectious when excreted, *C. cayetanensis* is excreted as an unsporulated oocyst. It requires at least seven days to begin sporulation and become infective (Sathyanarayanan & Ortega, 2006; CDC, 2013). Figure 2 illustrates the life cycle of *Cyclospora cayetanensis*, including the stages inside the human body (CDC, 2013).

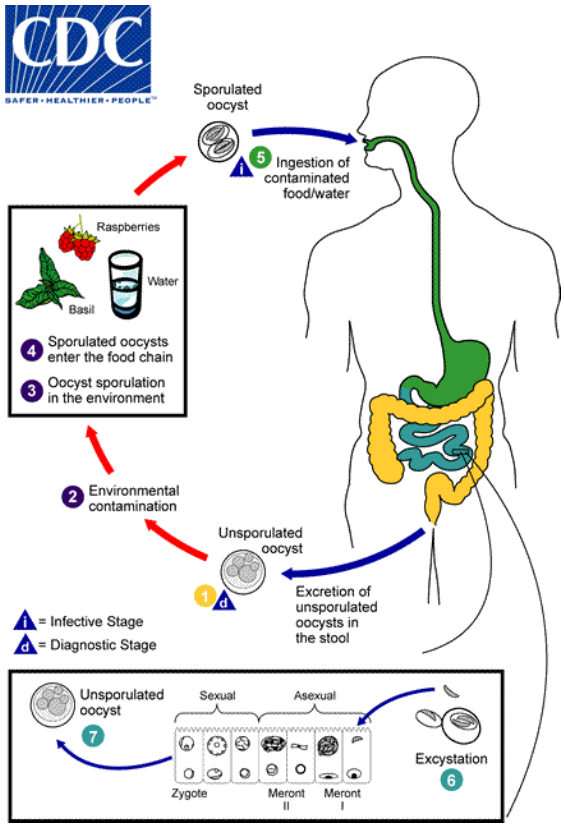

Figure 2. Life Cycle of *Cyclospora cayetanensis* from CDC, 2013.

2.1.4 Resiliency

In the environment, *Cyclospora* survives inside oocysts, which are hard, shell-like structures that provide protection against environmental conditions, such as varying temperatures and pH, as well as against chemicals and disinfectants. Other coccidian human pathogens, such as *Cryptosporidium spp.* and *Isospora spp.*, also exhibit typical oocyst resilience to natural decay in the environment and disinfection processes (U.S. EPA, 2001).

As *C. cayetanensis* has never been cultured or propagated in an animal model, the current best indicator for infectivity or viability is the ability of oocysts to sporulate. Many studies use the percent sporulation to indicate whether the organism is inactivated. *C. cayetanensis* can survive in water for two months at 4 °C and seven days at 37°C (Sathyanarayanan & Ortega,

2006). *Cyclospora* oocysts survive within the upper boundary of natural environmental temperatures, being shown to survive in temperatures of up to 80°C (Sathyanarayanan & Ortega, 2006). However, extreme temperatures can affect the viability and sporulation time of *C. cayetanensis* oocysts. *Cyclospora* spp. oocysts optimally sporulate between 22 and 30 °C in deionized water (Smith et al., 1997). Freezing temperatures (-20°C) severely limit oocyst sporulation in deionized water and other biological materials such as milk products and leafy greens (Sathyanarayanan & Ortega, 2006). Oocysts can also be rendered inactive at freezing temperatures. Temperatures of -20°C for 2 days or -70°C for an hour will inactivate *Cyclospora* oocysts (Ortega & Sanchez, 2010) based on the assumption that a reduction in sporulation indicates inactivation.

Compared to other coccidian human pathogens, chemicals have limited effect on inactivation of *C. cayetanensis*. *Cyclospora* is resistant to treatments commonly used in water treatment, such as chlorination and chlorine dioxide (ClO_2) gas (Ortega et al., 2008). Ortega et al. (2008) inoculated basil and lettuce with *C. cayetanensis* and applied gaseous chlorine dioxide at 4.1 mg/liter for 20 minutes. The authors noted there was no change in sporulation at this treatment level. Hydrogen peroxide is used to inactivate *Cryptosporidium*; however studies tend to evaluate chemical disinfectants on other, easier to study protozoa (Ortega, 2008), and therefore, little is known about the effect of chemical disinfectants on *Cyclospora*.

Pesticides appear to have inconsequential effects on *C. cayetanensis*. Sathyanarayanan and Ortega (2004) tested three fungicides (captan 50% W.P., benomyl 50% W.P., and zineb 75% W.P.) and two insecticides (malathion 25% W.P. and diazinon 4E 47.5%) at varying concentrations (below, at, and above recommended concentration levels for those pesticides). The effects of these treatments were evaluated at intervals between 30 minutes and 1 week.

There was no change in sporulation up to 24 hours, but the sporulation percentage was reduced with benomyl after 1 week of exposure (Sathyanarayanan and Ortega, 2004).

Ultraviolet radiation has the potential to inactivate *C. cayetanensis*. Broiler chickens fed *Eimeria acervulina* (as a surrogate for *C. cayetanensis*) oocysts from UV-treated raspberries and basil had a lower rate of infection than those fed untreated foods (Kniel et al., 2007).

Various other methods such as desiccation and high-pressure processing have been suggested as methods for reducing risk of cyclosporiasis infection. A coccidian organism thought to be *Cyclospora* was extremely sensitive to rupture when subjected to desiccation (Long et al., 1991). However, the identification of the organism was not confirmed. High-pressure processing (550 MPa at 40 °C for 2 min), a common food sterilization method, was used to treat *E. acervulina* oocyst-infected raspberries. The raspberries were fed to broiler chickens but the birds remained asymptomatic and did not shed oocysts (Kniel et al., 2007). It is unclear how these findings transfer to *C. cayetanensis*.

2.2 Human infection

Cyclosporiasis is a particular risk for young, elderly, and immunocompromised individuals. Infection is thought to occur with ingestion of as few as 10 to 100 oocysts. The number of cases of cyclosporiasis increased in 2018 from the previous years. As of October 4, 2017, 1065 cases were reported to the Center for Disease Control and Prevention (CDC) from the summer of 2017 alone. (CDC, 2017). In 2018, 2,299 cases were reported to the CDC as of October 1, 2018. This dramatic increase in cases in 2018 was attributed to an increase in the number of separate outbreaks and the use of new diagnostic techniques, such as multiplex PCR, to correctly diagnose cases that may not have been identified in previous years (CDC, 2018).

2.2.1 Treatment for cyclosporiasis

While cyclosporiasis often resolves itself given time, there are treatments available. Dehydration is a concern with extended periods of diarrhea, so it is important to ensure the individuals remain hydrated. Often individuals without additional health concerns resolve the infection on their own. When treatment is required, Trimethoprim/sulfamethoxazole (TMP/SMX) is the only option (CDC, 2013). There is no alternative treatment for individuals for whom TMP/SMX does not work, typically due to a sulfa allergy or because they did not respond to the treatment. HIV positive and other immunocompromised individuals are generally treated with TMP/SMX to minimize complications.

As with many intestinal parasites, such as *Giardia* and *Cryptosporidium*, patients are often provided treatment before the infection is identified. However, cyclosporiasis does not respond to any treatments that work for other intestinal parasites, and TMP/SMX does not treat other common protozoan infections. The difficulties associated with diagnosing the illness and successfully treating the individual can lead to longer infections compared to other parasitic diseases.

2.2.2 Outbreak surveillance

Cyclosporiasis is a reportable disease in more than 43 states, requiring health professionals to report cases of the disease to the state health department to help track and manage food-borne outbreaks (CDC, 2013). The CDC monitors clusters of cases as well as individual cases of *C. cayetanensis* infection to identify links between infections and sources of infection (CDC, 2013). All cases of cyclosporiasis must be reported in these states to the state health board, whether associated with a known outbreak or not, since outbreaks may span multiple states.

2.2.3 Food safety and detection

Currently there is no way to identify *Cyclospora* contaminated food as it is transported (Buss et al., 2015). Existing detection techniques involve destroying the food to concentrate the oocysts. More research needs to be done to develop methods of detecting *Cyclospora* and then inactivating or destroying oocysts on intact food.

2.3 Environmental Prevalence

2.3.1 Geographic distribution

Cyclospora cayetanensis is found throughout the world. Most endemic countries are located in tropical or semi-tropical environments. The United States is considered to have no endemic *Cyclospora* (CDC, 2013). There are still numerous infections of cyclosporiasis each year in these non-endemic areas due to travel and food-borne transmission from imported food (CDC, 2013).

Endemic regions have year round cases of cyclosporiasis because reinfection is common. However, in the United States and much of Europe, imported produce in the summer months may cause outbreaks, but with waste treatment facilities that can remove oocysts, reinfection from the environment is not such a concern. Some older sewage systems have Combined Sewage Outfalls (CSOs) which discharge untreated sewage into streams during high rainfall events. Chacín-Bonilla (2017), addressed *Cyclospora cayetanensis* distribution in developing countries, and created a map illustrating the distribution of *Cyclospora* across developing regions (Figure 3). The colors on the map in Figure 3 show different types of transmission: orange indicates an area has reported to have endemic infections, yellow indicates people have traveled to the country and have reported an infection, and green indicates residents have become infected with

no travel history. This map may be incomplete because cyclosporiasis may not be diagnosed or reported.

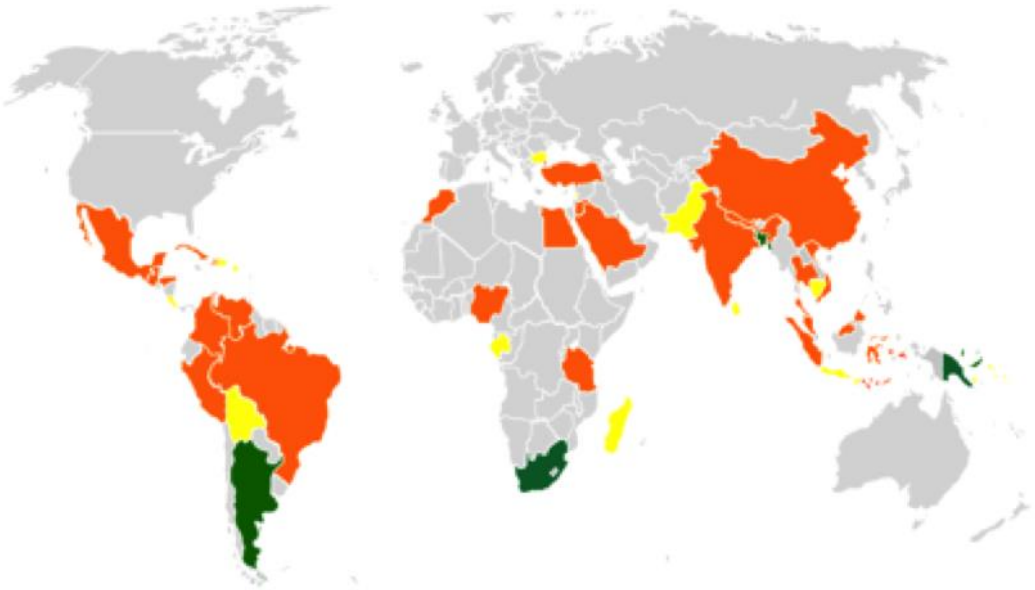

Figure 3. Geographic spread of *C. cayetanensis* in developing regions, Chacín-Bonilla, 2017. Orange indicates an area has reported to have endemic infections, yellow indicates people have traveled to the country and have reported an infection, and green indicates residents have become infected with no travel history.

2.3.2 Transmission in the environment

Cyclospora cayetanensis infection occurs via the fecal-oral route. However, the oocysts are transmitted in a variety of ways, depending on the environment. In areas with minimal sanitation infrastructure, and where the pathogen is endemic, the most common source of infection is contaminated water. Agricultural fields irrigated with contaminated water or harvested produce washed with contaminated water can be sources of infective oocysts.

