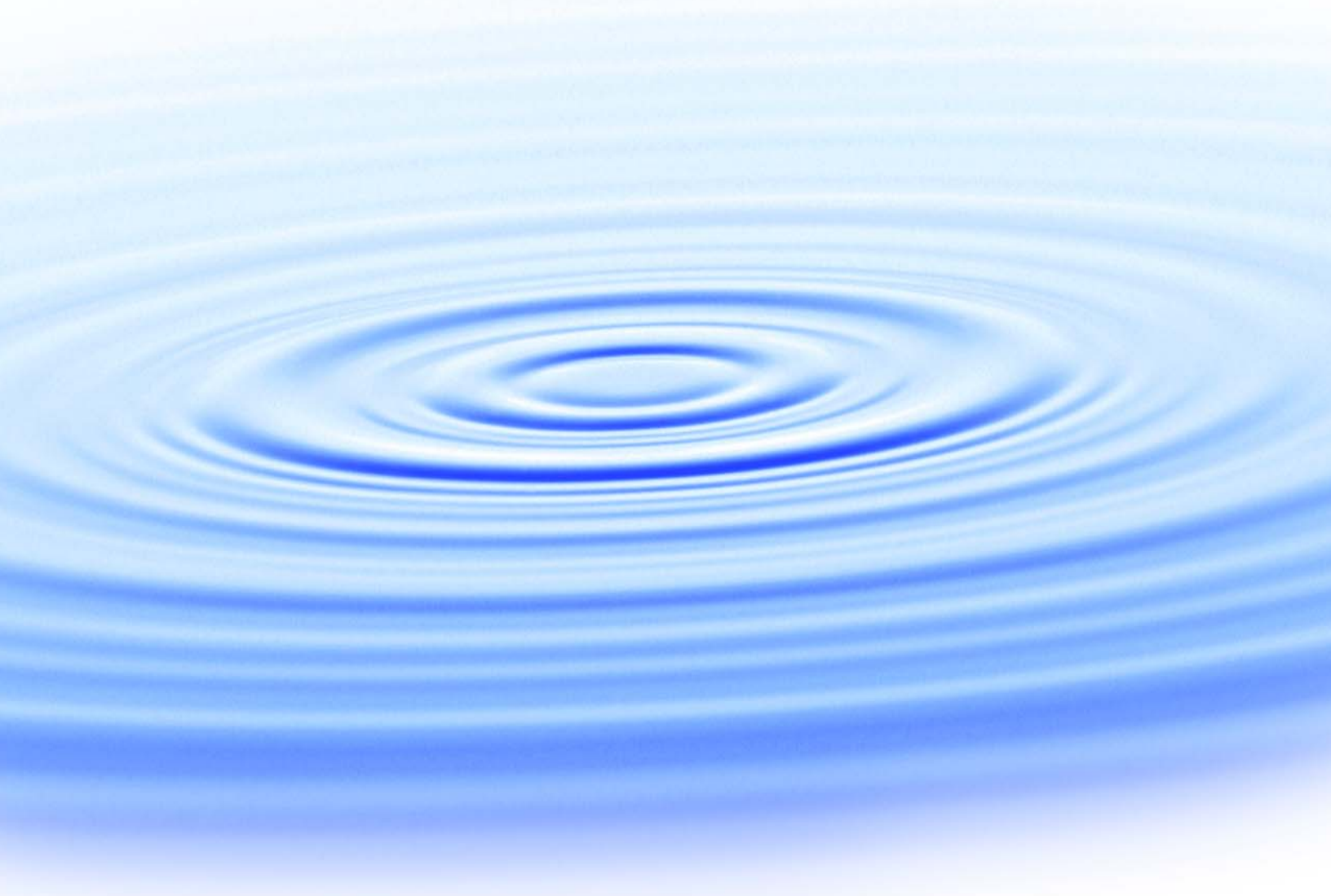
Determination of *Cryptosporidium* and *Giardia* Occurrence, Infectivity, and Genotyping in Wastewater Effluents



WateReuse Research Foundation

Determination of *Cryptosporidium* and *Giardia* Occurrence, Infectivity, and Genotyping in Wastewater Effluents

About the WateReuse Research Foundation

The mission of the WateReuse Research Foundation is to conduct and promote applied research on the reclamation, recycling, reuse, and desalination of water. The Foundation's research advances the science of water reuse and supports communities across the United States and abroad in their efforts to create new sources of high-quality water through reclamation, recycling, reuse, and desalination while protecting public health and the environment.

