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ASSESSMENT OF CURRENT AND NEW METHODS FOR INVESTIGATING THE OCCURRENCE OF ENTERIC PATHOGENS IN AMBIENT WATERS AND WATER SOURCES FOR REHYDRATION OF A FLORIDAN WETLAND

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ASSESSMENT OF CURRENT AND NEW METHODS FOR INVESTIGATING THE OCCURRENCE OF ENTERIC PATHOGENS IN AMBIENT WATERS AND WATER SOURCES FOR REHYDRATION OF A FLORIDAN WETLAND

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Assessment of Current and New Methods for Investigating the Occurrence of Enteric Pathogens in Ambient Waters and Water Sources for Rehydration of a Floridan Wetland

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ABSTRACT

Water quality assessment is currently used for determining the risks from contamination with waterborne pathogenic microorganisms. The public health significance of such microorganisms cannot be fully understood without the application of reliable environmental monitoring techniques that provide efficient recovery as well as information on the infectious potential of specific isolates. This research was carried out to assess the analytical performance of current and new methods for investigating the occurrence of waterborne *Cryptosporidium* oocysts in samples of surface water, groundwater and reclaimed water used for public access irrigation in the State of Florida.

Method 1623 of the U.S Environmental Protection Agency and a modified version of the Information Collection Rule protozoan method were evaluated using a combination of experimental conditions aimed at the optimization of the detection methods for the assessment of waterborne *Cryptosporidium*. Significant improvements on the recovery and detection of oocysts were obtained using Method 1623 with the Envirochek HV capsule filter. Additional modifications of the method components were

included in order to provide information on the infectious potential of oocysts. The results suggest that even without overcoming method inconsistencies, the introduction of better concentration methods and highly specific identification techniques provide the required tools for addressing the challenges of existing and new emerging waterborne pathogens.

Microbiological characterization of ambient waters and proposed waters for wetland and lake restoration in Section 21 Wellfield was carried out in order to identify microbial constituents of concern and to determine the potential public health risks associated with the future enhancement of the hydrologic conditions of the Wellfield. Naturally occurring levels of microorganisms were found in ambient waters indicating an ambient level of risk exposure to pathogens and vulnerability of groundwater to microbial contamination. The highest detection of microbial indicators and pathogens occurred in the Interceptor canal while the reclaimed water source had the lowest level of microbial contamination. *Cryptosporidium* and enteric viruses were chosen as microbial of concern for the risk assessment study as they were found in all sampled sites. Further characterization of the ambient water quality is necessary prior to completing the microbial risk assessment of the pilot rehydration project.

CHAPTER ONE

INTRODUCTION

Contamination of natural waters with pathogenic microorganisms directly affects public health because of the use of these sources for contact recreation, shellfishing and drinking water supply (Rose et al., 1996; Hurst, 1997; Francy et al., 2000; Scott et al., 2002). Most microbial waterborne pathogens of concern originate in the enteric tracts of humans or animals and enter the aquatic environment via fecal contamination.

Five critical elements in the transmission of waterborne infectious agents have been described: (i) source of the infectious agent, (ii) specific water-related modes of transmission, (iii) specific attributes of the organism that allow it to survive and possibly multiply and to move into and within the aquatic environment, (iv) infectious dose and virulence factors of the organism, and (v) host susceptibility factors (Moe, 1997).

Prevention and control of waterborne diseases require accurate and rapid detection methods to measure microbiological water quality and to identify and evaluate risk factors for waterborne disease (Moe, 1997). Microbial indicators have been used globally as a warning of possible contamination and as an index of water quality deterioration (Toranzos and McFeters, 1997; Francy et al., 2000). Heavy reliance has been placed on the total and fecal coliform group of bacteria as microbial indicators for determining the sanitary quality of water and the public health risks associated with the

possible presence of pathogenic microbes derived from fecal wastes such as protozoan parasites and enteric viruses (Gerba, 1999; Scott et al., 2002). The results of recent investigations, notwithstanding have demonstrated that the reliability of fecal coliforms to predict both the parasitological and virological quality of recreational water and drinking water is inadequate (Griffin et al., 1999; Quintero and Botero, 2000; Jiang et al., 2001).

Current investigations have also indicated that the coliforms' ecology, prevalence, and resistance to stress differ to a great extent from those of many of the pathogenic microorganisms they are proxy for, thereby limiting the utility of coliform bacteria as indicators of fecal pollution (Sobsey et al., 1990; Desmarais et al., 2002; Scott et al., 2002). *Escherichia coli*, enterococci, *Clostridium perfringens* and bacteriophages have been suggested as alternative indicators to signal conditions in recreational and shellfish-harvesting waters that might lead to adverse health consequences (Toranzos and McFeters, 1997; Ashbolt et al., 2001).

Although no single water quality indicator is perfect and there is considerable variety in the ways that different indicator organisms are applied in various geographical areas and situations, public health concerns have generally been well served. The presence of indicator organisms will likely continue to be used as a criterion of water quality that will be of value if attention is given to the development and use of optimal methods for the recovery of these microorganisms (Toranzos and McFeters, 1997; Gerba et al, 1999).

The application of reliable and rapid detection methods for the assessment of microbial indicators in natural water samples has been made possible with the

introduction of the emerging new molecular biological technologies (Bej et al., 1991; Behr et al., 2000; Frahm and Obst, 2003). However, researchers recommend further comparative evaluation with a significant number of samples in order to determine the potential for full contribution of these assays to the improved monitoring of the sanitary quality of water (Frahm and Obst, 2003).

Specialized methods have been developed for the detection of specific microbial pathogens, based on the concentration of large water volumes, use of antibodies, cell culture and PCR (Rose et al, 1996). The application of environmental pathogen monitoring may be used not only for assessing impairment of waters but also for developing a database on microbial occurrence that will be useful in characterizing public health risks and in risk management (Rose et al., 1996).

Microbial Indicators and their Significance to Public Health

Coliform Bacteria

Coliform bacteria belong to the family Enterobacteriaceae and include *Escherichia coli* as well as various members of the genera *Enterobacter*, *Klebsiella*, and *Citrobacter*. Among these, *E. coli* is generally considered the most reliable indicator since its presence directly relates to fecal contamination with its implied threat of presence of enteric disease (Toranzos and McFeters, 1997; Gerba, 1999; Geissier et al, 2000). The use of *E. coli* as an indicator of fecal pollution has been questioned due to the ability of such bacterium to survive and replicate in contaminated soils in tropical and subtropical areas (Hazen and Toranzos, 1990; Desmarais et al., 2002).

Some issues about the use of *E. coli* as an indicator arises from the fact that there are also pathogenic strains, i.e. toxigenic *E. coli* strains (Ohno et al. 1997). The toxigenic *E. coli* strain is also problematic to detect, as it forms viable but non-culturable cells in water (Kogure and Ikemoto, 1997, Ashbolt, 2001).

Enterococcus spp.

Enterococcus is a subgroup of the fecal streptococci (FS) currently used as an indicator of fecal contamination due to its occurrence in the feces of humans and warm-blooded animals (Godfree et al., 1997). The fecal streptococci belong to the genera *Enterococcus* and *Streptococcus* (Godfree et al., 1997; Gerba, 1999). The genus *Enterococcus* is differentiated from other streptococci by its ability to grow in 6.5% of sodium chloride at high pH 9.6 and a temperature of 41 °C. The majority of species currently assigned to the genus *Enterococcus* belong to one of three distinct groupings. The first group consists of *Ent. faecium*, *Ent. faecalis*, *Ent. durans*, *Ent. hirae* and *Ent. mundtii*. A second distinct grouping includes the species *Ent. avium*, *Ent. pseudoavium*, *Ent. raffinosus* and *Ent. malodaratus*. The species *Ent. casseliflavus* and *Ent. gallinarum* are closely related and form the third group (Godfree et al., 1997). Most species of fecal origin and formally classified as streptococci have been assigned to the genus *Enterococcus*. Six species of streptococci can be considered to be exclusively associated with the intestinal flora of humans or other animals, *Strep. bovis/Strep. equinus*, *Strep. alactolyticus*, *Strep. suis*, *Strep. intestinalis* and *Strep. hyointestinalis*. Of the genus *Streptococcus*, only *S. bovis* and *S. equines* are considered to be true fecal streptococci. These two species of *Enterococcus* are predominantly found in animals; *E. faecalis* and

E. faecium are more specific to the human gut. Enterococci are considered to have certain advantages over the coliform and fecal coliform bacteria as indicators: (i) they rarely multiply in water; (ii) they are more resistant to environmental stress and chlorination than coliforms, and (iii) they generally persist longer in the environment (Gerba, 1999). Enterococci are considered reliable indicators of health risks in marine environments (Cabelli et al., 1982; Cabelli et al., 1983).

Clostridium perfringens

C. perfringens is a sulfite-reducing, anaerobic spore former bacterium; it is gram positive, rod-shaped, and exclusively of fecal origin (Fujioka and Shizumura, 1985, Araujo et al., 2001). *C. perfringens* is present in large numbers in human and animal wastes (10^3 to 10^4/100 mL), and its spores are resistant to treatment practices (chlorination) and extreme in temperature (75°C for 15 minutes) (Payment and Franco, 1993). This bacterium is considered a good indicator of current fecal contamination as well as a conservative tracer of past fecal contamination (Fujioka and Shizumura, 1985). *C. perfringens* has been suggested as an indicator of removal of protozoan parasites or viruses during drinking water and wastewater treatment (Payment and Franco, 1993) as well as a suitable indicator for the presence of pathogens of faecal origin in surface waters (Sorensen et al., 1989).

Laboratory studies have indicated that the current method of recovering *C. perfringens* with the mCP medium is not the best choice for monitoring this bacterium in waters (Sartory et al., 1998; Araujo et al., 2001). The results of recent investigations demonstrated that the performance characteristics of the fluorogenic TSC agar were

much better than using the current mCP media as judged by the recovery efficiency of typical colonies of *C. perfringens* and specificity using different water samples (Araujo et al., 2001).

Bacteriophages

Bacteriophages are viruses that infect *E. coli* and other coliforms, as well as possibly other members of the Enterobacteriaceae (Leclerc, 2000). They usually consist of a nucleic acid molecule (genome) surrounded by a protein coat (capsid) (IAWPRC, 1991). The use of bacteriophages (or bacterial viruses) as appropriate indicators of fecal pollution has been proposed because of their constant presence in sewage and polluted waters (Havelaar et al., 1986; Havelaar et al., 1990, IAWPRC, 1991). These organisms have also been suggested as indicator of viral pollution because their structure, morphology, size as well as the behavior in the aquatic environment closely resemble those of enteric viruses (Hsu et al., 1995). Bacteriophages have been used extensively to evaluate virus resistance to disinfectants, to evaluate virus fate during water and wastewater treatment, and as surface and groundwater tracers (IAWPRC, 1991; Leclerc et al., 2000). The use of bacteriophages as indicators of fecal pollution is based on the assumption that their presence in water samples denotes the presence of bacteria capable of supporting the replication of the phage. Three groups of phage have been described: (i) the somatic coliphage, which infect *E. coli* host strains through cell wall receptors, (ii) the F-specific bacteriophage, which infect strains of *E. coli* and related bacteria through the F^+ or sex pili and, (iii) phages of anaerobes which infect the main genera of anaerobic

bacteria. A significant advantage of using coliphage is that they can be detected by simple and inexpensive techniques that yield results in 8-18 hours.

F-specific coliphage (male-specific phage) are considered as the indicator of choice for assessing the potential presence of human enteric viruses in estuarine and marine environments impacted by wastewater sources (Calci et al., 1998). Certain groups of F+ coliphages, in particular the F-specific RNA bacteriophages, are relatively resistant to conditions applied in water treatment and their resistance parallels that of some important groups of human viruses (IAWPRC, 1991).

Waterborne Enteric Pathogens

Cryptosporidium spp.

Cryptosporidium is an obligate, intracellular parasite that belongs to the phylum phylum Apicomplexa, suborder Eimeriorina and family Cryptosporidiidae. The transmissible stage parasite is a mature, thick-walled oocyst, 4 to 6 μm in size. Cryptosporidiosis, which is the illness caused by *Cryptosporidium parvum,* is a coccidian infection of humans, domestic animals, and other vertebrates. It is characterized by gastrointestinal illness that can lead to chronic or life-threatening diarrhea for immunocompromised (Andersen 1997; Fayer et al., 2000). When oocysts are ingested along with food or water, the oocyst wall opens (excystation) in the small intestine releasing sporozoites that attach to and invade epithelial cells of the gastrointestinal tract (Rose, 1988, Smith and Rose, 1998, Sterling and Marshall, 1999).

Transmission of infectious oocysts of *Cryptosporidium parvum* via surface and drinking water supplies has been reported (Rose, 1988, Gallaher et al., 1989, Smith and

Rose, 1998). *Cryptosporidium* oocysts can enter the environment via human and animal wastes (Rose et al., 1997, Smith and Rose, 1998, Tamburrini and Pozzio, 1999). Several studies, conducted in North America and in the United Kingdom, document the occurrence of *Cryptosporidium* oocyst contamination in surface water and outbreaks of water-associated and waterborne cryptosporidiosis (Casemore et al., 1997, Smith and Rose, 1998, Oppenheimer et al., 1999). Rivers, lakes, springs, and groundwater have all been implicated as sources, and contaminated water was linked to evidence of suboptimal treatment. Suboptimal coagulation, flocculation, filtration, and/or disinfection were partially responsible for epidemic levels of cryptosporidiosis in three communities in Georgia, Wisconsin, and Oregon (Rose et al., 1997). Recreational waterborne outbreaks have been associated with swimming pools as well (Rose et al., 1997).

The occurrence of *Cryptosporidium* spp. in animals has been published in a number of reviews (O'Donoghue, 1995; Fayer et al., 2000). *Cryptosporidum* spp. oocysts have also been detected in over 30 species of birds, including wild, exotic, and domestic fowl, at least 9 species of marine and freshwater fish, 40 species of snake, 15 species of lizards, and tortoises). However, the greatest risk to drinking water supplies and public health is presented by calves, which carry and shed large numbers of oocysts that are infectious for humans (primarily *C. parvum*) (Fayer et al., 2000; Rochelle et al., 2001).

Giardia spp.

The protozoan *Giardia lamblia* is a member of the order Diplomonadida that includes a variety of binucleate flagellate parasites typically inhabiting the intestinal tract of mammals, birds, and reptiles (Lindmark and Jarroll, 1984). *Giardia lamblia* has a

simple, direct life cycle, and is transmitted as a fecal contaminant directly or in food, water or on surfaces. Infection of the host results when the environmentally resistant cyst is ingested. After excystation, the trophozoite or vegetative form of the organism emerges in the proximal small intestine following exposure to the acidic environment of the stomach. Some of the trophozoites encyst more distally in the small intestine, allowing completion of the life cycle when the cyst are passed in the faeces and ingested by a subsequent host (Adam, 2000). *Giardia* is the most common protozoan parasite affecting the small intestine in humans. The illness caused by infection with *Giardia* may be acute and self-limiting or chronic; it may be asymptomatic or the cause of severe enteropathy with malabsorption. The *Giardia* cyst is round to oval with dimensions ranging from 8 to 18 μm long by 5 to 15 μm wide. Many mammalian reservoir hosts can carry the parasite. However, the degree to which *Giardia* infections in animal contribute to illness in humans remains unclear (Shaeffer, 1999). When *Giardia* cysts enter the environment they can survive for prolonged periods. Bingham et al., (1979), documented *G. lamblia* cysts survival for up to 77 days at 8 °C and 4 days at 37°C.

Giardia lamblia has been the most common cause of waterborne disease outbreaks in the United States when an agent could be identified (Craun et al, 1999, Schaeffer, 1999). Experiences with waterborne outbreaks of *G. lamblia* led to promulgation of the Surface Water Treatment Rule (SWTR) by the US Environmental Protection Agency (USEPA). The SWTR requires filtration of surface water sources that do not meet specified criteria for watershed protection, coliform bacteria, and turbidity (Craun et al., 1998).

Enteroviruses

The enteroviruses are members of the family Picornaviridae and are represented by poliovirus (3 serotypes), coxsackievirus A (23 types), coxsackievirus B (6 types), echovirus (32 types), and enteroviruses (4 types). Enteroviruses are icosahedral viruses approximately 27 to 32 nm in diameter. The nucleic acid of this type of virus consists of single-stranded RNA. These are the viruses most often detected in polluted waters. However, their apparent higher prevalence may be associated, in part, with available cell lines for their propagation, because, many pathogenic viruses such as HAV (hepatitis A virus), enteric adenoviruses, rotavirus, Norwalk virus, and other small round viruses are difficult to grow in conventional cell lines. Human enteroviruses cause a variety of clinical illnesses ranging from paralytic poliomyelitis, myocarditis (heart infection), and diabetes to the common cold. Enteroviruses may be transmitted by either the fecal-oral route or the respiratory route. One route may predominate depending on the serotype. All enteroviruses (except, possibly, enterovirus type 70) are believed to be capable of fecal-oral transmission.

Coxsackieviruses are the enteric viruses that have been most commonly isolated from water, even treated drinking water, with exception of vaccine poliovirus. Enteroviruses may not be completely removed by domestic sewage treatment, including disinfection as normally practiced, and they can usually be isolated from treated wastewater. Enteroviruses have been isolated in almost any environment that has been exposed to human fecal contamination—surface water and groundwater, marine water and sediments, shellfish, crabs, crops irrigated with sewage, domestic solid waste, soil, aerosols, and fields spray-irrigated with sewage water. The survival of enteroviruses and

other enteric viruses depends on many factors, including temperature, sewage pollution, microbial activity, and adsorption to solids (clays and sediments). Generally, the lower the temperature, the longer the survival times. Below 5 °C, enteroviruses may survive for years in the environment. They are stable at pH 3-5 for 1-3 hours and can tolerate pH 10-11 for several minutes (Gerba, 1999; American Water Works Association, 1999).

Statement of the Problem

Environmental pathogen monitoring is currently used for assessing impairment of waters used for recreation (primary and secondary contact), public water supplies, aquifer protection, and protection and propagation of fish, shellfish and wildlife. This approach is also useful for developing a database on microbial occurrence for further characterization of public health risks and risk management.

Specialized methods have been developed for detection of specific waterborne pathogenic microorganisms, based on the concentration of large water volumes, use of antibodies, cell culture infectivity assays and genetic/molecular techniques. Although these methods have been successfully used for the detection of several waterborne pathogens, they require additional testing in order to demonstrate feasible application to pathogen monitoring in a wide variety of waters.

Cryptosporidium is recognized worldwide as a waterborne pathogen, and the species *C. parvum* is the major cause of cryptosporidial infections in humans and livestock. Waterborne transmission of the oocysts and outbreaks of cryptosporidiosis either through drinking water or recreational use are well documented and have been listed by several authors. This research evaluated the analytical performance of method

1623 and a modified version of the ICR method for detection of *Cryptosporidium* oocysts in surface waters used as non-contact recreational water bodies, groundwater used as drinking water sources and reclaimed effluents used for public access irrigation in the State of Florida. The occurrence of *Cryptosporidium* was fully evaluated in reclaimed effluents using conventional techniques in conjunction with advanced molecular and culture based methods to determine the species, genotypes and infectious potential of *Cryptosporidium* isolates.

Wetlands and lakes within the section 21 Wellfield have experienced lowered water levels due to development, groundwater pumpage and drought conditions. These stressed conditions have affected the ecological functions and aesthetics of the site, which is also used as a public park. The City of St. Petersburg recommended using the Section 21 Wellfield as the site for a pilot project to test the feasibility of rehydrating stressed wetlands and lakes with stormwater and reclaimed water. A large multidisciplinary project was established to study Section 21 Wellfield Rehydration. In order to assess potential public health risks associated with the future enhancement of the hydrologic conditions within Section 21 Wellfield, the occurrence of microbial indicators of water quality and waterborne pathogenic microorganisms was evaluated in ambient waters (lakes and production wells) and proposed water sources (stormwater from the Interceptor Canal and reclaimed water) that will potentially be applied to the Section 21 Wellfield during project implementation for restoration of the site's lakes and wetlands.

CHAPTER TWO

ASSESSMENT OF CURRENT AND NEW METHODS FOR DETECTION OF WATERBORNE *Cryptosporidium* PARASITES

Introduction

Several methods have been developed and tested to evaluate *Cryptosporidium* occurrence in environmental waters. Concentration, purification, and detection are the three key steps in all methods that have been approved for routine monitoring of waterborne oocysts. These steps have been optimized to such an extent that low levels of naturally occurring *Cryptosporidium* oocysts can be efficiently recovered from water. The filtration systems developed in the US and Europe trap oocysts more efficiently and are part of the standard methodologies for environmental monitoring of *Cryptosporidium* oocysts in source and treated water. Purification techniques such as immunomagnetic separation and flow cytometry with fluorescent activated cell sorting impart high capture efficiency and selective separation of oocysts from sample debris. Monoclonal antibodies with higher avidity and specificity to oocysts in water concentrates have significantly improved the detection and enumeration steps. Cell culture techniques are now used to examine oocysts viability/infectivity while PCR-based detection methods allow differentiation of the human pathogenic *Cryptosporidium* parasites from those that do not infect humans, and tracking of the source of oocyst contamination in the environment (Quintero-Betancourt et al., 2002).

Although the methods described above have been successful in recovering and detecting waterborne *Cryptosporidium* oocysts, they require more testing to demonstrate feasible application to a wide variety of environmental waters.

Cryptosporidium spp.: Taxonomy

Cryptosporidium is an obligate, intracellular parasite that belongs to the phylum Apicomplexa (referred to as the Sporozoa one of 5000 species) to the class Coccidea and the family Cryptosporidiidae (Rose et al., 2002). In humans, the parasite infects the microvililllus border of the gastrointestinal epithelium causing acute self-limiting diarrhoea in immunocompetent individuals, and a chronic life-threatening disease in immunocompromised patients (Hijjawi et al., 2002). The oocyst is the infective stage responsible for transmission.

Over 20 different species of *Cryptosporidium* have been named based on host occurrence, however within the genus *Cryptosporidium* only 10 are currently recognized valid species: *C. parvum*, *C. wrairi*, *C. felis*, *C. muris* and *C. andersoni* in mammals, *C. meleagridis* and *C. baileyi* in birds, *C. serpentis* and *C. saurophilum* in reptiles, and *C. nasorum* in fish (Fayer et al., 2000). Two new species have been recently described: *C. canis* in dogs and *C. molnari* in fish (Fayer et al., 2001; Alvarez-Pellitero and Sitja-Bobadilla, 2002). Additionally, several host-adapted genotypes that include the human, bovine and mouse genotypes of *C. parvum*, and the ferret, pig, bear and marsupial genotypes have been described.

Studies (Carreno et al., 1999) based on small subunit ribosomal RNA (SSrRNA) sequencing have shown that the gregarines and *Cryptosporidium* form a clade separate

from the other major apicomplexan clade containing the coccidian. Hijjawi et al., 2002 studied the presence of novel stages in the *Cryptosporidium* life cycle and argued against its present classification within the coccidian and confirmed its affinity to the gregarines. The results of these studies supports the need to reconsider the classification of *Cryptosporidium* within the coccidia and point out the necessity of future work to specifically harvest novel stages from cell culture and from in vivo infection in order to study them at the structural level.

The genetic structure and relationship of *Cryptosporidium* parasites has been elucidated through molecular and phylogenetic analysis (Morgan et al., 1998, 1999, Sulaiman et al., 2000, 2002, Xiao et al., 1999, Xiao et al., 2002). DNA sequences of the small subunit rRNA (SSU rRNA), 70 Kilodalton (KDa) heat shock protein (HSP70) and actin genes obtained from all the established *Cryptosporidium* spp. has facilitated the assessment of the validity of various *Cryptosporidium* parasites and the public health significance of parasites of zoonotic origin (Xiao et al., 2002). The multi-locus phylogenetic approach has revealed extensive genetic diversity among *Cryptosporidium* parasites, and suggested that host adaptation and host-parasite co-evolution contribute to much of the parasite heterogeneity (Xiao et al., 2002).

At present, the identification and naming of genotypes are largely based on the host origin. When significant or consistent sequence differences from the existent genetic data are identified, a new genotype is named after a host from which it is isolated (Xiao et al., 2002).

The application of the current and new methods for genotyping and identifying subgenotypes of isolates has demonstrated the importance of understanding species

diversity in the identification and tracking of infectious genotypes/subgenotypes in different populations and geographic regions (Glaberman et al., 2001, Peng et al., 2001, Xiao et al., 2002).

Cryptosporidium Genotypes Infectious to Humans

The diversity of *Cryptosporidium* species that can infect humans has important implications for epidemiological studies and environmental screening (Reed et al., 2002). Two major *C. parvum* genotypes associated with human infection have been characterized by phenotypic (i.e., host specificity, severity of clinical symptoms) and genotypic methods: one genotype exclusively from humans and a single nonhuman primate (genotype 1 or human type) and a second genotype in livestock as well as humans (genotype 2 or calf type) (Peng et al., 1997; Spano et al., 1998, Widmer, 1998; McLauchlin et al., 1999; Morgan et al., 1999; Sulaiman et al., 2001; O'Connor et al., 2002). The occurrence of these two genotypes imply the existence of two separate transmission cycles for *C. parvum*: one of antroponotic origin (genotype 1) and one of zoonotic origin (genotype 2). Bovine play an important role as reservoirs for human cryptosporidiosis and also as a source of environmental contamination (Peng et al., 2001). Different genotyping approaches have demonstrated that the relative proportions of anthroponotic and zoonotic genotypes vary from country to country suggesting that genetic variation may be linked geographically (Xiao et al., 2000, Guyot et al., 2001; Peng et al., 2001). No evidence of recombination between the two genotypes has been reported, suggesting the possibility that these are two separate species (Akiyoshi et al., 2002).