In regions with advanced sanitation practices, *Cyclospora* is generally not endemic. In these regions, human waste rarely comes into contact with the environment, so the life cycle of

Cyclospora is inhibited by the inability to mature in the environment and then infect another host. Contaminated produce and berries from endemic areas infect the majority of individuals who become sick in non-endemic areas. Other people are infected while traveling in endemic regions through contact with contaminated food or water. Figure 4 illustrates the various ways oocysts can be transferred from different sources in the environment.

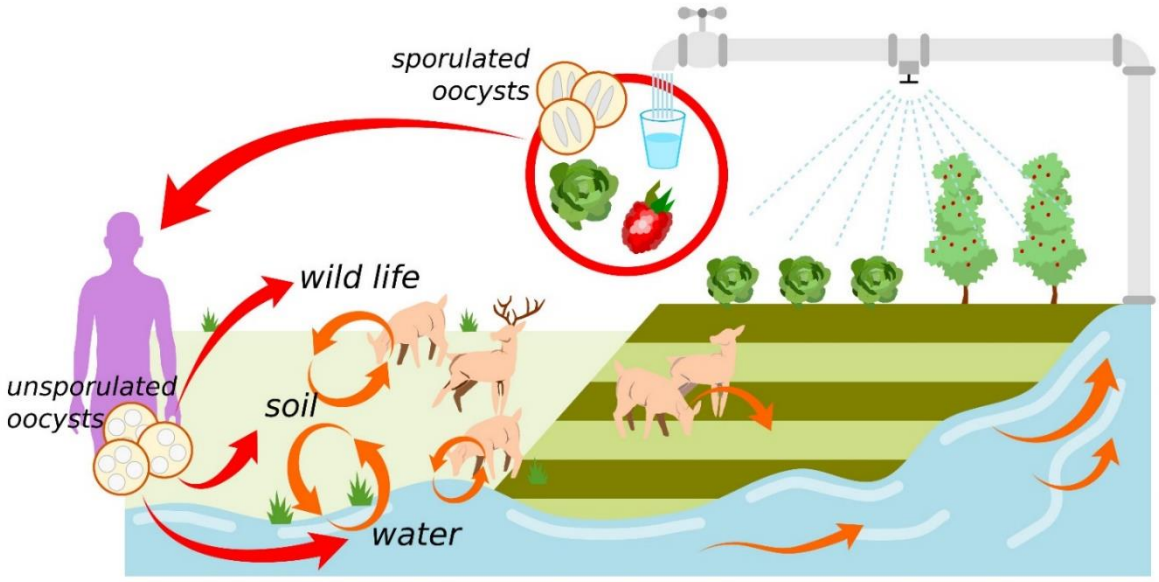

Figure 4. Potential routes of *C. cayetanensis* transport in the environment,

2.3.2.1 Wildlife

Studies have not yet identified domestic or wild animals as the source of infection in humans, although contact with animals has been associated with infection (Marangi et al., 2015). *C. cayetanensis* has been found in shellfish (Aksoy et al., 2014). Several fecal samples from animals in Nepal tested positive for *C. cayetanensis* (Chu et al., 2004). Two dogs, a monkey and a chicken had *C. cayetanensis* DNA in their feces, but infection could not be confirmed without

examining their tissue. From these studies, it is unclear whether animals could be infected by *C. cayetanensis* or if they could transport oocysts in the environment.

2.3.2.2 Soil

Contact with soil contaminated with feces was found to be associated with infection on San Carlos Island, Venezuela (Chacín-Bonilla *et al*., 2007). The authors suggested that ingesting soil may be a factor for infection. A review paper on soil transmission of *Cyclospora* was written by Chacín-Bonilla (2008). Contaminated soil was also detected in Italy, but transmission methods were unclear (Giangaspero et al., 2015)

Soil type can affect transmission of *Cryptosporidium* oocysts through the soil matrix (Davidson *et al*., 2014). Due to the similarity between *Cryptosporidium* and *Cyclospora*, this may be an important aspect of environmental transport and should be a focus of future work. Unfortunately, transmission of *C. cayetanensis* oocysts by contaminated soil is still not well understood. Further studies to identify risk factors and specific modes of transmission are necessary to understand the risk of infection due to soil.

2.3.2.3 Water

Cyclospora oocysts are large relative to most waterborne pathogens, and therefore, are easier to remove by physical treatment technologies (i.e., sand filtration) compared to chemical treatment (Ortega et al., 2008). However, when these standard treatment technologies are not present, or are not working properly due to inadequate routine maintenance, oocysts may pass through the treatment process and back into surface water systems. Once in surface water, oocysts can be transported long distances and reintroduced onto crops by processes like

irrigation, where water is sprayed onto the crops or applied using furrow or flood irrigation, dispersing oocysts to a wide area.

In regions with improved sanitation and wastewater management systems, water treatment is normally sufficient to remove *Cyclospora* oocysts. This makes it difficult for oocysts to enter a water supply in high quantities. Nevertheless, a study in Spain sampled several drinking water and wastewater treatment plants and locations of interest along a river over a 1 year period from 2008-2009 (Galván et al., 2012). Out of 223 samples, 9% (20) contained *Cyclospora* spp. and *C. cayetanensis* was found in 17 of those 20 samples. The authors concluded that existing water treatment plant regulations are insufficient to completely remove all human pathogens.

A study of two wastewater treatment plants in Arizona found *Cyclospora cayetanensis* in both the influent and effluent (Kitajima et al, 2014). Nine out of 48 samples collected from 2011-2012 were positive for *C. cayetanensis* using a novel qPCR technique, but they did not determine the removal efficiency of the wastewater treatment plants.

Most water treatment methods rely on a variety of biological, chemical and physical treatment processes. *Cyclospora* oocysts are very robust and can remain active even with chlorine disinfection (Kitajima *et al*., 2014). Chlorination is a common water treatment practice, but will not inactivate *Cyclospora* oocysts. Physical treatment practices appear to be most effective since the oocysts are relatively large. Sedimentation and flocculation can help remove oocysts in settling tanks.

2.3.2.4 Food

Foods that are eaten raw can be contaminated by contact with contaminated water or human waste. The major hurdle in managing the spread of *Cyclospora* is the inability to detect

contaminated produce in the supply chain and remove the pathogen once it is detected. There is still much that is not known about how *Cyclospora cayetanensis* adheres to foods, how long it persists, and how to minimize the risk to humans.

Many cases of cyclosporiasis in developed regions are due to contaminated, imported produce. There have been many reported outbreaks of cyclosporiasis in North America (Kozak *et al*., 2013). As of October 4, 2017, 1065 individuals were reported to have cyclosporiasis during the summer of 2017 in the United States. Of those individuals, 596 indicated they didn't travel internationally, and thus likely became infected domestically due to contaminated foods (CDC, 2017). Any produce imported from regions with endemic *C. cayetanensis* is a potential source for domestic outbreaks of cyclosporiasis in the US.

2.4 Conclusion

Cyclospora cayetanensis is an emerging pathogen of concern and yet little is known about how it is transported in the environment. Field-scale contamination is a major concern as *C. cayetanensis* requires time in the environment to mature before becoming infective. Water, soil and animals have been found to have oocysts, and all can transfer oocysts to crops grown on contaminated fields. When the produce is harvested and sent to non-endemic regions, the oocysts cause cyclosporiasis. Soil and wildlife are the least understood modes of transmission. More research is needed to not only understand the complete life cycle of *Cyclospora cayetanensis* in the environment, but also how to improve food safety and prevent outbreaks.

CHAPTER 3: PRESENCE IN THE ENVIRONMENT

Cyclospora cayetanensis is an emerging food-borne protozoan pathogen. Similar to other gastrointestinal pathogens, cyclosporiasis causes prolonged diarrhea. Unlike *Cryptosporidium*, *Cyclospora* oocysts are not infective when they are shed by infected individuals. Oocysts mature in the environment for 7-10 days before sporulating. Little is known about how *C. cayetanensis* is transported in the environment and which factors inhibit or promote sporulation. Water and fresh produce, such as leafy greens and berries, are common sources of infection. Contact with soil has also been correlated with cyclosporiasis infection. In addition to acting as a vector to transport oocysts from the environment to the body, water and soil may be important reservoirs to not only allow *C. cayetanensis* to persist, but also transport the oocysts from one location to another. This study examines a snapshot of an urban area where human waste sporadically enters the environment via Combined Sewer Outfalls. Nested PCR and two rounds of non-nested PCR analysis of soil, water, and wildlife scat identified possible *C. cayetanensis* DNA, but sequencing is needed to differentiate between *C. cayetanensis* and *Eimeria spp.*

3.1 Introduction

Cyclospora cayetanensis is a food- and water-borne protozoan pathogen (Huang et al., 1995). Infected individuals shed immature oocysts into the environment where they mature, given satisfactory environmental conditions (Sathyanarayanan & Ortega, 2006). The oocysts take between 7 and 10 days to mature into sporulated oocysts after which they can excyst and become infective (Ortega et al., 1994). During the environmental phase, oocysts are influenced by the

conditions of the environment and can be transported and deposited in new areas (Giangaspero et al., 2015).

In endemic regions, oocysts are generally transported by water. Humans can become infected after drinking contaminated water or after using the water to prepare food. Irrigation water can also contaminate food products in the field because it is sprayed directly onto produce and the oocysts can become trapped in small spaces. Similarly, produce can be contaminated if pesticides are diluted with contaminated water prior to field application (Sathyanarayanan & Ortega, 2004).

Areas with advanced sanitation practices are generally considered non-endemic for *Cyclospora*. Most people living in these regions acquire infections during travel to endemic regions or by consuming fresh produce imported from endemic areas (CDC, 2017; Chacín-Bonilla, 2017).

Soil is a potential route of transmission. Several studies have indicated a connection between contact with soil and rates of infection (Chacín-Bonilla, 2008; Chacín-Bonilla et al., 2007; Giangaspero et al., 2015). Chacín-Bonilla et al. (2007) determined that contact with fecal-contaminated soil was associated with cyclosporiasis on San Carlos Island, Venezuela. .

At this time, no natural animal hosts for *C. cayetanensis* have been identified (Eberhard et al., 2000). However, animals may be able to transport ingested oocysts to new locations. Chu et al. (2004) detected *C. cayetanensis* DNA in fecal samples of various animal species in Nepal. The DNA was found in fecal samples obtained from two dogs, a monkey, and a chicken (Chu et al., 2004) indicating thethe presence of the pathogen, but not whether it was infective or not.

Humans are currently the only known host of *C. cayetanensis* (Eberhard et al., 2000). Therefore, untreated human waste contaminated with *C. cayetanensis* oocysts must enter the

environment for environmental contamination to occur. In areas with advanced sanitation, there are fewer opportunities for environmental contamination. However, the opportunity arises during large rainfall events in communities that utilize combined sewage and stormwater management systems. During large or intense rain events, combined systems are unable to contain the excess volume of water and stormwater combined with untreated sewage is discharged into streams at combined sewer outfalls (CSOs). The extent to which CSO areas are contaminated with *C. cayetanensis* is unknown.

If CSOs do discharge *C. cayetanensis* into the environment, the oocsts can move to new locations that can be harmful to humans. While not infected, wildlife may still be able to transport *C. cayetanensis* oocysts from site to site. If an animal carrying *C. cayetanensis* oocysts defecates in a field, the *C. cayetanensis* can enter the food system. To help prevent cases of cyclosporiasis, potential sources of contamination need to be understood and managed. Water may be contaminated directly by CSO discharge. Wildlife may drink the water and defecate on soil or in other water sources, spreading oocysts further afield.