The Foundation sponsors research on all aspects of water reuse, including emerging chemical contaminants, microbiological agents, treatment technologies, salinity management and desalination, public perception and acceptance, economics, and marketing. The Foundation's research informs the public of the safety of reclaimed water and provides water professionals with the tools and knowledge to meet their commitment of increasing reliability and quality.

The Foundation's funding partners include the Bureau of Reclamation, the California State Water Resources Control Board, the California Energy Commission, and the California Department of Water Resources. Funding is also provided by the Foundation's subscribers, water and wastewater agencies, and other interested organizations.

Determination of *Cryptosporidium* and *Giardia* Occurrence, Infectivity, and Genotyping in Wastewater Effluents

Zia Bukhari, Ph.D.
Mark W. LeChevallier, Ph.D.
American Water

Giovanni Widmer, Ph.D.
Tufts University School of Veterinary Medicine

Udi Zuckerman, Ph.D.
Mekerot Water

Cosponsor
Bureau of Reclamation

WateReuse Research Foundation
Alexandria, VA

Disclaimer

This report was sponsored by the WateReuse Research Foundation and cosponsored by Bureau of Reclamation. The Foundation, its Board Members, and the project cosponsors assume no responsibility for the content of this publication or for the opinions or statements of facts expressed in the report. The mention of trade names of commercial products does not represent or imply the approval or endorsement of the WateReuse Research Foundation, its Board Members, or the cosponsors. This report is published solely for informational purposes.

For more information, contact:

WateReuse Research Foundation
1199 North Fairfax Street, Suite 410
Alexandria, VA 22314
703-548-0880
703-548-5085 (fax)
www.WateReuse.org/Foundation

WateReuse Research Foundation Project Number: WRF-06-003
WateReuse Research Foundation Product Number: 06-003-1

ISBN: 978-1-934183-57-1
Library of Congress Control Number: 2012948927

Printed in the United States of America

Printed on Recycled Paper

Contents

Figures

Tables

Acronyms

AHBSS	acidified Hanks' balanced salt solution
AR	annual risk
ATCC	American Type Culture Collection
AW	American Water
BOD	biochemical oxygen demand
BSA	bovine serum albumin
CBOD	carbonaceous biochemical oxygen demand
CC-IFA	cell culture–immunofluorescence assay
CC-PCR	cell culture–polymerase chain reaction
CO_2	carbon dioxide
COWP	*Cryptosporidium* oocyst wall protein
CT	concentration × time
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4′,6-diamidino-2-phenylindole
DI	deionized
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DPD	*N*,*N*-diethyl-*p*-phenylenediamine
DR	daily risk
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GDH	glutamate dehydrogenase
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HCT-8	human ileocecal adenocarcinoma
ICR	Information Collection Rule
IFA	immunofluorescent antibody
IgG	immunoglobulin G
IgM	immunoglobulin M
IMS	immunomagnetic separation
LPM	liter per minute
LT2ESWTR/LT2	Long-Term II Enhanced Surface Water Treatment Rule
MBR	membrane bioreactor
MGD	million gallons per day
MGW	molecular grade water
mJ	millijoule