Most human infections including recent waterborne outbreaks, have been caused by genotype 1 isolates (Peng et al., 1997; Patel et al., 1998; McLauchlin et al., 1999; Xiao et al., 2001c, O'Connor et al., 2002). Nevertheless, the results of recent investigations have revealed that multiple genotypes can occur within single outbreaks, indicating that single sources of exposure can contain mixed genotypes. Coinfection of humans with multiple genotypes is also possible, as multiple genotypes have been isolated from a single patient (McLauglin et al., 1999; Reed et al., 2002).

Recently, several studies have revealed that immunocompromised and immunocompetent humans can be infected with genetically diverse parasite populations (*C. parvum* dog genotype, *C. meleagridis* and *C. felis*) (Xiao et al., 2001). Therefore, extensive genotyping of water samples from various matrices (source water, finished water, wastewater, river stormwater, combined sewer overflow) and environmental settings (feral, rural, urban, recreational) is recommended in order to obtain a better understanding of the distribution of *Cryptosporidium* spp. in various waters, the human infection potential of waterborne *Cryptosporidium* oocysts, and the contribution of humans, farm animals, companion animals, wildlife, and other factors, such as sanitation, wastewater discharge, agriculture, recreation, and weather, to *Cryptosporidium* oocyst contamination of water in certain settings. Such information would be useful for scientific management of watersheds and for source water protection (Xiao et al., 2001a).

Waterborne *Cryptosporidium* in the US

Cryptosporidium is recognized worldwide as a waterborne pathogen, and the species *C. parvum* is the major cause of cryptosporidial infections in humans and

livestock (O'Donoghue, 1995). Waterborne transmission of the oocysts and outbreaks of cryptosporidiosis either through drinking water or recreational use are well documented and have been listed by several authors (Solo-Gabriele and Neumeister, 1996; Rose et al., 1997; Smith and Rose, 1998; Oppenheimer et al., 2000; Fayer et al., 2000). More than half (56%) of the 75 waterborne cryptosporidiosis outbreaks between 1984 and 1999 were associated with drinking water, while 33 outbreaks (44%) were related to use of recreational water facilities including pools, rivers, and lakes (Fayer et al, 2000).

Cryptosporidium and *Giardia* (a related enteric protozoa) occur widely in surface and drinking water supplies in the United States. In an extensive monitoring, LeChevalier and Norton (1995) reported that the occurrence of *Cryptosporidium* and *Giardia* in raw water samples was 60% and 54%, respectively. Examination of filtered drinking water showed that *Cryptosporidium* oocysts were detected in 13% of the water samples and *Giardia* cysts were detected in 17% of the time.

The effectiveness of treatment processes for removing both pathogens has been evaluated through monitoring (Rose et al., 1988b; LeChevalier et al., 1991a; LeChevalier et al., 1991b; Rose, 1991; Jakubowski et al., 1996; LeChevalier and Norton, 1995; Hass et al., 1999). The United States Environmental Protection Agency's "Interim Enhanced Surface Water Treatment Rule" (IESWTR) stipulates zero as the goal for the maximum contaminant level of protozoan parasites in drinking water. Compliance is defined by performance requirements for water-treatment plants and by monitoring indices (e.g. turbidity, performance of individual filters) that aim to optimize the filtration process and in some cases the disinfection process. The regulation was published in December 1998, the final revision was published in January 2001, and the rule was effective since January

2002. Key provisions in the IESWTR establish a Maximum Contaminant Level Goal (MCLG) of zero for *Cryptosporidium* and require a 2-$\log_{10}$ (99%) *Cryptosporidium* removal when using filtration only. The IESWTR applies to public water systems that use surface water or ground water under the direct influence of surface water and serve 10,000 or more people.

In the United Kingdom, the sampling and analysis requirements outlined in the Water Supply (Water Quality) (Amendment) Regulations 1999 dictate that treated water supplies be monitored daily for *Cryptosporidium*. The regulation stipulates a legally enforceable maximum of ten oocysts per 100 L, and is process (filtration) based. Detection of *Cryptosporidium* oocysts at any level above ten per 100 L constitutes a criminal offense (Fairley et al., 1999).

Studies on groundwater in the U.S (once thought to be more protected source) reported that between 9.5% and 22% of samples were positive for *Cryptosporidium* (Hancock et al., 1998, 2000). Waterborne *Cryptosporidium* outbreaks associated with groundwater or springs have been listed by Lisle and Rose (1995).

Cryptosporidium oocysts have been isolated from marine waters affected by primary treated sewage (Johnson et al., 1995; Johnson et al., 1997). Consequently, the risk of contracting cryptosporidiosis from accidentally ingesting oocysts while swimming in coastal areas contaminated with sewage cannot be dismissed. California sea lions have been suggested as potential reservoirs of *Cryptosporidium*, although the significance in environmental transmission of *Cryptosporidium* is yet to be investigated (Den et al., 2000).

Cryptosporidium parvum and *Giardia lamblia*, according to some public health officials, are no more prevalent in reclaimed effluents than in other high-quality irrigation waters (York and Walker-Coleman, 2001). Occasional findings of (oo)cysts in reclaimed water may, however, present a health risk due to the potential high viability and exposure. Public concern about the health risk has prompted many states, including Florida, to add periodic sampling for *Giardia* and *Cryptosporidium* to current reuse rules (Chapter 62-610 of the Florida Administrative Code, Florida Department of Environmental Protection, 1999).

Presently, there are no approved methods for sampling and detecting protozoan pathogens in reclaimed effluents. With the development of new methods for detecting waterborne *Cryptosporidium* and *Giardia*, there is a great interest to apply these methods for the evaluation of pathogen reductions by wastewater reclamation processes and for compliance monitoring of effluents from reclamation facilities that provide water for public access irrigation.

Current and Alternative Methods for Detection of Waterborne *Cryptosporidium*

Current methods for detection of waterborne *Cryptosporidium* include three basic steps: (i) concentration of the water sample (e.g. filtration) to recover low numbers of parasites typically found in the environment; (ii) purification (e.g. density gradients and/or immunomagnetic separation) to separate oocysts from non-target organisms and other debris; (iii) and immunofluorescent statining to detect oocysts microscopically in water sample concentrates.

During the 1990's, the United States Environmental Protection Agency (USEPA) approved a method for detection and quantification of *Cryptosporidium* oocysts in water samples as part of a monitoring rule known as the "Information collection Rule". The method was called the ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procecure (USEPA, 1996). This method has the advantage of being quantitative, but does not distinguish species or assess viability. While many have argued that the ICR method is difficult, tedious, inefficient, and of limited value, it appears that the criticisms are without any validity as the published literature demonstrates that the method is peer-reviewed and scientifically valid, and is used worldwide by water utilities, public health officials and regulators providing valuable information (Rose et al., 2002).

In 1996, the USEPA initiated an effort to identify new and innovative technologies for protozoan monitoring and analysis. After evaluating potential alternatives to the then-current method through literature searches, discussions with research and commercial laboratories, and meeting with experts in the field, the Engineering and Analysis Division within the Office of Science and Technology within EPA's Office of Water developed draft Method 1622 for *Cryptosporidium* detection. This *Cryptosporidium*-only method was validated through an interlaboratory study in August 1998, and was revised and approved as a valid method in January 1999. In October 1998, the USEPA validated a method for simultaneous detection of *Cryptosporidium* and *Giardia* and designated the new combined procedure as Method 1623: *Cryptosporidium* and *Giardia* in water by filtration, immunomagnetic separation (IMS) and immunofluorescence assay (IFA) microscopy.

Methods 1622 and 1623 require filtration, immunomagnetic separation of the (oo) cysts, and an immunofluorescence assay for determination of (oo) cysts concentrations, with confirmation through vital dye staining (4',6-diamidino-phenylindole (DAPI)) and differential interference contrast (DIC) microscopy (USEPA, 1999a,b; 2001). The interlaboratory validation of Methods 1622 and 1623 used Gelman (Envirochek^{TM}) capsule filtration, Dynal IMS, and Meridian staining protocol. Alternate procedures are allowed, provided that required quality control tests are performed and all quality control acceptance criteria are met.

Further modification and alternatives to methods 1622 and 1623 have been proposed. Such alternative methodologies have enabled laboratories to improve the efficiency of recovery of waterborne parasites. Overviews of the standard and alternative detection methods for *Cryptosporidium* in water are given in Tables 2.1 and 2.2, respectively.

New filtration devices have been tested for concentration of oocysts from water samples. Simmons et al. (2001) used a hollow-fiber ultrafilter system to concentrate oocysts in seeded surface and reagent water. The disposable ultrafilter consists of a series of polysulfone hollow fibers contained within a polycarbonate housing, which is portable and easy to use. Large volumes of water can be processed, and the concentration procedure is compatible with the subsequent purification and detection steps in EPA method 1622.

Genera Technologies and Seven Trent Water have developed another novel filter system, the Crypto-Dtect compressed filter system. The Crypto-Dtect system uses multiple layers of open cell, reticulated foam rings. When compressed, the rings act as

Table 2.1 Standard techniques for recovery, concentration, and detection of *Cryptosporidium* oocysts from environmental waters

Concentration technique	General approach for concentration and detection procedures	Recovery efficiency
Cartridge filtration ASTM, 1991; USEPA, 1996	100 L (raw water source), 1000 L (finished) Water sample filtered through a 10-inch-long polypropylene yarn-wound cartridge filter, 1.0 µm, nominal porosity Filter cut apart and particles eluted. Stomacher Eluting solution consists of: - Phosphate buffered saline (PBS), pH 7.4 - 0.1% polyoxyethylensorbitan mono-oleate (Tween-80) - 0.1% Sodium dodecyl sulfate (SDS) Sample concentrated by centrifugation (1,050 X g, 10 min) Percoll-sucrose Density-gradient centrifugation used to purify concentrated sample Oocysts detected by IFA and confirmed by DIC microscopy	0-100%
Method 1622/1623: (USEPA, 1999a,b)	10-1000 liter water samples Water sample filtered through the Envirochek™ HV sampling capsule Particles eluted through wrist action agitation Eluting buffer consists of: - Laureth-12 - 1 M Tris pH 7.4 - 0.5 M EDTA, 2 Na, pH 8.0 - Antifoam A - Deionized water Sample concentrated by centrifugation (1,000-1,100 X g for 15 minutes) to a final pellet Dynal IMS used to purify concentrated samples Oocysts detected by IFA and confirmed by DIC microscopy and vital dye staining characteristics (4',6-diamidino-2-phenylindole [DAPI])	12-93% (21-100%)[a]

Table 2.1 (Continued)

Standard Operating Protocol (SOP) for the Monitoring of *Cryptosporidium* oocysts in tretated water supplies 1999, SI No. 1524, 2000.	Water samples filtered through Genera Filta-Max™ filter membranes Particles eluted using appropriate wash station Eluting buffer consists of: - Phosphate buffered saline (PBS) - 0.1% polyoxyethylene (20) sorbitan monolaurate Sample concentrated by centrifugation (1,100 X g for 15 minutes) Dynal IMS procedure Oocysts detected by IFA and confirmed by DIC microscopy and vital dye staining characteristics (4',6-diamidino-2-phenylindole [DAPI])	30-50%[b]

[a] US EPA Acceptance criteria
[b] SOP Acceptance criteria

very efficient filters but, when decompressed, they allow simple removal of the entrapped particles. The compressed foam filter system is conveniently sized, easy to handle, simple to install and very efficient at entrapping *Cryptosporidium* oocysts from water and releasing them upon elution. More recently, Genera Technologies has patented the Filta-Max™ system for collection and recovery of oocysts as part of their complete detection process, along with Puri-Max™ (immunomagnetic separation) and Quanti-Max™ (automated detection) (Sartory et al. 1998).

An alternative collection method for the recovery of *Cryptosporidium* oocysts from large volumes of water involves continuous flow (CF) centrifugation. CF centrifugation concentrates particles by size and weight (Swales and Wright, 2000). Processing time is rapid, but this intensive equipment-based technique is not field applicable.

The methods of choice for detection of *Cryptosporidium* oocysts in water samples include EPA method 1623 in the US and the Standard Operating Protocol 1999, SI No. 1524 (Genera Filta-Max, Dynal IMS, and Celllab FITC) in the UK. Both methods rely on immunofluorescence assays for detection of oocysts. Lot-to-lot variation in commercially available antibodies and significant differences in properties such as avidity and cross-reactivity with algae and non-*parvum* species have been reported. Hoffman et al. (1999) identified differences among four of the currently used antibodies and indicated that while none is without its advantages and disadvantages, meticulous quality control and quality assurances are needed for routine detection of parasites in water. Ferrari et al. (1999) used different classes of monoclonal antibodies (IgG1, IgG3, and IgM) for

Table 2.2 Alternative techniques used for concentration of *Cryptosporidium* oocysts from environmental waters

Technique	Concentration and detection procedures	Recovery efficiency	Reference:
Membrane filtration	20-liter water samples Filtration through polycarbonate membranes (293 mm, pore sizes 5 and 1 μm) Purification through density gradient (40% potassium citrate) Detection by IFA	5-25%	(Ongerth and Stibbs, 1987)
Calcium carbonate flocculation method	10-liter water samples Calcium chloride (1 M) Sodium hydrogen carbonate (1 M) Sulfamic acid (10%) Purification and detection through flow cytometry and IFA	69%	(Vesey et al., 1993)
Membrane dissolution method	10-liter water samples Filtration through 142-mm-diameter, 1.2-μm-pore size mixed esters of cellulose membrane filter Purification by Percoll-sucrose density gradient flotation or Dynal IMS. Detection by IFA	50.2%	(Aldom and Chagla, 1995, McCuin et al., 2001)
Compressed foam filter system	10-20 liter water samples Filta-Max Purification by Chemunex modified Dynal IMS Detection by ChemScan	49-73%	(Sartory et al., 1998, Rushton et al., 2000)
Continuous flow centrifugation	100 liter water samples Continuous flow centrifuge Clarification through Percoll discontinuous gradients or flow cytometry Detection by IFA	13-14.8%	(Swales and Wright, 2000).
Size selective continuous flow filtration	100 liter water samples Size selective continuous flow filtration apparatus Detection by IFA	0-85%	(Oda et al., 2000)

environmental monitoring of *Cryptosporidium*. Their results revealed that staining with IgG1 antibodies generally produced fewer unwanted fluorescent particles than staining with IgM and IgG3 antibodies. It appears that IgG1 antibodies have higher avidity and specificity to oocysts in water concentrates than other commercially available antibodies (Weir et al., 2000).

Modifications to immunofluorescence detection of *Cryptosporidium* include the use of additional nonspecific fluorescent dyes such as 4',6-diamidino-2-phenylindole (DAPI) (Rochelle et al., 1995). The combination of DAPI with immunofluorescence provides an easy reproducible method for detecting sporulated oocysts in water samples and minimizes the need for DIC or phase contrast optics (Grimason et al., 1994).

Flow cytometry with fluorescence activated cell sorting (FACS) improves the sensitivity of the immunofluorescence assay, and the combination of FACS with epifluorescence microscopy enables enumeration of low numbers of *Cryptosporidium* oocysts in environmental samples (Vesey et al., 1993; Vesey et al., 1994; Vesey et al., 1997a; Medema et al., 1998). In this technique, fluorescing oocysts are separated from most other particles, which means that the oocysts can be detected easily and rapidly. It should be noted, however, that the presence of large numbers of fluorescent particles may still cause difficulties during microscopic confirmation of suspect oocysts. Interference from autofluorescent particles can be minimized when a FITC fluorochrome is used (Vesey et al., 1997b). CY3, phycoerythrin, and tetramethylrhodamine B isothiocyanate were the best fluorochromes for drinking water samples (Vesey et al., 1997b). More recently, Ferrari et al. (2000) tested a two-color immunofluorescence flow cytometric assay, which could potentially increase the specificity of current detection methods while

aiding confirmation steps. They evaluated six different combinations of *Cryptosporidium*-specific monoclonal antibodies and found that the combination containing CRY104-FITC and CRY104-PE produced a highly specific assay.

Laser scanning devices (e.g. ChemScan RDI, Chemunex, Paris, France) have also been used to detect oocysts (Rushton et al., 2000). After concentration with the Genera Crypto-Dtect filter system and purification by IMS, the final extract is absorbed onto a carrying membrane and labeled with FITC-monoclonal antibody. The membrane is then scanned on the ChemScan (CS), which records the location of every possible oocyst using the parameters of fluorescence, size, and shape. Subsequently, a conventional fluorescence microscope, fitted with an electronic stage, can be used to view the membrane. In parallel trials, CS performed consistently better with a 73% average recovery efficiency as compared with 49% for manual fluorescence microscopy (MM). The ChemScan is rapid, easy to operate, and unaffected by the presence of debris in the final concentrate.

Routine and accurate detection, enumeration, speciation, and viability assessment of waterborne protozoan parasites remains an ambitious goal in environmental microbiology.

Objectives

The public health significance of waterborne *Cryptosporidium* can be fully understood with the application of reliable environmental monitoring techniques that provide efficient recovery and accurate detection of the organisms in various types of waters. Most of the methods available for parasite monitoring have been designed for analyzing *Cryptosporidium* parasites in raw and finished drinking water. The main objective of this study was to evaluate the analytical performance of Method 1623 of the U. S Environmental Protection Agency and a modified version of the Information Collection Rule method for detection of *Cryptosporidium* and *Giardia* in water samples. A combination of experimental conditions was employed in order to optimize the recovery and enumeration steps involved in the detection of *Cryptosporidium* oocysts in surface water (freshwater and seawater), groundwater and reclaimed effluents used for public access irrigation in the State of Florida.

Materials and Methods

Water Samples

Samples of reclaimed effluents used for public access irrigation, groundwater sources used as drinking water and surface waters used as non-contact recreational water bodies were included in the study. The samples from reclaimed effluents were obtained from four water reclamation facilities located in one metropolitan area of Florida. These are conventional activated sludge facilities that provide filtration to secondary treated effluents for reduction of pathogens and particles prior to disinfection with chlorine gas. The filtration system of the reclamation process includes deep bed dual media filters (anthracite/sand/gravel; Facilities A and B) and shallow bed sand filter (anthracite and/or sand, Facilities C and D). Groundwater samples were collected from three production wells located within Section 21 Wellfield located in Hillsborough County. Surface waters were collected from four major lakes within Section 21 Wellfield and from Bayboro bay in St. Petersburg Campus.

Recovery Efficiency Experiments

These experiments were carried out as described in the April 1999 version of USEPA method 1623 to demonstrate acceptable method performance and included: (i) initial precision and recovery tests, (IPR) (ii) ongoing precision and recovery tests (OPR), (iii) matrix spikes (MS) and (iv) method blanks. A combination of filtered tap water and distilled water was used as the reagent water sample for blanks, IPR and OPR tests. Matrix spikes were carried out with tap water, reclaimed water, lake water and seawater. Two spike suspensions were used in these experiments as described below.

IPR tests are intended to establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery (USEPA, 1999). Each method requires a separate set of IPR samples. For this purpose, 10 and 100 L of reagent water samples were filtered, eluted, concentrated, purified (IMS), stained and examined by IFA using the three filtration devices described below. Four reagent water samples were processed with each filter and spike suspension and the results of these analyses were used to compute the average percent recovery (Mean) and the relative standard deviation (RSD) of the recovery. The RSD is the standard deviation divided by the mean times 100. The mean and RSD were compared with the corresponding limits for initial precision and recovery described in method 1623 (USEPA, 1999) in order to determine method performance. OPR tests were carried out throughout the study to verify all performance criteria. For this purpose, one spike reagent water sample was filtered, eluted, concentrated, purified (IMS), stained and examined by IFA. The frequency of OPR tests was one spiked reagent water sample for every 20 samples analyzed. MS along with matrix spike duplicates (MSD) were carried out with spiked field samples (surface water, groundwater, reclaimed water) to determine the effect of the method's oocyst and cyst recovery. For this purpose, three spiked water matrices were processed filtered, eluted, concentrated, purified (IMS) stained and examined by IFA as described above. Reclaimed water was the key water matrix used in the study to determine analytical performance and reproducibility of the different methods and filtration systems. Therefore, the number of samples processed was higher than the rest of the water matrices. MS for each water type were processed and the mean of the number of (oo)cysts were computed as follows: Matrix spike duplicate (MSD) or Mean =

(MS1+MS2/2). From these results, the relative percent difference (RPD) was computed as follows: RPD = 100 x [(oo)cysts detected in MS1 – oocysts detected in MS2]/Mean. The results of these calculations were then compared with the corresponding limits established in method 1623 (USEPA, 1999) to determine precision and recovery of the methods using different water matrices.

Three types of filters and two spike suspensions were used to evaluate mean (oo) cyst recoveries in the different water matrices. The reproducibility of the methods was thoroughly evaluated with reclaimed water and tap water using (oo)cyst recovery rates as an indication of method performance. Evaluation of recovery rates in reclaimed effluents and tap water was based on a three factorial treatment design with two levels for filter type and spike and three levels for filter type, spike, and organism. The three types of filtration devices used in the study were the standard Envirochek^{TM} sampling capsule (Pall Gelman Laboratory, Ann Arbor, MI), the Envirochek HV sampling capsule (Pall Gelman Laboratory, Ann Arbor, MI) and the polypropylene yarn wound cartridge filter (Filterite, Timonium, Maryland). ColorSeed^{TM} C&G (Biotechnology Frontiers, North Ryde, BC, Australia) and flow cytometer-sorted *Cryptosporidium* oocysts and *Giardia* cysts obtained from the Wisconsin State Laboratory of Hygiene Flow Cytometry Unit (WSLH) (Madison, WI, USA) were used as the spike suspensions in IPR tests and recovery efficiency experiments with tap water samples. ColorSeed^{TM} C&G was used in the recovery efficiency experiments carried out with samples of reclaimed water to differentiate indigenous and seeded (oo)cysts. ColorSeed C&G spikes consist of hundreds of red fluorescent labeled and gamma irradiated *Cryptosporidium* oocysts and *Giardia* cysts in approximately 1 mL of saline solution. They can be used as internal

quality control parameters to determine the performance or percent recovery achieved with every test (see http://www.biotechfrontiers.com). WSLH suspensions are composed of live and unlabeled (oo) cysts suspended in saline solution.

For seeded experiments, water volumes of 10 L were filtered through the standard Envirochek sampling capsule while 100 L were filtered through the Envirochek HV capsule and the polypropylene yarn-wound cartridge filter. Seeded samples were filtered on-site and the filters were transported to the laboratory on ice.

Method 1623

All water samples were filtered following standard procedures. The volume of sample filtered was similar for all water types (between 10 and 100 L) except for groundwater samples where large volumes (more than 1000 L) were required. Envirochek filtration was performed with an automatic-demand diaphragm pump and flow rates were maintained at 2-3.5 L m^{-1}. Samples obtained from groundwater sources were collected from a tap. After filtration, the capsules were placed on ice and transported to the laboratory for processing following the steps included in the April 1999 version of method 1623, with some modifications. The immunomagnetic separation (IMS) procedure was performed with a Dynal GC-Combo kit (Prod. No. 730.02, DYNAL A.S., Oslo, Norway) and included two dissociation steps with 100 µl of 0.1 N hydrochloric acid (HCl) instead of 50 µl as recommended in the manufacturer's instruction. For the initial recovery efficiency experiments, IMS concentrates were divided into four aliquots of 50 µl each, fixed with absolute methanol and stained with fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* and anti-*Giardia*

monoclonal antibodies following standard procedures. (Oo) cyst counts were obtained by epifluorescence microscopy and each count was recorded by slide number.

Two commercially available monoclonal antibodies were used in the immunofluorescence assay (IFA) and evaluated for detection sensitivity: (i) EasyStain™ (Biotechnology Frontiers, North Ryde, BC, Australia) and (ii) *Giardia*-a-Glo™/*Crypto*-a-Glo™ (Waterborne Inc., New Orleans, LA, USA). Confirmation of (oo) cysts was carried out by staining with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and Propidium iodide (PI) as described below. Nomarski differential interference contrast (DIC) microscopy was included to look at the internal morphology of (oo) cysts.

Modified ICR Protozoan Method

Filtration with the polypropylene yarn wound cartridge filter was carried out using a gasoline-powered portable water pump with flow rates maintained at 8-10 L m^{-1}. The method for processing samples (filtration, elution and sample concentration) included most of the steps of the Information Collection Rule Protozoan Method (ICR) for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure, EPA/814-B95-003 (USEPA, 1995) plus some modifications. IMS instead of Percoll/sucrose flotation was used as the clarification technique for selective separation of cysts and oocysts from debris. Identification and confirmation of (oo) cysts was accomplished through epifluorescence microscopy following the procedures described above.

Vital Dye Assay

Water sample concentrates were analyzed for the presence of potentially viable *Cryptosporidium* oocysts using the vital dye assay following Campbell et al. (1992) with slight modifications. The modifications included in the vital dye assay were related to the incubation period of (oo) cysts with DAPI and PI.For the vital dye staining assay, the modifiecations included incubation of (oo)cysts with the dyes for 10 minutes at room temperature. Previous experiments in our laboratory demonstrated that this time was optimal for maximal dye uptake and no differences have been found using the length of time described in the protocol of Campbell et al.