To increase our understanding of the fate of *C. cayetanensis* in the environment, we screened soil, water, and wildlife scat collected in CSO areas for the presence of *C. cayetanensis*. If Cyclospora cayetanensis is found in certain locations and types of samples, risk management strategies can be developed to minimize the risk of human infection.

3.2 Methods

3.2.1 Sample Collection

The Metropolitan Water Reclamation District of Greater Chicago monitors CSO activity and notifies the public when the CSOs actively discharge sewage. CSOs are located along

waterways throughout the Greater Chicago Area. All sampling waswas collected within one week after active discharge events. The following three CSO sites were selected because of their accessibility via public parks.

St. Paul Woods: Morton Grove, IL, along North Branch of the Chicago River

Caldwell Woods: Chicago, IL, along North Branch of the Chicago River

Devon Avenue: Forest Preserve of Cook County, at Devon Ave and S. Dee Rd, along Des Plaines River

Each site was divided into four sampling points, one upstream of the CSO and three downstream. Point Z was 30 m upstream of the CSO, and A, B, and C were downstream of the CSO at 30 m increments. Soil was collected at each sampling point, both at the edge of the water and in the upland area. Soil was collected from within 20 ft of the water's edge in a place that would cause minimal disruption to the vegetation. A visual inspection was used to find a representative soil sample from the sampling point. If there were multiple types of soil, then all types were collected from the top 1 inch of soil. Water samples were collected from each sampling point as well, within 5 ft of the bank for the safety of the researchers. All observed wildlife scat found between the extreme upstream and downstream sampling points was collected.

Using a disposable spoon, soil or scat was collected to fill a 50 ml conical tube. Each sample was collected with a new spoon to prevent contamination between samples. After sampling and during transport to the lab, collection tubes were stored in a lunchbox cooler containing an ice pack. Photographs were taken of all samples and a record of the GPS

coordinates for some of the sampling locations was included in the sampling data sheets, see Appendix A. A description of the samples was also recorded, including organic matter, fecal freshness, and any special features.

EPA method 1623 (U.S. EPA Office of Water, 2005), describes options to concentrate *Cryptosporidium* and *Giardia.* The method was modified to collect and concentrate *Cyclospora* because of similarity in morphology to *Cryptosporidium*. Some of the steps require agents that bind to the surface of the organisms, and there are no alternatives for *Cyclospora*. Thus, a modified approach was used where approximately 8-10 gallons of water were collected using a transfer pump. Later the water was transported back to the lab for mechanical concentration. The inlet hose was continually moved from the surface to the middle of the stream flow and back to the surface to achieve a depth-integrated water sample, about 3 feet from the bank, until the collection bucket (10 gal.) was full. During transport, the 10 gallon buckets of water were wrapped in foil thermal blankets and packed with ice.

3.2.2 Sample Preparation

ScatScat and soil samples were stored at -80°C for three or more days to render any *Cyclospora* oocysts non-infectious (Sathyanarayanan & Ortega, 2006). It was not possible to freeze the 10 gallon water buckets due to their size, so the water samples were concentrated before storing at -80°C. Large particulates were filtered out of the water by pouring the water through cheesecloth. Then, water was poured into 250 mL plastic Nalgene centrifuge bottles, and centrifuged at 2125 x g for 30 minutes. Much of the water was removed from the top and discarded, leaving approximately 10 mL of water with a film of fine particulates at the bottom. The particles were resuspended before transferring the concentrate to another storage container.

This process was repeated until all the particulates in the water were concentrated. Then, that water was centrifugedcentrifuged using the same process, again using 250 mL bottles. The third round of centrifugation used 50 mL conical tubes and all the supernatant was removed except for a thin layer to ensure that the pellet remained undisturbed. These pellets were resuspended and placed in one 50 mL conical tube, which was stored at -80°C to inactivate any *C. cayetanensis* oocysts.

3.2.3 DNA Extraction and PCR

DNA was extracted from each sample using the PowerSoil DNA Isolation Kit (MoBio) following the manufacturer's instructions with few exceptions. To mechanically break open the oocysts to release the DNA, a manufacturer representative suggested using a tissuelyzer for 5 minutes at 30 Hz rather than vortexing the samples for 10 minutes at maximum speed. All centrifugation steps were reduced from 10,000 x g to 9,500 x g due to centrifuge limitations. Additionally, to increase the genomic DNA concentration, the elution step was completed using 75 µL of Solution C6. DNA was stored at -20°C until PCR was performed.

In addition, DNA was isolated from a subset of samples with the QIAamp DNA Stool Mini Kit (QIAGEN) to compare the genomic DNA product from both kits. The initial heating step was done at 95°C, as suggested for cells that are difficult to lyse. Samples were stored at -20°C until PCR was performed.

C. cayetanensis 18S rRNA gene was amplified with nested PCR (Lee et al., 2010). The two sets of primers, listed in Table 2, detect *C. cayetanensis* as well as *Eimeria* spp., and therefore sequencing the final product is required to confirm the species. At the start of this study in 2016, the nested PCR procedure was the main published method that did not require

specialized equipment such as qPCR or single nucleotide polymorphism PCR, which was not feasible for this study.

Table 2. Primers for nested PCR (Lee et al., 2010)

Primer	Sequence	Amplicon lengthlength (base pairs)
CYCF1E	5′-TACCCAATGAAAACAGTTT-3′	636
CYCR2B	5′-CAGGAGAAGCCAAGGTAGG-3′	
CYCF3E	5′-CCTTCCGCGCTTCGCTGCGT-3′	294
CYCR4B	5′-CGTCTTCAAACCCCCTACTG-3′	

GoTaq Hot Start Green Master Mix (Promega) was used for both the first (primers CYCF1E and CYCR2B) and second (CYCF2E and CYCR4B) round of nested PCR reactions. A 50 µL reaction volume contained 25 µL of the master mix and 2 µL of each primer (forward and reverse). If the DNAconcentration was very low in a given sample, 5 µL of genomic DNA were added to the reaction volume and 16 µL of nuclease free water filled the volume to 50 µL. The initial denaturation was for 2 minutes at 94°C followed by, 35 cycles of denaturing at 94°C for 30 sec., annealing at 55°C for 30 sec., and extension at 72°C for 1 min. The polymerization lasted 5 min at 72°C. The reaction was held at 4°C. The same parameters were used for the second round of PCR (see Table 2).).

The amplified products were visualized using gel electrophoresis of a 2% agarose gel stained with ethidium bromide,.,. We loaded 10 µl of eacheach sample Land 10 µl of hyperladder 100bp ladder (Bioline).

Due to the low specificity with the primers from Lee et al. (2010), new primers were designed to specifically target only *C. cayetanensis*. With a limited number of oocysts from the CDC, very little could be used to validate and optimize the PCR methods. The novel primers were designed to produce a short fragment length to facilitate using qPCR in a second round of PCR after an initial amplification using PCR. As one round of PCR was unable to show low concentrations of *C. cayetanensis* DNA, another round of PCR using the same primers was needed to correctly identify a positive sample. Rather than having 35 cycles in both the first and second round of PCR, the first round only included 20 cycles to ensure that the concentrations of all the DNA did not plateau. This ensured that if *Cyclospora* was present, sufficient quantities of *Cyclospora* were amplified but that the non-target DNA was not amplified to the same extent. Table 3 shows the novel set of primers.

The new set of primers used with the two rounds of PCR is very specific, so if there is *C. cayetanensis*, the primers will bind to the target sequence. However, if the target sequence is not present, the primers may bind to other, less specific fragments, increasing the possibility of producing multiple bands.

Table 3. Primers for two step PCR

Primer	Sequence	Length (base pairs)
CYCLO18S_574	5′-CGGTAATTCCAGCTCCAATAGT-3′	100
CYCLO18S_673C	5′-CACACCCTACGGGCAAG-3′	

The procedure for the nested PCR (Lee et al., 2010) was modified for use with the CYCLO18S primers. The 20 μL PCR reaction volume contained 7.8 μL of nuclease-free water, 0.1 μL of each CYCLO18S 100 ng/μL forward and reverse primer, 10.0 μL of GoTaq G2 Hot Start Green Master Mix, and 2.0 μL of template DNA. After mixing, the PCR tubes were placed in the thermal cycler to run the following process (Table 4).

Table 4. Two Step PCR process for CYCLO18S

FREQUENCY	STEP	TEMPERTURE (°C)	TIME
1X	Initial Denature	95°C	2 minutes
20X	Denature Anneal	94°C 60°C	15 seconds 1 minute
1X	Polymerization Chill	72°C 10°C	5 minutes 5 minutes

For the second round of PCR, the same procedure as described in Table 4 with the same primers, except the product from the first round of PCR, was used as the DNA template. Also, the second round was run 35X during the denature/anneal step instead of 20X. The method for visualizing the PCR products using gel electrophoresis was the same as for the original primers.

Concentrated *Eimeria acervulina* was processed following the same procedure as the other samples and used to optomize the CYCLO18S primers. PCR was done at a variety of temperatures from 55-65°C during the annealing step to identify the optimal temperature for the primers to anneal to *C. cayetanensis* and not to *E. acervulina* samples. 60°C was found to be the optimal temperature.

3.3 Results and Discussion

We collected 16 samples from Devon Ave, 23 from Caldwell Woods, and 22 from ST. Paul Woods. The Devon Ave. site was very difficult to access due to heavy vegetation, also making it difficult to locate scat (feces). Unfortunately, one scat sample turned out to be a sponge saturated with muddy river water, so there are only five scat samples from the Devon Ave. site. Table 5 shows the number and types of samples collected from each site.

Table 5. Number of environmental samples collected at each site.

Site	Soil	Feces	Water	Total
Devon Ave.	8	5	3	16
Caldwell Woods	8	11	4	23
St. Paul Woods	8	10	4	22

Initially, samples were processed with both the MoBio (M) and Qiagen (Q) kits. However, Figure 5 illustrates that the DNA product from the Qiagen kit was less clear and there were fewer bands than the DNA product from the MoBio kit. After doing the majority of the Devon Ave. samples with both kits, the remaining samples were only processed with the MoBio kit to improve the chance of detecting *Cyclospora*. Unless noted, all samples were extracted with MoBio.

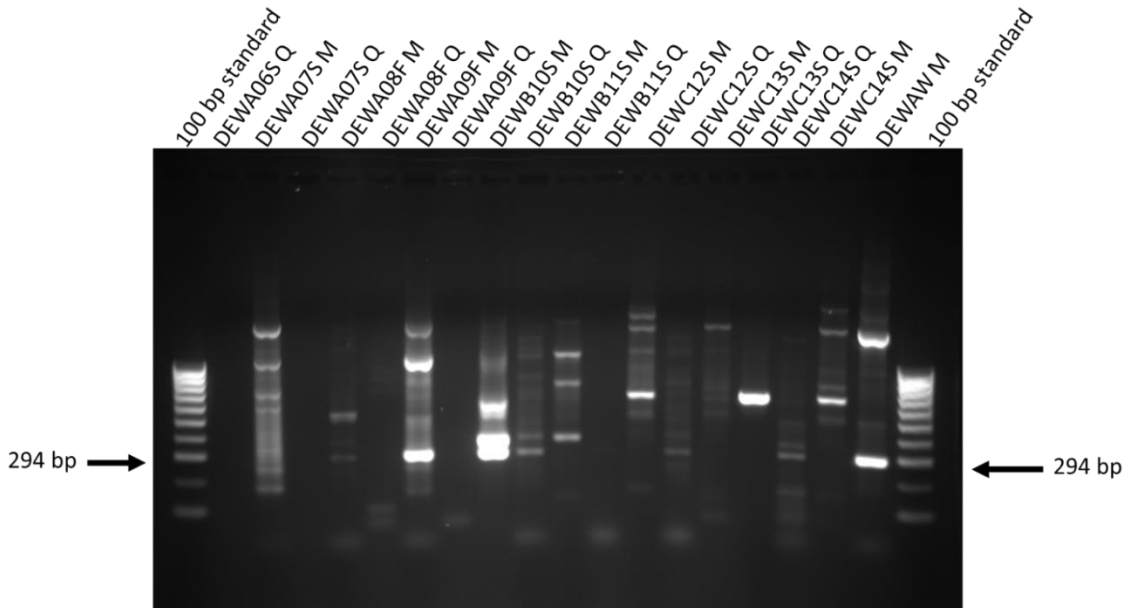

Figure 5. Mobio vs. Qiagen DNA extraction kits. MoBio produces more and brighter bands at the target length (294bp). The 100bp standards are located on either end of the gel, with the 294bp location marking the target amplicon length at the side of the figure. Sample IDs are listed across the top. DEW refers to the Devon Ave CSO site, followed by the sample location at the site and the sample number. F stands for feces, S for soil, and W for water. The final letter indicates the DNA extraction kit type.

