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Analysis of Variable Effects on Presence of Cryptosporidium Oocysts and Giardia Cysts in Effluent Water from Wastewater Treatment Utilities in Florida from 1998 to 2010

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Analysis of Variable Effects on Presence of Cryptosporidium Oocysts and Giardia Cysts in Effluent Water from Wastewater Treatment Utilities in Florida from 1998 to 2010

by

Katherine Jane Barkan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science Department of Global Health College of Public Health University of South Florida

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Abstract

The concern of a *Cryptosporidium* or *Giardia* waterborne outbreak due to treated wastewater has had water treatment utilities using some of the highest water cleansing technologies available. Cryptosporidiosis and *Giardiasis* are severe diarrheal diseases which can lead to death, thus it is important that appropriate steps are taken to assure these parasites are not present in the effluent of treated wastewater. This study examined the results of 863 assays for *Giardia* and *Cryptosporidium* on the effluent of wastewater treatment facilities and found that county of collection, watershed of collection, and laboratory analyzing the sample have the most significant impact on the detection of *Cryptosporidium* oocysts and *Giardia* cysts in wastewater effluent and that there were minimal but significant differences in method of treatment and method of filtration. To date no other comprehensive analysis of this data has been done.
Introduction

*Cryptosporidium*

*Cryptosporidium* is a protozoan coccidian parasite of the apicomplexa phylum found in the respiratory and gastrointestinal tracts of many hosts. *Cryptosporidium* species are ubiquitous in nature with worldwide distribution and have mammalian, avian, piscine, and amphibian hosts (Fayer, 2010). *Cryptosporidium* oocysts can be found in soil, surface water, recreational water, drinking water, and fecal-contaminated food.

Although many hosts are capable of acquiring this organism, it is thought that the main environmental reservoirs for this organism are cattle and rodents (Chalmers et al 1997). *Cryptosporidium* has been a recognized cause of disease for over a century but has widely garnered attention in the US since the 1970s as an increasing cause of infectious disease. *Cryptosporidium* was recognized as a disease causing parasite in 1907 by E.E. Tyzzer in mice and has been commonly detected by veterinarians ever since (U.S. E.P.A., 2001). The first episode of cryptosporidiosis in a human was recorded by Nime in 1976, in a 3-year old girl in Tennessee. Since then, the incidence and prevalence of this organism have been increasing. A large part of this is due to the discovery that much of the fatal diarrheal diseases in HIV and AIDS patients is caused by *Cryptosporidium* (Kim et al, 1998). Healthy individuals exhibit symptoms ranging from asymptomatic to severe, but the infection is almost always fatal in immunocompromised patients.
(Pieniazek, et al, 1999). This has been a leading cause for a major portion of the studies of Cryptosporidium species and pathogenesis as well studies for increasing the efficacy of diagnostic testing.

Cryptosporidiosis is a mainly zoonotic disease transmitted to humans from various mammals, birds, reptiles, and rodents through the fecal-oral route. It has also been demonstrated to be an anthroponotic disease further adding to its ability to be easily transmitted. The current number of species and the specific described genotypes varies constantly as they are described and identified through by polymerase chain reaction (PCR) and gene sequencing (Champliaud, et al, 1998). Methods for serological testing for antibodies to Cryptosporidium sp. have been effective in detecting different species of this organism from a variety of hosts (Priest, et al, 2006). Over 40 genotypes and 15 species of Cryptosporidium have been described (Ruecker, et al, 2007). Although there have been several species of Cryptosporidium there are currently eight that have been found to be infective to and pathogenic in humans: C. parvum, C. hominis, C. meleagridis, C. felis, C. canis, C. muris, C. suis and C. corvine (Kosek, et al, 2001). Of these species C. parvum, C. hominis, and C. meleagridis, respectively, are the organisms that cause the highest levels of disease in humans. An increasing number of species are being proven to infect humans, including strains thought as only host specific (Xiao, et al, 2001).

The species causing the highest rate of disease in humans is C. parvum although C. hominis is considered to be the human specific species. Most mammals are infected with host-specific species of Cryptosporidium. These are
generally infections throughout a domesticated animal community. Some wild mammals can become infected by another host-specific species through practices such as coprophagia, a demonstrated common behavior in foxes. The most likely explanation for this is that *C. parvum* is a widely infective species and is hosted by a variety of domesticated animals. Cows are a known host of this organism and transmission is thought to occur by general contact as well as through the ingestion of unpasteurized milk. Once infected, this organism is easily spread from human to human (Pieniazek, et al, 1999).

A topic of controversy has been the exact route through which humans are infected with this organism. The main question is whether the specific organisms are being shed by animals in water that eventually comes into contact with drinking water or if transmission occurs by another route. There have been several studies demonstrating the connection between *Cryptosporidium* harboring wild mammals and the occurrence of *Cryptosporidium* oocysts in runoff and storm water (Cizek et al, 2008) (LeChevallier, Norton, & Lee, 1991). Hence these animals can be a source of *Cryptosporidium* in watersheds and source water (U.S. E.P.A., 2001). Zhou, et al (2004) found that animals inhabiting areas where possible watershed contamination is present, were shown to be infected by species that cause no known illness in humans. To date few studies determining the differing concentrations of *Cryptosporidium* species infectious to humans in water sources have been completed. While non-pathogenic species may infect watersheds, and thus can be detected by the current standard assay, USEPA Method 1623, the ratio of non-pathogenic to pathogenic is not known
Any detection of *Cryptosporidium* oocysts can be seen as a public health threat.

After infection with *Cryptosporidium* some people may be completely asymptomatic while still shedding high levels of oocysts. For most infected people, the symptoms of watery diarrhea, stomach pain or cramps, dehydration, nausea, vomiting, fever, and weight loss start seven to ten days after being infected. These symptoms usually persist for one to two weeks, but can range from a few days to several weeks. In otherwise healthy immunocompetent persons the disease and infection is self-limiting (Pieniazek, et al, 1999). During the complicated lifecycle there is the possibility of autoinfection and thus patients may have symptoms that come and go for a period lasting up to a month.