Statistical Analysis

A three-way ANOVA task along with multiple-comparison procedures (Bonferroni and Tukey tests) were used to analyze the experimental data generated from the study. A level of significance of 5% was used to test for statistical differences. The statistical tools, where applicable, evaluated the effects of each factor (filter type, spike, organism) individually or the interactions between factors on the recovery efficiency of (oo) cysts. The same approach was used to test for statistical significance for the number of indigenous (oo) cysts detected in reclaimed effluents. The number of indigenous organisms detected in the reclaimed effluents was transformed to natural logarithm ($\ln y + 1$). Analysis was done using the Statistical Analysis System (SAS) software program 8.02 (SAS Institute, Inc.). All parametric assumptions were checked prior to analysis.

Results

Method Performance Criteria

Table 2.3 shows the results of the initial precision and recovery tests carried out to demonstrate acceptable method performance. Recoveries of (oo) cysts from both spikes suspensions were higher using method 1623 with the Envirochek capsule filters than using the modified ICR method with the polypropylene yarn wound cartridge filter. Both the Envirochek HV and the regular Envirochek filters recovered on average between 37% and 62% of spiked (oo) cysts while the polypropylene yarn wound cartridge recovered between 4% and 13%. Mean *Cryptosporidium* recoveries with the Envirochek HV filter were 57% (RSD 40%) with ColorSeed and 45% (RSD 39%) with WSLH suspensions, respectively. Mean percent recoveries for *Giardia* were 37% (RSD 40%) with ColorSeed and 57% (RSD 33%) with WSLH spikes. Similar data were obtained with the regular Envirochek capsule filter for *Cryptosporidium* oocysts. With ColorSeed suspensions mean *Cryptosporidium* and *Giardia* recoveries were 62% (RSD 38%) and 58% (RSD 40%), respectively. For *Giardia* cysts, the mean percentage efficiency of recovery with ColorSeed was 45% (RSD 41%); the mean percent recovery with WSLH suspensions was 49% (RSD 39%). With the polypropylene yarn wound cartridge filter, the mean percentage efficiency of *Cryptosporidium* and *Giardia* recoveries were notably lower than using the previous methods. With ColorSeed spikes, the mean percent recovery was 4% (RSD 200%) while the mean percent of oocysts using WSLH spikes was 13% (RSD 138%). Mean percent recovery of *Giardia* was 10% (RSD 150%) with ColorSeed and 5% (RSD 180%) with WSLH spikes, respectively.

Table 2.3 Summary table of the initial precision and recovery tests for *Cryptosporidium* and *Giardia* using different methods and spike suspensions

Method/Protozoa	Spike suspension[a]			
	ColorSeed[b]		WSLH[c]	
	Mean Recovery (Percent [n=4])	Precision (RSD)[d]	Mean Recovery (Percent [n=4])	Precision (RSD)
ICR[e] Yarn-wound polypropylene filter				
Cryptosporidium	4	200	13	138
Giardia	10	150	5	180
Method 1623 EnvirochekHV				
Cryptosporidium	57	40	45	39
Giardia	37	40	57	33
Method 1623 Envirochek				
Cryptosporidium	62	38	58	40
Giardia	45	41	49	39

[a] Spike suspensions: [b]Biotechnology Frontiers (North Ryde BC, Australia)
Cryptosporidium count = 98±1.5;
Giardia count = 98±1.5.
[c] Wisconsin State Laboratory of Hygiene Flow Cytometry Unit (Madison, WI, USA),
Cryptosporidium count = 198±2.30,
Giardia count = 201±3.83
[d] Relative standard deviation
[e] Information Collection Rule method (modified version)

Immunofluorescent Staining with Two FITC-Monoclonal Antibodies (FITC-mAbs)

The application of two different mAbs in the immunoflourescence assay was carried out initially with 10 samples collected from various sample sites (surface, reclaimed, groundwater). The results of this assay demonstrated that both mAbs were equally effective in detecting *Cryptosporidium* oocysts and *Giardia* cysts in positive controls and duplicate environmental samples, however staining water sample concentrates with the EasyStain mAb resulted in lower levels of background fluorescence and non-specific binding than when staining with Waterborne mAbs. As a result, the enumeration of (oo) cysts as well as the differentiation of (oo) cysts from non-target organisms and background debris was markedly improved with EasyStain. These improvements were more evident and useful when evaluating reclaimed and surface water samples, which contained high levels of algae and mineral particles. Therefore, the EasyStain mAb was used throughout the study.

Some other observations on the use of two different mAbs were noteworthy. The (oo) cysts used in the recovery efficiency experiments were analyzed in duplicate with the two mAbs before and after processing. The microscopic analysis of processed and unprocessed *Giardia* cysts revealed some degree of uneven immunofluorescence staining with the two antibodies tested, as if portions of the cysts wall had been partially removed. In some instances, the cysts did not stain well with the FITC-mAbs. This uneven staining pattern occurred only for *Giardia* cysts obtained from ColorSeed C&G spike suspensions. No differences were detected for *Cryptosporidium* oocysts, whose fluorescent intensity was similar in both processed and unprocessed controls. These

results indicated that none of the methods used for sample processing were detrimental to *Cryptosporidium* oocyst staining; however *Giardia* cysts are somehow affected by the combined immunofluorescent labeling. Using the appropriate filter, the (oo) cysts from ColorSeed could be easily identified against the background.

(Oo)cyst Recoveries in Tap Water and Reclaimed Water

The recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts in tap water varied among filters and spike suspensions used in the experiments; these results are summarized in Table 2.4. Five replicates for each filter and spike suspension were used in the experiments. Mean (oo) cyst recovery efficiency percentages and relative standard deviations (RSD) were 75%±16% for *Cryptosporidium* oocysts and 54%±22% for *Giardia* cysts using the Envirochek HV capsule filter and ColorSeed as the spike suspension. Mean percent recoveries of tap water samples seeded with WSLH spike suspensions were 76%±9% for *Cryptosporidium* and 49%±49% for *Giardia*. The standard Envirochek filter recovered on average 90%±6% of oocysts and 64%±16% of cysts from ColorSeed spike suspensions, while percent recoveries using WSLH suspensions averaged 63%±5% and 39%±26% for oocysts and cysts, respectively. The polypropylene yarn wound cartridge filter recovered on average 25%±46% of oocysts and 23%±39% of cysts from ColorSeed; the recoveries obtained with WSLH suspensions were 11%±54% and 13%±46% for oocysts and cysts, respectively. The results of the statistical tests demonstrated that mean percent recoveries with the two different spike

Table 2.4 Mean (oo)cyst percent recoveries and relative standard deviations of tap water samples processed by Method 1623 and the modified ICR method

Method (Filter type)	Spike suspension			
	ColorSeed[a]		WSLH[b]	
Method 1623	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
HV (n = 5)	75±16 (60 - 90)	55±22 (39 - 70)	76±9 (68 - 84)	49±49 (17 - 81)
Standard Envirochek (n = 5)	90±6 (82 - 97)	64±16 (51 - 77)	63±5 (60 - 66)	39±26 (29 - 56)
Modified ICR Yarn-wound (n = 5)	25±44 (10 - 39)	23±39 (11 - 34)	11±55 (4 - 19)	13±46 (6 - 21)

[a] *Cryptosporidium* count: 98±1.5; *Giardia* count: 98±1.5 cysts

[b] *Cryptosporidium* count: 198.25±2.3; *Giardia* count:174±1.6 cysts

The number in parenthesis corresponds to minimum and maximum oocyst recovery rates

suspensions were significantly higher using the Envirochek capsule filters than using the polypropylene cartridge filter ($p<0.0001$). Statistical significance was found when the interaction between filter and organism was evaluated ($p=0.0008$); these results demonstrated that Envirochek filtration provided significantly higher mean recoveries of *Cryptosporidium* oocysts than *Giardia* cysts in samples of spiked tap water ($p<0.0001$). Percent recoveries of *Cryptosporidium* and *Giardia* were both low using the modified version of the ICR method with the polypropylene yarn wound cartridge filter.

The interaction between filter and spike suspension was analyzed and the results demonstrated that such an interaction was statistically significant ($p=0.0013$). ColorSeed C&G performed better than WSLH suspensions using the Envirochek capsule filter ($p<0.0001$) and the polypropylene cartridge filter ($p<0.0001$). However, there were no statistical differences between spike suspensions when the Envirochek HV capsule filter was used.

When IMS concentrates obtained from spiked tap water samples were divided into four aliquots of 50 µl each, more than 80% of the (oo) cysts detected were found in the first two slides. For spiked samples of reclaimed water the same pattern was observed with more than 90% of the (oo) cysts detected in the first two slides. As a result, the first and third aliquots were used for IFA, while the second and fourth aliquots were further divided into two more aliquots for the cell culture infectivity assay and PCR.

Table 2.5 summarizes the results of the recovery efficiency experiments in samples of reclaimed water. The recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts varied among methods and filtration devices used in the study. Most of the

Table 2.5 Mean (oo)cyst recoveries and levels of indigenous (oo)cysts in samples of reclaimed water processed by Method 1623 and the modified ICR method

Method (Filter type)	Protozoa					
	Cryptosporidium			*Giardia*		
Method 1623	Oocysts/100 L	%	Adjusted Levels	Cysts/100 L	%	Adjusted Levels
HV	74	28	264	36	24	150
(n = 5)	20	19	95	34	11	309
	2	1	200	<1	1	NA[b]
	2	28	7	6	34	18
	107	84	127	34	68	50
Mean		32			27	
RSD[a]		97			96	
Envirochek	20	25	80	20	29	69
(n = 5)	90	41	219	90	5	1800
	<10	16	NA	<10	2	NA
	<10	34	NA	70	47	149
	14	85	16	130	49	265
Mean		40			26	
RSD		67			84	
Modified	1	2	50	2	0	NA
ICR	26	27	96	14	20	70
Yarn-wound	2	19	10	<1	13	NA
(n = 5)	<1	12	NA	<1	9	NA
	5	16	31	5	12	42
Mean		15			11	
RSD		60			66	

[a] Relative standard deviation
[b] Not applicable

samples were collected and processed simultaneously using the three types of filters; 100% of the IMS concentrate was analyzed and the concentration of (oo)cysts was expressed by the equivalent volume examined. Pre-stained (oo) cysts from ColorSeed C&G spike suspensions were included as internal quality controls to differentiate spiked from indigenous (oo) cysts. Table 2.5 includes the number of indigenous oocysts and estimations of the adjusted level of indigenous oocysts detected based on the mean recovery rates and relative standard deviation obtained for every sample. For these experiments, the percent recovery efficiency of *Cryptosporidium* oocysts varied from 1% to 84% with the HV filter (32±97, Mean±RSD). The number of oocysts detected ranged from 2 to 107 per 100 L and the adjusted levels ranged from 7 to 264 oocysts. The percent recovery efficiency of *Giardia* cysts with the same filter varied from 1% to 68% (27±96, Mean±RSD) with levels of cysts detected between 6 and 36 and adjusted levels of cysts ranging from 18 to 309.

The percent recovery efficiency of (oo) cysts obtained with the standard Envirochek filter was slightly similar to the results obtained with the HV filter. The recovery rates ranged from 16% to 85% (40±67, Mean±RSD) for *Cryptosporidium* while recovery rates for *Giardia* ranged from 2% to 49% (26±84, Mean±RSD). The corresponding number of oocysts detected ranged from 14 to 90 and the adjusted numbers ranged from 16 to 219 per 100 L. The number of cysts detected ranged from 20 to 130 while the adjusted number ranged from 69 to 1800. With the polypropylene cartridge filter, the percent recovery efficiencies of (oo) cysts were relatively lower than with previous methods.

Cryptosporidium recovery rates ranged from 2% to 27% (15±60, Mean±RSD) and the number of oocysts detected plus the adjusted numbers ranged from 1 to 26 and from 10 to 96, respectively. *Giardia* recovery rates ranged from 0% to 20% (11±66, Mean±RSD) and the number of cysts detected ranged from 2 to 14. Adjusted levels of cysts with the polypropylene cartridge filter were between 42 and 70 per 100 L. Despite large variations observed in samples of reclaimed water, the results of these experiments indicated that filtration with the Envirochek capsule filters produced significantly higher recovery rates than filtration with the polypropylene cartridge filters ($p<0.0001$). Both the HV filter and polypropylene cartridge filter allowed the collection of 100 L of reclaimed water, however due to low recoveries obtained with th polypropylene cartridge filter its use for protozoa analysis in reclaimed effluents is not recommended. The volume of reclaimed water achievable with the standard Envirochek filter never exceeded 20 L. In some instances, the increased pressure throughout the filtration components precluded the collection of 10 L of reclaimed water. and even at 10 L the filtration of water was restricted. Therefore, volumes of 10 L were consistently filtered at all facilities with the standard Envirochek filter and the concentrations were further expressed per 100 L for comparison with the HV and yarn wound filter.

The data obtained from these analysis revealed that the concentrations of indigenous (oo)cysts expressed per 100 L were relatively similar with the Envirochek capsule filters and that there were no statistical differences when comparing concentrations of (oo)cysts with both filters. However, there were significant differences between concentrations of (oo) cysts detected with the Envirochek capsule filters and the polypropylene cartridge filter ($p<0.0001$).

Indigenous (Oo)cysts in Reclaimed Effluents

Based on previous results, method 1623 with the Envirochek HV capsule filter was chosen for further analysis of protozoa in reclaimed effluents. Table 2.6 summarizes the results of the pathogen-monitoring program aimed at the determination of *Cryptosporidium* oocysts and *Giardia* cysts occurrence in the final effluent of four water reclamation facilities that eemploy two different type of filtration bed systems for removal of protozoa. ColorSeed C&G spike suspensions were used as internal quality controls in these analyses. IMS concentrates (200 µl) were stained with fluorescent monoclonal antibodies plus fluorochromes (DAPI/PI) and analyzed for oocysts using epifluorescence microscopy. Three samples per facility were analyzed and the level of (oo) cysts was expressed per 100 L plus the adjusted levels of (oo) cysts based on the recovery efficiency percentages obtained for the same batch of samples. All of the samples collected were positive for *Cryptosporidium* and *Giardia* regardless of the type of filtration bed system used at each facility. The levels of *Cryptosporidium* oocysts detected without adjustment ranged from 2 to 209 per 100 L while the number of *Giardia* cysts ranged from 13 to 118 per 100 L. There were no statistical differences in the number of *Cryptosporidium* oocysts detected in the facilities using deep bed or shallow bed filtration. However, the number of *Giardia* cysts per 100 L was significantly different between facilities ($p = 0.1$).

Recoveries of *Cryptosporidium* and *Giardia* from Various Water Matrices

Table 2.7 summarizes the results of the recovery efficiency experiments for *Cryptosporidium* oocysts and *Giardia* cysts in various water matrices. Again, ColorSeed

Table 2.6 Levels of *Cryptosporidium* oocysts and *Giardia* cysts in reclaimed effluents based on results obtained from recovery efficiency experiments using ColorSeed™ as an internal quality control system

Filtration system at the facility	Protozoa	Level of indigenous (Oo)cysts per 100 L	Recovery Efficiency (%[a])	Adjusted Level of (oo)cysts per 100 L
Deep bed, multiple media	*Cryptosporidium*[b]	8	31	26
		209	31	674
		36	14	257
		2	8	25
		35	11	318
		40	5	800
Shallow bed automatic backwash		28	27	102
		11	10	110
		77	31	248
		75	18	417
		20	19	105
		106	14	757
Deep bed, multiple media	*Giardia*[c]	35	24	146
		13	2	650
		11	10	110
		66	24	275
		20	19	105
		40	42	95
Shallow bed automatic backwash		10	31	32
		85	43	198
		118	38	310
		106	14	757
		35	11	318
		75	18	417

[a] Percentage recovery efficiency

[b] Geometric mean: 23 oocysts/100 L (deep bed filters); 39 oocysts/100 L (shallow bed filters). [c] Geometric mean: 25 cysts/100 L (deep bed filters); 54 oocysts/100 L (shallow bed filters). The t test revealed statistical differences in the number of cysts per 100 L between facilities using deep bed and shallow bed filters ($p = 0.1$).

was the spike suspension used for all these experiments. For groundwater matrices, 100 L of water were filtered through the HV and yarn wound filter. The standard Envirochek filter that has been designed for volumes lower than or equal to 10 L was not used in these water matrices.

The relative percent difference (RPD) of the recovery was used to determine the precision of each method with the different water matrices according to the specifications described in the 1999 version of method 1623. For *Cryptosporidium*, the mean percent recovery and RPD obtained with the HV filter was 71% and 25%, respectively. Mean percent recovery for *Giardia* was 37% while the RPD was 13%. With the modified ICR method, mean percent recoveries for *Cryptosporidium* and *Giardia* were 20% and 14%; RPDs for the same organisms were 50% and 57%.

Surface water matrices obtained from Bayboro bay and lakes within Section 21 Wellfield were processed with the three filters. Mean percent and RPD of recovery of *Cryptosporidium* with the HV filter in water samples from Bayboro bay were respectively 39% and 41%. For *Giardia*, the mean recovery was 11% and the RPD was 72%. The results obtained with the standard Envirochek filter were slightly better for *Cryptosporidium*, however the variability in the recovery rate of *Giardia* cysts did not fall within the method's quality control criteria (30%). The mean percent recovery and RPD were 29% and 38%, respectively. With the modified ICR method neither *Cryptosporidium* nor *Giardia* were efficiently recovered from spiked samples; the mean percent recovery for oocysts was 11% and 7% for cysts while the maximum RPD for each organism was 109% and 116%, respectively.

Table 2.7 *Cryptosporidium* and *Giardia* recoveries in various water matrices

Method (Filter type)	Water Matrix (Volume)	*Cryptosporidium*[a]			*Giardia*[b]		
		%[c]	MPR[d]	RPD[e]	%	MPR	RPD
	Groundwater						
Method 1623	MS1	62			35		
HV filter	MS2	80	71	25	40	37	13
	MS3	85			67		
Modified ICR	MS1	15			10		
	MS2	25	20	50	18	14	57
	MS3	19			24		
Method 1623	Surface water						
HV filter	(Bayboro bay)						
	MS1	31			7		
	MS2	47	39	41	15	7	72
	MS3	39			22		
Envirochek	MS1	58			24		
	MS2	91	74	45	35	29	38
	MS3	75			29		
Modified ICR	MS1	5			3		
	MS2	17	11	109	12	7	116
	MS3	15			9		
Method 1623	Lakes						
HV filter	MS1	12			7		
	MS2	22	17	59	15	11	73
	MS3	15			9		
Envirochek	MS1	15			10		
	MS2	35	25	80	20	15	67
	MS3	27			22		
Modified ICR	MS1	5			3		
	MS2	12	8	82	10	6	107
	MS3	12			5		

[a] Spike dose of *Cryptosporidium* oocysts: 99±0.7 (ColorSeed)
[b] Spike dose of *Giardia* cysts: 100±0.3 (ColorSeed)
[c] Percent recovery
[d] Mean percent recovery
[e] Relative percent difference

All of the spiked water samples from the lakes had low and variable recovery rates. The method's quality control criterion for *Cryptosporidium* was only met using method 1623 with the Envirochek HV filter. With the standard Envirochek filter and the modified ICR method neither *Cryptosporidium* oocysts nor *Giardia* cysts were efficiently recovered.

Potential Viability of *Cryptosporidium* Oocysts Using the Vital Dye Assay

Three populations or categories of oocysts were present in water sample concentrates obtained from reclaimed effluents: (i) PI-positive (PI+) oocysts, (ii) DAPI-negative PI-negative (DAPI- PI-) oocysts, and (iii) DAPI-positive PI-negative (DAPI+ PI-) oocysts. PI+ oocysts were the population most frequently detected and accounted for more than 80% of the oocysts present in all reclaimed effluent samples analyzed. These are considered dead oocysts by the criteria established for this assay by Campbell et al. (1992). PI+ oocysts seen under DIC microscopy were characterized for having a disorganized appearance and lack of any distinct sporozoite structure. All DAPI- PI- oocysts were empty with no internal sporozoite structure; these oocysts have been described as "ghosts" in previous studies (Campbell et al. 1992, Anguish and Ghiorse, 1997). DAPI+ PI - oocysts were marginally detected in the water samples analyzed; these oocysts satisfied the criteria of Campbell et al. for potentially infectious oocysts and DIC microscopy revealed intact sporozoite structures.

Giardia cyst populations or categories were predominantly PI+ (>95%), which indicated that most of the cysts were inactivated or nonviable. DIC microscopy revealed that for the majority of the cysts the internal morphology was completely destroyed.

The molecular characterization and potential infectivity of *Cryptosporidium* isolates was carried out in this study and the results are presented in a separate chapter.

Discussion

Numerous innovative technologies for analysis of waterborne protozoan parasites have been described. The present study was designed to evaluate the reproducibility of USEPA method 1623 and a modified version of the ICR method using a combination of experimental conditions aimed at the detection of *Cryptosporidium* oocysts in four different water matrices. Additional information on the recovery and detection of *Giardia* cysts was also included.

The performance of both methods was initially evaluated with reagent water (IPR tests) and the results obtained indicated that the acceptance criteria described in the 1999 version of method 1623 for *Cryptosporidium* (RSD, 40%) and *Giardia* (RSD, 41%) was met using both the standard Envirochek and HV filters. Conversely, the modified ICR method with the polypropylene yarn wound cartridge filter did not generate acceptable recovery rates. Despite the latter results, further analyses were carried out to evaluate analytical performance of the methods in samples of tap, reclaimed water, groundwater, lake water and seawater.

(Oo) cyst recovery efficiency in the different water matrices was the key parameter used to assess analytical performance (method reproducibility) while the volume of water sample achievable with each filter determined filtration performance. Among the filters used in this study, the Envirochek HV capsule filter performed better in all water types as demonstrated by the recovery rates obtained for both *Cryptosporidium* oocysts and *Giardia* cysts. The RPD of *Cryptosporidium* recovery in spiked groundwater and surface water samples were all within the method's quality control criteria (40%) using method 1623 with the HV filter. Much more variability in *Cryptosporidium* recovery was observed in reclaimed water samples with all filters. *Giardia* cysts were also efficiently recovered from spiked groundwater and tap water samples with the HV filter; the relative percent difference (13%) was far below the value outlined in the method's quality control criteria (30%). Again, low and variable recovery efficiencies of cysts were observed in reclaimed and surface water samples.

Water quality parameters in the final effluent (data not shown) such as turbidity, TSS, pH, NH_3-N determined at the water reclamation facilities were not associated with variations in (oo) cyst recoveries. However, a significant correlation was found between the level of indigenous *Cryptosporidium* oocysts and CBOD concentrations ($r = 0.84$, $p<0.0001$). Further research is required to understand more about the significance of this relationship. CBOD is the amount of oxygen required to oxidize any organic matter present in the water biochemically; it is an indirect indication of the concentration of organic contamination in the water (Ray, 1999).

Despite large variations observed in samples of reclaimed water, the results of these experiments indicated that filtration with the Envirochek capsule filters produced

significantly higher recovery rates than filtration with the polypropylene cartridge filters ($p<0.0001$). Both the HV filter and polypropylene cartridge filter allowed the collection of water volumes greater than 100 L, however the filtration performance of these filters was affected by the turbidity of the lake waters.

The volume of water achievable with the standard Envirochek filter never exceeded 20 L. In some instances, the increased pressure throughout the filtration components precluded the collection of 10 L of water. The recovery efficiency experiments, therefore, were carried out with 20 L of lake water for both the HV filter and the polypropylene wound cartridge filter to maintain a consistent sample volume. Only method 1623 with the HV filter allowed recoveries of *Cryptosporidium* that fall within the EPA's QA/QC method guidelines. *Giardia* cysts were not efficiently recovered with any of the methods and filters tested in spiked surface water. Similar results were obtained with spiked seawater samples, however method 1623 with the Envirochek capsule filters performed better than the modified ICR method.

The use of the appropriate filtration system is critical in the analysis of waterborne protozoan pathogens since it is the initial step involved in the concentration of the organisms. The Envirochek HV capsule filter has been demonstrated to perform better than the standard Envirochek filter in natural waters (DiGiorgio et al., 2002) and the results of this study with samples of reclaimed water, groundwater and seawater support those observations. Nevertheless, the filtration capacity of the HV filter can be affected by water turbidity and water matrix components that can lead to low and variable (oo) cyst recovery rates (DiGiorgio et al., 2002) as it occurred in this study with samples of surface water. Water matrix components associated with samples of reclaimed water

might account for inconsistencies in method reproducibility, since the turbidity of these samples never exceeded 1.75 NTU. Further research is needed to determine the matrix components of environmental waters that affect the recovery efficiency of (oo) cysts in order to provide better methods for the assessment of water quality.

The effects on the analytical performance of both methods with seawater samples are more complex and require further investigations. The volume of seawater achievable with the HV filter was 100 L and the turbidity of the water ranged from 0.65 NTU to 1.5 NTU. Matrix effects such as high concentrations of dissolved iron (between 4 and 20 mg/L) and organic carbon (up to 38 mg/L) have been found to interfere with the IMS-IFA portion of EPA method 1623 (Yakub and Stadterman-Knauer, 2000; DiGiorgio et al., 2002). None these water quality parameters were measured in water samples collected from Bayboro bay. However, the results obtained with the Envirochek filters were relatively better than with the polypropylene cartridge filters, thereby suggesting the potential use of the capsule filters for environmental monitoring of protozoan pathogens in marine waters.

Despite variations in method reproducibility, it was possible to determine that the occurrence of indigenous protozoa in environmental samples was significantly improved using method 1623 with the HV filter, which allowed the detection of significantly higher numbers of (oo) cysts than with the standard Envirochek filter and the polypropylene yarn wound cartridge filter. These observations were made in reclaimed water samples where most of the indigenous (oo) cysts were detected.