Figures 6-9 illustrate the CYCLO18S PCR that was used to distinguish *C. cayetanensis* from *Eimeria* spp. Most samples produce many non-specific bands, indicating that the PCR technique needs further optimization. Samples were designated positive if there was a discrete band at 100 bp, for example CDW21W in Figure 6. If the smear of fragmented DNA did not have a clear band, but ran through the 200 and below range with no change in the 100 region, it was deemed negative, see CDWZ3S in Figure 6.

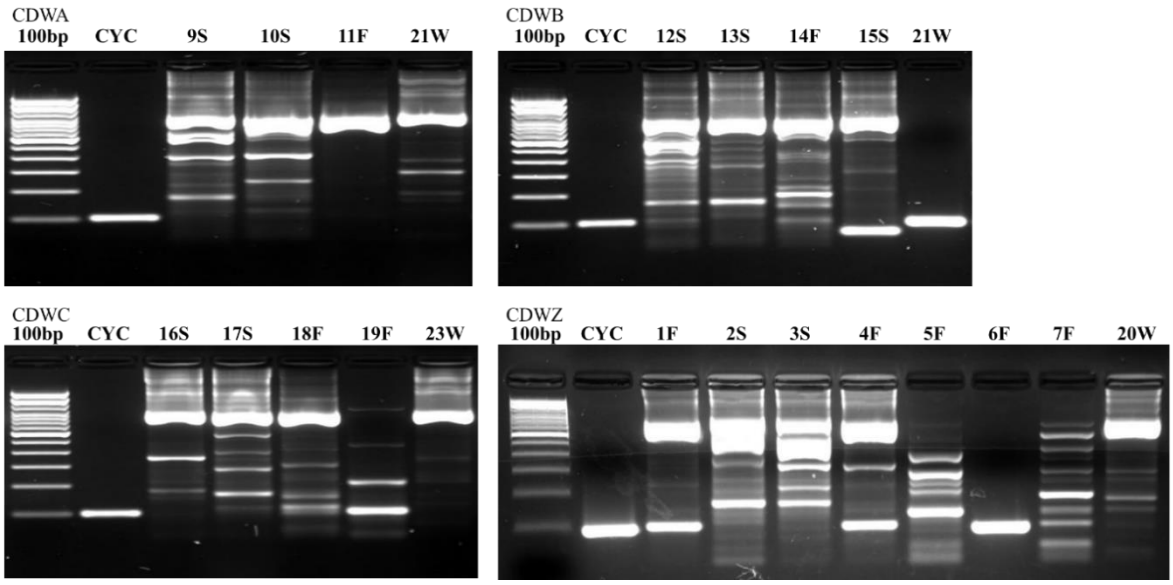

Figure 6. Caldwell Woods Two Rounds of PCR with transects A-C and Z. The 100pb standard (far left in each image) is used to visualize the amplicon size. The *Cyclospora* (CYC) DNA represents the correct positive band position at 100bp. Positive samples, labeled with the sample number and type (F=feces, S=soil, and W=water), are identified by a positive band at 100bp.

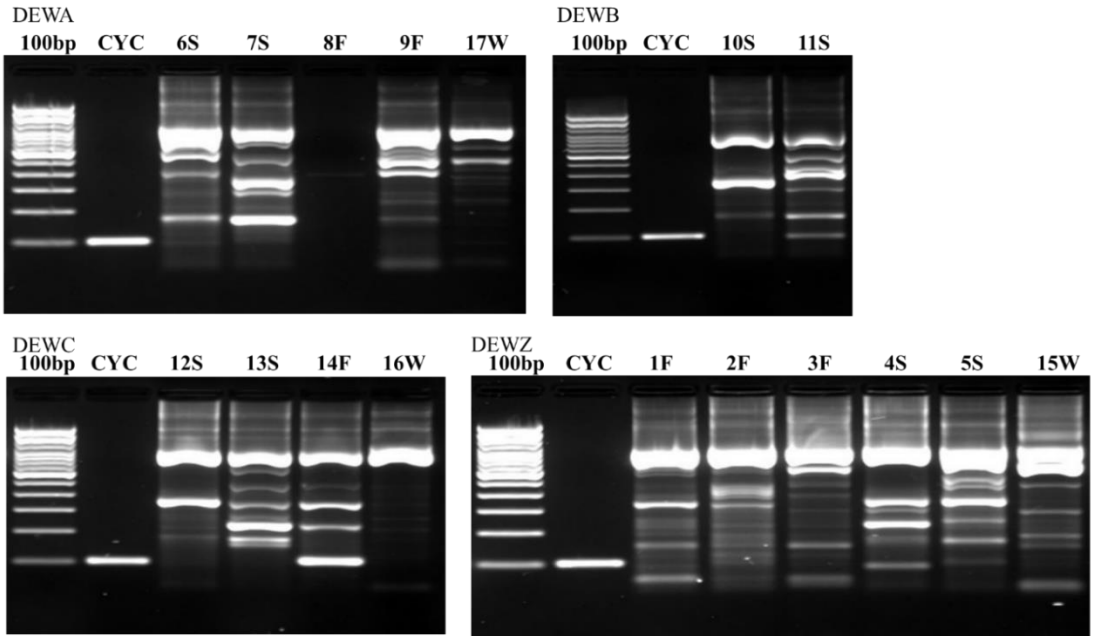

Figure 7. Devon Avenue Two Rounds of PCR, with transects A, C, and Z. The 100pb standard (far left in each image) is used to visualize the amplicon size. The *Cyclospora* (CYC) DNA represents the correct positive band position at 100bp. Positive samples, labeled with the sample number and type (F=feces, S=soil, and W=water), are identified by a positive band at 100bp.

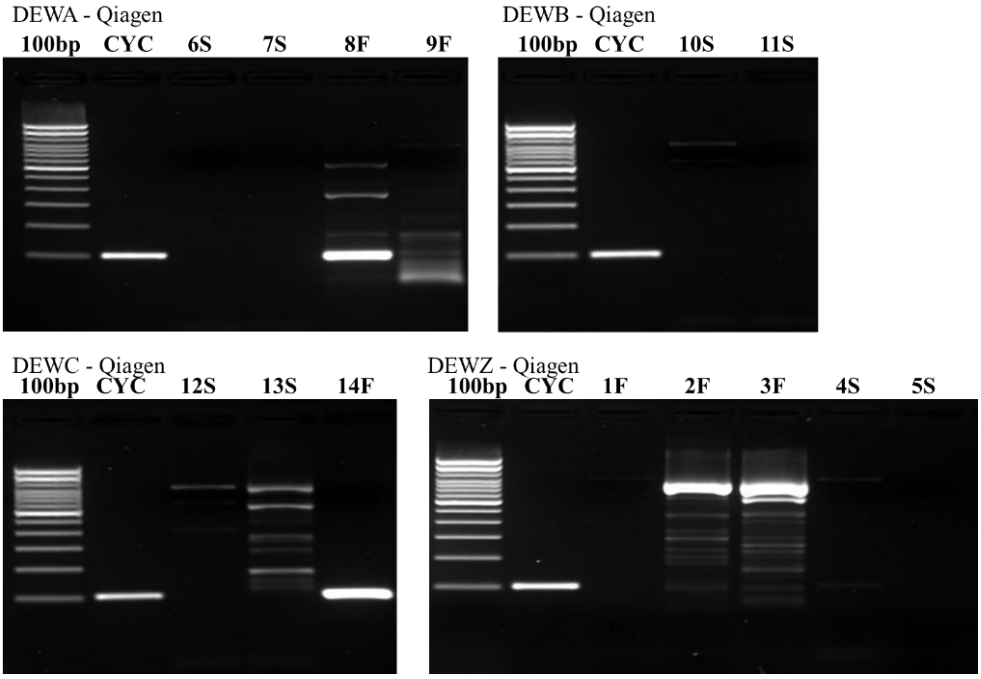

Figure 8. Devon Avenue Two Rounds of PCR, with transects A, C, and Z. Using DNA from Qiagen kit. The 100pb standard (far left in each image) is used to visualize the amplicon size. The *Cyclospora* (CYC) DNA represents the correct positive band position at 100bp. Positive samples, labeled with the sample number and type (F=feces, S=soil, and W=water), are identified by a positive band at 100bp.

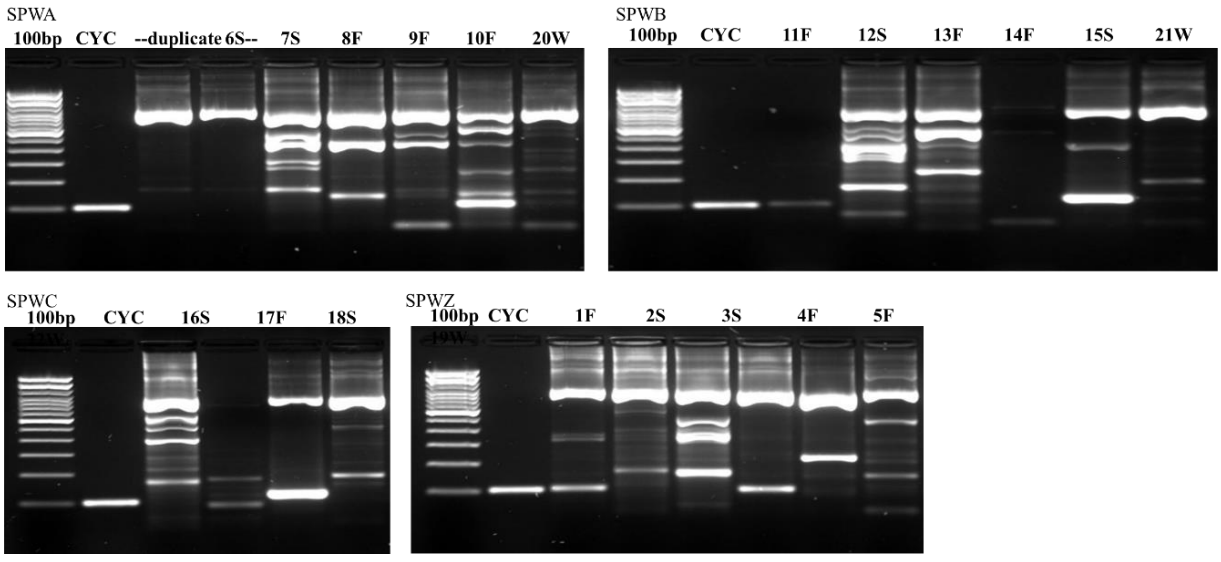

Figure 9. St. Paul Woods Two Rounds of PCR with transects A-C, Z. The 100pb standard (far left in each image) is used to visualize the amplicon size. The *Cyclospora* (CYC) DNA represents the correct positive band position at 100bp. Positive samples, labeled with the sample number and type (F=feces, S=soil, and W=water), are identified by a positive band at 100bp.