When the immune system is compromised or the infected person has another infection or disease, the *Cryptosporidium* infection may last longer and have more severe symptoms (Pozio, et al, 1997). In many HIV positive and AIDS patients cryptosporidiosis is a fatal disease causing unrelenting diarrhea (Pieniazek, et al, 1999).

An infected host sheds the infectious thick-walled oocysts of *Cryptosporidium* which then contaminates food and water sources. The complicated lifecycle of *Cryptosporidium* begins with ingestion of the thick-walled oocysts. The oocyst releases sporozoites which then invade the lumen and lungs and undergo asexual reproduction. The sexual reproduction phase then takes place with the female macrogamonts and male microgamonts resulting in fertilization. The following zygote develops into thin-walled oocysts that autoinfect
the host and thick-walled oocysts that exit the host through feces (Current & Garcia, 1991).

Cryptosporidiosis diagnosis is done with various microscopy, staining, and antibody detection procedures used to detect oocysts in feces. Multiple staining techniques such as an acid-fast staining, giemsa staining, and auramine staining can be used to detect oocysts. Tissue biopsies of the small intestine can be stained with hematoxylin and eosin which shows oocysts attached to the epithelial cells. Antigen/antibody detection techniques such as direct fluorescent antibody (DFA), immunofluorescent assays enzyme-linked immunosorbent assay (ELISA), and polymerase chain reactions (PCR) are extremely useful in diagnosis (Jex, et al, 2008).

There is no proven preventative or curative treatment for cryptosporidiosis (U.S. E.P.A. 2001), but nitazonaxide and parmomycin are showing promise in treatment of cryptosporidiosis (Chakrabarti and Chakrabarti, 2009). While mammalian infections caused from Cryptosporidium are generally limited to the gastrointestinal tract, there have been respiratory tract infections caused by this organism as well. Respiratory tract infections have been found in avian and mammalian species (Akiyoshi et al, 2003). Cama in 2007 noted that HIV-positive patients commonly acquire the respiratory version of this disease (Cama, et al, 2007). Studies, such as that of Mercado et al (2007) and Mor et al (2010), indicate the potential for transmission of Cryptosporidium by respiratory secretions. Those studies also demonstrate that healthy immunocompetent people testing positive for gastrointestinal Cryptosporidium have also tested
positive for *Cryptosporidium* in their sputum. In a study of 926 children in Uganda by Mor et al. (2010), *Cryptosporidium* DNA was found in the sputum of 35% of the children who were positive for gastrointestinal *Cryptosporidium*. Of those children, 94% of them were HIV-seronegative. In the control population of children who were negative for gastrointestinal *Cryptosporidium* and tested for respiratory *Cryptosporidium* 100% of them were negative for respiratory Cryptosporidiosis. In this study, the sputum was analyzed by molecular methods for the detection of *Cryptosporidium* DNA; microscopic assays for the detection of *Cryptosporidium* oocysts or sporozoites were not performed. This means while present in the respiratory tract *Cryptosporidium* may not be transmissible by respiratory secretions. There has been no evidence found that respiratory *Cryptosporidium* is caused by gastrointestinal cryptosporidiosis. The third most common species of *Cryptosporidium* to infect humans, *C. meleagridis*, is the most common organism found in many avian species. In several studies respiratory transmission of this organism in avian species has been established. This species of *Cryptosporidium* has been shown to infect humans as well as avian species and infects non-human species primarily by respiratory secretions (Akiyoshi, 2003). Since this leads to the possibility of a higher than known respiratory transmission of *Cryptosporidium*, further examination of the possible public health threats this poses is warranted.
Giardia

*Giardia duodenalis*, also known as *G. lamblia* and *G. intestinalis*, is a flagellated protozoan parasite in the Metamonada phylum. *Giardia* is ubiquitous worldwide with a wide range of mammalian and avian hosts. There are an estimated 2.5 million cases of *Giardiasis* annually. While the highest burden of disease caused by *Giardia* is in developing countries, in developed countries *Giardia* is the most prevalent intestinal parasite (Furness, 2000). Humans are a main reservoir for *Giardia* although it is found in many small aquatic and semi-aquatic mammals (Baker, 2007). *Giardia* can be found in surface water, lake and pond water, water from shallow wells, and recreational water facilities such as pools, beaches, and fountains. As with *Cryptosporidium*, *Giardia* is a zoonotic disease (Feng & Xiao, 2011). Different infective species of *Giardia* have been found in cats, dogs, cattle, deer, and beavers. Numerous cases of campers acquiring the disease from water inhabited by beavers have given this disease the nickname “beaver fever”.

*Giardia* was first noted by Antony van Leeuwenhoek in 1681 after an examination of his own fecal matter. In 1859 Vilem Lambl described and illustrated its morphology and was again described in 1895 by Alfred Giard. Over two centuries since its first description passed before *Giardia* was associated with diarrheal diseases in 1902 by Charles Stiles (Cox, 2002). In 1954 Rendtorff officially linked the parasite with diarrheal disease.

Over 40 species of *Giardia* have been described but only six morphologically distinct species are recognized: *G. lamblia*, *G. muris*, *G. ardeae*, *G. intestinalis*.
*G. psittaci, G. agilis, and G. microti.* The species *G. lamblia* is the main species thought to infect humans. *G. lamblia, G. microti,* and *G. muris* are found in mammals, *G. ardeae* and *G. psittaci* in birds, and *G. agilis* has been isolated from amphibians. Many species of *Giardia* appear morphologically identical and thus naming and identifying different species has been difficult (Adam, 2001).

A *Giardia* infection starts with the ingestion of water or food that has been contaminated with fecal matter containing cysts. Once a cyst is ingested, excystation occurs in the stomach or in the duodenum. The excysted trophozoites undergo asexual reproduction through binary fission in the small intestines. At this point some of the trophozoites continue to asexually reproduce causing autoinfection. The rest of the trophozoites encyst while in the large intestines and get passed through the remainder of the digestive tract in feces. While trophozoites and cysts get passed out of the body in feces only the cyst form is able to survive outside the host (Adam, 2001).