The results of an extensive two-year monitoring program with the modified ICR method to determine the occurrence of *Cryptosporidium* and *Giardia* in reclaimed effluents, deep injection monitoring wells and surface water samples throughout Florida revealed lower concentration of these organisms (0.5 (oo)cysts/100 L – 15 (oo) cysts/100 L) than the concentrations reported in the present study. Therefore, the inclusion of method 1623 with the HV filter in current monitoring programs represents an improvement towards the assessment of protozoan parasites in the environment.

Further modifications and alternatives to method 1623 and ICR method have been proposed in an attempt to optimize the initial concentration process involving filtration. For example, Simmons et al. (2001) reported 42-46% recovery for *Cryptosporidium* oocysts when they used a disposable polysulfone hollow-fiber ultrafilter for concentrating oocysts from seeded surface and reagent water. Kuhn and Oshima, (2002) reported mean oocysts recoveries of 55% using hollow-fiber ultrafiltration for concentrating oocysts from seeded tap and surface water of various turbidities. The results of collaborative trials of (oo) cyst recovery from source water with a modified Filta-Max elution and concentration technique (McCuin and Clancy, 2003) generated recoveries of *Cryptosporidium* oocysts that ranged from 12.4% to 36.5%, while *Giardia* cysts recovery percentages ranged from 22.7% to 68.3%. An alternative collection method for the recovery of *Cryptosporidium* oocysts from large volumes of water involves continuous flow centrifugation. CF centrifugation concentrates particles by size weight, and recovery of oocysts averages 14% (Swales and Wright, 2000). Process time is rapid, but this intensive equipment-based technique is not field applicable.

The inclusion of pre-stained (oo) cysts(ColorSeed C&G) as an internal quality control enabled the assessment of method performance at the point and time of sample collection, which is important to evaluate not only ongoing method and laboratory performance but also to identify methodology deficiencies that can occur when evaluating protozoan pathogens for monitoring compliance. The latter has important implications for the establishment of pathogenic numeric standards for natural waters, reclaimed waters and drinking water.

CHAPTER THREE

MICROBIOLOGICAL CHARACTERIZATION OF PROPOSED WATER SOURCES FOR REHYDRATION OF A FLORIDAN WETLAND

Introduction

The term "wetlands" is defined by the United States Environmental Protection Agency (USEPA) in 40CFR 232.2(r) as: Those areas that are inundated or saturated by surface or groundwater at a frequency and duration sufficient to support and that under normal circumstances do support, a prevalence of vegetation typically adapted for life in saturated soil conditions.

The many benefits that wetlands provide has been extensively recognized and programs have been developed to restore and protect wetland resources at the local, State and Federal levels of government (USEPA, 2000) http://www.epa.gov/OWO/wetlands/science/hgm.html).

As the link between land and water, wetlands play a vital role in water quality management programs. Wetlands provide a wide array of functions including shoreline stabilization, nonpoint source runoff filtration, and erosion control, which directly benefit adjacent and downstream waters. In addition, wetlands provide important biological habitat, including nursery areas for aquatic life and wildlife, and other benefits such as groundwater recharge and recreation.

Wetlands are present in landscapes that favor the pounding or slow runoff of surface water, discharge of groundwater, or both. Some wetlands depend almost exclusively on precipitation, surface water, or groundwater, but most wetlands receive water from a combination of these sources. Studies on selected wetlands and their contiguous watersheds in four landscapes in the US indicated that their sources of water are appreciably different and that management or protection of these wetlands would require different managements (Winter et al., 2001).

The City of St. Petersburg (City) owns and operates the Section 21 Wellfield, which is located on 583 acres at the Southwest corner of Dale Mabry Highway and Van Dyke Road in northwest Hillsborough County (Figure 1), Tampa Bay Water (TBW) under a Consolidated Water Use Permit issued by the Southwest Florida Water Management District (SWFWMD) operates the wellfield. The Section 21 Wellfield is currently permitted to withdraw 9.6 million gallons per day (mgd), based on a 36-month running average. The City leases all of the property to Hillsborough County for use as a public park (Lake Park), and Tampa Bay Water owns the one-acre parcels that surround each of six production wells (Section 21 Wellfield Wetland Restoration Project: Interim Report).

Wetlands and lakes comprise approximately half of the land within the section 21 Wellfield. During the past several years these wetlands and lakes have experienced lowered water levels due to development, groundwater pumpage and drought conditions. These stressed conditions have affected the ecological functions and aesthetics of Lake Park. The City of St. Petersburg recommended using the Section 21 Wellfield as the site for a pilot project to test the effectiveness of rehydrating stressed wetlands and lakes

using reclaimed water and or surface water from a major drainage canal, the Interceptor Canal.

The Interceptor Canal is a storm water canal that was constructed to divert excess surface water flows from the northern portion of the Sweetwater Creek and Brushy Creek basins to downstream areas of the Brushy Creek system. Tampa Bay Water has been monitoring the flow in the Interceptor Canal since 1995. An analysis of the first portion of this data, from June 1995 through September 1996 indicated that between 0.61 and 0.89 mgd may be available during the wet season for diversion from the Interceptor Canal to the Section 21 Wellfield (Section 21 Wellfield wetland Restoration Project, 2002).

The proposed water source for rehydration is the reclaimed effluent from the Hillsborough County's Dale Mabry Advanced Wastewater Treatment Plant. This plant provides advanced secondary treatment of wastewater and currently disposes of its effluent through a surface water discharge into Brushy Creek south of South Village Drive. The reclaimed water system contains storage facilities that serve to hold reclaimed water until it is used or discharged into Brushy Creek. The Northdale storage tanks (two 5-million gallon reclaimed water storage tanks) are located south of the Section 21 Wellfield in a public recreation area located along Northdale Boulevard just north of the Interceptor Canal. The Dale Mabry AWWTP, owned and operated by Hillsborough County, is the nearest wastewater treatment plant that produces reclaimed water that meets the standards established criteria for the discharged of reclaimed water to and from receiving wetlands (Rule 62-611, Florida Administrative Code [F.A.C.]). The potential

for reclaimed water availability for the pilot program has been estimated to be 2 mgd of reclaimed water from the Dale Mabry AWWTP to the wellfield.

Wetland restoration is proposed in three general areas: one wetland located northwest of Lake Starvation (NW wetland), a wetland located southwest of Lake Jackson (W. wetland), and one wetland located west of Lake Simmons (SW wetland). Restoration is also proposed for all four of the lakes on the wellfield, an area of approximately 80 acres (Figure 3.1).

Figure 3.1 Sampling sites within Section 21 Wellfield

Objectives of the Study

A large multidisciplinary project was established to study Section 21 Wellfield Rehydration. Overall the objective was to evaluate potential public health risks associated with the future enhancement of the hydrologic conditions within Section 21 Wellfield using surface water from the Interceptor canal and/or reclaimed water from the Dale Mabry Advanced Wastewater Treatment Plant as sources for restoration of the site's lakes and wetlands. Work elements in support of the public health risk assessment of the Pilot Restoration Project included: (i) site characterization; (ii) computer-based hydrologic modeling; (iii) ambient and source water quality sampling; and (iv) the development of a conceptual model of the surface water features and subsurface characteristics of the wellfield.

This work focuses on the microbiological characterization of the proposed source waters for rehydration and ambient waters or background waters of the wellfield (lakes and groundwater). This corresponds to the third work element of the overall public health risk assessment described above (ambient and source water quality sampling). The remaining project goals (site characterization, computer-based hydrologic modeling) were coordinated by HDR Engineering. The information was included in a report submitted to Tampa Bay Water and Southwest Florida Water Management District.

The specific objectives of this work focused on the microbial characterization of ambient and proposed source waters through the assessment of: (i) bacterial indicators (total and fecal coliforms, enterococci, heterotrophic bacteria, *Clostridium perfringens*), (ii) waterborne pathogenic protozoa (*Cryptosporidium* and *Giardia*) (iii) viruses and bacteriophages.

The main objectives of this study were:

- To assess the microbial quality of the potential water sources (Interceptor Canal and reclaimed water) proposed for wetland and lake restoration in the Section 21 Wellfield
- To assess the microbial quality of ambient waters (lakes and groundwater) within the Section 21 Wellfield, and
- To identify microbiological constituents of concern for the risk assessment plan of study.

Materials and Methods

Sample Locations

Water samples were collected from storm waters from the Interceptor canal (n = 4); reclaimed effluent samples from Hillisborough County's Northdale reclaimed water storage tanks (n = 4); surface water samples from four of the major lakes (Jackson, Starvation, Crum, Simmons) (n = 12) and groundwater samples from four permitted production wells (wells: 21-2, 21-6, 21-8, 21-10) (n = 11). Dates of sample collection and site location are provided in Table 3.1. Three out of twelve samples obtained from the lakes consisted of grab samples that were evaluated for microbial indicators; these sample events did not include parasites and enteroviruses.

Sample Collection

Two protocols for sample collection were used: (i) grab samples for microbial indicators (total and fecal coliforms, enterococci, *Clostridium perfringens*, heterotrophic bacteria, bacteriophages) (ii) large-volume analysis by cartridge filtration for protozoa (*Cryptosporidium* and *Giardia*) and enteroviruses.

Grab samples for microbial indicators were collected using sterile 1000-mL plastic bottles. All samples were immediately placed in an ice chest containing ice and transported to the laboratory for analysis within four hours of collection.

For protozoa, water samples were filtered through a 10-inch-long polypropylene yarn-wound cartridge filter, (1.0 μm, nominal porosity, Filterite, Timonium, Maryland) using a gasoline-powered portable water pump with flow rates maintained at 1-3 gal/min. For enteroviruses a 0.2 μm, positively charged zeta pleated cartridge filter (1 MDS [Mark

Table 3.1 Data on sample collection: Section 21 Wellfield Pilot Rehydration Project

Sample ID	Date of Sample collection	Site ID	Rainfall (cm)
HDR-1	04/06/99	WELL 21-10	0
HDR-2	04/06/99	WELL 21-6	0
HDR-3	04/06/99	LAKE STARVATION	0
HDR-4	04/06/99	LAKE JACKSON	0
HDR-5	04/07/99	R.E DM-WWTP[a]	0
HDR-6	04/07/99	WELL 21-2	0
HDR-7	06/19/99	WELL 21-10	N.A[b]
HDR-8	06/19/99	WELL 21-6	N.A
HDR-9	06/19/99	LAKE STARVATION	N.A
HDR-10	06/19/99	LAKE CRUM	N.A
HDR-11	06/27/99	R.E DM-WWTP	N.A
HDR-12	06/27/99	LAKE SIMMONS	N.A
HDR-13	09/25/99	R.E DM-WWTP	5.43[c]
HDR-14	09/25/99	INTERCEPTOR CANAL	5.43[d]
HDR-15	09/25/99	WELL 21-10	5.43[e]
HDR-16	09/25/99	WELL 21-8	5.43[f]
HDR-17	09/25/99	LAKE SIMMONS	5.43[g]
HDR-18	09/25/99	LAKE CRUM	5.43[h]
HDR-19	01/17/2000	WELL 21-8	0
HDR-20	01/17/2000	WELL 21-10	0
HDR-21	01/17/2000	LAKE CRUM	0
HDR-22	01/17/2000	LAKE SIMMONS	0
HDR-23	01/17/2000	LAKE STARVATION	0
HDR-24	06/06/2000	WELL 21-2	0
HDR-25	06/06/2000	WELL 21-10	0
HDR-26	09/21/2000	INTERCEPTOR CANAL	N.A
HDR-27	08/01/2001	LAKE JACKSON	0
HDR-28	08/01/2001	INTERCEPTOR CANAL	N.A
HDR-29	08/07/2001	INTERCEPTOR CANAL	N.A

[a] Reclaimed effluent: Dale Mabry Wastewater Treatment Plant

[b]Not available

[c,d,e,f,g,h] average of total precipitation two or three days previous to the date of sample collection

D. Sobsey], Cuno Inc., Meriden, Conn.) was used. After collection, the filters were placed on ice and transported to the laboratory for processing.

Bacterial Indicators

Water samples were filtered through membrane filters (0.45 μm [pore size] and 47 mm diameter; Gelman Sciences) according to procedures described in the *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998). Sample size was governed by expected bacterial density and degree of turbidity. For reclaimed and groundwater analysis between 100 and 1750 mL were filtered. For surface water samples (lakes) the volumes filtered were 0.1 and 10 mL. Following filtration, membrane filters were placed on specific media and incubated at specific temperatures depending on the type of bacterial indicator evaluated.

For detection of total coliform bacteria, membrane filters were placed on M-Endo medium and incubated for 24 hours at 37 ±0.2°C. The colonies that produced a metallic sheen were enumerated as total coliforms according to the *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998). The number of total coliform bacteria was expressed as colony forming unit per 100 mL (CFU/100 mL).

Filtered samples for detection of fecal coliform bacteria were grown on mFC medium (modified medium for fecal coliform bacteria; Difco Laboratories, Detroit, MI) and sealed in plastic bags within 30 min of filtration. The plates were incubated for 24 hours in a water bath at 44.5±0.2 °C. The bacterial colonies with various shades of blue were counted as fecal coliform bacteria and concentrations expressed as CFU/100 mL (APHA, 1998).

For enumeration of Enterococci, membrane filters were placed on mEI agar plates (modified medium for detection of enterococci; Difco Laboratories, Detroit, MI) and incubated for 18 to 24 hours at 41±0.5°C. Those colonies exhibiting a blue halo were counted as enterococci and concentrations expressed as CFU/100 mL.

Clostridium perfringens was enumerated by growth on mCP medium (Acumedia, Baltimore, Maryland). Sample sizes were increased to 20 and 50 mL since lower densities of *C. perfringens* are found in environmental waters. Membrane filters were incubated anaerobically in GasPak jars (BBL [Baltimore Biological Laboratories] GasPak, Beckton Dickinson) for 18 to 24 hours at 45 ±0.5 °C. Yellow colonies were exposed to ammonium hydroxide fumes; colonies that turned red or dark pink were enumerated as *C. perfringens* and concentrations expressed as CFU/100 mL (Bisson and Cabelli, 1979).

Bacteriophages

Bacteriophages were analyzed by two methods: the agar overlay method of Adams (1959) and the enrichment protocol obtained from Bill Yanko (Director of Wastewater, County Sanitation Districts of Los Angeles County, San Jose Creek Water Quality Laboratory, Whittier, California). Two bacterial hosts were used separately to analyze the samples: *E. coli* host bacterial culture (host strain ATCC [American Type Culture Collection] 15597 and *E. coli* HS (pFamp)R (male specific coliphage host).

The host bacterium, *E. coli* (ATCC strain 15597) was used in the agar overlay method. Cultures were grown to logarithmic phase in tryptic soy broth (TSB) at 37 °C for

4 to 6 hours under constant agitation. The agar overlay method was performed with two-ml aliquots of sampled water. Aliquots were mixed with molten trypticase soy agar (TSA) (3 mL) and 1 mL of host bacteria. The mixture was then poured on solid plates of TSA, allowed to solidify and incubated 24 h at 37 °C. Plates were observed for plaque formation and scored. Five replicates for each sample were analyzed to reduce the limit of detection to 10 plaque-forming units (PFU/100 mL).

In order to assess the potential for viruses to impact wells located within Section 21 Wellfield, the enrichment protocol (presence/absence test) was used. Low-level concentrations of bacteriophages occur in groundwater; therefore large sample volumes need to be assayed (Leclerc et al., 2000). The enrichment protocol increases the number of phages to the point that they can be detected by direct plating. This protocol was also applied to those surface water samples that were negative with the agar overlay method. The host bacterium *E. coli* p(Famp)R was used for enumerating F-specific or male-specific bacteriophages. F-specific bacteriophages can be further characterized as F+RNA or F+DNA bacteriophages and used as indicator of groundwater vulnerability to contamination (USEPA, 1999). F-specific bacteriophages are used as a valuable model for viral inactivation by chemical disinfection (IAWPRC, 1991).

E. coli p(Famp)R cultures were grown, maintained an assayed on tryptone medium containing ampicilin and streptomycin as described by Debartolomeis and Cabelli (1991). Media preparation followed modifications described by Yanko et al., 1999. Briefly, 11X broth medium used for large volume enrichment cultures and containing 11-fold concentration of each ingredient was filter sterilized with a GV Durapore membrane filter (0.22 μm) to prevent burning. TTC (2,3,5-triphenyl

tetrazolium chloride) was added to a final concentration of 0.1% in tryptone agar plates (1.2% agar) and used for spot confirmation and plaque assay (Yanko et al., 1999). Water samples (1 L) were inoculated with 10 mL of host bacterial culture [*E. coli* HS (pFamp)R] and 100 mL of 11X Tryptone broth with antibiotics (Ampicillin-Streptomycin). After a 48-hour period incubation at 35 °C, ten µl of the enriched sample was then spotted onto a lawn of host bacteria, incubated at 37 °C, and examined for lysis zones. Results were expressed as presence/absence. To determine if the isolates were F+DNA or F+RNA coliphage, RNAse was incorporated in the plating growth medium. One plate contained RNAse and one did not. Aliquots of 5-10 µl were spotted onto a lawn of host bacteria, incubated at 37 °C, and examined for lysis zones. The coliphage neutralized in the presence of RNAse were F+RNA coliphage.

Protozoan Analysis (*Cryptosporidium* spp. and *Giardia* spp.)

Samples were processed and assayed for enteric protozoa by filtration, concentration/purification (immunomagnetic separation [IMS] and Percoll/sucrose) and immunofluorescence microscopy techniques according to a modified version of United States Environmental Protection Agency Information Collection Rule method (USEPA/ICR) (USEPA, 1995). Modifications included the use of the IMS technique instead of Percoll/sucrose flotation due to better recovery efficiencies. Sample size varied according the type of water analyzed. For reclaimed effluent and groundwater samples between 500 L and 3700 L were filtered. For surface waters (lakes and stormwater) the volume of sample filtered ranged from 100 to 600 L. Volumes were monitored by

attached flow meters. The volume of sample in this case depended on the turbidity of the water since some of the filters from surface water sources clogged during collection of the samples. The volumes for the different water types correspond to those included and recommended in the ICR method.

After collection, the filters were placed on ice for transport to the Water Pollution Laboratory (University of South Florida, College of Marine Science) where they were processed by cutting the filter and washing the collected material from the filter using eluting solution to recover the protozoan cysts and oocysts. The eluting solution consisted of phosphate-buffered saline (PBS) pH 7.4, containing 0.1% polyoxyethylenesorbitan (20) mono-oleate (Tween 80) and 0.1% sodium dodecyl sulphate (Sigma Chemical Co., St. Louis, MI) The resultant eluate (1.8 L) was centrifuged to a concentrated pellet representing the initial volume of water collected. An aliquot of concentrated pellet was clarified by using Percoll/Sucrose (P/S) (USEPA, 1995) and Immunomagnetic separation (IMS) according to the Dynal IMS protocol described below. Sample concentrates clarified by P/S showed a high concentration of microalgae and debris that interfered with the visualization and enumeration of cysts and oocysts using the immunofluorescence technique. Dynal IMS is a rapid and selective separation technique included in method 1623 of the USEPA to accurately assess *Cryptosporidium* and *Giardia* occurrence in raw surface waters used as source waters for drinking water treatment plants (USEPA, 1999a).

The Dynal IMS (Dynabeads anti-*Cryptosporidium* and anti-*Giardia*; product 730.02; Dynal A.S., Oslo, Norway) procedure was performed according to the manufacturer's specifications with some modifications. Briefly, 10 mL of a diluted filter

concentrate was placed in a screw cap Leighton tube, and 1 mL of 10X buffer A, 1 mL of 10X buffer B and 100 µl of the bead conjugate for each protozoa were added (buffer A and B and dynabeads are included in the Dynal kit).

Each sample was rotated through 360° for 1 hour at room temperature, and the tube was placed in a magnetic particle concentrator (MPC-1) to separate the bead-(oo)cyst complex from the contaminating debris. The beads were resuspended in 1 mL of 1X buffer A, transferred into an Eppendorf tube, and separated by using a magnetic particle concentrator (MPC-M), and the supernatant was removed and discarded.

The dissociation of the bead-(oo)cyst complex was accomplished by using 200 µl of 0.1 N HCl. The final concentrate was stained with fluorescein isothiacyanate-conjugated anti-*Cryptosporidium* sp. immunoglobulin M (IgM) and anti-*Giardia* sp. immunoglobulin G (IgG) monoclonal antibodies (FITC-mAbs; Waterborne, Inc, New Orleans, USA) and a solution of 4',6'-diamidino-2-phenylindole (DAPI).

An Olympus BH2 fluorescence microscope equipped with a blue filter block (excitation, 490 nm; emission, 510 nm) was used for detection of FITC-monoclonal antibody-labeled (oo)cysts at a magnification of X 200. The presence of (oo)cysts was confirmed at a magnification of X400 by using a UV excitation (excitation 400 nm; emission, 420 nm) to determine the presence of DAPI-stained sporozoite nuclei. Nomarski-Differential Interference Contrast (DIC) optics were used to determine the presence of the internal morphology of (oo)cysts, which was undertaken at either x400 or x1,000 magnification. Equivalent concentrations of (oo)cysts per 100 L were then calculated.

Enteroviruses Analysis

The isolation and detection of enteroviruses was carried out by the production of cytopathogenic effects (CPE) in cell culture. Sample sizes were similar to those described previously for protozoa. Water samples were concentrated by filtering through 0.2 μm, positively charged zeta-pleated filters (1 MDS, Cuno). Enteroviruses were eluted from the filters using a 1.5% beef extract 0.05 M glycine solution (pH 9.5). One liter of the solution was passed into the filter housing and was allowed to sit for 1 minute. Pressurized N_2 was used to force the beef extract solution out of the filter housing. The eluate containing desorbed viruses was collected in a sterile 1-liter bottle. This procedure was carried out twice. The pH of the eluate was then brought to neutral using 1 N HCl. and further concentrated by organic flocculation according to the ICR protocol (USEPA, 1995). Concentrated samples were filter sterilized and inoculated onto Buffalo Green Monkey (BGM), Rhabdosarcoma and MA-104 cells and cytopathogenic effects (CPE) indicated the presence of infectious viruses. Enteroviruses were expressed as most probable number per 100 L (MPN/100 L) taking into account the equivalent volume examined. Cell culture analyses took place in the laboratory of Microbiology of Dr. Samuel R. Farrah (University of Florida).

Statistical Analysis

Arithmetic averages were calculated at each site for each variable. Values less than the limit of detection were considered as zero in these calculations. For those sites where all values were less than the limit of detection, the individual limits of detection for

each sample were averaged. The differences in the limits of sensitivity for different samples reflect differences in sample volumes. The biological data were transformed to $\log_{10}$ data ($\log_{10}$ of Y+1) and geometric averages were calculated. For those samples that generated microbial counts (lakes, stormwater and reclaimed effluents) a statistical test (Spearman rank order test) was performed to determine the relationship among concentrations of the various indicators. Because total coliform, fecal coliform, enterococci and *C. perfringens* data set did not follow a normal distribution, correlations between counts were evaluated using the Spearman rank order test. This nonparametric test measures the strength of association between pairs of variables without specifying which variable is dependent or independent and assumes that error distributions in the compared data set are the same.

Results

Bacterial Indicators in Section 21 Wellfield

Arithmetic and geometric means for levels of each bacterial indicator found in the lakes, production wells, stormwater samples and reclaimed effluents are given in Tables 3.2, 3.3, 3.4 and 3.5, respectively and are reported as CFU/100 mL. The geometric averages reflect the median values and elicit a better indication of central tendencies. The arithmetic averages may be 10 to 100 times greater than geometric levels and are influenced by the frequency of peak levels detected. Large concentrations for one sample will increase the overall mean and strongly influence the data (Rose at al., 1996). The analysis of the data in this study was based on the arithmetic mean. The arithmetic mean

in the evaluation of data for risk assessment may be a better measure because it may generate a higher central tendency value and possibly provide a greater safety factor (Hass et al., 1999).

Lakes

Among the sampled lakes, Lake Starvation had the greatest mean concentration of bacterial indicators (Table 3.2). The arithmetic means for total and fecal coliforms, enterococci and *C. perfringens* were $1.27x10^4$ CFU/100 mL, $5.17x10^3$ CFU/100 mL, $5.26x10^1$ CFU/100 mL, and $1.0x10^0$ CFU/100 mL. The arithmetic means for bacterial indicators in Lake Jackson were $4.30x10^3$ CFU/100 mL (TC), $2.2x10^4$ CFU/100 mL (FC) $1.12x10^3$ CFU/100 mL (E).

C. perfringens was below the detection limits in all samples collected at this site. Lake Crum had arithmetic means of $1.89x10^3$ CFU/100 mL (TC); $2.74x10^2$ CFU/100 mL (FC); $1.92x10^1$ CFU/100 mL (E), and $2.29x10^1$ CFU/100 mL (*C. perfringens*). The arithmetic means for Lake Simmons were $1.95x10^3$ CFU/100 mL (TC), $1.64x10^3$ CFU/100 mL (FC), $5.6x10^1$ CFU/100 mL (E), $3.9x10^1$ CFU/100 mL (*C. perfringens*) (Table 3.2).