Many samples have artifacts around 100bp in length. With many artifacts, the smaller size bands can move more slowly through the agarose, so may appear to have a slightly larger size than their true value. Bands that appear to be at 100 bp or just slightly larger (in the presence of artifacts) were considered positive. A good example of this difference in position can be found in Figure 6 with sample CDWZ1F beside CYC, but slightly higher. Future work requires additional optimization and validation of the new primer, and potentially sequencing of the suspected bands to ensure that the targeted sequence is *C. cayetanensis*.

The results from examining the gels are consolidated in Tables 6-11, below. The "–" indicates there is no band at the target DNA size, meaning the primers did not amplify any DNA in the correct range that is expected to be *C. cayetanensis.* The "+" indicates that there was a band of the appropriate size to be the target sequence, which could be *C. cayetanensis*, or in the case of nested PCR, *Eimeria spp*. Further sequencing will be needed to differentiate the two genera.

Appendix B includes all the gels illustrating the nested PCR results. These gels do not include the water sample from the A sampling point at St. Paul Woods (SPWA20W) and a scat sample from the C sampling point at Devon Ave. CSO from the MoBio kit (DEWC14F M) because the DNA from those samples was not extracted from the samples in time to run the PCR and produce the gels. Each image shows the bands from the samples compared to the 294 bp ladder from the nested PCR method.

Table 6. Caldwell Woods Site Nested PCR Results

	Z MoBio	A MoBio	B MoBio	C MoBio
Water	-	-	-	+
Soil	+	-	-	-
	-	-	-	-
Feces	+	+	+	-
	+		-	-
	+			
	-			
	-			
	-			

Table 7. Devon Ave. Site Nested PCR Results

	Z		A		B		C	
	MoBio	Qiagen	MoBio	Qiagen	MoBio	Qiagen	MoBio	Qiagen
Water	-		+		-		-	
Soil	+	-	-	-	+	+	-	+
	-	-	-	-	-	-	-	+
Feces	+	+	+	-				-
	-	-	+	-				
	-	-						

Table 8. St. Paul Woods Site Nested PCR Results

	Z MoBio	A MoBio	B MoBio	C MoBio
Water	-		-	-
Soil	-	+	+	-
	-	-	-	-
Feces	-	+	+	-
	-	+	+	
	-	-	-	

Table 9. Caldwell Woods Site CYCLO18S PCR Results

	Z MoBio	A MoBio	B MoBio	C MoBio
Water	-	-	+	-
Soil	-	+	+	+
	-	-	-	-
Feces	+	-	+	+
	+		-	+
	+			
	+			
	-			

Table 10. Devon Ave. Site CYCLO18S PCR Results

	Z		A		B		C	
	MoBio	Qiagen	MoBio	Qiagen	MoBio	Qiagen	MoBio	Qiagen
Water	-		-				-	
Soil	+	-	-	-	+	-	-	-
	-	-	-	-	-	-	-	-
Feces	-	+	-	+			+	+
	-	+	-	-				
	-	-						

Table 11. St. Paul Woods Site CYCLO18S PCR Results

	Z MoBio	A MoBio	B MoBio	C MoBio
Water	-	-	-	-
Soil	-	-	+	+
	-	-	-	-
Feces	+	+	+	+
	+	-	-	
	-	-	-	

As shown in Tables 6-11, there did not appear to be much clustering of positive samples with either the nested or two rounds of PCR. Nearly all sampling points at each site had at least one positive band. None of the upstream locations (Z) had any positive water samples. The CSOs cannot directly contaminate that area, it indicates that wildlife may be responsible for the transport of oocysts upstream.

Figure 12 consolidates all the positive samples from each method. The middle column lists the samples that were found positive using both the nested and non-nested PCR. The left column shows the samples found positive with the nested PCR only, while the right column shows the ones that were found positive with only the non-nested PCR.

Table 12. Frequencies of positive samples for each testing method

	Nested PCR only	**Both PCR techniques**	**Two round PCR only**
CDW	2S 5F 11F 23W	6F 7F 14F	1F 4F 10S 12S 17S 18F 19F 21W
DEW MoBio	1F 8F 9F 10S 15W	4S	11S 14F
DEW Qiagen	10S 12S	14F	2F 3F 8F
SPW	7S 8F 13F	10F 11F 15S	1F 4F 17F 18S

Most of the positive samples (from any method) are wildlife scat (n=22), and a few are soil (n=10), see Table 12. There are two positive water samples from the nested PCR technique and one from the two rounds of PCR method. Note that the results in Tables 6-11 cannot be used to determine the likelihood that both techniques agree on the identification of a given sample, this must be done by examining Table 12. This is because that different samples may be responsible for the positive mark and the methods do not actually agree. Neither method produced more positive samples in every location.

Table 12 displays the frequencies for positive samples being identified by one or both PCR techniques. Out of 61 samples, 8 (13%) were positive in both tests. Six (23%) of the 26 fecal samples were positive in both tests. There were likely fewer positive samples found in the water compared to soil and feces because sampling occurred up to one week after the CSO discharge events. By the sampling time, much of the discharged *Cyclospora*, if it existed, was probably carried far downstream or significantly diluted.

3.4 Conclusions

This study evaluated 61 environmental samples during three discrete CSO discharge events. While small, this study does support past research (Kitajima et al., 2014) indicating that *C. cayetanensis* may be more common in North America than previously thought. Future work should include an in depth study looking at temporal and spatial variability. Additional work should confirm where the organism is found in the environment and identify risks to the public based on the location and concentration.

While this paper details methods to collect and detect *Cyclospora*, it was performed on a very limited scale. The preliminary results, based on the PCR products with gel electrophoresis,

indicate that there is potentially *C. cayetanensis* in the environment near CSOs. However, *Eimeria* spp. may be causing the positive bands using the nested PCR method, so Sanger sequencing will be performed on any positive samples to confirm the presence or absence of *Cyclospora*. Future work would need different *Eimeria* spp. to further evaluate the specificity of the new primers. These were developed to be specific to *C. cayetanensis*, but were not able to be validated in practice. A major hurdle for validating the primers was the limited amount of *C. cayetanensis* oocysts and DNA available for testing.

CHAPTER 4: SOIL ADSORPTION

Transport of *C. cayetanensis* oocysts in contaminated soil is a challenging problem. Most studies on *Cyclospora* in soil have found a correlation between contaminated soil and cases of cyclosporiasis. This study was a proof of concept for soil adsorption of *C. cayetanensis*. Sandy, silty and clayey soils were inoculated with *C. cayetanensis* oocysts obtained from the Center for Disease Control and Prevention (CDC). Samples were incubated for 0 to 20 days before testing for oocysts that were not adhered to soil particles. The results were inconclusive. The most important limitation was not having enough *C. cayetanensis* to inoculate the soil in the experiment. Another difficulty was not having an optimized and reliable method to detect the presence of *C. cayetanensis*. Both of these limitations prevented us from determining relative quantities of DNA from each sample.

4.1 Introduction

Although a review of soil transmission of *Cyclospora* was written by Chacín-Bonilla (2008), transport of *C. cayetanensis* oocysts in contaminated soil is not well understood. Most studies on *Cyclospora* in soil have found a correlation between contaminated soil and cases of cyclosporiasis but have failed to prove causation. Contact with soil contaminated with feces was found to be associated with infection on San Carlos Island, Venezuela (Chacín-Bonilla *et al*., 2007). The authors suggested that ingesting soil may be a factor for infection.

There is evidence that microbes may adhere to clay particles (Cuadros, 2017). Soil type can affect transmission of *Cryptosporidium* oocysts through the soil matrix (Davidson *et al*., 2014). Due to the similarity between *Cryptosporidium* and *Cyclospora*, this may be an important

aspect of environmental transport and should be a focus of future work. This study is an attempt to understand whether *C. cayetanensis* adheres to different soil types.

4.2 Materials and Methods

4.2.1 Soil Preparation

Soil was collected from sites in south and central Illinois from locations identified by using the Web Soil Survey (https://websoilsurvey.sc.egov.usda.gov, retrieved 04/12/2018). Three soils were selected to range from sandy to clayey and are the same as used by Davidson et al. (2013). The Catlin soil was collected from Urbana, IL. It is a moderately drained silt-loam (Catlin series, mesic Oxyaquic Argiudolls: 24% sand, 50% silt, 26% clay). The Alvin fine sandy loam (60% sand, 25% silt, 15% clay) and Darwin poorly drained silty-clay (5% sand, 50% silt, 45% clay) soils were collected in rural Jasper County, IL. Before collecting the soil, all vegetation and organic material were removed from the soil surface and the top approximately 7.6 mm (3 inches) of soil was collected so that the soil texture would remain constant. Large debris and organic materials were removed with a 4 mm sieve. Any material that went through the sieve was dried at 105°C for 24 hours then stored in air-tight containers.

4.2.2 Soil and Sample Preparation

A PBS-Tween 80 buffer solution was prepared as described in Dixon et al. (2013). PBS and Tween 80 were mixed to create a 0.01% Tween 80 solution. The solution was adjusted to a pH of 7.4.

The *Cyclospora* working group at the Center for Disease Control and Prevention (CDC) provided $1.2x10^6$ oocysts in 1 mL of potassium dichromate preservative. Several conical tubes of

Cyclospora-positive buffer solution were prepared by adding 200 µL of *Cyclospora* oocysts to 50 mL of buffer solution.

Each 2 mL microcentrifuge tube was filled with 1.0 g of soil. As indicated in Table 13, each time point was completed in triplicate for each of the three soil types at room temperature (22-25°C). Additionally, on days 0, 1, 5, 10, and 20, there were negative controls to control for existing levels of *Cyclospora*. Positive controls consisting of only buffer with oocysts were only examined on days 0, 1, and 20.

Either the pure buffer (negative control) or buffer with oocysts was added to each tube. 400 µL of buffer was added to the Alvin soil. 700 µL of buffer was added to the Catlin and Darwin soils. The volume added to each tube was soil type dependent and determined by the amount needed to bring the soil to nearly 100% saturation. Complete saturation of the soil increased the opportunity for the oocysts to interact with individual soil particles.

Table 13. Number of samples for each treatment and control group for each type of soil.

Day	Treatment (soil and buffer with oocysts)	Negative Control (soil and buffer)	Positive Control (buffer with oocysts)
0	3	3	3
1	3	3	3
2	3		
3	3		
4	3		
5	3	3	
6	3		
7	3		
8	3		
9	3		
10	3	3	3
15	3		
20	3	3	3

4.2.3 Oocyst Recovery

Each of the tubes for the given time point were removed from the incubator and an additional volume (400 or 700 µL) of the pure buffer was added to each tube. Plastic toothpick swords were used to stir the mixture 10x, then the samples were vortexed for 5 sec at medium speed. Tubes were centrifuged at 1240 x g for 10 minutes, then the original volume of buffer (400 or 700 µL) was removed from the supernatant. Supernatant was collected from as close to the soil-fluid interface as possible to collect the oocysts which would float above the soil, but remain at the base of the fluid. This also avoided collecting any organic matter floating on the surface. Additional buffer was added and spun down, then removed following the preceding steps twice more. All three extractions were combined, providing a total volume of 1.2 mL of fluid from the Alvin soil and 2.1 mL from Catlin and Darwin. Three extractions were chosen because assuming 100% availability of the oocysts, three extractions would yield 87.5% of the initial number of oocysts. With uniform mixing of either 400 or 700 µL of oocyst buffer and an equal amount of pure buffer, 50% of the oocysts would be removed during the first and subsequent extractions. A combination of the three extracts should, therefore, contain 87.5% of the original number of oocysts. Further extractions would yield more oocysts but would substantially dilute the sample, making it even more challenging to enumerate such a low concentration of oocysts and introducing more error into the analysis. The data were normalized for the remaining 12.5% that was not extracted. The recovered supernatant was placed in 15 mL plastic conical tubes and stored at 4°C.