Unlike *Cryptosporidium,* *Giardia* only colonizes the lumen of an infected host. The major of infections are asymptomatic but they can cause acute or chronic diarrheal illnesses. The primary symptoms of infection are diarrhea, malaise, foul smelling flatulence, steatorrhoea (pale, foul, greasy stools), abdominal cramps, bloating, nausea, vomiting, and weight loss start one to three weeks after exposure. *Giardiasis* is generally a self-limiting disease with dehydration being the main health concern (Gardner & Hill, 2001).

Diagnosis of *Giardiasis* entails an ova and parasite examination of feces that is routinely repeated for a total of three specimens. Cyst detection from feces
aids in diagnosis in over 80% of patients. While ELISA assays are available, they are much more costly and should not take the place of stool examination. A majority of *Giardiasis* cases can be treated with anti-parasitic nitroimidazole medications (Gardner and Hill, 2001). Because of the dramatic fluid loss *Giardiasis* induced diarrhea can cause a main component of treatment is fluid and electrolyte management (Guerrant et al 2001). When the immune system is compromised or the infected person has another infection or disease, the infection may last longer and have more severe symptoms, but unlike *Cryptosporidium* it is not usually fatal as effective treatment therapies are widely available (Gardner & Hill, 2001).

*Cryptosporidium* and *Giardia* Water Safety Threat

The infectious oocysts and cysts of *Cryptosporidium* and *Giardia*, respectively, are found in the feces of infected animals (Hunter & Thompson, 2005). Both *Cryptosporidium* and *Giardia* are organisms that pose a health threat in developed, as well as developing, countries. The World Health Organization placed these parasites on its list of neglected diseases due to the need for further preventative and treatment method research (Chakrabarti and Chakrabarti, 2009).

The main public health threat currently posed by these organisms is a waterborne outbreak. *Cryptosporidium* and *Giardia* concerns arise because: they are easily spread through ingestion of contaminated drinking water, recreational water, and food; easily spread by fecal-oral routes involving animals and soil; and
Cryptosporidium is highly resistant to disinfection by chlorination at levels that are not toxic to people (Kosek, et al, 2001). Due to these concerns both organisms have been labeled as Category B Bioterrorism Agents. A Category B Bioterrorism Agent is defined as an agent that is moderately easy to disseminate, results in moderate morbidity and low mortality rates, and requires specific enhancements of the CDC’s diagnostic capacity and enhanced disease surveillance. Some examples of other Category B agents are brucella, epsilon toxin (clostridium perfringens), salmonella, E. coli 0157:H7, Q fever, ricin toxin, typhus fever, and other water safety threats (Rotz et al, 2002).

An example of the devastating effects of an outbreak (not due to bioterrorism) is the 1993 waterborne outbreak of Cryptosporidium in Milwaukee, Wisconsin. The outbreak occurred as a result of ineffective water filtration of a municipal potable water supply. While the obvious health effects of this outbreak were monitored it is one of the few Cryptosporidium outbreaks that had a retrospective cost-of-illness analysis. An estimated 403,000 people become ill and 104 died with the total cost of illness being approximately $96.2 million: $31.7 million in direct medical costs and $64.6 million in productivity losses (Corso, et al, 2003). In 1998 the main water supply in Sydney, Australia was contaminated with Cryptosporidium and Giardia, affecting over three million residents (Stein, 2000). In Florida there have been over 1,000 cases of Giardiasis reported annually for more than a decade. In 2006 a neighborhood water fountain became contaminated resulting in an outbreak with 38 cases of Giardiasis, 9 cases of cryptosporidiosis, and 2 cases of co-infection (Eisenstein,
According to the US EPA almost all of the Cryptosporidium outbreaks in the US after 1985 occurred as a result of a fault in treatment and/or filtration of water (US EPA, 2001). It is apparent that cryptosporidiosis and Giardiasis are diseases of major concern due to their health and economic impacts.

Enhanced water purification protocols were put into place in an effort to stem the spread and occurrence of Giardia and to prevent Cryptosporidium from getting into any potable water source. As both of these diseases are generally spread in a fecal-oral route, the highest degree of action was taken in the treatment of source water and wastewater that are open to the environment. Current requirements apply to public water systems that use ground or surface water that is under the direct influence of surface water, the types of water that are most susceptible to protozoan contamination.

In 1974 the U. S. Congress enacted the Safe Drinking Water Act as a public health measure to assure the safety of the nation’s drinking water supply. The regulations enacted examine chemical, microbiological, physical, and radiological contamination in water. This act was later amended and in 1989, Giardia was added as a contaminant for regulation. In 1998 the Interim Enhanced Surface Water Act was created to further control Cryptosporidium. The long Term 1 Enhanced Surface Water Treatment Rule was enacted in 2002 and lead to the current Long Term 2 (LT2) Enhanced Surface Water Treatment Rule developed in 2006. The goal of these rules is to control microbial pathogens in water while minimizing the public health risks of disinfectants, disinfection
byproducts, and chemical contaminants. Under the LT2 rule, source water treatment facilities must submit monthly samples for two years or semimonthly samples for one year for examination of Cryptosporidium and Giardia if they fail initial E. coli coliform or state-approved alternate indicator monitoring. If the levels of E. coli detected exceed the acceptable limits source water monitoring of Cryptosporidium is required.

The Safe Drinking Water Act Amendments of 1996 require the US EPA to evaluate public health risks posed by drinking water contaminants including the parasites Cryptosporidium and Giardia. The US EPA is responsible for assessing the parasite occurrence in raw surface waters used as source water for drinking water treatment plants. EPA Method 1623 was designed as the detection method for Cryptosporidium and Giardia in water from the treatment plants. The results were submitted to the US EPA for the purpose of monitoring the source water and to determine if additional treatment is required. This is essential to monitor increases in microbial risk that may occur when systems implement the Stage 2 Disinfectants and Disinfection Byproducts Rule (Stage 2 DBPR). Depending on the results of the tests facilities may have to install new or additional treatment and filtration systems as well as submit additional samples for testing. E. coli detection is enacted first as a cost saving method due to the high costs of collection and analysis of water samples for Cryptosporidium and Giardia. Price variance occurs between labs, but in general a total coliform analysis costs less than $70 while EPA Method 1623 Cryptosporidium and Giardia testing costs over $500. E. coli coliform testing detects the presence of
fecal contamination in water. As Cryptosporidium and Giardia are passed into water through contaminated feces monitoring first for the presence of fecal contamination is a time and cost saving measure. In order to assure that steps taken to minimize cyst and oocysts presence in the wastewater effluent are successful facilities that have had a positive test result are required to submit subsequent samples for testing in a sped-up timeframe until they have negative results.