The agar overlay method and the *E. coli* host bacterial culture (ATCC 15597) allowed the detection and enumeration of bacteriophages in one lake (Lake Jackson) and the level was 5 PFU/100 mL. When the bacterial host *E. coli* HS (pFamp)R and the agar overlay method were used no F-specific coliphage were detected in the lakes. However, the presence/absence test based on the enrichment protocol enabled the detection of F-specific coliphage in some of the sampled lakes that were negative by the agar overlay

Table 3.2 Microbial indicators in water samples collected in four lakes within Section 21 Wellfield

	Sampled lake			
Indicator	Jackson	*Starvation*	Crum	Simmons
TC (CFU/100 mL)				
No of samples	2	4	4	3
Percent Positive	100% (2/2)	100% (4/4)	100% (4/4)	100% (3/3)
Maximum value	$7x10^3$	$4.75x10^4$	$6.45x10^3$	$3.9x10^3$
Minimum value	$1.6x10^3$	$8.0x10^1$	$2.59x10^2$	$3.58x10^2$
Arithmetic Mean	$4.3x10^3$	$1.27x10^4$	$1.89x10^3$	$1.95x10^3$
Geometric Mean	$3.3x10^3$	$1.65x10^3$	$7.45x10^2$	$131x10^3$
FC (CFU/100 mL)				
No of samples	2	4	4	3
Percent Positive	100% (2/2)	100% (4/4)	100% (4/4)	100% (3/3)
Maximum value	$4.75x10^4$	$1.95x10^4$	$6.0x10^2$	$4.05x10^3$
Minimum value	$3.87x10^1$	$2.3x10^1$	$2.5x10^1$	$2.63x10^2$
Arithmetic Mean	$2.25x10^3$	$2.96x10^3$	$2.74x10^2$	$1.64x10^3$
Geometric Mean	$1.3x10^3$	$4.36x10^2$	$1.68x10^2$	$8.61x10^2$
E (CFU/100 mL)				
No of samples	2	4	4	3
Percent Positive	100% (2/2)	50% (2/4)	100% (4/4)	66% (2/3)
Maximum value	$2.20x10^3$	$4.75x10^4$	$4.0x10^1$	$8.8x10^1$
Minimum value	$4.5x10^1$	<1	$4x10^0$	<1
Arithmetic Mean	$1.12x10^3$	$5.26x10^1$	$1.95x10^1$	$5.6x10^1$
Geometric Mean	$3.15x10^2$	$5.4x10^0$	$1.42x10^1$	$1.02x10^2$
C. perfringens (CFU/100 mL)				
No of samples	2	4	4	3
Percent Positive	0% (0/2)	75% (3/4)	100% (4/4)	100% (3/3)
Maximum value	<1.3	$1.7x10^1$	$6.0x10^1$	$9.2x10^1$
Minimum value	<0.45	<0.45	$6.0x10^0$	$8.0x10^0$
Arithmetic Mean	<0.875	$9.54x10^0$	$2.3x10^1$	$3.9x10^1$
Geometric Mean	<0.7649	$8.3x10^0$	$1.4x10^1$	$2.4x10^1$

method. Lake Crum and Starvation were positive for bacteriophages in one out of four samples collected at each site using the enrichment protocol. This is an indication of very low levels of F- coliphage. F-specific coliphage in this sample were found to be RNA bacteriophages.

Groundwater

Table 3.3 summarizes the results of bacterial indicators determined for groundwater samples obtained from four production wells. Bacterial indicators were not detected in any of the sampled wells in a total filtered volume of 1750 mL. Values are reported as less than the limit of detection and averaged. According to the Standard Methods negative samples must be reported as less than one (<1) divided by the corresponding largest sample volume used (APHA, 1998). Mean bacterial counts of negative samples are therefore reported as <0.15 CFU/100 mL and <0.30 CFU/100 mL as described in Table 3.3. The variations in the limits of sensitivity for different samples reflect differences in sample volumes. Colilert medium was used for the last two samples collected from wells 21-2 and 21-10 (HDR-26 and HDR-27) in June 2000 and no coliforms were detected.

Interceptor Canal

Bacterial indicators were detected at high levels in water samples collected from the interceptor canal (Table 3.4). The arithmetic means for the different indicator organisms were: $1.2x10^4$ CFU/100 mL (TC), $8.72x10^2$ CFU/100 mL (FC), $1.90x10^3$

Table 3.3 Microbial indicators in water samples collected from four production wells within Section 21 Wellfield

	Sampled well			
Indicator	21-2	*21-6*	21-8	21-10
TC (CFU/100 mL)				
No of samples	2	2	2	5
Percent Positive	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/5)
Maximum value	<0.25	<0.45	<0.5	<0.5
Minimum value	<0.058	<0.058	<0.058	<0.058
Arithmetic Mean	<0.15	<0.25	<0.30	<0.30
Geometric Mean	NA[a]	NA	NA	NA
FC (CFU/100 mL)				
No of samples	2	2	2	5
Percent Positive	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/5)
Maximum value	<0.25	<0.25	<0.25	<0.25
Minimum value	<0.058	<0.058	<0.058	<0.058
Arithmetic Mean	<0.15	<0.15	<0.15	<0.15
Geometric Mean	NA	NA	NA	NA
E (CFU/100 mL)				
No of samples	2	2	2	5
Percent Positive	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/5)
Maximum value	<0.25	<0.25	<0.25	<0.25
Minimum value	<0.058	<0.058	<0.058	<0.058
Arithmetic Mean	<0.15	<0.15	<0.15	<0.15
Geometric Mean	NA	NA	NA	NA
C. perfringens (CFU/100 mL)				
No of samples	2	2	2	5
Percent Positive	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/5)
Maximum value	<0.25	<0.25	<0.25	<0.25
Minimum value	<0.058	<0.058	<0.058	<0.058
Arithmetic Mean	<0.15	<0.15	<0.15	<0.15
Geometric Mean	NA	NA	NA	NA

[a] Not applicable

Table 3.4 Microbial indicators in samples from the Interceptor Canal

	Stormwater site
Indicator	Interceptor Canal
TC (CFU/100 mL)	
No of samples	4
Percent Positive	100% (4/4)
Maximum value	$2.35x10^4$
Minimum value	$1.2x10^3$
Arithmetic Mean	$1.2x10^4$
Geometric Mean	$6.59x10^3$
FC (CFU/100 mL)	
No of samples	4
Percent Positive	100% (4/4)
Maximum value	$1.55x10^3$
Minimum value	$7.0x10^1$
Arithmetic Mean	$8.73x10^2$
Geometric Mean	$5.25x10^2$
E (CFU/100 mL)	
No of samples	4
Percent Positive	100% (4/4)
Maximum value	$3.35x10^3$
Minimum value	$3.0x10^1$
Arithmetic Mean	$1.90x10^3$
Geometric Mean	$8.08x10^2$
C. perfringens (CFU/100 mL)	
No of samples	4
Percent Positive	100% (4/4)
Maximum value	$3.0x10^1$
Minimum value	$9.3x10^0$
Arithmetic Mean	$2.1x10^1$
Geometric Mean	$1.92x10^1$

CFU/100 mL (E) and $2.1x10^1$ CFU/100 mL (*C. perfringens*). Bacteriophages were detected by the agar overlay method in two of four samples collected from the interceptor canal and levels ranged from 40 to 121 PFU/100 mL. These results were obtained using *E. coli* host bacterial culture ATCC [American Type Culture Collection] 15597, which enables the detection of somatic coliphage. The enrichment protocol and the bacterial host *E. coli* HS (pFampR) enabled the detection of F-specific RNA coliphage in three of the four stormwater sampling events.

Hillsborough County's Reclaimed Water Storage Tanks

The arithmetic means for total and fecal coliform in reclaimed water samples collected from Hillsborough County's Northdale reclaimed water storage tanks were 8 CFU/100 mL and 0.82 CFU/100 mL, respectively (Table 3.5). Enterococci were not detected and *C. perfringens* was present in one sample at a level of 10 CFU/100 mL. Bacteriophages were not detected in the reclaimed effluent.

Enteroviruses

Lakes

All lakes (Jackson, Starvation, Crum and Simmons) were positive for enteroviruses (Table 3.6) Lake Jackson was positive in one of two samples collected and the level was 1.7 MPN/100 L. Lake Starvation had a level of 0.63 MPN/100 L and was positive in one out of two samples collected. The level of enteroviruses found in lake Crum was 0.63 MPN/100 L; one of two samples was positive. Lake Simmons was

Table 3.5 Microbial indicators in samples fron the reclaimed water storage tank

Indicator	Reclaimed Effluent Dale Mabry
TC (CFU/100 mL)	
No of samples	4
Percent Positive	75% (3/4)
Maximum value	10
Minimum value	<0.058
Arithmetic Mean	8
Geometric Mean	7
FC (CFU/100 mL)	
No of samples	4
Percent Positive	75% (3/4)
Maximum value	1
Minimum value	0.45
Arithmetic Mean	0.82
Geometric Mean	0.52
E (CFU/100 mL)	
No of samples	4
Percent Positive	0% (0/4)
Maximum value	<0.8
Minimum value	<0.058
Arithmetic Mean	NA[a]
Geometric Mean	NA
C. perfringens (CFU/100 mL)	
No of samples	4
Percent Positive	25% (1/4)
Maximum value	10
Minimum value	<0.058
Arithmetic Mean	NA
Geometric Mean	NA

[a]Not applicable

Table 3.6 Number of enteroviruses observed in lake waters

	Sampled lake			
Enteroviruses	Jackson	*Starvation*	Crum	Simmons
MPN/100 L				
No of samples	2	3	3	2
Percent Positive	50% (1/2)	33% (1/3)	33% (1/3)	100% (2/2)
Sample 1	<0.20	<0.28	<0.71	0.96
Sample 2	1.7	<1.34	<0.02	1.82
Sample 3	-[a]	0.63	0.63	-

[a] No sample collected

positive in two of two samples collected with levels ranging from 0.96 to 1.82 MPN/100 L.

Groundwater

Infectious enteroviruses were isolated from the production wells (Table 3.7). The number of enteroviruses isolated from well 21-2 was 0.14 MPN/100 L and only one of two samples collected was positive. Well 21-8 had levels of 0.07 and 0.15 MPN/100 L and two of two samples collected were positive. Well 21-10 was positive in one of five samples and the level was 0.12 MPN/100 L. The wells of the Section 21 Wellfield are used as sources of drinking water and the treatment provided at the water treatment plant is appropriate for virus inactivation.

Interceptor Canal

Four of four stormwater samples obtained from the interceptor canal were positive for infectious enteroviruses. The levels of enteroviruses observed at this site were 0.73 MPN/100 L, 0.48 MPN/100 L, 4.4 MPN/100 L and 1.2 MPN/100 L (Table 3.8).

Hillsborough County's Reclaimed Water Storage Tanks

The level of enteroviruses found in reclaimed water samples was 0.09 MPN/100 L (Table 3.9). Only one of four samples was positive for enteroviruses.

Table 3.7 Number of enteroviruses observed in the production wells

	Sampled well			
Enteroviruses	21-2	*21-6*	21-8	21-10
MPN/100 L				
No of samples	2	2	2	5
Percent Positive	50% (1/2)	0%	100% (2/2)	20% (1/5)
Sample 1	<0.06	<0.025	0.07	<0.02
Sample 2	0.14	<0.02	0.15	<0.03
Sample 3	-[a]	-	-	<0.04
Sample 4	-	-	-	0.12
Sample 5				<0.10

[a] No sample collected

Table 3.8 Number of enteroviruses observed in samples from the Interceptor Canal

	Stormwater site
Enteroviruses	Interceptor Canal
MPN/100 L	
No of samples	4
Percent Positive	100% (4/4)
Sample 1	0.73
Sample 2	0.48
Sample 3	4.40
Sample 4	1.20

Table 3.9 Number of enteroviruses in the reclaimed water storage tank

	Reclaimed Effluent
Enteroviruses	Dale Mabry
MPN/100 L	
No of samples	4
Percent Positive	25% (1/4)
Sample 1	0.09
Sample 2	<0.16
Sample 3	<0.11
Sample 4	<0.08

Protozoa (*Giardia* and *Cryptosporidium*)

Lakes

The level of *Giardia* cysts and *Cryptosporidium* oocysts varied among the four lakes that were sampled (Table 3.10). *Giardia* was not detected in Lake Jackson, however 4.27 *Cryptosporidium* oocysts per 100 L were detected in one of two samples.

Lake Starvation had levels of *Cryptosporidium* oocysts between 2.33 and 112.7 oocysts/100 L and three of three samples were positive. *Giardia* cysts were detected at a level of 3.75/100 L. Lakes Crum and Simmons were positive for *Cryptosporidium* in one of two and two of two samples collected, respectively. The levels of oocysts were 20.7/100 L (Crum) and 1.62-4.30/100 L (Simmons).

The results presented in Table 3.10 would indicate that a higher level of protozoan pathogens was obtained with Percoll/sucrose when the number of cysts and oocysts were related to the equivalent volume examined. Similar equivalent volumes have to be examined in order to obtain similar counts of *Giardia* and *Cryptosporidium* after clarification with either Percoll/Sucrose or IMS. The volume of sample concentrate used in flotation Percoll/sucrose was 0.5 mL and still a lot of interference (debris and unspecific fluorescence) was observed. The volume of sample concentrate used in IMS can be increased to 1 or 2 mL. The highest volume used in this work was 2 mL for surface waters and the interference was much lower and slides were much faster and easier to read than using Percoll/Sucrose flotation. For that reason, IMS was chosen as the clarification technique. Comparisons between recovery efficiency of cysts and oocysts using Percoll/sucrose and IMS have been performed in the laboratory and higher recovery efficiencies have been obtained with IMS. The results of five seeding

Table 3.10 Number of protozoan parasites in lake waters

Protozoa	Sampled lake			
	Jackson	*Starvation*	Crum	Simmons
Oo(cysts)/100 L				
No of samples	2	3	2	2
Per cent Positive	50% (1/2)	100% (3/3)	50% (1/2)	100% (2/2)
Sample 1				
Giardia	<1.06	<0.96	<2.25	<2.41
Cryptosporidium	4.27	19.2[a], 4.8[b]	20.7	4.30
Sample 2				
Giardia	<2.03	3.75[a]	<1.04	<1.64
Cryptosporidium	<2.03	30[a], 112.7[b]	<1.04	1.64
Sample 3				
Giardia	-[c]	<1.16	-	-
Cryptosporidium	-	2.33	-	-

[a] Percoll/sucrose
[b] Immunomagnetic separation
[c] Indicates that no sample was collected

Table 3.11 Recovery efficiencies of *Cryptosporidium* oocysts and *Giardia* cysts using flotation Percoll/sucrose and DYNAL-IMS

Protozoa	Spike Doses	Recoveries (P/S)[a]		Mean Recoveries (DYNAL-IMS)	
Cryptosporidium		Number	%Recovery	Number	%Recovery
	148.42±1.05	66	45	120	82
	148.42±1.05	57	39	90	61
	148.42±1.05	35	24	120	82
	148.42±1.05	61	44	138	93
	148.42±1.05	44	30	115	77
Giardia	151.33±1.50	82	55	60	40
	151.33±1.50	55	37	90	59
	151.33±1.50	33	22	40	26
	151.33±1.50	37	25	60	40
	151.33±1.50	28	19	120	79

[a] Percoll-sucrose

experiments performed to determine the recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts are presented in Table 3.11. These results indicated that between 26% and 82% of (oo)cysts were recovered using IMS while 24% and 55% of seeded (oo)cysts were recovered using Percoll/sucrose flotation.

Groundwater

Two of the wells sampled were positive for the presence of protozoa (Table 3.12). One *Giardia* cyst was isolated from well 21-2 in one of two samples with a level of cysts of 1.46/100 L. Well 21-6 was positive for *Cryptosporidium* in two of two samples and the levels found were 0.1/100 L and 0.53/100 L. Wells 21-8 and 21-10 were negative for protozoa.

Interceptor Canal

Table 3.13 shows the level of enteric protozoa isolated from the Interceptor Canal. This level was the highest among the water samples analyzed in this study and corresponded to 287.67 *Cryptosporidium* oocysts/100 L. No *Giardia* cysts were found at this site.

Hillisborough County's Reclaimed Water Storage Tanks

The reclaimed effluent sample obtained from the storage tanks in Northdale was positive for *Cryptosporidium* in two of the four samples collected. No *Giardia* cysts were detected. The level of *Cryptosporidium* oocysts found at this site ranged from 1.91/100 L to 6.50/100 L (Table 3.14).

Table 3.12 Number of protozoan parasites in the production wells

	Sampled well			
Protozoa	21-2	21-6	21-8	21-10
Oo(cysts)/100 L				
No of samples	2	2	2	5
Percent Positive	50% (1/2)	100% (2/2)	NA[a]	NA
Sample 1				
Giardia	<0.22	<0.1	<0.12	<0.1
Cryptosporidium	<0.22	0.1	<0.12	<0.1
Sample 2				
Giardia	1.46	<0.53	<0.14	<0.05
Cryptosporidium	<0.12	0.53	<0.14	<0.05
Sample 3				
Giardia	-[b]	-	-	<0.05
Cryptosporidium	-	-	-	<0.05
Sample 4				
Giardia	-	-	-	<0.13
Cryptosporidium	-	-	-	<0.13
Sample 5				
Giardia	-	-	-	<0.12
Cryptosporidium	-	-	-	<0.12

[a] Not applicable
[b] Indicate that no sample was collected

Table 3.13 Number of protozoan parasites in samples from the Interceptor Canal

	Stormwater site
Protozoa	Interceptor Canal
Oo(cysts)/100 L	
No of samples	4
Percent Positive	25% (1/4)
Sample 1	
Giardia	<13.69
Cryptosporidium	287.67
Sample 2	
Giardia	<2.0
Cryptosporidium	<2.0
Sample 3	
Giardia	<1.65
Cryptosporidium	<1.65
Sample 4	
Giardia	<5.43
Cryptosporidium	<5.43

Table 3.14 Number of protozoan parasites in the reclaimed effluent storage tank

	Reclaimed Effluent
Protozoa	Dale Mabry
Oo(cysts)/100 L	
No of samples	4
Percent Positive	50% (2/4)
Sample 1	
Giardia	<0.63
Cryptosporidium	1.91
Sample 2	
Giardia	<0.39
Cryptosporidium	<0.39
Sample 3	
Giardia	<1.30
Cryptosporidium	6.50
Sample 4	
Giardia	<0.24
Cryptosporidium	<0.24

Discussion

The City of St. Petersburg currently owns Section 21 Wellfield and leases the property to Hillsborough County for use as a public park (Lake Park). Lake Park contains various recreational facilities including picnic and recreation areas, archery course, BMX track, and radio control car track, along with various jogging, hiking and equestrian trails. The northern two lakes (Lake Jackson and Starvation Lake) are used for nonmotorized recreational boating. There is also a horse stable that supports the equestrian use of the facility. Hillsborough County has constructed paved roadways and parking areas and has provided other facilities (play equipment, rest rooms) to support the recreational use of the park.

In order to assess potential public health risks associated with the future enhancement of the hydrologic conditions within Section 21 Wellfield, the occurrence of microbial indicators of water quality and waterborne pathogenic microorganisms was evaluated in the water sources (stormwater from the Interceptor Canal and reclaimed water) that will potentially be applied to the Section 21 Wellfield during project implementation for restoration of the site's lakes and wetlands. Overall, the source water characterization included the selection of Chemicals of Potential Concern (COPCs) and microbial constituents of concern in the source waters that may have the potential to cause adverse human health impacts through various exposure scenarios. This study focused on the microbial characterization of ambient waters and proposed source waters for rehydration. Exposure scenarios include drinking and showering with tap water that originated from the wellfield, or contact and ingestion associated with recreational use of the lakes at the wellfield.

Four major lakes (Jackson, Starvation, Crum and Simmons) and four existing production wells were sampled quarterly to provide microbial water quality data of background waters within Section 21 Wellfield. The frequency of sampling in the wells depended on the service status of a particular well. The potential reclaimed water source from the Northdale Storage Tanks was sampled four times during the one-year sample period. The Interceptor Canal (stormwater source) was scheduled for monthly sampling during the entire one-year sampling period, and twice monthly during the rainy season storm events. Due to low rainfall and lower water levels in the Interceptor Canal during the study period, only one sample was obtained from the Interceptor Canal in 1999. One additional sample was collected from the Interceptor Canal in 2000 and two more in 2001 to supplement the 1999 data.

The results of the microbiological sampling program demonstrated different levels of microbial occurrence in ambient waters and water sources proposed for rehydration of Section 21 wellfield (Figure 3.2). The highest level of microbial indicators was found in the lakes and stormwater samples from the Interceptor Canal. The evaluation of the water quality data obtained from these sites were based on the regulatory standards of the USEPA and the State of Florida Class III surface water standards for freshwater per Chapter 62-302.530 F.A.C. (Criteria for Surface Water Quality Classifications). These water-quality criteria are based on concentration of total and fecal coliforms and are expressed in two tiers in recognition of the fact that sampling

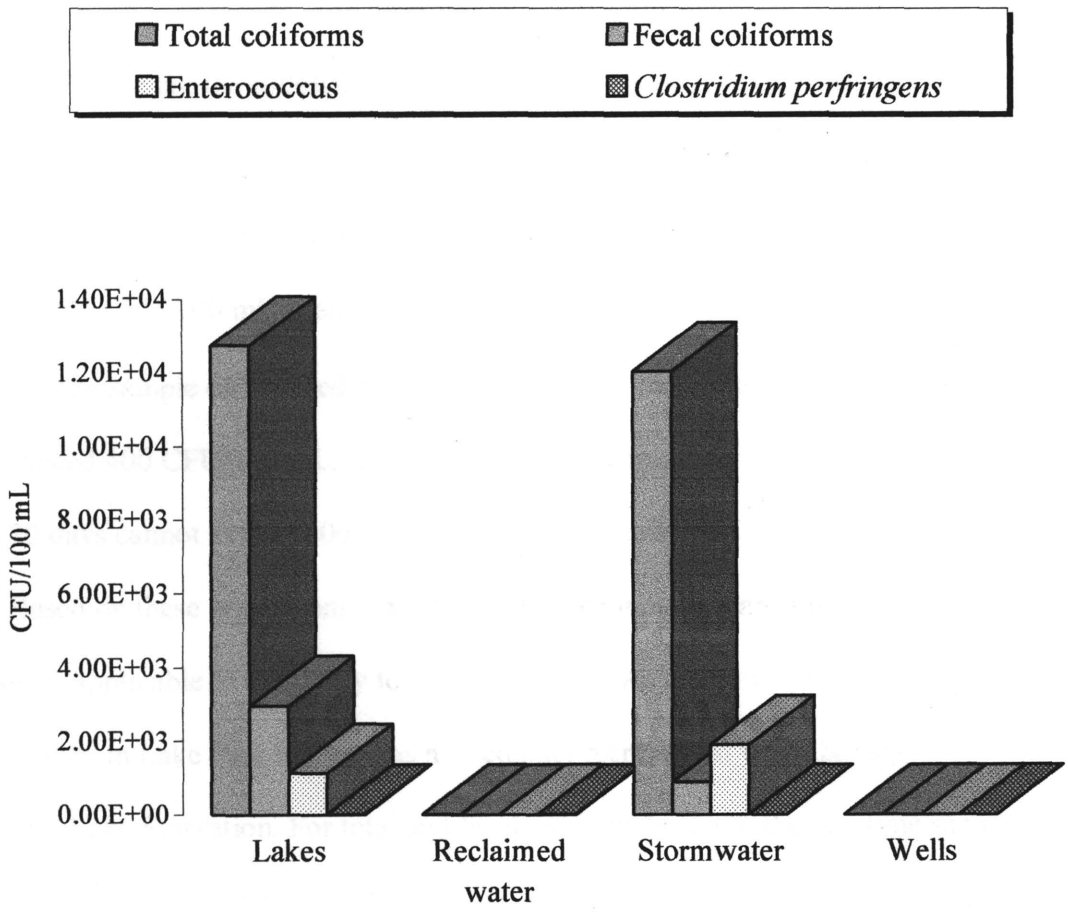

Figure 3.2 Levels of microbial indicators in ambient and proposed source waters for rehydration

will produce a range in results. The total and fecal coliform standards currently used in the state of Florida are the followings:

- 1,000 CFU/100 mL as a monthly average (geometric mean); nor exceed 1,000 CFU/100 mL in more than 20% of the samples examined during any month; ≤2,400 CFU/100 mL at any time (total coliforms);
- No one sample can exceed 800 CFU/100 mL, no more than 10% of samples can exceed 400 CFU/100 mL, and the geometric mean of ten samples collected within 30 days cannot exceed 200 CFU/100 mL (fecal coliforms).

Based on these criteria only the one time maximum standard for total and fecal coliforms is applicable to this study for surface waters. As mentioned before, some surface waters in Lake Park are used as non-contact recreational water bodies, specifically Lake Starvation. For total and fecal coliform bacteria, the one-time sample threshold limit is respectively <2,400 CFU/100 mL and 800 CFU/100 mL in recreational (Class III) waters. This maximum total and fecal coliform limit was exceeded in Lake Starvation, Jackson and Simmons and in samples collected from the Interceptor Canal (Figure 3.3). USEPA (1986) has established a one-time sample threshold for enterococci of 104 CFU/100 mL. This guidance threshold was exceeded in those sites where total and fecal coliform levels were also exceeding the guideline limit.