mL	milliliter
MLE	modified Ludzack–Ettinger
MPC	magnetic particle concentrator
MRA	microbial risk assessment
NaOH	sodium hydroxide
NGS	normal goat serum
NH_3	ammonia
NTU	nephelometric turbidity unit
O_3	ozone
PBMS	performance-based measurement system
PBS	phosphate buffered saline
PCFC	portable continuous-flow centrifuge
PCR	polymerase chain reaction
POTW	publicly owned treatment works
QA / QC	quality assurance/quality control
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
SBR	sequencing batch reactor
SD	standard deviation
TNTC	too numerous to count
TOC	total organic carbon
TPI	triosephosphate isomerase
TSS	total suspended solids
UF	ultrafiltration
µL	microliter
U.S. EPA	U.S. Environmental Protection Agency
USDA	U.S. Department of Agriculture
UV	ultraviolet
WSLH	Wisconsin State Laboratory of Hygiene
°C	degrees Celsius

Foreword

The WateReuse Research Foundation, a nonprofit corporation, sponsors research that advances the science of water reclamation, recycling, reuse, and desalination. The Foundation funds projects that meet the water reuse and desalination research needs of water and wastewater agencies and the public. The goal of the Foundation's research is to ensure that water reuse and desalination projects provide high-quality water, protect public health, and improve the environment.

An Operating Plan guides the Foundation's research program. Under the plan, a research agenda of high-priority topics is maintained. The agenda is developed in cooperation with the water reuse and desalination communities including water professionals, academics, and Foundation subscribers. The Foundation's research focuses on a broad range of water reuse research topics including:

- Defining and addressing emerging contaminants
- Public perceptions of the benefits and risks of water reuse
- Management practices related to indirect potable reuse
- Groundwater recharge and aquifer storage and recovery
- Evaluation and methods for managing salinity and desalination
- Economics and marketing of water reuse

The Operating Plan outlines the role of the Foundation's Research Advisory Committee (RAC), Project Advisory Committees (PACs), and Foundation staff. The RAC sets priorities, recommends projects for funding, and provides advice and recommendations on the Foundation's research agenda and other related efforts. PACs are convened for each project and provide technical review and oversight. The Foundation's RAC and PACs consist of experts in their fields and provide the Foundation with an independent review, which ensures the credibility of the Foundation's research results. The Foundation's Project Managers facilitate the efforts of the RAC and PACs and provide overall management of projects.

Treatment of drinking water was once considered adequate for reducing risk of infection from pathogenic organisms. However, with the increased need for water reuse, attention has focused on the potential of wastewater treatment processes to reduce pathogenic organisms to acceptable levels. The objective of this project was to accrue information to better understand the occurrence of *Cryptosporidium* in reuse effluents from plants employing various upstream treatment processes (i.e., secondary clarification, cloth filtration, sand filtration, and membrane bioreactors). This information is pertinent to development of future risk assessment models for human cryptosporidiosis that could arise from exposure to reuse effluents. To ensure that these data would facilitate development of robust risk assessment models, key parameters for inclusion in the model need to be organism concentration, infectivity, and species.

Joseph Jacangelo
Chair
WateReuse Research Foundation

G. Wade Miller
Executive Director
WateReuse Research Foundation

Acknowledgments

This study was funded by the WateReuse Research Foundation in cooperation with the Bureau of Reclamation. Completion of this project would not have been possible without the dedicated support of many individuals. The research team greatly appreciates their assistance. Specifically we thank Dr. Nkinin Stephenson for the initial cell culture–infectivity assays, and Yousef Ghotok, Mathew Lutz, and Ryan Tuck (interns) for support in the bench-scale disinfection studies, coordination of sampling, and data collection from the analytical laboratories. William Johnson performed the *Giardia* assemblage characterization work, and his support is gratefully acknowledged.

The research team gratefully acknowledges the support provided by Rita Scanlon, Allison Hill, and Bill Deckelmann (American Water Belleville Laboratory, Belleville, IL) for the LT2 analysis. Rubin Bonilla Santiago and Julia Dilo (Tufts Cummings School of Veterinary Medicine) are thanked for conducting the in vitro infectivity and genotyping analysis.