4.2.4 Microscopy

Several different microscopy techniques were used to enumerate the oocysts that were not adhered to the soil particles. A Brightline hemacytometer was loaded with 15 µL of recovered supernatant that was vortexed for 5 sec at medium speed to homogenize the fluid. All oocysts within the entire gridded area were counted. The area was scanned at 10x magnification, and any potential oocysts were confirmed at 20x. The Sedgewick Rafter counting chamber was filled with 1 mL of recovered supernatant and allowed to settle for 15 minutes before examining at 20x magnification.

4.2.5 DNA Extraction and PCR

The DNA was extracted using the MoBio kit as discussed in chapter 3. PCR was performed with both the nested PCR primers and CYCLO18S primers. At the time that the nested PCR was run, only three days from each soil type were available. All the samples were amplifiedanalyzed using the new CYCLO18S primers.

4.3 Results and Discussion

The microscopy techniques were not possible with the limited number of oocysts in the soil. The concentration was too low for the available equipment to quantify an actual value; with the hemacytometer it was only possible to examine four 15 µL aliquots of the recovered supernatant. It was unlikely an oocyst was in the 0.9 µL viewing area of the hemacytometer, and only one oocyst was observed, but it was outside the gridded area. Figure 10 shows an oocyst on the hemacytometer.

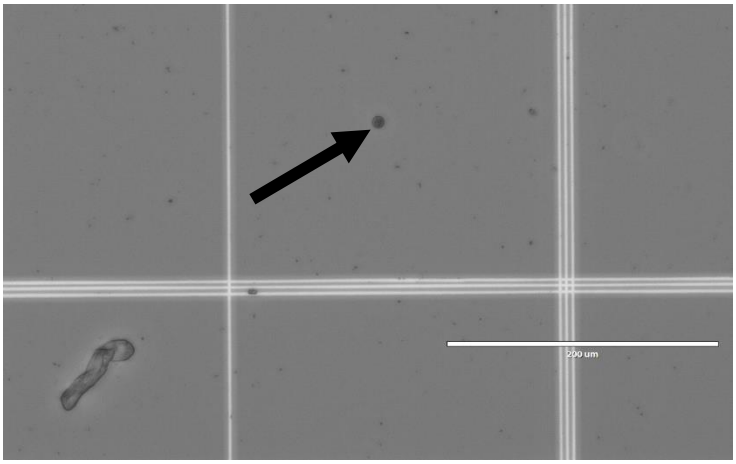

Figure 10. *C. cayetanensis* oocyst at 20x magnification on hemacytometer.

Figure 11 shows the PCR product from both rounds of nested PCR. The samples from day 0 have bright bands at the same location as the positive control and the expected fragment length of 294 bp, indicating that the oocysts had not yet bound to the soil. The only other sample that had a faint band is day 20 for Alvin soil. The lack of bands indicate that there was not enough *Cyclospora* DNA in the recovered supernatant to appear in the gel and thus most or all of the oocysts were adhered to the soil. At the far right of Figure 11, there is a positive (*C. cayetanensis* oocysts) and negative control (water). Additional days are included in the CYCLO18S process.

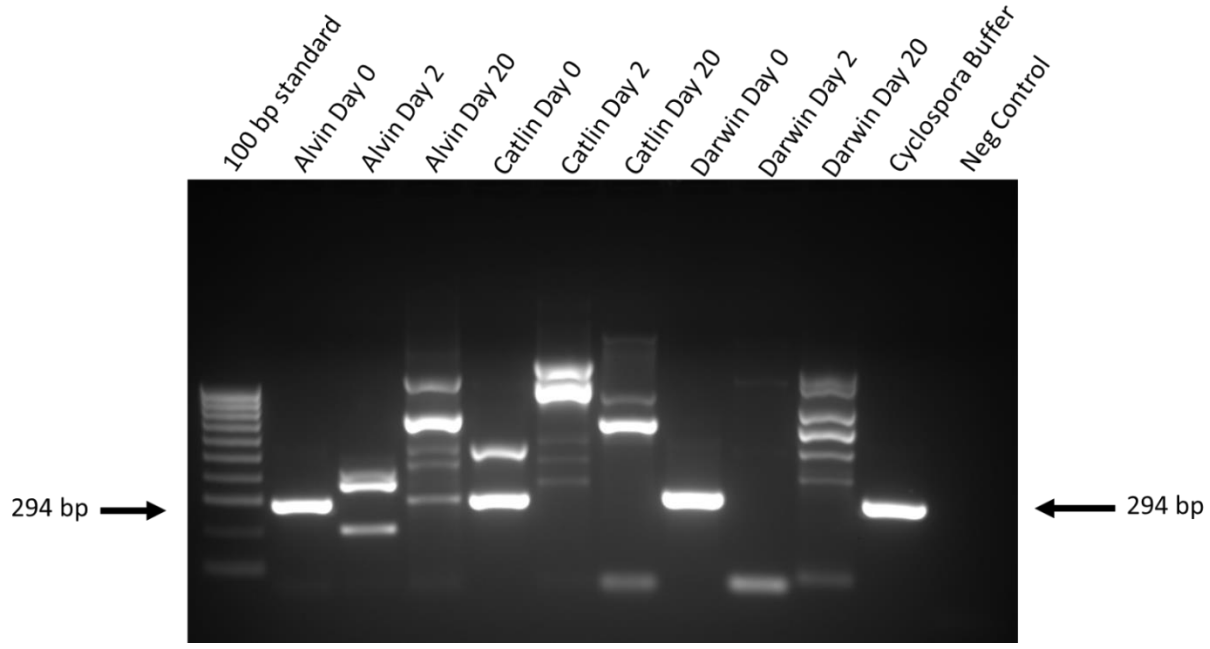

Figure 11. Gel electrophoresis of nested PCR for three soil types over time. The positive control (Cyclospora Buffer) is at the correct

The CYCLO18S PCR products are shown in Figures 12-14. The numbers indicate the number of days after inoculation that the oocysts were recovered from the supernatant. The "+" indicates that oocysts were used to inoculate the sample and "-" indicates a negative control where only pure buffer without any oocysts was used to inoculate the samples.

For Alvin soil, day 16 was the only positive sample. The Catlin soil had a positive sample on day 0, but also the negative control from day 10 produced a positive result. This may be due to switching the wells while loading the gels or due to contamination during one of the earlier steps. The Darwin soil did not have any positive samples.

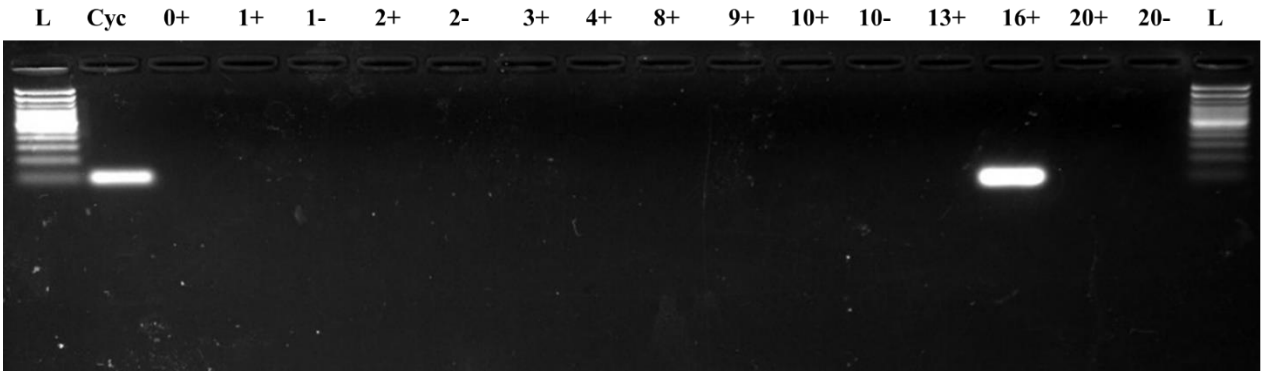

Figure 12. Gel electrophoresis after second round of PCR showing Alvin soil over time. The positive control is *C. cayetanensis* (Cyc). Each well is marked with the day and whether it is the positive or negative control

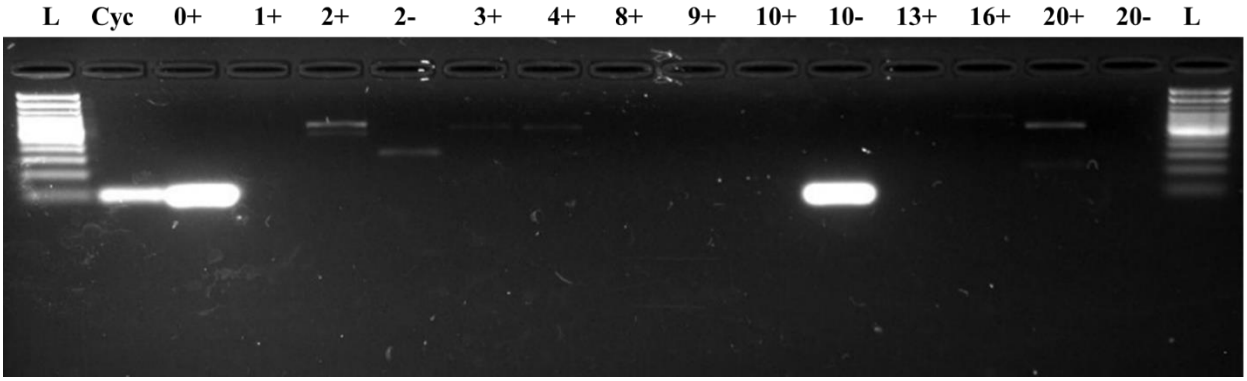

Figure 13. Gel electrophoresis after second round of PCR showing Catlin soil over time. The positive control is *C. cayetanensis* (Cyc). Each well is marked with the day and whether it is the positive or negative control

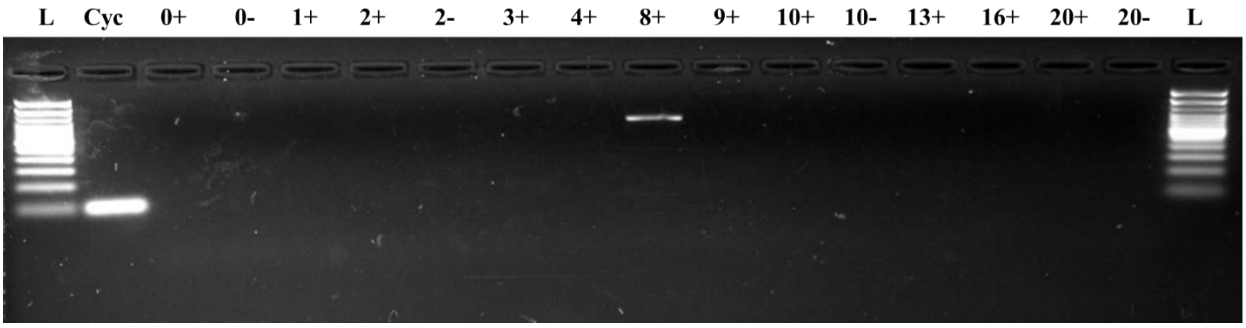

Figure 14. Gel electrophoresis after second round of PCR showing Darwin soil over time. The positive control is *C. cayetanensis* (Cyc). Each well is marked with the day and whether it is the positive or negative control

4.4 Conclusions

The second experiment described in this thesis demonstrates a method to study soil adsorption of *C. cayetanensis*. This study indicates that it is possible to recover oocysts that were mixed with the soil. This finding is significant given the small number of oocysts actually inoculated in the soil. Future work should ensure that sufficient oocysts are available to correctly estimate the concentration and number of oocysts applied to the soil and that the number is high enough to be reliably detected with microscopy or PCR techniques. Alternatively, new methods to detect small quantities of oocysts would allow the study to be performed with a limited oocyst supply.