Currently, there is no established threshold or recommended limits for bacteriophages. Although somatic coliphage may not be found exclusively in human fecal waste, their presence has been used as an indication of the extent of general contamination in an area (Lipp et al., 2001). A level of 100 PFU/100 mL based on previous research in our laboratory has been proposed as a guidance level for evaluation

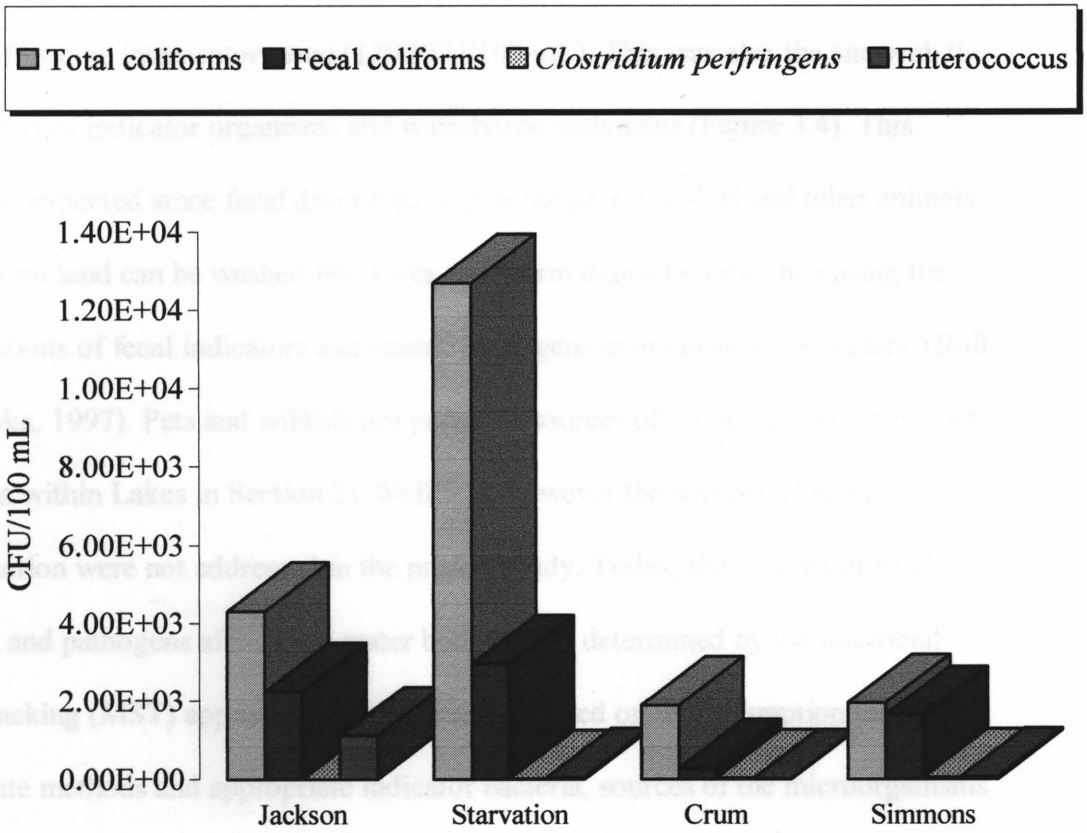

Figure 3.3 Level of microbial indicators in lakes within Section 21 Wellfield

of water quality. In the present study, this level was exceeded in one stormwater sample collected from the interceptor canal (120 PFU/100 mL). This was also the site with the highest level of indicator organisms and waterborne pathogens (Figure 3.4). This situation is expected since fecal droppings of pets (dogs, cats) birds and other animals that occur on land can be washed into lakes and storm drains by rain, increasing the concentrations of fecal indicators and enteric pathogens in environmental waters (Roll and Fujioka, 1997). Pets and wildlife are potential sources of microbial indicators and pathogens within Lakes in Section 21 Wellfield, however the sources of fecal contamination were not addressed in the present study. Today, the sources of fecal pollution and pathogens affecting a water body can be determined by the microbial source tracking (MST) approach. This approach is based on the assumption that, using appropriate methods and appropriate indicator bacteria, sources of the microorganisms can be found and characterized as to animal or human origin (Wiggins, 1996, Harwood et al., 2000; Simpson et al., 2002).

The bacterium *Clostridium perfringens* has been suggested as an indicator of sewage pollution. Based on work done in Hawaiian streams, Fujioka and Shizumura (1985) recommended a guideline of 50 CFU/100 mL for recreational waters. This recommended threshold was exceeded in Lake Simmons and Crum, but not in Lake Starvation. Similar levels of this bacterium have been reported in other areas of Florida (Lipp, 1999). However, the results of this and other investigations (Rose et al., 2000) have demonstrated that *C. perfringens* while indicative of fecal pollution only has limited value as an alternative indicator in areas of large dilution. More studies are needed to consider this bacterium as a microbial indicator in Florida or other subtropical waters.

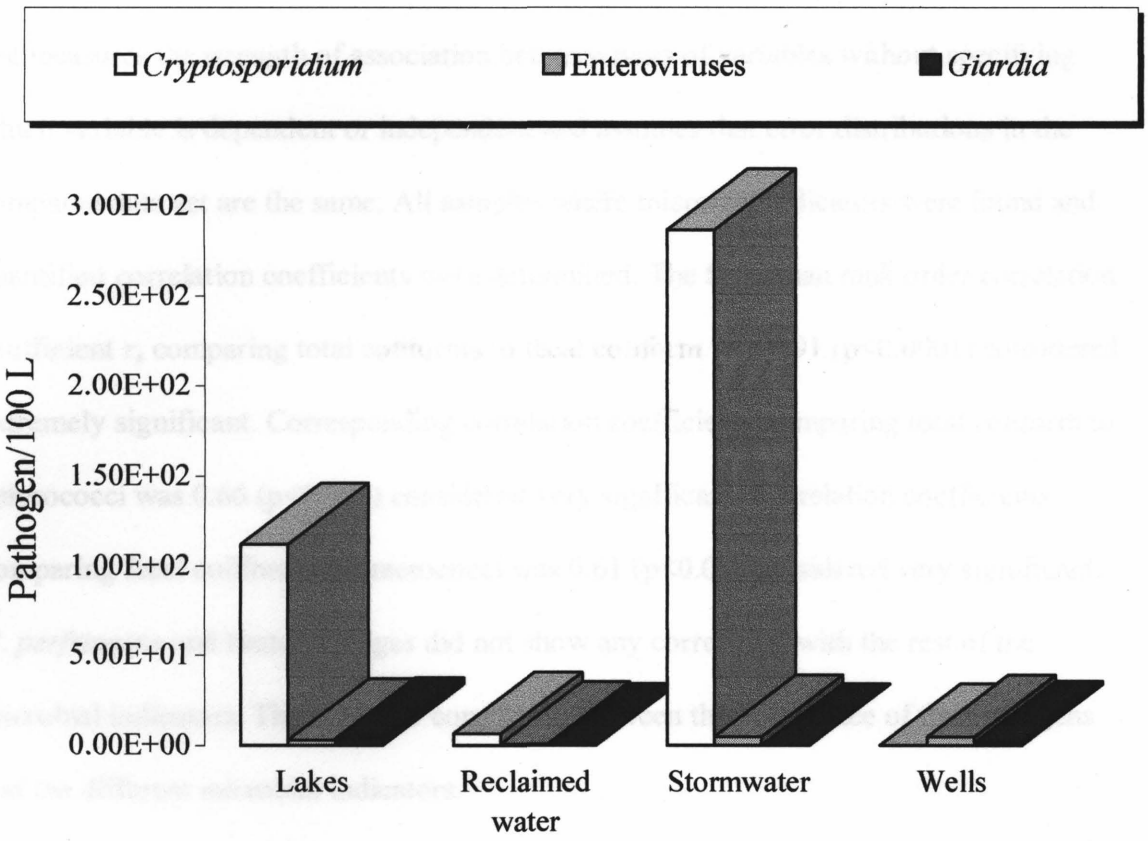

Figure 3.4 Level of enteric pathogens isolated from ambient waters and source waters proposed for rehydration

Correlations between microbial indicator counts obtained in this study were evaluated using the Spearman rank order test. As mentioned before, this nonparametric test measures the strength of association between pairs of variables without specifying which variable is dependent or independent and assumes that error distributions in the compared data set are the same. All samples where microbial indicators were found and quantified correlation coefficients were determined. The Spearman rank order correlation coefficient r_s comparing total coliforms to fecal coliform was 0.91 ($p<0.0001$) considered extremely significant. Corresponding correlation coefficients comparing total coliform to enterococci was 0.66 ($p<0.001$) considered very significant. Correlation coefficients comparing fecal coliforms to enterococci was 0.61 ($p<0.05$) considered very significant. *C. perfringens* and bacteriophages did not show any correlation with the rest of the microbial indicators. There was no correlation between the occurrence of the pathogens and the different microbial indicators.

The data obtained from this study indicated that the current recommended one-time threshold concentration for the different indicator organisms did not represent an adequate threshold for pathogen occurrence. These results would be demonstrating that compliance with water quality standards based on the current recommended one-time threshold concentrations for total and fecal coliforms, *C. perfringens* and enterococci are inadequate predictors of the occurrence of waterborne pathogens and public health risk from waterborne disease.

Previous investigations have demonstrated that the presence of enteric pathogens is not significantly correlated with levels of indicators in surface waters around Florida and other areas in the US (Rose et al., 1988, Callahan et al., 2001, Lipp et al., 2001).

Therefore, the assessment of *Cryptosporidium*, *Giardia*, and infectious enteroviruses is the preferred approach to evaluate the true evidence of fecal contamination, risk of exposure to contaminated water and potential health impacts.

Surface waters in Southern Florida have been analyzed for protozoan parasites and enteroviruses; the levels found in those studies (Lipp et al., 1999; Rose et al., 2000; Callahan et al., 2001) were relatively low compared to levels found in the present investigation.

The reclaimed effluent had the lowest level of microbial indicators; these levels were below the recommended threshold established for reuse activities in the state of Florida (25 fecal coliforms per 100 mL, Chapter 62-610 of the Florida Administrative Code [F.A.C]) in all occasions.

Groundwater samples were negative for the current microbial indicators except well 21-10 which was positive for FRNA coliphage. The enrichment protocol and the bacterial host used (*E. coli* strain HS(pFamp)R) enabled the detection of low number of these phages. Under laboratory testing conditions up to 1 PFU of FRNA coliphage added to 1,000 L has been detected (Fujioka and Yoneyama, 2001) therefore this technique is sensitive enough to detect low levels of bacteriophages. FRNA coliphage are consistently present in domestic raw sewage and are more similar to human enteric viruses with respect to environmental persistence and treatment process resistance than are indicator bacteria. FRNA phages are considered good indicators for monitoring the virological quality of water because they superficially resemble enteroviruses, caliciviruses, and hepatitis A virus (Hsu et al., 1995). Waterborne pathogens such as *Cryptosporidium*,

Giardia and enteroviruses were also detected in these sites indicating groundwater vulnerability to fecal contamination.

Monitoring groundwater samples for several potential microbial fecal indicators, including FRNA coliphage, is currently used to characterize the quality of the water with respect to vulnerability to contamination (USEPA, 1999; Fujioka and Yonemama, 2001). Yanko et al., (1999) have suggested the uses of FRNA coliphage as tracers to assess the potential for virus migration in soil.

The results of this study indicated that there are some low naturally occurring levels of microorganisms within the Section 21 Wellfield (i.e., lakes and groundwater) resulting in an ambient level of risk exposure to pathogens. *Cryptosporidium* is found widely distributed in surface waters in the U.S. and elsewhere (Smith and Rose, 1998). The parasite is introduced into the water environment of concern through fecal matter containing the protozoa. This could potentially include direct sewage discharge, rainfall runoff from agricultural and forested lands, or animal feeding operations. *Cryptosporidium* could also be introduced through recreational activities such as swimming. In Lake Park, horses, pets such as dogs, and the wildlife (birds) could also be potential sources of the protozoan. Deposition through mechanical vectors of *C. parvum*, such as the documented transport by birds, can also provide a means of introducing the protozoan to the source water (Graczyk, 1998).

To discuss about the potential health risks associated with the application of proposed source waters for restoration, the oocysts found in reclaimed water were assumed to be of human origin. However, these assumptions could not be made for the Interceptor Canal and the lakes where the highest levels of oocysts were found. There are

different potential sources of non-pathogenic *Cryptosporidium* species coming from animals that occur in ambient waters and this study did not distinguish between these types.

The presence of *Cryptosporidium* in the production wells suggests vulnerability. Studies on groundwater in the U.S (once thought to be a more protected source) reported that between 9.5% and 22% of samples were positive for *Cryptosporidium* (Hancock et al., 1998, 2000). In the present study, *Giardia* was detected once at well 21-2 and *Cryptosporidium* was detected once at well 21-6. These wells have been taken out of service by Tampa Bay Water pending further investigation. To date, additional testing has been carried out in four active production wells (21-5, 21-8, 21-9, 21-10) to determine if groundwater sources in the wellfield are under direct influence from surface water systems (lakes and wetlands). The testing is based on the Consensus Method (Microscopic Particulate Analysis) for Determining Ground Waters Under the Direct Influence of Surface Water (EPA, 1992). The preliminary results have demonstrated that the production wells have either no risk or moderate risk of surface water contamination.

The production wells at the Section 21 Wellfield were also positive for enteroviruses. Virus occurrence in the production wells was intermittent. Enteric viruses are species specific so that human health risks are only significantly associated with viruses from human fecal sources. The methodologies employed tend to favor detection of human enteroviruses, however the cell lines can possibly pick up other mammalian viruses, for example viruses from cows that would not represent a human health risk. Perhaps viruses from horses could be detected, which could be a source at the Section 21

Wellfield. There was no positive identification of the virus types for the Section 21 Wellfield restoration project; therefore the viruses cannot be definitively described.

The occurrence of viruses in aquifers is possible due to the resistant nature of the viral structure and the colloidal size (20 nm), which allows this group of pathogenic agents to potentially be transported through soil systems (Gerba and Bitton, 1984). Also, no samples were collected from the wells after disinfection, which would reduce the virus levels further due to their sensitivity to chlorination.

Various factors influence the die-off of oocysts during transport in surface and groundwater. These include predation (Fayer et al., 2000), physical removal by the aquifer (Marly et al., 2001), and natural die-off as influenced by environmental pressures such as naturally occurring organic acids, pH, sunlight, and temperature (Robertson et al., 1992). Under ideal conditions (temperature of 20 °C or lower and preservation in a chemically neutral solution such as distilled water), *Cryptosporidium* oocysts remain infectious after 24 weeks of storage (Fayer et al., 1998). The Section 21 Wellfield modeling predicted that the restoration water would first reach the well 21-9 after 175 days (25 weeks). Competing environmental pressures can serve to decrease or increase oocyst survivability and may be the most significant factor to evaluate in determining the risk posed by *Cryptosporidium.*

Perhaps the most important environmental factor that influences the survival of *Cryptosporidium* is temperature. *Cryptosporidium* oocysts have been proven to remain viable for up to 16 months (approximately 480 days) when stored in water at 4 °C (with antibiotics added). As temperatures approach the 20-25 °C, a temperature that would be expected in the lakes at the Section 21 Wellfield, viability decreases. The average

temperature in groundwater at the Section 21 Wellfield was estimated to be 22 °C and the surrounding surface waters to be 25 °C (sampling data). At these temperatures the estimated dieoff rates are 0.03944 and 0.0589 natural log/day (Walker and Stedinger, 1999). During the rainy summer months, the surface water temperatures can be as high as 33 °C, increasing the dieoff rate during this time period.

This rate constant can be used to predict the decrease in the concentration of viable *Cryptosporidium* oocysts over time that in turn can be related to distance if the groundwater flow velocity is known. Using a first order decay function ($C = C\ e^{-\lambda t}$), an estimate of the time to reach a target level can be calculated as follows:

$$T = - [\ln (C/C_o)]/\lambda$$

Where: C = concentration in groundwater at time "t"

C_o = initial concentration

λ = dieoff rate

t = time

Based on the decay function above, oocyst viability can be examined. The hydrologic modeling results have shown that the first arrival of restoration source water to the production wells is 175 days. Assuming that the oocyst levels found in the Interceptor Canal (288/100 L, average 74/100 L) were viable/infectious, and that the canal was used to restore the lakes, then the oocysts in groundwater would be reduced to approximately 0.3 viable/infectious oocysts per 100 L after 175 days. Therefore the numbers of viable oocysts would be reduced by almost 1000 fold due to dieoff during subsurface transport.

Similar to the protozoa, various functions are associated with the attenuation of viruses in surface and groundwaters. These include temperature as one of the key factors

influencing survival in groundwaters and surface waters. Each type of virus however, may have different inactivation rates. In groundwater systems viruses can be removed in the soil through adsorption, again the amount of removal is influenced by virus type and soil type.

Yates and Yates (1988) proposed a model to estimate inactivation of viruses in soils using the coliphage MS2 as a standard (Log_{10} inactivation = -0.181 + 0.0214 x T °C). The observed water temperature at the Section 21 Wellfield averaged 22 °C. Using the observed groundwater temperature 22 °C, the inactivation rate using the model is 0.289 log_{10}/day. Bitton et al. (1983) estimated a Polio 1 virus inactivation rate in Florida groundwater of 0.0456 log_{10}/day. The equation derived from this study was: y=0.0019X + 4.84, where the decay rate (k) estimated from the slope of the linear regression of the log transformed data was 0.0019 hr^{-1} (r^2 = 0.99). The level of virus reductions can be calculated using these inactivation rates, the virus migration rates, and groundwater travel time (model predicted) in days. Based on the hydrologic modeling results, the reductions of viruses would be 7.98 log_{10} reductions based on the rate described by Bitton et al. (1983).

In summary both viruses and *Cryptosporidium* numbers will be reduced in the environment. These microorganisms will dieoff over time, and numbers of viable organisms will be reduced by approximately 100,000,000 and 1000 fold, respectively after 175 days, which is the model-predicted time that it takes the restoration water to migrate to the first production well. In addition, physical removal through adsorption is estimated for viruses at a minimum of 99%. Oocyst removal through filtration may take place in the soil column, but was not quantified in this study. Based on the decay function

discussed above, oocyst dieoff would be expected as the source waters migrate to the Floridan Aquifer.

CHAPTER FOUR

ASSESSMENT OF POTENTIAL INFECTIVITY OF *Cryptosporidium* ISOLATES USING MOLECULAR TECHNIQUES AND TISSUE CELL CULTURE

Introduction

Molecular Assays for Detection of Waterborne *Cryptosporidium*

The development of sensitive and specific molecular detection methods such as the polymerase chain reaction (PCR) has greatly increased our knowledge about the type of *Cryptosporidium* in the environment. Many PCR assays for detecting waterborne oocysts have been described (Johnson et al., 1995; Mayer and Palmer, 1996; Stinear et al., 1996; Rochelle et al., 1997a; Rochelle et al., 1997b; Shiana et al., 1998; Chung et al., 1999; Kostrzynnska et al.,1999). In 1998, Wiedenmann et al. compared different PCR assays and demonstrated that problems inherent to the technique such as PCR inhibition, the requirement for extreme sensitivity, and viability assessment were all solved. In other words, Wiedenmann et al. (1998) demonstrated the suitability of PCR-based assays for routine environmental monitoring of *Cryptosporidium*. Some of the molecular approaches that have been developed to improve conventional detection methodologies are summarized in Table 4.1. One advantage of the molecular assays is that the PCR and RT-PCR products can be used to determine the genus, species, and genotype of the

Table 4.1 Molecular approaches for detection of *Cryptosporidium* oocysts in water

Limit of detection	Target sequence	Approach:	Reference:
1 oocyst	*C. parvum hsp70*	Seeded samples concentrated by calcium carbonate flocculation and Percoll-sucrose density centrifugation. Nucleic acid released by freeze and thaw cycles. mRNA isolated with Oligo$(dT)_{25}$ –coated magnetic beads. RT-PCR	Stinear et al., 1996
10 oocysts	*C. parvum hsp70* (hsp mRNA)	Seeded finished water concentrated by ICR method. In vitro cell culture (Cac0-2 cells), RNA extraction from cell culture and further isolation with Oligo (dT) cellulose kit. RT-PCR	Rochelle et al., 1997b
1-10 oocysts	Gene fragment CPR1 encoding a repetitive *C. parvum* oocysts cell wall protein	Seeded municipal water samples processed by the membrane filter dissolution method. DNA extraction: Lysis in TE-sarcosyl-proteinase K-buffer plus freeze and thaw cycles. Further DNA purification using QIAmp spin columns PCR, nested PCR, detection by Digene SHARP Signal™ System Assay.	Chung et al., 1999

Table 4.1 (Continued)

	C. parvum hsp70	Grab samples concentrated by centrifugation, DYNAL IMS plus acidified Hanks' balanced salt Solution-1% trypsin used to isolate oocysts. Purified oocysts inoculated into HCT-8 cells. Nucleic acid released by freeze and thaw cycles of harvested cells. Standard PCR	Di Giovanni et al., 1999
1 oocyst	Unknown genomic region	Seeded samples processed by method 1622. DNA extracted with Chelex 100 plus freeze and thaw cycles. Single tube nested PCR test and dot blot hybridization with an internal digoxigenin-labelled probe used for identification.	Haller-Soulier and Guillot, 1999.
10 oocysts/0.5 mL 1 oocyst/L	dsRNA	Seeded samples processed by ICR method. DsRNA extracted through Xtra Bind Capture System (Xtrana Inc., Denver, Colo) plus further extraction and purification procedures. Nested set RT-PCR amplification. Lateral flow chromatography format for detection	Kozwich et al., 2000

parasite. The genotype information can then be used to determine the specific strain or outbreak source.

The most common genotypic analysis are based on PCR-restriction length polymorphism (PCR-RFLP) analysis and/or sequencing of several genes: the small subunit ribosomal rRNA (Morgan et al., 1997; Xiao et al., 1999), 70 kDa heat shock protein (Sulaiman et al., 2000) β-tubulin (Widmer et al., 1998), *Cryptosporidium* oocyst wall protein (COWP) (Xiao et al., 2000) or thrombospondin-relative adhesive protein *Cryptosporidium*-1 (TRAP C1) (Spano et al., 1998) or TRAP C2 (Sulaiman et al., 1998). The small-subunit (SSU) rRNA-based nested PCR restriction fragment length polymorphism (RFLP) method of Xiao et al (2001a, 2001b) that targets the 18S small-subunit (SSU) rRNA gene locus of *Cryptosporidium* has been successfully applied to differentiate *Cryptosporidium* species and *C. parvum* genotypes in storm water, surface water, and wastewater samples. This technique offers a high level of sensitivity (Gobe et al., 2001) and can be used to differentiate the human pathogenic *Cryptosporidium* parasites from those that do not infect humans, and to track the source of oocyst contamination in the environment.

The feasibility of PCR-based detection methods for environmental monitoring of *Cryptosporidium* oocysts was recently described by Sturbaum et al. (2001). The authors were interested in detection sensitivity, and used micromanipulation techniques to deliver a desired number of oocysts (1 or 10) into PCR tubes for subsequent DNA liberation and PCR detection. They then demonstrated by using nested PCR-RFLP primers that the amplification rates increased from 38% to 100% for test samples containing 1 and 10 oocysts, respectively. These results suggest that

PCR-based detection methods may be sensitive enough to detect the low numbers of oocysts in environmental samples.

Quantitative methods for monitoring of waterborne *Cryptosporidium* have been described (Heid et al., 1996; Higgins et al., 2001; Di Giovanni et al., 2002; Fontaine and Guillot, 2002). Real-time PCR uses the TaqMan fluorogenic detection system for a continuous measurement of products throughout the reaction. This assay includes the primers and an oligonucleotide probe labeled with two fluorescent dyes that hybridizes to the target DNA. Quantitative PCR is performed with the Perkin-Elmer Applied Biosystem 7700 Sequence Detection System; quantification occurs in a real time during the amplification process without the need for electrophoresis or restriction digest for identification of species and genotypes. Di Giovanni et al., 2002, developed and applied a quantitative sequence detection (QSD) assay that targeted the *C. parvum* heat schock protein (*hsp70*) gene to quantify *C. parvum* oocysts in wastewater samples. Fontaine and Guillot, 2002, developed a TaqMan quantitative PCR assay that employs selected primer-probe set to identify a 138-bp section specific to a *C. parvum* genomic DNA squence.

DNA microarray technology has been developed for the detection of *Cryptosporidium* in environmental samples (Straub et al., 2002). Straub et. al. used a microarray of 68 capture probes targeting seven single-nucleotide polymorphisms (SNPs) within a 190-bp region of the *hsp*70 gene of *C. parvum*. Labeled *hsp*70 targets were gnerated by PCR with biotin- or Cy3- labeled primers. Hybridization patterns between genotypes were imaged using an Array-WoRx microarray scanner. Identification of SNPs required statistical analysis of the signal intensity data. The

microarray unequivocally differentiated *C. parvum* genotypes. Notwithstanding, some researchers point out that while the microarray technology is attractive, the obstacles of sample purity, sensitivity in the presence of non-target sequences and high cost makes its current application on a routine basis to environmental samples doubtful (Di Giovanni et al., 2002).

Viability and Infectivity Assays for Waterborne *Cryptosporidium*

Fluorescent antibody-based detection methods do not distinguish *C. parvum* from other *Cryptosporidium* oocysts that have no public health significance. In addition, IFA provides no information about the viability, infectivity, and virulence of recovered oocysts. These types of data are required to assess the public health risks of waterborne transmission of *Cryptosporidium* (Rochelle et al., 1997b; Matheson et al., 1998; Widmer et al., 1999; Fayer et al., 2000).