The different policies enacted monitor potable and surface water sources primarily due to the use of reclaimed water. While the US EPA has established guidelines for reclaimed water it does not regulate its use. Regulation is left to each individual state and the state’s interpretation of the guidelines, leading to varying definitions and uses. Reclaimed or “reuse” water is considered to be wastewater (sewage) that is treated to remove certain solids and impurities. The practices of “re-using” water were implemented to ensure freshwater conservation and sustainability for the alleviation of water shortages (Levine and Asano, 2004). The increasing municipal water demand in populated areas is another reason wastewater reclamation is being enacted. Reclaimed water is not treated to the same degree as potable water before use. Thorough treatment methods are needed to ensure that there is no contamination by Cryptosporidium and Giardia. Reclaimed water that is not properly treated may contain infectious cysts and oocysts. Reclaimed water is used for fire suppression, dust control, and irrigation of agricultural and recreational areas such as golf courses, playgrounds, and public parks. Agricultural use of contaminated reclaimed water
can lead to contaminated food. Recreational irrigation with contaminated reclaimed water can lead to increased exposure to humans. According to one statistic EPA released online: approximately 673 million gallons per day of reclaimed water was reused for beneficial purposes in 2009 in Florida. This highlights the importance of monitoring wastewater effluent in order to decrease any possible transmission of protozoan parasites to humans.

**Wastewater Treatment**

Many different methods are utilized in the treatment of wastewater, but almost all of them are 5-stage biological nutrient removal (BNR) processes. BNR removes nitrogen and phosphorus from wastewater through the use of various microorganic conditions (U.S. E.PA, 2007). The 5-stage BNR process has been shown to remove more wastewater nutrients, such as nitrogen, phosphorus, and oxygen, and thus provides better treatment of the water (Uygur, 2004). Often the activated sludge method is used in treatment due to its role in the breakdown of biomass (Fenu, et al, 2010) and its relative simplicity (Vigne, et al, 2010). Through a series of aerobic, anaerobic, and anoxic tanks carbon, nitrogen, phosphorus, metals, and pathogens are removed from the water (Song, et al, 2010; Bok, et al, 2002). Throughout each stage several biochemical reactions take place in the treatment of various contaminants (Goel, et al, 1998). The anaerobic stage is primarily responsible for pathogen removal (Novak, et al, 2010).
Tertiary treatment, such as filtration and disinfection, are necessary for removal of microorganisms from wastewater systems (Koivunen, 2003). Through the entire process most of the microorganisms in the water are removed in the filtration stage. To date no filtration method has been developed that is capable of eliminating all of the cysts and oocysts; the best methods filter out 99% of Cryptosporidium oocysts (U.S. E.P.A., 2001).

Continuous backwash filtration is a method of water filtration that employs granular media filter beds. The basic principle is that effluent water flow is reversed in a section of the filter bed media and pumped through the media in the opposite direction of regular flow (England, 1994). This dislodges waste from the media and mixes the media to a more optimal density allowing better water flow and filtration. In most systems the backwash water is discarded due to the high amount of waste in it. Backwash water is commonly discarded by discharged into a sanitary sewer, transport to a sewer plant or landfill, or sent to a basin where the solids are settled out. It is also mixed with the plant influent and re-cycled through the plant. All of the methods for disposal of backwash water are highly regulated due to the presence of contaminants. A continuous backwash method keeps the filter media from getting clogged and also eliminates the need to shut down for filter cleaning. It also eliminates the rush of “dirty” water that happens when the flow is restored.

A method that is gaining popularity is continuous backwash, upflow, deep bed, granular media (sand) filtration. This combines several proven effective filtration methods into one system. Influent water is pumped into the central feed
chamber of the tank. From there it is injected into the bottom layer of granular media and flows upward to the top of the tank. As the water travels upward the waste is caught in the media. The water is cleaned during the entire travel through the media because the sand is clean at the top of the tank. The heavy waste-laden sand sinks to the bottom of the tank, as it sinks to a level below the influent injection it is pumped vertically through an airlift where it is heavily scoured and the waste is removed. Once the sand reaches the top of the airlift it flows into a reject compartment where the lighter weight waste floats into the reject pipe as the sand falls into the washbox. Due to the difference in water level between the filtrate pool and the reject weir a small amount of polished water flows upward creating a counter-current that carries the remaining waste back into the reject weir while further cleaning the returning sand. The clean sand falls out of the washbox and forms the top layer of the media bed. This process allows for influent water to constantly flow through continuously cleaned sand while it is filtered (England, Darby, and Tchobanoglous, 1994).

Shallow bed backwash systems in use have a media depth of one to two feet and are generally traveling bridge systems. These systems can be continuous backwash systems but are generally downflow as opposed to the upflow system mentioned previously. While the upflow methods utilize narrow, deep beds downflow methods utilize a wide, shallow horizontal media bed. Systems that are not continuous backwash need to have cells routinely shut down for a backwash when necessary. As this process can lead to large drops in water pressure, can be extremely time consuming, and is not optimal for large
systems, it has been losing popularity (England, Darby, and Tchobanoglous, 1994). Larger facilities with shallow bed filters can be continuous backwash facilities when employing a traveling bridge filter. A traveling bridge filter does not require periodic shut down and it does not use backwash water holding tanks. The traveling bridge moves along the media bed and backwashes one section at a time.