The research team would like to express their sincere gratitude to the wastewater utilities and their staff, especially the following:

Jim Huntington, Edward Kwityn, and Chris Wilson (NJ), Steve Curtis, Jim Schilling, and Keith Koziatek (NJ/NY), John Tekula (NY), Bill McGinty (PA), Darrel Graham (NC), Karl Merbach (IA), Tim Lantz, John Stein, Tom Chinske, and Steve Phillips (IL), Jeffery Marlow and Paul Cornejo (AZ), and Bruce Zhang and Augustine Sandoval (HI).

Without the cooperation and hard work of these individuals, this project would not have been feasible.

This study was managed by Julie Minton and Burnett King (WateReuse Research Foundation) and conducted under the guidance of the Project Advisory Committee (PAC): Walter Jakubowski (WaltJay Consulting), Dr. Ron Fayer (USDA), Michael Ware (U.S. EPA), Dr. Rhodes Trussell (Trussell Technologies), and Yuliana Porras (Bureau of Reclamation). The project team is truly grateful for the support provided by these individuals.

Executive Summary

Treatment of drinking water was once considered adequate for reducing risk of infection from pathogenic organisms; however, with the increased need for water reuse, attention has focused on the potential of wastewater treatment processes to reduce pathogenic organisms to acceptable levels. A public health concern with reusing wastewater is the risk of transmitting infectious agents originating from human and animal feces. Depending upon the diseases present in the contributing communities, wastewaters may contain various pathogenic organisms including viruses, bacteria, helminths, and protozoa. Protozoan parasites of major health significance to humans include *Entamoeba histolytica*, *Giardia intestinalis* (synonymous with *G. lamblia* and *G. duodenalis*), and *Cryptosporidium* species (Straub et al., 1993).

To monitor *Cryptosporidium*, an intensive sampling program was launched for 14 geographically dispersed wastewater utilities over a 12-month period, with 8 utilities employing secondary clarification, 2 using sand filtration, 3 employing membrane bioreactors (MBRs), and 1 using cloth filtration. Sampling was designed to measure *Cryptosporidium* concentrations, infectivity, and genotype information after physical removal (i.e., predisinfection) and immediately postdisinfection. Method 1623 was used for sampling, with specific organism isolation using immunomagnetic separation (IMS) followed by immunofluorescence assay (IFA)-based detection and enumeration. As Method 1623 allows *Giardia* enumeration, monitoring of this protozoan organism was also included. During the sampling campaign, quality assurance was performed at the frequency defined in the Long Term II Enhanced Surface Water Treatment Rule, and matrix spiking indicated that overall recoveries with Method 1623 were highly variable for *Cryptosporidium* (6.9–75%) and *Giardia* (7.9–90%). Mean recoveries were lower in effluents from plants employing secondary clarification or sand filtration than in those from plants using cloth filtration or MBRs.

Eight plants employing secondary treatment were frequently positive for *Cryptosporidium* (20–66.7%) and *Giardia* (83–100%). One of the two plants employing sand filtration demonstrated that 8.3% of the postdisinfection samples were *Cryptosporidium*-positive. *Giardia* cysts were detected at both sand filtration plants, with one plant demonstrating a higher occurrence (i.e., predisinfection, 33.3%; postdisinfection, 50%) than the second plant (8.3% of samples positive). Of three MBR plants, only one plant demonstrated *Cryptosporidium*-positive samples (postdisinfection, 8.3%). In contrast, two of the three MBR plants contained 58.3–83.3% *Giardia*-positive samples. Effluents from one plant employing cloth filtration were positive for both *Cryptosporidium* and *Giardia*. The predisinfection samples for this plant contained a substantially higher frequency of *Giardia*-positive samples (66.7%) than of *Cryptosporidium*-positive samples (33.3%); however, 16.7% of the postdisinfection samples were positive for both organisms. Overall, 21.9% (73 of 333) of the samples were *Cryptosporidium*-positive and 70.1% (234 of 333) of the samples were positive for *Giardia* by IFA. Comparing protozoan concentrations with various water quality parameters (i.e., turbidity, total suspended solids, ammonia, chlorine, fecal coliforms, dissolved oxygen, pH, and carbonaceous biochemical oxygen demand) indicated no obvious correlation.