CHAPTER 5: CONCLUSIONS

Cyclospora cayetanensis is an emerging food-borne pathogen. This research adds to the limited knowledge about *Cyclospora cayetanensis* by considering the environmental life stage of *C. cayetanensis* as oocysts. To understand more about *C. cayetanensis* in the environment, we addressed several issues.

We performed a field study to determine whether *C. cayetanensis* is present in soil, water, or animal scat near Combined Sewer Outfalls (CSOs) in northern Illinois. Field scale contamination is a major concern as *C. cayetanensis* requires time in the environment to mature before becoming infective. Water, soil and animals have been found to have oocysts. Soil and wildlife are the least understood modes of transmission. Out of 61 samples from three locations in the Chicago metropolitan area, 8 (13%) were positive in two PCR tests. Six (23%) of the 26 fecal samples were positive in both tests. We likely found fewer positive samples in water compared to soil and feces, because sampling occurred up to one week after the CSO discharge events. By the sampling time, much of the discharged *Cyclospora*, if it existed, was probably carried far downstream. Nevertheless, the frequency of positive samples and having several samples test positive under both methods indicate that the organism is likely ubiquitous in the environment and not from a specific point-source.

While this paper details methods to collect and detect *Cyclospora*, the study was performed on a very limited scale. With the scale of this studydifficult to draw any conclusions about how the organism moves from one section of the stream to another, though animals may play a roll in transporting oocysts upstream. A more in depth study looking at temporal and spatial variability is needed. The preliminary results, just looking at the PCR products with gel electrophoresis indicate that there is potentially *C. cayetanensis* in the environment near CSOs.

However, *Eimeria* spp. may be causing the positive bands using the nested PCR method, so Sanger sequencing will be performed on any positive samples to confirm the presence or absence of *Cyclospora*. Future work would need different *Eimeria* spp. to further evaluate the specificity of the new primers. These were developed to be specific to *C. cayetanensis*, but were not able to be validated in practice. A major hurdle for validating the primers was the limited amount of *C. cayetanensis* oocysts and DNA available for testing.

The second experiment described in this thesis demonstrates a method to study soil adsorption of *C. cayetanensis*. This study indicates that it is possible to recover oocysts that were mixed with the soil. This finding is significant given the small number of oocysts actually inoculated in the soil. Future work should ensure that sufficient oocysts are available to correctly estimate the concentration and number of oocysts applied to the soil and that the number is high enough to be reliably detected with microscopy or PCR techniques.

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APPENDIX A: ENVIRONMENTAL SAMPLE DATA SHEETS

Table 14: Caldwell Woods CSO Site, part A.

				SAMPLE ID				F- Scat		
Date (month/day) 2017	Time	Site	Air or Water ºC	CSO acronym	Transect	Sample #	Sample Type	Fresh code**	GPS?	Initials/Notes
6/21	12:07	Caldwell Woods	21.80	CDW	Z	1	F	3	N42.00293 W087.78174	Located ~4 ft. from water.
6/21	12:09	Caldwell Woods	21.80	CDW	Z	2	S	NA	N42.00293, W087.28176	Found on slope, 3 ft from water.
6/21	12:11	Caldwell Woods	21.80	CDW	Z	3	S	NA		
6/21	12:15	Caldwell Woods	21.80	CDW	Z	4	F	3	N42.0030, W087.78178	May be decomposed mushroom. Located 10 ft. from water.
6/21		Caldwell Woods	21.80	CDW	Z	5	F	2	N41.9951, W087.8591	
6/21		Caldwell Woods	21.80	CDW	Z	6	F	1	N42.00308, W087.78125	Contains wood chips, fish scales. Possibly otter. Located ~20 ft. from water.
6/21		Caldwell Woods	21.80	CDW	Z	7	F	3		Raccoon. No distinct shape, mashed down in a pile.
6/21		Caldwell Woods	21.80	CDW	Z	8	F	2		wood). Found 10m from CSO, between CSO and transect Z.
6/21		Caldwell Woods	21.80	CDW	A	9	S	NA	N42.0029, W087.78092	Sandy.
6/21	12:40	Caldwell Woods	21.80	CDW	A	10	S	NA	N42.0029, W087.78092	
6/21	1:02	Caldwell Woods	21.80	CDW	A	11	F	4	N42.00291, W087.78086	Contains hair. Located ~15 ft. from water.
6/21	2:28	Caldwell Woods	21.80	CDW	B	12	S	NA	N42.00274, W087.78062	Located up on slope near water. Located ~1 ft. from water.
6/21	2:30	Caldwell Woods	21.80	CDW	B	13	S	NA	N42.00274, W087.78063	Located up on slope near water. Located ~1 ft. from water.
6/21	2:41	Caldwell Woods	21.80	CDW	B	14	F	3	N42.00273, W087.88064	Contains long hairs. Located ~7 ft. from water.
6/21	3:41	Caldwell Woods	21.80	CDW	B	15	F	2	N42.00273, W087.88065	Contains few short hairs. Located ~7 ft. from water, near sample 14.
6/21	3:50	Caldwell Woods	21.80	CDW	C	16	S	NA	N42.00243, W087.78049	Located <1 ft. from water.
6/21	3:55	Caldwell Woods	21.80	CDW	C	17	S	NA	N42.00243, W087.78050	Located on top of slope, ~5 ft. from water.

Table 14 (cont.)

				SAMPLE ID				F- Scat		
Date (month/day) 2017	Time	Site	Air or Water ºC	CSO acronym	Transect	Sample #	Sample Type	Fresh code**	GPS?	Initials/Notes
6/21	4:02	Caldwell Woods	21.80	CDW	C	18	F	4	N42.00264, W087.78068	Old. Contains few hairs. Clumpy, in small granules. Located 15m upstream of transect C and ~10 ft. from water.
6/21	4:08	Caldwell Woods	21.80	CDW	C	19	F	1	N42.0026, W087.78041	Not much shape, liquidy in center. Located ~30 ft. from water.
6/21	12:34	Caldwell Woods	19.10	CDW	Z	20	W	NA		Taken from center of river. Quick flow.
6/21	1:10	Caldwell Woods	19.10	CDW	A	21	W	NA		Taken from center of river. Quick flow.
6/21	3:45	Caldwell Woods	19.10	CDW	B	22	W	NA		Taken from center of river. Quick flow.
6/21	4:16	Caldwell Woods	19.10	CDW	C	23	W	NA		Taken from center of river. Quick flow.

Table 15: Devon Ave. CSO Site

				SAMPLE ID				F- Scat		
Date (month/day) 2017	Time	Site	Air or Water ºC	CSO acronym	Transect	Sample #	Sample Type	Fresh code**	GPS?	Initials/Notes
5/25	12:00	Devon St. East Woods	19.20	DEW	Z	1	F	3	N41.9951, W087.8591	Many raccoon piles. All located within ~3 ft.
5/25		Devon St. East Woods	19.20	DEW	Z	2	F	2	N41.9951, W087.8591	Located near feather.
5/25		Devon St. East Woods	19.20	DEW	Z	3	F	4	N41.9951, W087.85931	Clay-like.
5/25	12:12	Devon St. East Woods	19.20	DEW	Z	4	S	NA	N41.9951, W087.8591	Riverbank mud. ~6 ft. from water
5/25		Devon St. East Woods	19.20	DEW	Z	5	S	NA	N41.9951, W087.8591	Loose organic clay. ~20 ft. from water
5/25	12:30	Devon St. East Woods	19.20	DEW	A	6	S	NA	N41.99477, W087.8589	Located on slope ~5 ft. above water. Loose organic clay. ~6 ft. from water.
5/25		Devon St. East Woods	19.20	DEW	A	7	S	NA	N41.99477, W087.8589	
5/25		Devon St. East Woods	19.20	DEW	A	8	F	5	N41.99487, W087.85867	Possibly raccoon?
5/25	12:40	Devon St. East Woods	19.20	DEW	A	9	F	2	N41.99483, W087.85876	Located 14 m downstream. Furry. Contains white, waxy clumps
5/25	1:00	Devon St. East Woods	19.20	DEW	B	10	S	NA	N41.9944, W087.85857	Riverbank mud. ~3 ft. from water
5/25		Devon St. East Woods	19.20	DEW	B	11	S	NA	N41.99434, W087.85859	Light vegetation. ~8 ft. from water
5/25	1:15	Devon St. East Woods	19.20	DEW	C	12	S	NA	N41.99421, W087.85861	Spongey soil. ~8 ft. from water
5/25	1:17	Devon St. East Woods	19.20	DEW	C	13	S	NA	N41.99421, W087.85861	Twig/wood degraded debris
5/25		Devon St. East Woods	19.20	DEW	C	14	F	NA		Probably sponge, not scat
5/25	3:15	Devon St. East Woods	15.80	DEW	Z	15	W	NA		Decent flow. Near easily accessible bank with many animal tracks. ~7 ft. from bank.
5/25	3:30	Devon St. East Woods	15.80	DEW	C	16	W	NA		Decent flow. ~7 ft. from bank.
5/25	3:45	Devon St. East Woods	15.80	DEW	A	17	W	NA		Near logs in water. In an area with low flow

Table 16: St. Paul Woods CSO Site

				SAMPLE ID				F- Scat		
Date (month/day)2017	Time	Site	Air or Water ºC	CSO acronym	Transect	Sample #	Sample Type	Fresh code**	GPS?	Initials/Notes
6/27	12:44	St. Paul Woods	22.2°C	SPW	Z	1	F	4	N42.030387 W-87.794813	Located ~4 ft. from water. Looks fairly old, and had a few hairs nearby
6/27	12:48	St. Paul Woods	22.2°C	SPW	Z	2	S	NA		Right next to the river. Light brown and mostly clay. Found near animal tracks
6/27	12:52	St. Paul Woods	22.2°C	SPW	Z	3	S	NA		10ft from the river. Densely packed and dry. Might have been on a path.
6/27	12:55	St. Paul Woods	22.2°C	SPW	Z	4	F	4		Very dry. Might be too old.
6/27	1:07	St. Paul Woods	22.2°C	SPW	Z	5	F	3		Very close to the river. Non-uniform shape
6/27	1:33	St. Paul Woods	22.2°C	SPW	A	6	S	NA	N42.029866 W-87.793655	6" from water. Animal tracks nearby. Clay like.
6/27	1:38	St. Paul Woods	22.2°C	SPW	A	7	S	NA		Dry and crumbly. 15 ft from water on a slope
6/27	1:44	St. Paul Woods	22.2°C	SPW	A	8	F	2		10 ft from water on a slope. Has seeds in it.
6/27	1:46	St. Paul Woods	22.2°C	SPW	A	9	F	1		Very seedy. Moist. Brownish green. Possibly mullberry seeds.
6/27	1:53	St. Paul Woods	22.2°C	SPW	A	10	F	4		Found near a tree. Could be a pet dogs.
6/27	2:22	St. Paul Woods	22.2°C	SPW	B	11	F	2	N42.029668 W-87.793477	Near a tree. 20 ft from water. Many large seeds.
6/27	2:27	St. Paul Woods	22.2°C	SPW	B	12	S	NA		Soil was under leaves. 15ft from water. Moist clay.
6/27	2:29	St. Paul Woods	22.2°C	SPW	B	13	F	3		20ft from soil. Flaky. Near suspected racoon burrow. May be soil.
6/27	2:38	St. Paul Woods	22.2°C	SPW	B	14	F	1		Potentially Racoon scat. Contains many seeds. Very fresh.
6/27	2:46	St. Paul Woods	22.2°C	SPW	B	15	S	NA		Very moist. Light brown. Mud/clay. Very close to the water
6/27	3:02	St. Paul Woods	22.2°C	SPW	C	16	S	NA	N42.029400 W-87.793202	1 ft from water. Moist on a bareground.
6/27	3:09	St. Paul Woods	22.2°C	SPW	C	17	F	1 or 2		A bit mashed down. Hard outside, very fresh inside. 40 ft downstream of C

Table 16 (cont.)