Deep bed multimedia filtration consists of granular media that is over four feet deep. Most deep bed filters are downflow with layers of granules that are consecutively smaller in diameter with each new layer. The media most commonly used in various multimedia filters are sand, anthracite, garnet, ilmenite, alumina, and magnetite (Sutherland, 2009). The number and amounts of different media used varies with each facility. The cost of each media as well as transportation and storage costs of high density media are factors for determining the media used. Anthracite coal is widely used because it has a unique density allowing it to stay above other filter mediums easily, it has high carbon content, and its low uniformity allows for differing levels of solid waste penetration resulting in high flow rate and prefiltration. Granulated garnet is used because it has some of the same properties as anthracite and is a high density media. Ilmenite is used to promote coagulation. Alumina is used primarily as a flocculating agent and also reduces the amount of fluoride in the water. Coagulation and flocculating agents are important as they cause organic materials to agglomerate forming larger and heavier particles which are then caught in the various filters. Magnetite is used as a sorbent for arsenic. As the
water flows through the media it is cleaned and the polished effluent exits at the bottom of the tank. Backwashing for these systems is an involved and time consuming process as clean water is pumped backward through the filter to dislodge waste. The waste is resuspended resulting in a highly contaminated liquid that must be specially disposed of.
Statement of the problem

The purpose of this study was to determine if county of collection, watershed of collection, facility of collection, method of treatment, method of filtration, and laboratory examining the sample were factors in the quality of wastewater effluent through the presence of *Cryptosporidium* oocysts and *Giardia* cysts in samples submitted for testing.
Materials and Method

From 1998 to 2010 there were 234 facilities that submitted samples to 11 different laboratories for testing to detect *Cryptosporidium* oocysts and *Giardia* cysts. The 863 samples selected were samples that had been analyzed for both *Cryptosporidium* and *Giardia*. All of those samples were from effluent of wastewater facilities and thus were from facilities that may pose a public health risk if their effluent was contaminated. The records for the following information were reportedly submitted and recorded when samples were submitted: facility name, address, identification number, and permit number; facility water treatment and water filtration methods; sample collection, submission, and analysis dates; organization collecting the sample; organization analyzing the sample; and the sample results. Due to discrepancies such as submitters filling out submission inadequately, incorrectly and/or inconsistently not all of the information was available. Information for the total daily volume and capacity for each facility at the time of sample collection was not tracked and thus was not examined.

Linear multivariate regression analysis of the different variables was unable to be done due to multiple gaps in information. The results obtained were from different databases for sample information, facility information, and contact information. The information for facility size and capacity was not enough for significant data to be obtained. Because there was such a scarcity of information for facility size and capacity in general, let alone the specific size and capacity of
the facility at the time the sample was collected, no analysis of impact of the facility size and capacity was able to be done. Unless otherwise stated the program used to compile and compare the data was Microsoft Excel 2003. With this program charts, pivot tables, percentages, sums, linear regression lines, and correlation coefficients were done.

In order to properly compare the different factors involving quality of wastewater effluent supplemental data for county of collection, watershed of collection, county population, and rate and incidence of cryptosporidiosis and Giardiasis per county were needed. The county for each facility was determined using the facility address and the county boundaries as defined by the State of Florida. The watershed of each sample was determined by comparing the facility collection address with the boundaries of the 29 major watersheds of Florida as defined by The Florida Water Resource Management Division and Environmental Assessment and Restoration Division of the Florida Department of Environmental Protection (FL DEP, 2011). Information for the population per county, rates of disease, and counts of disease were obtained from the Florida Community Health Assessment Resource Tool Set established by the Florida Department of Health Office of Health Statistics and Assessment (FLCHARTS, 2011).

Starting in the 1990s the EPA evaluated methods for the detection, monitoring, and analysis of Cryptosporidium. The first procedure adopted was the Information Collection Rule (ICR) Protozoan Method for Detecting Giardia Cysts and Cryptosporidium Oocysts in Water by a Fluorescent Antibody Procedure in 1995 (U.S. E.P.A., 1995). Method 1622 was released as final in
1999, then updated in 2005 but it only included detection for *Cryptosporidium* (U.S. E.P.A., 2005). In 1999 a revised method for simultaneous detection of *Cryptosporidium* and *Giardia*, Method 1623, was validated and released (U.S. E.P.A., 1999). Both Method 1622 and Method 1623 were revised in 2001 with method updates including new filter and antibody stain components, clarified sample acceptance criteria, modified sample testing procedures, updated quality control acceptance criteria, and inclusion of spiking suspensions. To support the Long Term 2 Enhanced Surface Water Treatment Rule revisions to both Method 1622 and Method 1623 were made in 2003 and 2005 (U.S. E.P.A. Method 1622, 2005) (U.S. E.P.A. Method 1623, 2005). The 2003 revisions included a modified version of the methods using a new type of filter, approval oocysts and cysts for quality control samples from a different manufacturer, new rejection criteria for samples, guide for measuring sample temperatures, quality control sample requirements and results clarification, guidance for staining slides, and other minor corrections. For 2005 the revisions include approval of a continuous-flow centrifugation method, addition of a new monoclonal antibody stain, and clarification of analyst verification and sample condition criteria. The sample collection, storage, and analysis procedures changed by varying degrees with each revision. Due to these revisions sample quality that was acceptable in 2000, may not have been unacceptable in 2007. Because of the various changes in product vendors and method procedures the samples themselves would not have been processed the same over the course of the revisions. In order to reduce some of the bias effect this may have caused only samples that were
simultaneously tested for *Cryptosporidium* and *Giardia* were analyzed. To minimize possible detection bias only the overall results of positive (cysts or oocysts detected) and negative (no cysts or oocysts detected) were used, overall organism count and organism viability were not factored into the results.

Only sample results for samples that were simultaneously examined for *Cryptosporidium* and *Giardia* were used in this study. The EPA ICR Protozoan Method and the EPA Method 1623 are the rules that explicitly cover this type of analysis and detection. In the earlier ICR Protozoan Method a large volume of water is passed through a yarn-wound filter. Protozoa are eluted from the filter and cysts and oocysts are separated from other particulate debris by flotation on a Percoll-sucrose solution. The water layer/Percoll-sucrose interface is placed on a membrane filter, stained, and then examined under a microscope. A major drawback to this method is that it is a very time consuming process.