In addition to occurrence and concentrations, it is necessary to examine the infectivity and genotyping information of the recovered oocysts and cysts to understand the public health significance of the protozoa detected in wastewater effluent samples. Although no suitable in vitro infectivity assays for *Giardia* cysts exist, a previously validated cell culture–IFA (CC-IFA) procedure was used to measure infectivity of *Cryptosporidium* oocysts and indicated that 6.3%

(18 of 285) of the samples were potentially infectious. The majority of the CC-IFA positive samples (n = 15) were derived from treatment plants employing secondary clarification. The infectivity data also demonstrated that infectious oocysts were detectable postchlorination; however, no infectious oocysts were detected following UV disinfection.

Molecular detection of *Cryptosporidium* utilized a polymerase chain reaction (PCR) with primers specific for the *Cryptosporidium* oocyst wall protein (COWP) gene. The frequency of PCR positives was compared with the frequency obtained by microscopic detection using Method 1623. Agreement between the two detection methods was observed for only 5% of the samples. In contrast, 21% of the samples were positive by IFA only, whereas 13% were positive by the molecular method (PCR). Overall 39% of the samples analyzed by either IFA or PCR were *Cryptosporidium*-positive. Comparing these data according to wastewater treatment processes revealed an interesting pattern. Following secondary clarification and cloth filtration, oocyst detection was higher using IFA than using PCR, whereas following sand filtration and MBR treatment the converse was true. This suggested a possible impact of PCR inhibitory compounds in effluents generated from systems employing conventional treatment. Despite the limitations of utilizing PCR following certain processes, sequencing analysis of PCR products frequently detected *Cryptosporidium* species infectious to humans (*C. parvum* and *C. hominis*). *C. parvum* was the more frequently detected species (31 of 40 samples taken from 12 utilities); *C. hominis* was detected in 9 of 40 samples taken from six utilities. Both species were detected in samples from five utilities. *Giardia* assemblages A and B, which are responsible for human infections, were detected in 100%, 97.6%, and 100% of the samples by use of the β-giardin, glutamate dehydrogenase (GDH), and triosephosphate isomerase (TPI) genes, respectively. Nonhuman assemblages were detected in two samples with the GDH gene. One sample contained the dog assemblage (assemblage D), and the second sample contained the rodent assemblage (assemblage G). These two samples were from separate plants; however, both plants employed secondary clarification.

Data presented in this report will facilitate advanced microbial risk assessments in the future; however, some preliminary risk analyses were performed with the assumption that all effluents were reused in unrestricted zones. As may be expected, the greatest risk of human cryptosporidiosis was calculated for reuse of effluents from plants employing secondary treatment (36–3672 infections per 10,000 persons). Advanced treatments such as sand filtration (1–3.5 infections per 10,000 persons) and MBR (0.6–1.3 infections per 10,000 persons) showed marked reduction in the calculated risk.

In summary, even advanced wastewater treatment processes (i.e., MBRs) can develop integrity issues (especially with aging) that can lead to discharge of *Cryptosporidium* oocysts and *Giardia* cysts, which are capable of causing human disease. Based on the data presented in this report, the risks of infection with *Cryptosporidium* appear to be low for advanced processes such as MBRs and may be reduced further by adopting simple operational practices. In conjunction with a multibarrier approach, continuous monitoring of judiciously selected water quality indicators can help to optimize performance of the physical treatment processes and assist in reducing protozoa pass through. Coupling this with disinfectants that are highly effective for inactivating protozoa (i.e., UV light) should ensure that the risk of human cryptosporidiosis can meet or exceed acceptable levels.