The classical definition of viability is the ability of an organism to reproduce, metabolize, and in the case of obligate parasites, to infect. Seven methods have been used to evaluate the viability of enteric protozoa including *C. parvum*. These include: (i) in vitro methods such as excystation (Bingham and Meyer, 1979; Smith and Smith, 1989; Rose, 1990; Robertson et al., 1993; Black et al., 1996; Schaefer, 1997; Vesey et al., 1997b); (ii) inclusion or exclusion of fluorogenic dyes (Schupp and Erlandsen, 1987; Campbell et al., 1992; Robertson et al., 1992; Campbell et al., 1993); (iii) nucleic acid stains (Belosevic et al., 1997a; Belosevic et al., 1997b; Neumannn et al., 2000); (iv) reverse transcriptase polymerase chain reaction (RT-PCR) (Stinear et al., 1996; Rochelle et al., 1997b; Kaucher and Stinear, 1998;

Widmer et al., 1999; Jenkins et al., 2000); (v) fluorescence *in situ* hybridization (Vesey et al., 1995; Vesey et al., 1998); (vi) infectivity methods using mice models (Roberts-Thomson et al., 1976; Korich et al., 1990; Enriquez and Sterling, 1991; Finch et al., 1993; Tzipori, 1998); and (vii) cell culture (Slifko et al., 1997; Rochelle et al., 1997b; Di Giovani et al., 1999). Fluorogenic dyes and cell culture have the greatest application to environmental samples.

Mice models have been used in the past to determine infectivity for *C. parvum* genotype 2 (Korich et al., 1990). The animal infectivity method is, however, tedious, difficult, and expensive, and is not readily amenable to normal laboratory analysis in the water industry (Neumann et al., 2000). Moreover, the *C. parvum* human genotype (genotype 1) will not infect standard animal models (Widmer et al., 2000). It is only recently that serial propagation of type 1 *C. parvum* in gnotobiotic piglets has been successful (Widmer et al., 2000).

Excystation is a method that has been used in laboratory disinfection and survival studies, but cannot be used to study oocyst viability in environmental samples because it requires large concentrations of oocysts (10^5 cysts/mL or greater) for the analysis. In addition, in vitro excystation methods have limited use in samples where there is high microbial abundance and diversity (Neumann et al., 2000).

The inclusion or exclusion of vital dyes has been used as a marker of intact membranes in *Cryptosporidium* oocysts and as an indicator of the presence of internal features such as nuclei. The vital dyes are fluorogenic, which makes them amenable to the IFA and microscopic procedures, and they may be useful tools for assessing the viability and infectivity of small numbers of oocysts found in environmental samples

(Smith et al., 1991;Campbell et al., 1992; Smith, 1996; Jenkins et al., 1997). One commonly used dye permeability assay tests the differential uptake of the fluorochromes 4'-6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by the oocysts (Gasser and O'Donoghue, 1999). Sporozoite nuclei, which take up DAPI but fail to stain with PI are viable, while nuclear material that stains with both fluorochromes is non-viable. The interpretation of viability from fluorogenic dye inclusion or exclusion must be undertaken cautiously, since the dye tests are known to overestimate viability and the staining can be variable with a portion of the oocysts not staining with either dye (Campbell et al., 1992; Smith, 1996; Jenkins et al., 1997; Black et al., 1996; Neumann et al., 2000). Nonetheless, results from dye permeability assays correlate well with results from in vitro excystation assays and the standard mouse infectivity assay under specific conditions.

Nucleic acid stains with SYTO dyes (SYTO-9, hexidium, and SYTO-59) have also been tested as tools for identifying infectious *C. parvum* oocysts in source drinking water (Belosevic et al., 1997a; Neumann et al., 2000). SYTO-59 is particularly useful, because its fluorescence spectrum does not overlap with that of FITC. It may therefore be used in conjunction with commercially available FITC-labeled anti-*C. parvum* monoclonal antibodies to detect and determine the viability or infectivity of oocysts in environmental samples (Neumann et al., 2000).

RT-PCR is a molecular method that is based on the amplification of RNA and often specifically messenger RNA (mRNA) (Abbaszadegan et al., 1997; Wiedenmann et al., 1998; Widmer et al., 1999). The method involves several steps: (i) isolation of RNA from the (oo)cyst, (ii) purification of the RNA, (iii) reverse

transcription to a DNA complementary strand, (iv) amplification, and (v) detection of the amplified product. One of the advantages of RT-PCR is that it does not depend on a preceding biological process like in vitro excystation (Wiedenmann et al., 1998). RT-PCR suffers from many of the same disadvantages as PCR such as: (i) inefficient extraction of RNA from the cysts or oocysts, (ii) interferences in the transcription and amplification steps by environmental constituents, (iii) small processed volumes for RT-PCR, and (iv) non-quantitative nature of the test.

Cryptosporidium oocyst infectivity can also be determined by cell culture methods, which has shown to be sensitive to low numbers of oocysts (Slifko et al., 1997; Rochelle et al., 1997b; Slifko et al., 1999; Di Giovanni et al., 1999). Numerous cell lines and detection schemes have been used (Table 4.2), but most investigators use the Human illeocecal adenocarcinoma cells (HCT-8) cell line. Standard filtration and IMS procedures have been used to prepare environmental concentrates for inoculation onto cell monolayers, where the oocysts undergo excystation and initiate infection in the cells (Di Giovanni et al., 1999). A quantitative procedure has been developed whereby the cells are fixed and infection is observed using labeled antibodies and microscopy (Slifko et al., 1997; Slifko et al., 1999). This has been successfully used to study UV inactivation of *Cryptosporidium* oocysts (Huffman et al., 2000). Alternatively, the cells are extracted and PCR or RT-PCR is used to detect infection (Rochelle et al., 1997a; Di Giovanni et al., 1999).

Water laboratories have already demonstrated (through peer-reviewed literature) the high sensitivity of cell culture to a single oocyst (DiGiovanni, et al., 1999; Rochelle, et al., 1997b; Slifko, et al., 1997, 1999). Other benefits of using cell

culture to study *C. parvum* infectivity include the following: (i) the method is applicable to environmental oocysts from treated and untreated waters, (ii) both genotypes 1 and 2 will infect HCT-8 cells, (iii) results are available in 48 hours, and (iv) the method is fairly easy and less labor intensive than animal infectivity studies. The correlation between cell culture data and animal infectivity data is significant ($r = 0.78$) for both untreated and treated (disinfected) oocysts (Slifko, 2001).

A fluorescent in situ hybridization (FISH) technique developed by Vesey et al. (1998) shows considerable promise as an indicator of *C. parvum* oocyst viability. In these assays, a fluorescent DNA probe is targeted to the 18S rRNA of *C. parvum*. The 18S rRNA is usually present in viable organisms and is degraded by cellular RNases in dead or dying cells. Existing FISH techniques, however, are limited to measuring the viability of *C. parvum* oocysts and not their infectivity (Neumann et al., 2000).

Novel approaches for measuring viability of oocysts in environmental samples have been described. For example, Call et al. (2001) developed a quantitative immunoassay that can detect low numbers of excystable, sporozoite-releasing *C. parvum* oocysts in turbid water samples. The CP7 viability assay uses a monoclonal antibody (CP7) to capture soluble *C. parvum* sporozoite antigen that has been released by in vitro excystation. The captured antigen is measured via electrochemiluminescence (ECL) using a ruthenium-labeled anti-rabbit antibody. The ECL counts derived from the CP7 viability assay are directly related to the number of viable oocysts. The configuration of the CP7 viability assay permits the evaluation of samples from turbid environmental water sources (as high as 200 NTU) at a detection limit as low as 50 viable oocysts/mL of concentrated sample.

Table 4.2 Cell lines used to study *Cryptosporidium parvum* genotype 2 infectivity

Cell Line (origin)	Isolate or outbreak source	Original Isolating Host	Infectivity Detection Method	Reference
Caco-2 (human)	Ames, IA	Bovine	RT-PCR	Rochelle, 1997b
BS-C-1 (African green monkey)	NA	Bovine	Giemsa stain	Deng and Cliver, 1998
BFTE (bovine)	Ames, IA	Bovine	SEM and TEM	Yang, 1996
HCT-8 (human)	KSU-1, Ames, IA, TAMU, UCP	Bovine, Equine	ELISA, PCR, RT-PCR, Specific IF, FDM-MPN, In-situ Hybridization	Woods, 1996; DiGiovanni, 1999; Okhuysen, 1999; Slifko et al., 1997, 1999; Rochelle et al., 2000
MDBK (bovine)	GCH1, Ames, IA	Human, Bovine	IF, PCR	Theodos, 1998
MDCK (canine)	Ames, IA	Bovine	Giemsa stain	Yang, 1996

Nucleic acid sequence-based amplification (NASBA) has been recently applied as a novel method to detect viable oocysts (Baeumner et al., 2001; Cook, 2003). The approach relies on NASBA amplification of heat schock protein mRNA (*hsp70*). Transcripts are produced by incubating oocysts at 42 °C for 20 minutes, potentially facilitating the detection efficiency of the amplification method. Detection of the NASBA signal is accomplished by hybridization to labeled probe followed by electrochemiluminiscence (ECL).

Esch et. al. 2001 developed a test-strip assay for the detection of amplicons produced NASBA technique from *C. parvum* mRNA. The detection is based on a competitive binding assay and signal generation by liposomes. Dye-containing liposomes are tagged with biotin and oligonucleotides (reporter probes). The probes are complementary to a specific region in the amplicon sequence and to a synthetic oligonucleotide sequence (antisense-reporter probe) immobilized on a nitrocellulose membrane strip. *C. parvum* amplicon is mixed with the probe-tagged liposomes. If the target sequence is present, the liposomes will bind via the reporter probes to the target and the mixture is allowed to migrate along the membrane strip. If not target sequence is present, liposomes will bind (via the receptor probe) to the antisense-reporter probe immobilized in the first capture zone on the strip. The liposomes contain biotin on ther surfaces, which enables their binding to a second zone in which antibiotine antibodies are immobilized. Gray-scale densitometre is employed to quantify the amount of liposomes present in either zone. The assay can be conducted in 30 min and is, therefore, much faster than oligonucleotide detection by Southern blotting or agarose gel staining.

Esch et al. (2001) also developed a sensitive microfluidic chip that detects viable *C. parvum*. The microfluidic chip (microfluidic RNA sensor) detects amplicons generated by nucleic-acid-base-amplification (NASBA) from mRNA produced by viable oocysts as a response to heat schock. The RNA sensor uses oligonucleotides-tagged liposomess as hybridization markers in a sandwich-hybridization assay. The detection conducted with the chip is specific and target concentrations as low as 0.4 fmol/μl can be measured.

Objectives

Overall the objective of this study was to apply molecular and tissue culture assays for sensitive detection, characterization and quantification of infectious *Cryptosporidium* species and *Cryptosporidium parvum* genotypes in samples of surface water, groundwater, drinking water and reclaimed water.

The specific objectives of this study were:

(i) To assess the efficacy of two DNA extraction protocols to maximize the sensitivity of the molecular assays

(ii) To assess the sensitivity of a nested PCR protocol for detection and characterization of *Cryptosporidium* species and genotypes in various water matrices

(iii) To provide information on the infectious potential of *Cryptosporidium* isolates using in vitro cell culture

Materials and Methods

Water Samples

The samples used for molecular characterization of *Cryptosporidium* isolates consisted of 25% of the IMS concentrates obtained from samples of surface water, groundwater sources, finished drinking water and reclaimed water processed following the steps of the April 1999 version of USEPA method 1623 and modified procedures described in Chapter two.

DNA Extraction

DNA was extracted from IMS concentrates using two methods: (i) the method described in the QIAamp DNA Mini kit (Qiagen, Valencia, Calif.) and (ii) the Chelex resin freeze/thaw method described by Di Giovanni et al. (2002). Firstly, IMS concentrates were centrifuged (10,000 rpm 3 minutes) and resuspended in either 50 µl molecular grade water or 400 µl of 1X PCR Buffer depending on the DNA extraction procedure used. Water concentrates resuspended into molecular grade water were mixed with 10 µl of 1:1 ratio (v/v) of Chelex 100 resin/TE buffer and subjected to eight cycles of freezing and thawing. DNA was recovered from the supernatant after a quick spin step and stored at –20 °C before it was used for PCR analysis.

Water concentrates resuspended into 400 µl of 1X PCR Buffer were subjected to eight cycles of freezing and thawing, spun at 10,000 X g for 3 minutes to recover oocyst DNA from the supernatant (200 µl) and incubated with 1 mg of proteinase K per mL at 56 °C for 1 hour. Following one-hour period of incubation, the samples were diluted with an equal volume of pure ethanol and oocyst DNA was extracted by

passing the oocyst-ethanol suspension through QIAamp DNA Mini isolate columns. The entire QIAGEN extraction procedure was performed as recommended by the manufacturer, except that 50 µl of Buffer AE (instead of 200 µl) were used during the final elution step. During the DNA extraction procedures, samples of *Cryptosporidium*-free molecular grade water were included randomly as negative controls.

SSU rRNA-based Nested PCR-RFLP Technique

The two-nested PCR-restriction fragment length polymorhism (RFLP) technique of Xiao et al (2000) that targets the 18S small-subunit (SSU) rRNA gene locus of *Cryptosporidium* was used for molecular characterization of oocysts isolated from water samples.

PCR amplifications were performed using two volumes of DNA preparation (5 and 50 µl). Reaction mixtures contained 1X PCR buffer (Qiagen 10X PCR buffer with 15 mM $MgCl_2$), 200 µM (each) deoxynucleoside triphosphate (Amersham Biosciences, Piscataway, NJ), 100 nM (each) primer, 2.5 U of Hot Start *Taq* polymerase (Qiagen Valencia, CA) and 5 or 50 µl of DNA template in a total 50 and 100-µl reaction mixture, respectively. Positive and negative PCR controls were run in parallel with each set of samples. PCR positive controls for the initial amplification reaction consisted of molecular-grade water and various amounts of *C. parvum* template DNA. PCR negative controls contained various amounts of molecular grade water. Primary PCR was performed with primers 5'-TTCTAGACCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3'.

Forty PCR cycles (94 °C for 45 s, 55 °C for 60 s, 72 °C for 90 s) were carried out in an eppendorf thermal cycler (Eppendorf AG) with an initial host start at 95 °C for 15 min, and a final extension at 72 °C for 1:30 min For the secondary PCR product, 5 μl of the primary PCR product was amplified with nested primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3'. Cycling conditions were identical to those used for the primary PCR. PCR products were analyzed on 1.5% agarose gels containing 0.5 μg ml^{-1} ethidium bromide. Resulting bands were visualized by UV transillumination.

The detection limit of the PCR techniques was tested with flow-cytometer counted oocysts suspensions (Wisconsin State Laboratory of Hygiene) containing 10 and 100 oocysts.

Secondary PCR products were purified using the QIAquick PCR purification kit (Qiagen Ltd, Valencia, CA) and eluted in Tris Buffer (10 mM Tris.Cl, pH 8.5) prior to restriction fragment analysis and sequencing to remove dNTPs, polymerases, salts and primers. For restriction fragment analysis, 20 μl of the secondary PCR product was digested in a 25-μl (total volume) reaction mixture containing 20 U of *Ssp*I (New England BioLabs, Beverly, Mass) for species diagnosis or 20 U of *Vsp*I (MBI Fermentas Inc) for genotyping of *C. parvum* and the appropriate amount of restriction buffer at 37 °C for 1 h. Digested products were fractioned on a 2.0% agarose gel and visualized by ethidium bromide staining. The patterns of DNA bands were used to differentiate the species and genotypes of *Cryptosporidium* parasites according to methodology described by Xiao et. al. (1999, 2000, 2001). Automated

sequencing was performed on the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem) of the Genomic Technology Support Facility at Michigan State University. The resulting sequences were compared with partial sequences available in the Gen Bank Data Base to identify possible matches with the sequences of the species of *Cryptosporidium* obtained from samples of reclaimed effluents used for public access irrigation.

Cell Culture Infectivity Assay

Water sample concentrates were analyzed for the presence of infectious *Cryptosporidium* oocysts using the Foci Detection Method-Most Probably Number (FDM-MPN) Assay developed by Slifko et al (1999) and modified by Gennaccaro et al (2002). Briefly, 25% of the IMS concentrate was bleach treated (8 min at room temperature) using 10.5% (vol/vol) sodium hypoclorite (Sigma) in phosphate buffered solution (pH 7.2). The samples were washed once by centrifugation and suspended in 1 mL of growth medium (RPMI 1640, Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and other additives (2% 1 M HEPES, and 1% 200 mm L-glutamine). Aliquots of this suspension were inoculated onto human ileocecal adenocarcinoma cell (HCT-8 cells ATCC CCL-244) monolayers cultivated in eight-well chamber slides (LabTech II, Nalgene Nunc, Naperville, Ill). Uninoculated cell monolayers were included on each well slide as negative controls. After 90 minutes, more growth medium was added to each well and the slides were incubated in a 5% CO_2 atmosphere at 37 °C for 48 hours. Well chamber slides were fixed with 100% methanol for 8 minutes and labeled

using the previously described indirect antibody procedure FDM (Slifko et al., 1997; Slifko et al., 1999). Staining was performed with rat anti-*C. parvum* sporozoite (Waterborne Inc., New Orleans, LA, USA) followed by a second labeling with anti rat IgG FITC (Sigma Aldrich, Inc., St. Louis, MO, USA). Chamber slides were examined under epifluorescence and differential interference contrast microscopy (DIC) and each well was scored as positive or negative for infection. The numbers of positive wells for each sample was entered into the most probable number (MPN) program (www.epa.gov/nerlcwww/mpn/htm) to determine the number of infectious oocysts per milliliter. The MPN program determined the detection limit for the assay where no infectious oocysts were observed. The concentration of infectious oocysts was expressed as number of infectious oocysts per 100 L on the basis of the equivalent volume examined.

Results

Comparison of Two DNA Extraction Procedures

Table 4.3 summarizes the results of the assessment of two methods for extraction of *Cryptosporidium* DNA from spiked milliQ water and IMS concentrates obtained from samples of reclaimed water, surface water and groundwater. Flow cytometer sorted oocysts (10 oocysts) were spiked into water concentrates and the DNA was extracted with the QIAamp DNA Mini kit and the Chelex method. A positive or negative score was given to the specific extraction procedure on the basis

Table 4.3 Summary table on the assessment of DNA extraction procedures and sensitivity of PCR techniques tested on spiked milli Q water and environmental water concentrates

DNA extraction protocol/PCR technique	Water matrix	Number of Oocysts seeded	PCR results	Average QSD quantitation
Qiagen columns				
	MilliQ water	10±2.9[b]	+	
Nested PCR	MilliQ water	10±2.9	+	
	MilliQ water	10±2.9	+	NA[c]
	MilliQ water	10±3.9	-	
	MilliQ water	10±3.9	+	
	MilliQ water	98±1.5[c]	-	
	MilliQ water	98±1.5	+	
	Reclaimed	10±2.9	-	
	Reclaimed	10±2.9	+	
	Groundwater	10±2.9	+	
	Groundwater	10±3.9	+	
	Surface	10±3.9	-	
	Surface	10±3.9	+	
Chelex method				
	MilliQ water	10±2.9	+	
Nested PCR	MilliQ water	10±2.9	+	
	MilliQ water	10±2.9	+	
	MilliQ water	10±3.9	+	NA
	MilliQ water	10±3.9	+	
	MilliQ water	10±3.9	+	
	MilliQ water	10±3.9	+	
	Reclaimed	10±2.9	+	
	Reclaimed	10±2.9	+	
	Groundwater	10±2.9	+	
	Groundwater	10±2.9	+	
	Surface	10±3.9	+	
	Surface	10±3.9	+	
Chelex method	MilliQ water	10±2.9	+	
	MilliQ water	10±2.9	+	
QSD[a]	MilliQ water	10±2.9	+	
	MilliQ water	10±3.9	+	8
	MilliQ water	10±3.9	+	

[a] Quantitative Sequence Detection
[b] Wisconsin State Laboratory Hygiene spike suspensions
[c] Not applicable

of the presence/absence of ethidium-bromide stained bands (820 bp) on agarose gel after nested PCR. The DNA extraction procedure detailed in the QIAamp DNA Mini kit yielded ethidium-bromide stained bands in five of the seven spiked milliQ water samples and in two of the six spiked IMS concentrates. DNA of *Cryptosporidium* extracted with the Chelex method produced positive PCR amplification in all of spiked milli Q water samples and IMS concentrates.

Quantitative PCR was used to confirm the number of oocysts seeded into milli Q water and IMS concentrates. The Ct values and the equation derived from the standard curve allowed the quantitation of spiked oocysts. All milli Q water samples were spiked and the DNA was extracted using the Chelex method. Five of the five spiked water samples produced positive PCR amplification with a mean density of 8 oocysts detected per reaction, which is close to the number of oocysts provided by the spike suspension. These results demonstrated that the chelex based method consistently made available DNA for PCR amplification from low numbers of oocysts seeded into natural waters.

Characterization of *Cryptosporidium* by Nested PCR-RFLP and DNA Sequencing

Table 4.4 describes the species and genotypes of *Cryptosporidium* isolates characterized at the SSU rRNA locus. Seven of the thirty-two samples (22%) analyzed by nested PCR produced positive PCR amplification with SSU rRNA specific primers (Figure 4.1). Six of the seven samples were collected from reclaimed effluents and one corresponded to a sample collected from the final effluent of a

Table 4.4 *Cryptosporidium* concentrations and genotypes isolated from water samples using IFA and nested PCR

Site	Sample	IFA counts	Oocysts per 100 L	PCR results	Species and/or Genotype[a]
Reclaimed Effluent	1	4	8	-	
	2	107	209	+	*C. parvum* genotype 2
	3	5	61	-	-
	4	5	11	-	-
	5	83	162	+	*C. parvum* genotype 2
	6	151	319	+	*C. parvum* genotypes 1 and 2
	7	26	55	-	-
	8	3	6	-	-
	9	2	8	-	-
	10	40	120	+	*C. parvum* genotypes 1 and 2
	11	26	53	+	*C. parvum* genotype 1 and 2
	12	26	104	+	*C. parvum* genotype 2
Finished	1	0	<0.25	+	*C. parvum* genotype 1
	2	0	<0.25	-	-[b]
	3	0	<0.25	-	-
	4	0	<0.25	-	-
Production well	1	0	<0.20	-	-
	2	0	<0.20	-	-
	3	0	<0.22	-	-
	4	0	<0.22	-	-
	5	0	<0.14	-	-
	6	0	<0.15	-	-
	7	0	<0.15	-	-
	8	0	<0.26	-	-
Lake Water	1	0	<20	-	-
	2	0	<22	-	-
	3	0	<12	-	-
	4	0	<22	-	-
	5	0	<44	-	-
	6	0	<25	-	-
	7	0	<15	-	-
	8	0	<26	-	-

[a]Differentiation of *Cryptosporidium* species and genotypes achieved by digestion of the secondary product with restriction enzymes (*Ssp*I and *Vsp*I) and direct sequencing of the secondary PCR product. [b] Indicate negative result

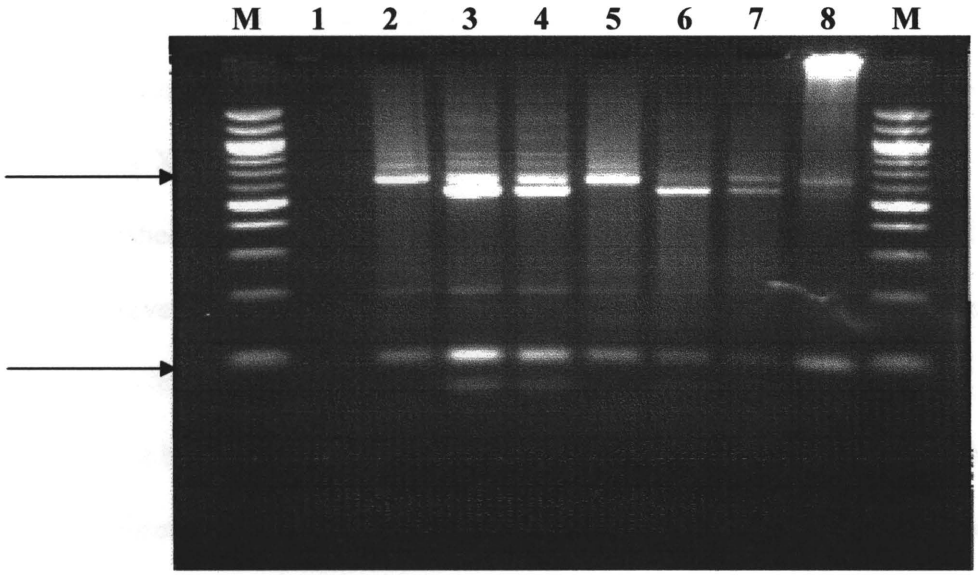

Figure 4.1 Genotyping of *Cryptosporidium* isolates in water samples using the SSU rRNA-based PCR-RFLP technique[a]

[a] Differentiation of *Cryptosporidium* genotypes was demonstrated by digestion of the secondary PCR product with *Vsp*I. Lanes 2, 5 and 8, *C. parvum* bovine genotype 2 (Reclaimed water); lanes 3, 4 and 7, *C. parvum* genotypes 1 and 2 (Reclaimed water); lane 6, *C. parvum* human genotype 1 (Finished drinking water). M, 100 bp DNA ladder. Upper and lower arrows correspond to band sizes of 600 bp and 100 bp, respectively.

drinking water treatment plant that is currently monitored for *Cryptosporidium* and *Giardia* using IFA.

Figure 4.1 shows the restriction patterns of the *Cryptosporidium* isolates obtained from reclaimed water samples and one finished drinking water sample. Restriction digestion of the secondary PCR products with *Ssp*I yielded restriction patterns (data not shown) similar to those previously described for the species *C. parvum* by Xiao et al (2001). Differentiation of *C. parvum* genotypes was accomplished through digestion with *Vsp*I. The restriction patterns obtained with this enzyme revealed the presence of the *C. parvum* human and bovine genotypes in the samples. Three of the seven samples contained both *C. parvum* genotypes 1 and 2 as determined by the presence of four bands that were similar in size to those described for the aforementioned *C. parvum* genotypes by Xiao et al. The sequence identities as determined by BlastN search for the amplicons obtained from these isolates were found to be between 91% and 100% homologous to nucleotide sequences of the species *C. parvum* (Table 4.5).