An advanced protozoan detection method, Method 1623, was created as a performance based method, meaning that modifications of the method may be used if they show equivalent or better performance. For this method a sample of approximately 10 liters is pumped through an approved 1-micron membrane filter. The filters are submitted to an approved lab for analysis. To retard any degradation of cysts or oocysts captured on the filter samples are shipped on ice and stored in a cooler prior to analysis. Within 96 hours of sample collection filters are eluted with an aqueous buffered salt and detergent solution to wash cysts and oocysts from the filter. The eluate is then concentrated and purified. Magnetic beads that are conjugated to antibodies for *Cryptosporidium* and
Giardia are added to the concentrate along with buffering agents and the mixture is then rotated. After rotation a magnetic particle concentrator is used to separate the magnetized cysts and oocysts from extraneous material. The resulting bead/cyst bead/oocysts complexes are washed with acid to dissociate the cysts and oocysts from the beads. The now purified sample is applied to a slide with a small amount of base solution and allowed to dry. Within 72 hours of application of purified sample to the slide the slide is stained with fluorescently labeled monoclonal antibodies to Cryptosporidium and to Giardia and 4′,6-diamidino-2-phenylindole (DAPI). The stained sample slide is then examined within 7 days by an experienced laboratory technician using epifluorescence and differential interference contrast (DIC) microscopy. The results of the slides are recorded and reported.

The ICR Protozoan Method and Method 1623 have similar limitations: neither method is able to identify the species of organism or the host species; organic and inorganic debris can interfere with the sample analysis; organisms and debris that fluoresce can interfere with cyst and oocysts microscopic detection; freezing of the sample, filter, eluate, concentrate, or slides can interfere with detection; and inappropriately maintained laboratory equipment can affect detection. An advancement of the Method 1623 is that with the DAPI staining it is possible to determine the viability of detected cysts and oocysts whereas the ICR Protozoan Method cannot.
Results and Discussion

Due to current state regulations positive tests necessitate multiple further samples be submitted for testing. It follows that original effluent quality is a major determinant in the number of samples submitted. To examine if this was in fact true, a comparison of the number of positive samples to the overall number of samples submitted for each county was done. The data was fit to a linear regression line with a 0.78 coefficient of correlation. A considerable positive correlation between the number of positive samples and the overall number of samples submitted per county exists. Another comparison of the percent positive samples to the overall number of sample submitted for each county was done. This yielded a coefficient of correlation of 0.02 for this data set. This suggests that the most significant relation between a county having positive samples is the overall number of samples submitted, with the percent positive having little to no significant relation.

Each sample submitted was simultaneously tested for *Cryptosporidium* oocysts and *Giardia* cysts. Therefore each sample had two results but for the purpose of analysis was only considered as a single sample. In order to more accurately reflect this in the results the data for certain analysis was grouped together. As seen in Table 1 of the 863 samples submitted *Cryptosporidium* oocysts were detected in 52% and *Giardia* cysts were detected in 65% of the samples. For 99% of the samples that had *Giardia* cysts detected,
Cryptosporidium oocysts were also detected. For almost every county Giardia cysts were detected in more wastewater effluent samples than Cryptosporidium oocysts and the occurrence of Giardiasis was higher than that of Cryptosporidiosis. This is easily explained by two facts 1) Giardiasis has long been a disease with a much higher incidence and prevalence than Cryptosporidiosis and 2) Cryptosporidium oocysts are harder to detect than Giardia cysts.

**Table 1.** A comparison of the percent of samples testing positive for detection of Cryptosporidium oocysts and/or Giardia cysts for all samples submitted for Cryptosporidium and Giardia testing (1998 – 2010).

<table>
<thead>
<tr>
<th></th>
<th>Percent Positive</th>
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<tbody>
<tr>
<td>Cryptosporidium</td>
<td>52%</td>
</tr>
<tr>
<td>Giardia</td>
<td>65%</td>
</tr>
</tbody>
</table>

A comparative analysis of results to determine if a significant difference in detection was noted with respect to sample collection and analysis method for the ICR Protozoan Method and Method 1623 was attempted. Due to lack of information for collection and analysis method for specific samples an exact analysis of the difference in methods was not possible. However, in the first years of sampling the prevalent method was the ICR Protozoan Method with Method 1623 being the prominent method in recent years. Thus in an attempt to analyze a possible difference in the efficacy of detection methods sampling data from 1998 to 2001 was compared with sampling data from 2009 to 2010. In
Table 2 this data, including the different percents positive, for samples submitted for testing is presented. For the time periods the 2009 to 2010 period had an overall percent positive of 73.0 % compared to that of 36.7 % for 1998 to 2001. This difference suggests that the detection methods, the ICR Protozoan Method and Method 1623, may play a role in the detection of oocysts and cysts in the samples. This does not rule out the possibility that during that amount of time the actual presence of oocysts and cysts increased. A list of the counties submitting samples for the two time periods is available in Appendix E.

**Table 2.** A comparison of counties submitting samples for *Cryptosporidium* and *Giardia* testing by number of counties submitting samples, total number of sample submitted, overall percent positive for all counties, and the percent positive of the 18 counties that submitted samples for 1998 to 2001 and for 2009 to 2010.

<table>
<thead>
<tr>
<th>Data for All Samples Submitted for <em>Cryptosporidium</em> and <em>Giardia</em> Testing for 1998 to 2001 Compared to That of 2009 to 2010</th>
<th>1998 - 2001</th>
<th>2009 - 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Counties Submitting Samples</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Total Number of Samples Submitted for Testing</td>
<td>166</td>
<td>248</td>
</tr>
<tr>
<td>Overall Percent Positive of All Counties</td>
<td>36.7 %</td>
<td>73.0 %</td>
</tr>
<tr>
<td>Percent Positive of the Same 18 Counties</td>
<td>37.0 %</td>
<td>69.5 %</td>
</tr>
</tbody>
</table>

Of the 67 counties in Florida: 27 did not submit any samples; of the 40 counties that did submit samples 15 counties submitted ten or less samples; and only
Hillsborough, Pinellas, Sarasota, and Volusia counties submitted over 50 samples. It is incredibly important to note the 40 counties that did not account for any samples. In Florida there are currently over 3,700 individually permitted wastewater facilities. Treatment facilities not under the influence of surface water, that serve less than 10,000 people, and that are filtered are not necessarily required to submit samples. The samples in this study were collected from 234 wastewater facilities. A limitation of this study is that it only had test results for approximately 5% of the wastewater treatment facilities in Florida. Analysis of the difference between the variables examined for the facilities that submitted samples versus those that did not was unable to be done. It is possible that significant information on the efficacy of treatment and filtration methods is therefore undetected.