Chapter 1

Introduction

1.1 Background

Treatment of drinking water was once considered adequate for reducing the risk of infection from pathogenic organisms. However, the increased need for wastewater reuse has focused attention on the potential of wastewater treatment processes for reducing the numbers of pathogenic organisms to acceptable levels. It is estimated that 1.7 billion gal/day are reused in at least 27 states in the United States. California and Florida are among the highest reusers (>650 MGD) in the United States (Florida DEP, 2010; SWRCB, 2009). Florida plans to reuse effluents from 65% of its facilities using >0.1 MGD by 2020. A public health concern with reusing wastewater is the risk of transmitting infectious agents originating from human and animal feces. Depending on the diseases present in the contributing communities, wastewaters may contain varying numbers of pathogenic organisms including viruses, bacteria, helminths, and protozoa. Protozoan parasites of major public health significance include *Entamoeba histolytica*, *Giardia intestinalis* (synonymous with *G. lamblia* and *G. duodenalis*), and *Cryptosporidium* species (Straub et al., 1993).

Cryptosporidium belongs to the family Cryptosporidiidae, class Sporozoasida, and phylum Apicomplexa. It was originally thought that *Cryptosporidium* organisms were highly host-specific, and almost 20 species were named according to the species of the infected host from which they were isolated (Current et al., 1986; Fayer and Ungar, 1986; Levine, 1984; Tyzzer, 1912). Later, cross-transmission studies with mammalian isolates of *Cryptosporidium* indicated low host specificity, which first prompted Tzipori et al. (1980) to consider *Cryptosporidium* a single-species genus and then led Levine (1984) to suggest that only four species were valid. Later the number of accepted species increased to six with *C. parvum*, causing respiratory and intestinal infections, and *C. muris*, causing stomach infections. Utilization of molecular characterization procedures over the last 15 years has facilitated considerable reorganization of *Cryptosporidium* taxonomy. Two species (*C. hominis* and *C. parvum*) have emerged as of most significance from a public health perspective, and as such, of the greatest concern to the water and wastewater industry. Of these species, *C. parvum* infects both humans and animals, whereas *C. hominis* infects only humans (Xiao et al., 2004). Additional *Cryptosporidium* species capable of infecting humans and causing disease (mainly in children and immunocompromised persons) include *C. felis*, *C. muris*, *C. canis*, *C. suis*, and *C. meleagridis* (Muthusamy et al., 2006; Pieniazek et al., 1999; Xiao et al., 2001). Fayer (2009) highlighted that 12 valid species infected mammals (*C. hominis*, *C. parvum*, *C. muris*, *C. wrairi*, *C. felis*, *C. andersoni*, *C. canis*, *C. suis*, *C. bovis*, *C. fayeri*, *C. ryanae*, and *C. macropodum*); however, this list has been extended to include three additional species (*C. xiaoi*, *C. ubiquitum*, and *C. cuniculus*). Currently three species are considered to infect birds (*C. meleagridis*, *C. baileyi*, and *C. galli*), and three species infect amphibians and reptiles (*C. serpentis*, *C. varanii*, and *C. fragile*). A number of other *Cryptosporidium* genotypes also exist and may become recognized as valid species as further evidence is accrued.

Cryptosporidium oocysts are well adapted for environmental disease transmission, and numerous waterborne outbreaks have occurred over the last 20 years. With a dramatic increase in world population and increased urbanization, it would be reasonable to assume that the environmental

burdens of this ubiquitous organism would also increase in wastewaters. An improved understanding of the occurrence, origin, removal, and inactivation of oocysts in wastewater effluents would allow an assessment of their significance with respect to human disease either as a consequence of direct exposure (during agricultural or recreational reuse) or indirectly by ingestion of oocysts in drinking water. Such data would also facilitate risk assessments and watershed management strategies by downstream water utilities that are engaged in site-specific treatment strategies to comply with the requirements of the Safe Drinking Water Act. In the past 20 years, numerous surveys have attempted to elucidate the impact of biosolids and wastewater effluents on environmental contamination with oocysts. For example, *Cryptosporidium* oocyst concentrations in wastewater influents have been reported to range between 4.1 and 13,700 oocysts/L in the United States (Madore et al., 1987) and between 2.5 and 800 oocysts/L in the United Kingdom (Parker, 1993). Similarly, treated wastewaters have also shown variable and sometimes very high oocyst concentrations (i.e., 0–3930 oocysts/L for the United States and 0.024–38 oocysts/L for the United Kingdom).