IFA vs. Nested PCR

The presence of *Cryptosporidium* oocysts was evaluated by the combination of IFA and nested PCR in order to determine the percentage of correspondence between the total number of samples positive as determined by the two methods. *Cryptosporidium* oocysts were detected in twelve of the twelve samples (100%) of reclaimed water analyzed by IFA. The oocyst counts ranged from 2 to 151 and the level of oocysts expressed by the equivalent volume examined ranged between 8 and

Table 4.5 Summary of the results obtained from the GenBank BLAST search

Sequence[a]	Identities	Species	e-score[b]	*Vsp*I[c]
1	429/431 (99%)	*C. parvum*	0.0	Genotype 2
2	458/458 (100%)	*C. parvum*	0.0	Genotypes 1 and 2
3	371/404 (91%)	*C. parvum*	e^{-150}	Genotypes 1 and 2
4	278/279 (99%)	*C. parvum*	e^{-150}	Genotype 2
5	284/284 (100%)	*C. parvum*	e^{-158}	Genotype 1
6	259/270 (95%)	*C. parvum*	e^{-123}	Genotypes 1 and 2
7	428/434 (98%)	*C. parvum*	0.0	Genotype 2
8	385/385 (100%)	*C. parvum*	0.0	Genotype 2
9	828/833 (98%)	*C. muris*	0.0	ND

[a] Sequences 1 through 6 corresspond to *Cryptosporidium* oocysts isolated from reclaimed effluents. Sequence 7 corresponds to a *Cryptosporidium* isolate obtained from finished drinking water. Sequences 8 and 9 correspond to *C. parvum* oocysts (Iowa isolate, University of Arizona) and *C. muris* oocysts purchased from Waterborne Inc.

[b] In a database similarity search, the e-score is the number of matches expected by chance between the query sequence and random or unrelated database sequences from a database of the size used.

[c] Restriction patterns obtained with the enzyme *Vsp*I are depicted on Figure 4.1

319 oocysts/100 L, respectively. With nested PCR, six of the twelve samples of reclaimed water (50%) were positive for *Cryptosporidium*. Oocyst counts that produced positive PCR amplification were equal to or greater than 26 oocysts, which corresponded to levels of 53 and 319 oocysts/100 L, respectively. Four samples of finished drinking water were negative for *Cryptosporidium* oocysts by IFA, however one sample contained oocysts of the species *C. parvum* as determined by the band patterns obtained after RFLP and sequencing of the secondary PCR product. Based on these results, 58% correspondence was found between the number of samples positive as determined by IFA and nested PCR.

Cell Culture Assay for Testing Potential Infectivity of *Cryptosporidium* Oocysts

The FDM-MPN assay allowed the detection and quantification of infectious oocysts in 6 of 24 (25%) of the samples analyzed. The samples that contained infectious oocysts were all collected from reclaimed effluents (Table 4.6). Neither the production wells (groundwater sources) nor the lakes were positive for infectious *Cryptosporidium* oocysts. Table 4.6 summarizes the result of the IFA counts, the concentration of oocysts (oocysts/100 L) detected in reclaimed effluents and the recovery rates of oocysts obtained from duplicate samples. Pre-stained (oo)cysts from ColorSeed C&G spike suspensions were included as internal quality controls to differentiate spiked from indigenous (oo) cysts. The numbers of indigenous oocysts detected by IFA were adjusted to the recovery rates as well as the number of infectious oocysts detected by the FDM-MPN assay.

Table 4.6 Occurrence of infectious *Cryptosporidium* oocysts in samples of reclaimed water, groundwater and lake water

Sample type	IFA[a] results				Infectious oocysts MPN/100 L[e]
	No. oocysts	%RE[b]	Levels[c]	Adjusted Levels[d]	
Reclaimed					
Facility 1	4	-[f]	8	-	<1.3
	107	31	209	674	8 **(26)** (0.8 , 22)
	5	22	61	277	<2.5
	5	-	11	-	<1.5
Facility 2	83	29	162	286	5 **(17)** (0.3 ,12)
Facility 3	151	12	319	1258	2 **(17)** (0.4 , 8)
	26	27	55	204	<3.2
Facility 4	3	8	6	75	<1.8
	2	5	8	160	<2.5
	40	15	120	800	4 **(27)** (0.5 , 25)
	26	-	53	-	7.3 (0.8 – 16.9)
	26	-	104	-	18.4 (5.5 – 41.4)
Groundwater	0	62	<0.20	NA[g]	<11
	0	-	<0.22	-	<8
	0	-	<0.22	-	<9
	0	80	<0.22	NA	<8
	0	-	<0.14	-	<10
	0	85	<0.15	NA	<12
	0	-	<0.15	-	<15
	0	-	<0.26	-	<7
Lakes					
Jackson	0	31	<20	NA	<10
Jackson	0	47	<12	NA	<8
Starvation	0	39	<11	NA	<9
Starvation	0	29	<14	NA	<8

[a]Immunofluorescence assay
[b] Recovery efficiency
[c] oocysts/100 L
[d] Adjusted levels based on recovery efficiency
[e] Most Probable Number/100 L
[f] Not done
[g] Not applicable
The numbers in bold correspond to levels of infectious oocysts adjusted to recovery efficiency

Cryptosporidium IFA counts between 26 and 152 oocysts produced foci in HCT-8 cells, which corresponded to the numbers of oocysts that produced positive PCR amplification by nested PCR. These oocyst counts occurred in 50% of the IMS concentrate; the remaining 50% was further divided into two aliquots and used for nested PCR and tissue cell culture. Since 25% of the IMS concentrate obtained from these samples could potentially hold between 5 and 10 oocysts, the results of this study would suggest that a minimum of DNA equivalent to approximately 5 - 10 oocysts are required for molecular characterization of *Cryptosporidium* isolates and assessment of oocyst infectious potential using tissue cell culture.

Discussion

In this study, two DNA extraction procedures were evaluated in different water matrices in order to provide information on the efficacy and consistency of *Cryptosporidium* DNA extraction methods for sensitive detection of waterborne oocysts using nested PCR. The efficacy of the DNA extraction procedures was assessed by spiking water sample concentrates with low concentrations of *Cryptosporidium* oocysts followed by nested PCR and further visualization of ethidium bromide stained bands in agarose gels. The results showed that DNA extracted with the chelex method was less prone to contain PCR inhibitors as determined by the number of spiked milliQ water and natural samples that produced positive PCR amplification. DNA extraction with the QIAamp DNA Mini kit was found to be less efficient than the chelex method in providing *Cryptosporidium* DNA for subsequent PCR amplification. This method of extraction requires more

preparation time as well as several centrifugation steps that might result in DNA losses. The amount of starting DNA (DNA yield), however, was not estimated in this study.

Matrix-derived factors carried through the DNA isolation and purification procedure might have also been responsible for the inhibition in PCR amplification. Humic acids have been demonstrated to be one of the most important inhibitors for PCR amplification of *Cryptosporidium* DNA (Sluter et al., 1997).

Efficient and consistent DNA extraction procedures are required for PCR amplification of *Cryptosporidium* DNA from environmental samples due to the nature of the organism, its resistance to disruption and lysis, and the matrix in which the protozoa is present (Orlandi and Lambell, 2000). IMS is the most common technique used for selective separation of waterborne oocysts from environmental debris prior to DNA extraction and PCR amplification in order to minimize and/or remove the contaminants that inhibit PCR enzymes and other enzymes required for molecular analysis (Johnson et al., 1995; Di Giovanni et al., 1999; Hallier-Soulier and Guillot, 1999; Kostrzynska et al., 1999). Freeze-thaw disruption in the presence of Chelex 100 has been demonstrated to be an efficient and feasible technique for providing *Cryptosporidium* DNA for subsequent molecular characterization (Johnson et al., 1995; Di Giovanni et al, 1999). The chelex-based method provides certain advantages over several other methods used for extracting DNA from complex matrices: (i) Chelex sequesters divalent heavy metals that would otherwise introduce DNA damage, (ii) the boiling treatment with Chelex is useful for releasing DNA from

low numbers of cells, and (iii) Chelex protects the extracted DNA from the effects of boiling (Walsh et al., 1991; Hallier-Soulier and Guillot, 1999).

The results of the present investigation demonstrate the feasibility of the oocyst purification and DNA extraction procedures for the subsequent assessment by molecular techniques of the infectious potential of *Cryptosporidium* oocysts isolated from natural waters. This type of information is highly important for reclaimed waters used for unrestricted irrigation since recent implementation of periodic sampling for protozoan pathogens to current reuse rules in the state of Florida (Chapter 62-610 of the Florida Administrative Code, Florida Department of Environmental Protection, 1999) requires not only the enumeration of the parasite in the final effluent but also the characterization of the species of public health concern and the assessment of potential oocyst infectivity.

Nested PCR provides the sensitivity and reproducibility required for rapid screening of large numbers of samples and several of these assays have been described for detection of waterborne *Cryptosporidium* oocysts (Mayer and Palmer, 1996; Xiao et al.,1999; Monis and Saint, 2001; Sturbaum et al., 2001). The SSU rRNA-based nested PCR-RFLP technique developed by Xiao et al (1999, 2000, 2001) has the advantage over other molecular techniques of detecting and differentiating all known *Cryptosporidium* spp. and divergent *C. parvum* parasites from various animals. This technique was used in the present investigation along with IFA and tissue cell culture to evaluate the occurrence of infectious waterborne *Cryptosporidium* oocysts in various water matrices.

C. parvum was the predominant species isolated from samples of reclaimed water, which is congruent with the environmental setting (urban) and sampling location (reclamation facilities in a major metropolitan area). The identification of *C. parvum* oocysts by nested PCR in finished drinking water indicates a potential breakthrough in the water treatment process. The effluent of this drinking water facility is monitored periodically for protozoan parasites using IFA and *Cryptosporidium* oocysts have been occasionally detected at very low concentrations (0.3/100 L) as determined by IFA. These results suggest that *Cryptosporidium* monitoring programs that combine conventional assays (IFA) with advanced molecular fingerprinting techniques provides a better approach for assessing the occurrence and persistence of *Cryptosporidium* contamination as well as the efficacy of drinking water treatment practices.

Previous investigations have shown that the FDM-MPN method is an excellent and reproducible assay for quantifying oocyst infectivity in vitro (Slifko et al., 1999). The results of this and an additional study (Gennaccaro et al., submitted) demonstrate the sensitivity of the cell culture infectivity assay for determining low numbers of infectious oocysts in reclaimed effluents. The infectivity measured in HCT-8 cell cultures, however, is prone to variability and the percent oocyst infectivity in our laboratory can range between 0.3% and 40%. Among others, variations in infectivity within the same *Cryptosporidium* genotype have been reported (Slifko et al., 1999; Chapell et al., 1999; DiGiovanni et al., 1999; Rochelle et al., 2002). Therefore, it is expected to find that the *Cryptosporidium* isolates obtained from reclaimed effluents may show the same degree of variability in infectivity.

Rochelle et al. (2002) indicated that incubation of oocysts in 0.75% sodium taurocholate prior to inoculation of cell monolayers resulted in increased infectivity in cell cultures. The application of this approach to maximize the sensitivity of the assay and determine the potential infectivity of naturally occurring *Cryptosporidium* isolates needs further research. Notwithstanding and despite the variations in recovery efficiency and infectivity in cell culture, the results of this investigation revealed the potential application of current and emerging *Cryptosporidium* detection methods for ascertaining sources and occurrence of this protozoa under the most stringent sampling and analysis requirements outlined in the IESWTR and Total Maximum Daily Load (TMDL) criteria.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Are Current and New Detection Methods Reliable to Determine the Occurrence of Waterborne *Cryptosporidium*?

During the course of this investigation several method components of current and new methods for detection of waterborne *Cryptosporidium* were assessed. The filtration performance of the Envirochek capsule filters (method 1623) and the polypropylene cartridge filter (modified ICR protozoan method) was evaluated in different water matrices in order to determine feasible application of either filter for continuous and reliable monitoring of *Cryptosporidium*. The purification step using IMS was subjected to slight modifications in order to provide quantitative information on the limits of detection sensitivity of the different assays. The detection of oocysts was evaluated through epifluorescence microscopy incorporating a new monoclonal antibody that target specific antigens on the surface of *Cryptosporidium* oocysts. Information on the potential infectivity of the isolates was provided by the SSU rRNA-based PCR-RFLP technique and the FDM-MPN assay.

As discussed previously, the use of the appropriate filtration system is critical in the analysis of waterborne *Cryptosporidium* since it is the initial step involved in the concentration of the oocysts. In this context, the volume of water achievable with a

specific filter may have important implications in the sensitivity and reliability of subsequent steps aimed at the detection and identification of the organism. Sufficient numbers of oocysts are required in order to determine the concentration of oocysts in the sample, the infectivity of the isolates and the species of public health significance. The concentration of oocysts is determined using epifluorescence microscopy while the potential infectivity and species of public health significance required more advanced techniques such as cell culture infectivity assays and PCR methods. The application of efficient concentration schemes and sensitive detection methodologies are therefore required for adequate monitoring of *Cryptosporidium* in the environment.

The results of this study suggest that the Envirochek HV capsule filter is the best choice among the other filters for initial concentration of waterborne oocysts as determined by the recovery rates obtained with spiked natural waters and the number of indigenous waterborne oocysts detected after filtration, concentration, purification and IFA. The HV filter incorporates a new track-etched membrane with a unique pore structure that allows sampling of high volumes of water. Such large volumes of water are required for recovering low numbers of oocysts that may be present in natural waters or highly treated water in the absence of conventional microbial indicators used to assess the efficiency of the treatment process. It is well known that protozoan parasites such as *Cryptosporidium* are more resistant than the indicator bacteria to removal and inactivation by conventional drinking water treatment processes. The United States Environmental Protection Agency's "Interim Enhanced Surface Water Treatment Rule" (IESWTR) stipulates zero as the goal for the maximum contamination level of protozoan parasites in water. Key provisions in the IEWSTR establish a Maximum Contaminant

Level Goal (MCLG) of zero for *Cryptosporidium* and require a 2 – $\log_{10}$ *Cryptosporidium* removal when using filtration only. Through application of efficient concentration methods and sensitive detection techniques compliance with monitoring requirements can be assured.

The polypropylene cartridge filter allowed the filtration of equivalent and even higher volumes of water than the HV filter, however the recovery efficiency of oocysts was consistently low as well as the number of indigenous oocysts detected. The standard Envirochek capsule filter has been designed for filtration of ≤10 L of water.

The introduction of EasyStain into the immunofluorescence assay helped to improve the sensitivity of oocyst detection as determined by the reduction in the level of background fluorescence and non-specific binding observed when evaluating samples of surface water (seawater and freshwater) and reclaimed water containing high levels of microalgae. Even though species other than *C. parvum* can be detected with this antibody, the specificity of the assay is improved with the elimination of fluorescence from non-target organisms. This is an improvement over the current available monoclonal antibodies recommended in method 1623 for evaluating the occurrence of waterborne *Cryptosporidium* and *Giardia*.

The results of this study suggest that the limits of detection sensitivity for the nested PCR and tissue cell culture assay may range between 5 and 10 oocysts. These numbers of oocysts were present in a small percentage of the water concentrate (25%) obtained after filtration-elution, centrifugation and purification by IMS.

Regardless of the low and variable oocyst recovery rates obtained with the HV filter in samples of reclaimed water, the number of oocysts provided with method 1623 and the HV filter was adequate for assessing the infectious potential of *Cryptosporidium* oocysts, which is highly significant for better public health assessment of treated wastewater used for unrestricted irrigation.

The incorporation of method 1623 with the HV filter along with nested PCR-RFLP revealed the presence of the species *C. parvum* in the effluent of a drinking water facility that had been consistently negative for *Cryptosporidium* using the conventional immunofluorescence assay, thereby indicating that the inclusion of molecular genotyping techniques into current monitoring programs provide better assessment on pathogen occurrence and operating conditions at the facility (i.e., filtration) that lead to breakthrough of the organism.

Further evaluation of new filtration devices (hollow-fiber ultrafilter, Filta-Max) for recovery of waterborne oocysts would help to determine the feasibility of incorporating novel concentration methods as efficient as the Envirochek HV filter into current monitoring programs. The advantage of the HV filter over the hollow fiber ultrafilter and Filta Max filter is that it is completely disposable, thereby extremely useful in the field where assembling housings and cleaning filter holders after each test can lead to cross contamination.

The rapid and accurate identification and enumeration of waterborne parasites remains an ambitious goal in environmental microbiology. Even though standard methodologies are currently available for detection of *Cryptosporidium* in environmental

waters, alternative methods, which are simpler, more efficient and reliable, are still being evaluated. Standard approaches are needed for other emerging parasites such as *Cyclospora*. Extremely sensitive molecular techniques, which are already available, are particularly important for low level of detection, and identification of species and source of contamination. Still the most sensitive concentration and detection techniques required to assess virulence and viability/infectivity of waterborne parasites are either expensive or not practical for routine monitoring of waterborne parasites. Therefore, investments in new techniques for rapid, sensitive and specific detection of microbial pathogens in water are needed. In addition, the development of a consensus approach for applying PCR methods to environmental samples is needed. Finally, application of these methods to a variety of environs for developing occurrence databases will be the ultimate test.

What is the level of microbial contamination within Section 21 Wellfield? Is Rehydration with reclaimed water and stormwater feasible?

The results of the microbiological monitoring program of Section 21 Wellfield Pilot Rehydration Project suggest that between the two proposed source waters for restoration, water from the Interceptor Canal had the highest detection of microbial indicators and pathogens. The reclaimed water had the lowest level of microbial contaminants.

Stormwater is expected to hold high concentration of microbial constituents since fecal droppings of pets (dogs, cats) birds and other animals that occur on land can be washed into storm drains by rain, increasing the concentrations of fecal indicators and

enteric pathogens in water sources. Therefore, the potential use of stormwater as a source for rehydration can be compromised due to the presence of high levels of fecal contamination. This issue will be considered in the risk assessment plan of study.

The acceptability of reclaimed water for any particular use is dependent on the physical, chemical, and microbiological quality of the water. Factors that affect the quality of reclaimed water include source water quality, wastewater treatment processes and treatment effectiveness, treatment reliability, and distribution system design and operation (Crook and Surampalli, 1996). The presence of pathogenic microorganisms in wastewater creates the potential for adverse health effects where there is contact, inhalation, or ingestion of microbiological constituents of health concern. The criteria established for the discharge of reclaimed water to and from receiving wetlands (rule 62-611, F.A.C.) do not specify any criteria for microbiological constituents of health concern. Therefore, the recommended threshold established for reuse activities in the state of Florida was used to evaluate the adequacy of reclaimed water from the storage tanks of the DM Wastewater treatment plant for wetland and lake rehydration. The threshold limit for reuse activities were all met. However, low levels of infectious enteroviruses and protozoan parasites were found in these samples. As a result, these microorganisms were chosen as microbial constituents of concern for the risk assessment plan of study.

Among the lakes, Lake Starvation was positive for all enteric pathogens (*Cryptosporidium*, *Giardia* and enteroviruses) with levels of *Cryptosporidium* as high as 112.7 oocysts/100 L. Lake Simmons had the highest percent detection and levels of infectious enteroviruses.

Eleven groundwater samples from the production wells at thee Section 21 Wellfield were analyzed for enteroviruses, of which four were positive. Of the 11 production well samples analyzed for protozoa, *Giardia* was detected once at well 21-2, and *Cryptosporidium* was detected once at well 21-6. Although levels of protozoan parasites (*Cryptosporidium* and *Giardia*) were consistently lower than the lake samples, the levels of enteroviruses were similar to those present in the lakes.

This study did not attempt to determine the source of fecal-derived organisms to the groundwater production wells at the Section 21 Wellfield, although some land use activities at the site may be suspects, including equestrian activities. A number of newer tools (bacterial source tracking, coliphage enrichment procedure followed by genotyping and direct pathogen monitoring for viruses and parasites including speciation) are available. The application of these tools at Section 21 Wellfield will help to better determine and define microbial water quality and help to estimate any potential public health risks.

In order to undertake the microbial risk assessment, the actual waterborne pathogens that cause disease in humans are examined. The assessment of microbial indicators is used to look at overall bacteriological quality and the potential for fecal contamination. The direct relationship of these indicators to public health risks and health effects has not been shown. For purposes of evaluating the potential human health risks associated with the restoration project, two of the major groups of waterborne pathogens of concern (protozoan and viruses) were addressed.

As mentioned before, *Cryptosporidium* was monitored during the water quality sampling performed for the Section 21 Wellfield Restoration Project and was found in Section 21 Wellfield more often and at a higher prevalence than *Giardia*. *Cryptosporidium* oocysts are smaller (4 to 6 μm in diameter) than *Giardia* cysts. In addition, the oocysts are resistant to chlorination. Therefore, *Cryptosporidium* was selected as a conservative microorganism over *Giardia* for further evaluation in the risk assessment plan of study.

Limitations in using *Cryptosporidium* for the risk assessment plan of study must be considered. Several methods exist for *Cryptosporidium*, including the ICR method (USEPA, 1995) and method 1623 (USEPA, 1999, 2001), however the matrix components present in environmental waters may affect the efficiency of the methods causing low and variable oocyst recovery rates. Additionally, speciation or viability of the detected oocyst cannot be made using either cited methods. This means that the bird species of *Cryptosporidium* that does not cause disease in humans cannot be distinguished from the mammalian species that is pathogenic to humans. These uncertainties can possible lead to an overestimation of the risk as the assumption is usually made that all oocysts detected have the potential to cause disease in humans and are viable and infectious.

The enteric viruses were also included as pathogenic agents of concern for the risk assessment plan of study because: (i) They are small in size and have the ability to migrate to the groundwater as they move readily through the subsurface soils; (ii) They cause numerous diseases with acute impacts (diarrhea) and also chronic diseases (myocarditis); (iii) They have a low infectious dose, which means exposure to very few

viruses can cause disease; (iv) Their presence and risk are not correlated to typical indicator bacteria.

Impacts to groundwater by viruses are of great concern due to the resistant nature of the viral structure and the colloidal size (20 nm), which makes this group of microorganisms easily transported through soil systems (Gerba and Bitton, 1984). Viruses can also survive several months in groundwater and are more resistant to water disinfection than are the coliforms (Gerba and Rose, 1985; Yates and Yates, 1988). Studies in the United States have found viruses in 20% to 30% of the groundwater where coliforms were not predictive of viral contamination (Abbaszadegan et al., 1993). New techniques using PCR have shown that there is much more viral occurrence in groundwater than previously recognized (Borchardt et al. 2003).

Enteroviruses can be found in wastewater and are removed to various levels depending on the treatment (Rose et al., 1996). The enteroviruses were monitored during the Section 21 Wellfield Restoration project using standard cell culture techniques, which allows for determination of infectivity and further identification if necessary. The treatment provided to this groundwater is adequate to inactivate these viruses.

The occurrence of protozoan parasites and enteroviruses in groundwaters indicated: (i) that there is some low naturally occurring level of microorganisms within the Wellfield resulting in an ambient level of risk exposure to pathogens, (ii) there is some level of vulnerability of groundwater to microbiological contamination.

The general hydrology of the Section 21 Wellfield consists of the surficial aquifer and the Upper Floridan aquifer separated by a clay semi-confining unit. The production

wells at Section 21 Wellfield are approximately 400 to 600 feet deep and withdraw water from the Upper Floridan aquifer. Several investigations have demonstrated that microbial contaminants may enter the subsurface directly via structures which by-pass the soil zone such as septic tanks. There are some septic tanks systems operating within the Wellfield and all of them are located far from the production wells where *Cryptosporidium*, *Giardia* and enteroviruses were found. *Cryptosporidium* oocysts are spherical particles 4 to 6 μm in diameter, with a specific gravity of 1.07 g cm^3 (Current, 1987). Like many microbes, *Cryptosporidium* oocysts have been classified as colloidal particles. Recent investigations have shown that *Cryptosporidium* oocysts entrained in surface or subsurface water will travel at the same velocity as the water (Brush et al., 1999). An understanding of the mechanisms governing waterborne transport of oocysts is needed to develop management practices that reduce or eliminate oocysts migration from the source of contamination (fecal deposition in the soil surface or structures which by-pass the soil zone such as septic tanks) and to facilitate assessment of aquifer's vulnerability to contamination with oocysts.

The results of additional testing carried out in four active production wells (21-5, 21-8, 21-9, 21-10) to determine if groundwater sources in the wellfield are under direct influence from surface water systems (Microscopic Particulate Analysis) have indicated that the production wells may have either no risk or moderate risk of surface water contamination.

Model-derived travel times to the production wells and dilution factors will be used in the exposure assessment for microbial risks. However, the limited water quality sampling results to date suggest that there may be some low, naturally occurring level of

microorganisms in the groundwater resulting in an ambient level risk of exposure to pathogens. The two *Cryptosporidium* oocysts found in well 21-6 suggest vulnerability as it was mentioned before, even if these oocysts are not viable or a strain that is not infectious to humans. Depending on the average concentration of oocysts in groundwaters, this ambient risk could outweigh future risks due to the small percentage of source waters (3.3 percent) migrating to the production wells. Due to limited number of samples and uncertainties in computing average *Cryptosporidium* concentrations, additional characterization of the ambient water quality is necessary prior to completing the microbial risk assessment.

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