The number of facilities in any area depends on the water usage and consumption for that area as well as the capacity of the facilities in that area. The amount of wastewater produced is directly proportional to the water use in an area. Because water usage is a function of the population of an area the rate of samples submitted per 100,000 people per county could demonstrate if the population of a county could be the most important determiner in the number of samples submitted for that county. This accounts for the difference of population density of the areas submitting samples. Figure 1 is a graph of the rate of samples submitted for Cryptosporidium and Giardia testing per 100,000 people per county (1998-2010). As previously described the population information was
taken from Florida CHARTS. The data used was an average of the population in each county per year for the years 1999 to 2010.

If the factors affecting the effluent quality of wastewater treatment facilities were equivalent the rate of samples per 100,000 people per county would be similar. Figure 1 demonstrates that there is a difference, but not does not demonstrate the reason for the difference. This difference could be explained by the number of facilities submitting samples and the size of facilities submitting samples. In an effort to examine if there was an obvious geographic link to the distribution of counties in which a higher rate of samples was submitted a map of Florida counties with the associated rate was made, this is represented in Figure 2. There was no obvious link between the geographical location of a county and the rate of samples that were submitted in that county per 100,000 people. Some counties that have the larger populations are among the counties submitting the lower numbers of samples, such as Monroe, Miami-Dade, Palm Beach, Duval, Hillsborough, and Pinellas counties. A possible explanation for this is that counties with larger populations have facilities that account for a greater proportion of the population. It is also possible that counties with greater populations have better means of maintaining their treatment facilities. Comparing Figure 2 and Figure 3 shows that counties with higher rates of samples submitted are not always the counties with the greater number of facilities submitting samples. This leads to the conclusion that there may be a difference between the sizes of the facilities or that certain counties with larger populations are better able to maintain their treatment facilities.
Rate of Samples Submitted for Cryptosporidium and Giardia Testing per 100,000 People per County (1998-2010)

Figure 1. Rate of samples submitted for Cryptosporidium and Giardia testing per 100,000 people per county 1998-2010. The population used was an average population for each county for 1998 through 2010.

A comparison of the rate of samples submitted for Cryptosporidium and Giardia per 100,000 for each county versus the rate for each watershed was not able to be done. There was no population information available for the watershed to do a rate comparison with.

Figure 3 is a map of the number of facilities submitting samples for Cryptosporidium and Giardia testing per county for 1998 through 2010. A calculation for the rate of facilities submitting samples per 100,000 for each county was unable to be done due to limitations of the data available. There was no distinct pattern of the number of facilities that submitted samples per county seen on the map in Figure 3.
Figure 2. Map of Rate of samples submitted for Cryptosporidium and Giardia testing per 100,000 people per county 1998-2010. The population used was an average population for each county for 1998 through 2010.
Figure 3. Map of the number of facilities submitting samples for Cryptosporidium and Giardia testing per county for 1998 through 2010.

The map of the number of facilities submitting samples for Cryptosporidium and Giardia testing per watershed for 1998 through 2010, Figure 4, also shows no distinct pattern of distribution for the areas submitting a greater number of samples. However, the map demonstrates that for the southern tip of Florida a wide area of the Florida Everglades accounts for an area where no samples were taken from. Much of this area is either rural, agricultural, or protected land with no or few wastewater treatment plants and is therefore not expected to be submitting samples for testing. When comparing this with Figure 3 it demonstrates the idea that for certain areas where there is a greater population, such as coastal south Florida, there will be more facilities submitting
samples. The corresponding watersheds for areas with high sample levels show this distinct division between coastal areas and inland areas. A watershed is defined as an area or region that is drained by a river, river system, or other body of water. This means that watersheds right next to each other will have waters that flow into different rivers, basins, or bodies of water. When a river or lake is contaminated by wastewater effluent that has cysts and/or oocysts in it the entire watershed it resides in is at an increased risk of becoming contaminated. If a specific county had a greater number of samples submitted by a small number of facilities it could be inferred that the facilities were then unable to appropriately treat their water.

Of the 29 main watersheds in Florida three did not have any samples submitted: Chipola River, Fisheating Creek, and Perdido River. Figure 5 is a map of the distribution of the number of samples submitted for Cryptosporidium and Giardia testing per watershed between 1998 and 2010.

When examining Figures 4 and 5 it is apparent that the watersheds where there were a greater number of samples collected coincided with the areas where there were a greater number of facilities that submitted samples for Cryptosporidium and Giardia testing. This also demonstrates that in the watersheds the areas where a greater number of samples were submitted did not have a small number of facilities accounting for the samples.

Three different filtration methods, continuous backwash (18.8%), deep bed multi media (20.7%), and shallow bed backwash (36.9%), accounted for 76.4% of the total samples. Of the remaining samples nine different filtration
methods were used, due to the small number of facilities utilizing these methods no further analyses was performed on those filtration methods.

Figure 4. Map of the number of facilities submitting samples for Cryptosporidium and Giardia testing by watershed for 1998 through 2010.
Continuous backwash, deep bed multi media, and shallow bed backwash filtration systems had similar positive percentage rates of detection for *Cryptosporidium* and/or *Giardia* of 58.3%, 52.6%, and 59.8%, respectively. The differences in the percentages were not large enough to be considered statistically significant: p-values were all greater than 0.5, meaning the hypothesis that they were not significantly different was correct.

When examining the distribution of the three main filtration methods by county Figures 6A and 6B shows that all three filtration methods had some overlapping areas of numbers of facilities submitting samples. The number of facilities within each county that used continuous backwash filtration, deep-bed multi media filtration, and shallow-bed automatic backwash were grouped to...
compare whether any area had a more prominent number of a specific filtration method, this is shown in Figure 6A. In order to examine a geographic pattern of the distribution of the three different filtration methods a map of the information in Figure 6A was made, Figure 6B.