				SAMPLE ID				F- Scat		
Date (month/day) 2017	Time	Site	Air or Water ºC	CSO acronym	Transect	Sample #	Sample Type	Fresh code**	GPS?	Initials/Notes
6/27	3:20	St. Paul Woods	22.20	SPW	C	18	S	NA		6" from water. Moist. Mud/clay
6/27		St. Paul Woods	20.80	SPW	Z	19	W	NA		Water from site Z. Might have some oil due to the oil in the pump.
6/27		St. Paul Woods	20.80	SPW	A	20	W	NA		Water from site A
6/27		St. Paul Woods	20.80	SPW	B	21	W	NA		Water from site B
6/27		St. Paul Woods	20.80	SPW	C	22	W	NA		Water from site C

APPENDIX B: ENVIRONMENTAL GEL IMAGES FROM NESTED PCR

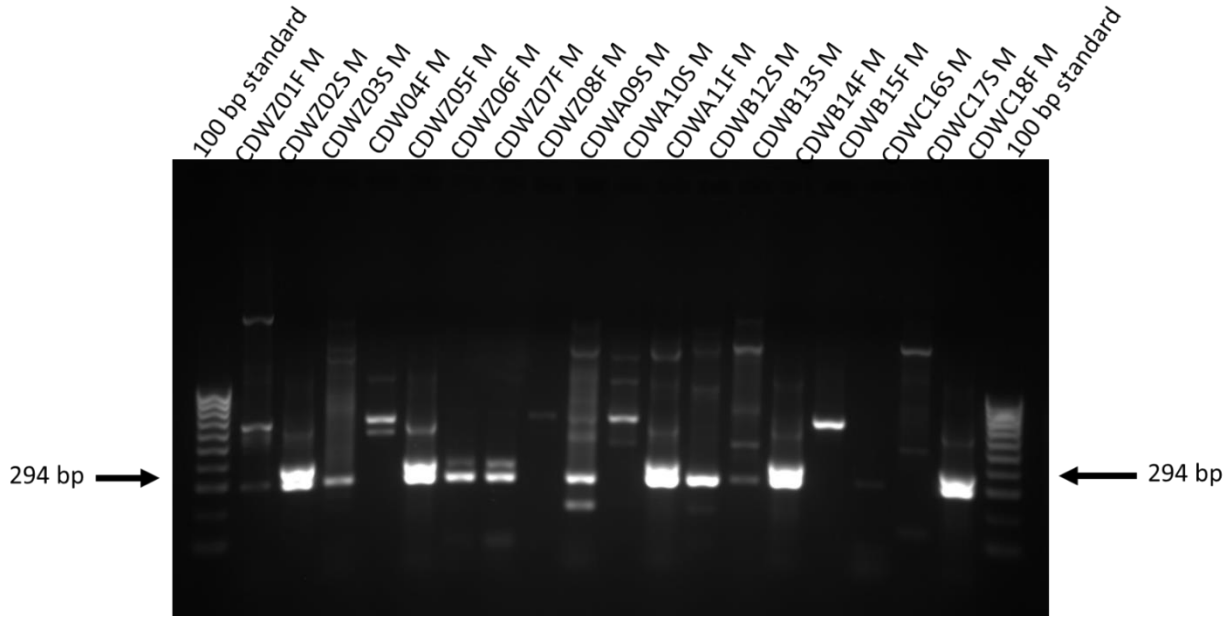

Figure 15: Caldwaell wood Nested PCR samples

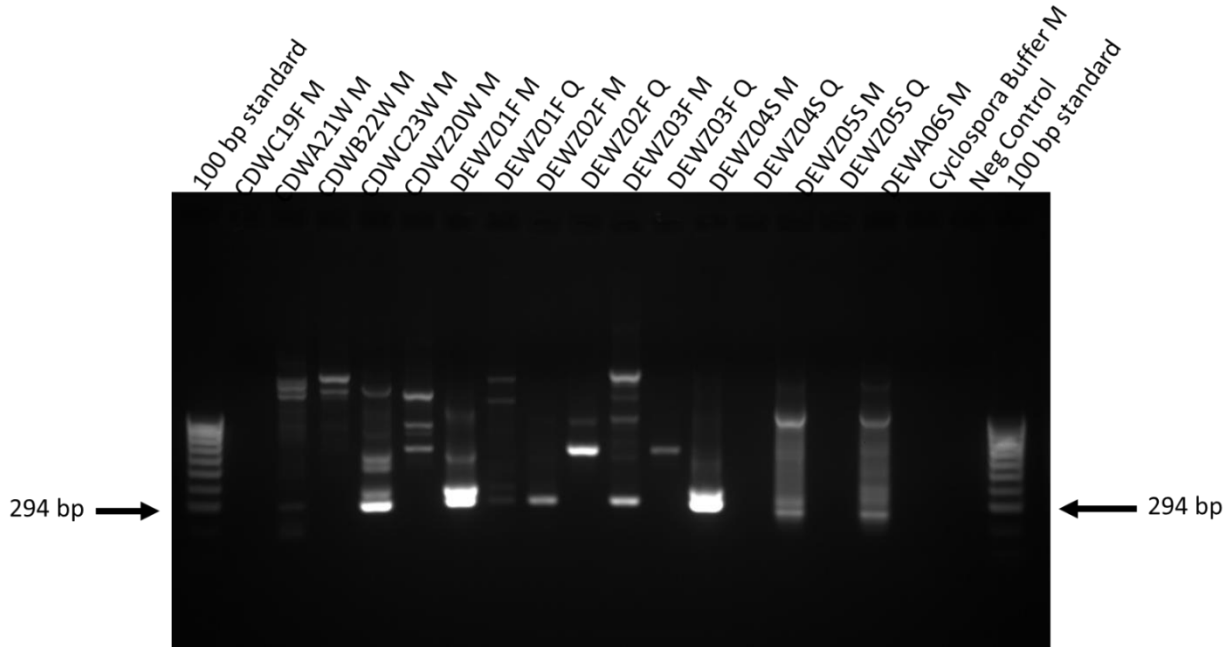

Figure 16: Caldwell and Devon Ave. Nested PCR samples with positive and negative controls

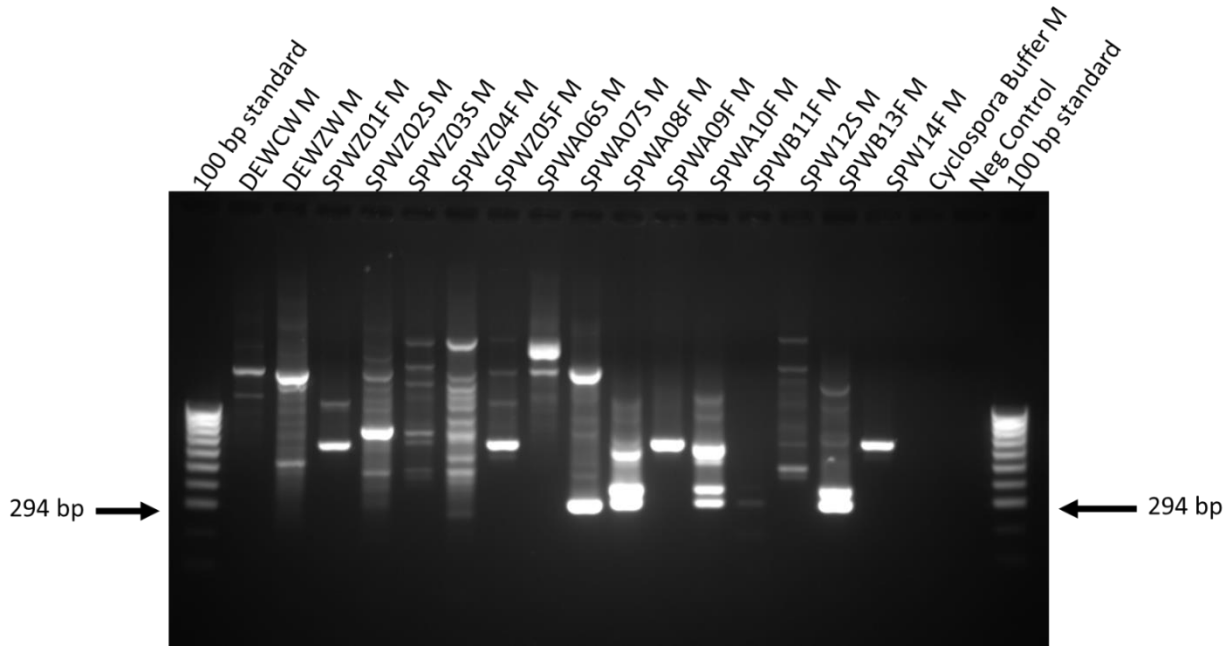

Figure 17: Devon Ave. and St. Paul Woods samples with positive and negative controls.

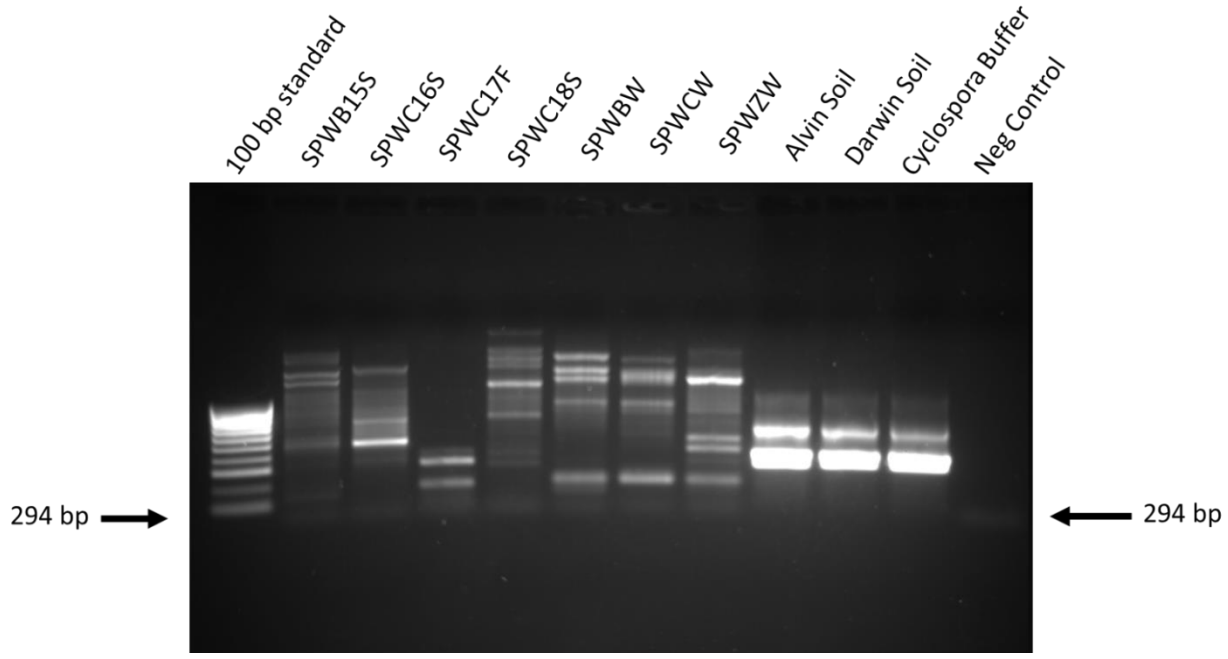

Figure 18: St. Paul Woods samples and test soil samples of different soil texures.