Where comparisons between wastewater influents and effluents at various stages of the treatment processes have been conducted, data indicate wide variations in oocyst removal efficiencies, ranging between 75% and 97.7% (DiBenedetto et al., 2005; Gennaccaro et al., 2003; Kfir et al., 1994; Madore et al., 1987; Mayer and Palmer, 1996; Ottoson et al., 2006; Parker, 1993). In a bench-scale study simulating the secondary treatment process, oocyst removal efficiency was reported to be 83–84% (Villacorta et al., 1992). More important, this study demonstrated that oocysts in the effluent retained their infectivity to neonatal mice. Another study conducted at American Water (AW) used a modified version of the U.S. Environmental Protection Agency (U.S. EPA) Method 1622 to recover *Cryptosporidium* oocysts from secondary effluents and identified the presence of a mixture of *C. parvum* subtypes (infecting cattle and mice) as well as *C. meleagridis* (Di Giovanni et al., 2002).

Tertiary filtration without coagulation typically removes 90–99% of *Cryptosporidium* oocysts (Madore et al., 1987; Ottoson et al., 2006; Rose et al, 1996; Suwa and Suzuki, 2003), whereas application of coagulants before tertiary filtration has been shown to further improve oocyst removal (i.e., 99.9–99.99%) in pilot studies (Suwa and Suzuki, 2003). Normally the role of tertiary treatment is to remove suspended solids that pass through the secondary clarification step, and although it is uncommon for tertiary filtration to use prior coagulation, there appear to be obvious benefits from this practice. Another trend in the wastewater industry is the use of low-pressure ultrafiltration (UF) membranes, referred to as membrane bioreactors (MBRs) , which replace clarifiers and tertiary filters. MBRs present a physical barrier to *Cryptosporidium* oocysts because of their submicrometer pore size and are considered highly effective for *Cryptosporidium* removal (Ottoson et al., 2006); however, there is some evidence to suggest that low levels of oocysts may occur in MBR effluents (Karim and LeChevallier, 2005).

Historical data from various studies indicate that *Cryptosporidium* oocysts can pass through various stages of the treatment train, possibly including MBR systems. However, the levels of risks associated with organisms that are usually recovered in these effluents have not been elucidated. One reason has been the absence of appropriate methods. In addition to quantitative oocyst occurrence data, information on oocyst infectivity and genotypic characterization of the environmental isolates is needed to develop robust microbial risk assessment (MRA) models. Because of methodological limitations, accrual of such information had not been possible until the methods that are discussed in Section 1.2 became available.

1.2 Environmental Methods for Isolation of Protozoa

For *Cryptosporidium* oocysts in wastewater, the potential to cause human disease will depend not only on their concentration and species, but also on their infectivity. The majority of previous studies have used methods for environmental isolation and detection of oocysts that could not differentiate among *Cryptosporidium* species or determine oocyst infectivity. Studies conducted prior to 1997 typically used a permutation of a method known in the United States as the Information Collection Rule (ICR) method. ICR itself was a monitoring program, which was implemented between July 1997 and December 1998 and utilized yarn-wound filters for sample collection, followed by oocyst clarification by density gradient flotation. In the ICR program, 5838 samples were analyzed, and 7% of these samples were *Cryptosporidium*-positive, with a mean oocyst concentration of 0.067 oocysts/L (Messner and Wolpert, 2002). The ICR method was known to be very cumbersome and often yielded poor and highly variable overall oocyst recovery efficiencies (i.e., 12% ± 11%; range, 1%–30%; n = 140) (Scheller et al., 2002). The ICR method used immunofluorescence-based detection of *Cryptosporidium* oocysts, and although it could provide morphometric information on oocyst integrity, it provided no indication of whether the oocysts were dead or alive (i.e., viability information).