When comparing the percent positive and areas of location of different filtration methods in place no discernable pattern emerged as to one filtration method having better or worse detection limits or geographical association.

Figure 6A. The number of facilities per county for the three different filtration methods of continuous backwash, deep-bed multiple media, and shallow-bed automatic backwash grouped together.
A comparison was done for the four laboratories where the greatest number of samples submitted for *Cryptosporidium* and *Giardia* testing were analyzed. Figure 7 shows the overall number of samples that were negative, number of samples that were positive, and the percent positive of samples for those laboratories. The frequency of detection of cysts and oocysts could be a function of the specific lab that did the analysis. Each facility did not submit samples to the same laboratory for testing each time a sample was taken. This eliminates some of the bias present for each facility specifically accounting for positive samples. There were 11 laboratories that analyzed samples. Of those 11 only four laboratories had analyzed enough samples to be statistically comparable. These labs will be referred to as Laboratory A (a county water utility), B (a commercial laboratory), C (a state public health laboratory), and D (a university laboratory). The percent positive of samples was not statistically significant between Laboratory A and Laboratory C (p-value = 0.15). There was a
significant difference in the percent positive of samples between Laboratory A and Laboratory B (p-value < 0.0001), as well as Between Laboratory B and Laboratory C (p-value < 0.0001). Laboratory D had a significant difference from all of the others labs, but was statistically closer to the results from Laboratory C than any other laboratory. Of these results the most intriguing is that of the differences between Laboratories A, B, and C. Laboratory C analyzed a greater quantity of samples but still had a similar percent positive to that of Laboratory A. Laboratory B had a significantly smaller percent positive than all the other labs. These results point towards a significant difference in the number of samples in which oocysts and cysts are detected depending on the laboratory analyzing the samples. Tying these results in with those shown in Table 2 a cause of the significant difference in the percent positives of samples may be the laboratory that does the testing as well as which method of detection that laboratory utilizes.

![Results of Samples Submitted for Cryptosporidium and Giardia Testing per Laboratory](image)

**Figure 7.** The overall number of samples that were negative, number of samples that were positive, and the percent positive of samples for Laboratory A, Laboratory B, Laboratory C, and Laboratory D.
Because of the health risks posed by wastewater contaminated with Cryptosporidium oocysts and Giardia cysts, the rates of samples positive for each county were compared with the rates of cryptosporidiosis and giardiasis for 1998 through 2010, see Figure 8 for rate of cryptosporidiosis and Figure 9 for giardiasis. As described previously, the rates of cryptosporidiosis and giardiasis for all Florida counties were obtained from Florida Charts. Madison County was the only county that had no reported cases of cryptosporidiosis and has one of the smallest levels of Giardiasis. A possible explanation for this is human to human spread of disease as opposed to individual cases all being caused by contact with contaminated water.

**Figure 8.** Map of the average rate of cryptosporidiosis per 100,000 people by county during 1998 through 2010.
The quality of effluent water from wastewater treatment facilities in Florida varies by county and by watershed. When examining the rate of samples submitted for a county and the rate of disease of that county there seems to be an almost inverse proportion. This would seem to suggest that areas in which there is a higher burden of disease may be in areas where it seems that the facilities do not need to do sample testing or are areas in which there are no large treatment facilities that are required to submit samples for testing. This could also mean that areas with smaller treatment facilities that are not required to submit samples under the current rule do, in fact, need to be doing sample testing. It could also suggest that in areas that the quality of the effluent wastewater is inadequate, as evidenced by detection of cysts and oocysts, the
route of transmission for the disease is not necessarily from contaminated wastewater effluent but possibly human to human contact. It is evident that further monitoring of the differences between laboratories analyzing samples, as well as the method of analysis, is needed.

Significant public health risks are still posed by Cryptosporidium and Giardia, further monitoring and evaluation of source water treatment and filtration methods is necessary to accurately address their levels in water supplies.


Appendices

Appendix A. List of Florida Counties

Alachua       Lee
Baker         Leon
Bay           Levy
Bradford      Liberty
Brevard       Madison
Broward       Manatee
Calhoun       Marion
Charlotte     Martin
Citrus        Miami-Dade
Clay          Monroe
Collier       Nassau
Columbia      Okaloosa
DeSoto        Okeechobee
Dixie         Orange
Duval         Osceola
Escambia      Palm Beach
Flagler       Pasco
Franklin      Pinellas
Gadsden       Polk
Gilchrist     Putnam
Glades        Saint Johns
Gulf          Saint Lucie
Hamilton      Santa Rosa
Hardee        Sarasota
Hendry        Seminole
Hernando      Sumter
Highlands     Suwannee
Hillsborough  Taylor
Holmes        Union
Indian River  Volusia
Jackson       Wakulla
Jefferson     Walton
Lafayette     Washington
Lake
Appendix B. Map of Florida Counties

Figure A1. Map of Florida Counties
Appendix C. List of Florida Watersheds

Apalachicola – Chipola
Caloosahatchee
Charlotte Harbor
Choctawhatchee – St. Andrew
Everglades
Everglades West Coast
Fisheating Creek
Florida Keys
Indian River Lagoon
Kissimmee River
Lake Okeechobee
Lake Worth Lagoon – Palm Beach Coast
Lower St. Johns Nassau – St. Marys
Middle St. Johns
Ocklawaha
Ochlockonee – St. Marks
Pensacola
Perdido
St. Lucie – Loxahatchee
Sarasota Bay – Peace - Myakka
Springs Coast
Southeast Coast – Biscayne Bay
Suwannee
Tampa Bay
Tampa Bay Tributaries
Upper East Coast
Upper St. Johns
Withlacoochee
Appendix D. Map of Florida Watersheds

Figure A2. Map of Florida Watersheds
Appendix E. Lists of Counties Submitting Samples for *Cryptosporidium* and *Giardia* Testing for 1998 to 2001 and 2009 to 2010

<table>
<thead>
<tr>
<th>1998 to 2001</th>
<th>2009 to 2010</th>
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<tbody>
<tr>
<td>Bay</td>
<td>Alachua</td>
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<td>Bay</td>
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