Following substantial method refinements and multilaboratory validations, U.S. EPA Method 1622/1623 (U.S. EPA, 2005) emerged as a more reliable alternative for monitoring *Cryptosporidium* and *Giardia*. By this method, recoveries with the sample collection step (using filtration) were improved to >90%. Instead of the highly variable flotation procedures for sample clarification, specific oocyst capture was achieved by use of the highly efficient (>70%) immunomagnetic separation (IMS) procedure. Use of IMS was also able to provide substantially cleaner oocyst concentrates (compared with flotation procedures), which eliminated or reduced environmental inhibitors and increased the probability of successfully deploying molecular procedures for subsequent detection or verification. The presence of fewer environmental contaminants also facilitated the integration of U.S. EPA Method 1623 with cell culture to determine the infectivity of environmentally derived oocysts. Two studies extensively used Method 1623 in conjunction with cell culture–polymerase chain reaction (CC-PCR) to establish *Cryptosporidium* occurrence and infectivity in both surface water (LeChevallier et al., 2003) and finished drinking water (Aboytes et al., 2004). In addition, the U.S. EPA has incorporated Method 1623 as a part of its Long Term II Enhanced Surface Water Treatment Rule (LT2ESWTR or LT2 rule) to supplement existing regulations.

Method 1623 uses immunofluorescence-conjugated antibodies (IFA) to specifically detect *Cryptosporidium* oocysts and *Giardia* cysts. To better understand the potential of oocysts recovered from treated wastewater effluents to cause human disease transmission, it is necessary to assess whether oocysts are dead or alive (viability/infectivity) and whether they belong to the species known to cause disease in humans (molecular characterization).

1.2.1 In Vitro Viability and Infectivity

Oocyst viability assays such as in vitro excystation (Robertson et al., 1993), fluorogenic vital dyes (Campbell et al., 1992), and fluorescence in situ hybridization (Smith et al., 2004) were developed in the 1990s to determine whether oocysts were dead or alive. Several studies utilized these assays to examine the viability of environmentally derived oocysts (Robertson et al., 1992) as well as the efficiency of various disinfectants for oocyst inactivation (Bukhari et al., 1999, 2000). Although these in vitro viability assays offered several advantages over traditional animal infectivity models in that they required significantly less time to produce results, were easy to use, and were relatively inexpensive, it has been demonstrated, particularly with UV inactivation,

that some in vitro viability assays could lead to grossly erroneous results (Bukhari et al., 1999). As a result, it became clear that the demonstration of intracellular developmental stages of the parasite was critical in determining whether *Cryptosporidium* oocysts were dead or alive (Bukhari et al., 1999). This prompted studies to evaluate the suitability of cell culture procedures as a less expensive and more user-friendly alternative to neonatal mouse infectivity assays. Later studies demonstrated that oocyst isolation procedures (i.e., U.S. EPA methods 1622 and 1623) could be combined successfully with cell culture for determining infectious oocyst occurrence in raw (LeChevallier et al., 2003) and finished (Aboytes et al., 2004) waters.

To utilize *Cryptosporidium* occurrence data (from environmental monitoring programs) for MRA, it is imperative to use standardized or well-established protocols for oocyst enumeration and infectivity. The literature described numerous variations in oocyst pretreatment, oocyst inoculation, incubation times for assessing infectivity, and variations in the procedure for detecting in vitro developmental stages of the parasite in cell monolayers. With these various permutations, comparison of oocyst infectivity data from various in vitro infectivity protocols has not been feasible. Recognizing that independent validations, which assess both the reproducibility and the predictive capacity of a method, are a key to developing oocyst infectivity standards, an international consortium (consisting of American Water Works Association Research Foundation, now Water Research Foundation; Drinking Water Inspectorate, United Kingdom; KIWA, the Netherlands; U.K. Water Industry Research Ltd.; U.S. EPA; and Water Services Association, Australia) supported a study to validate the sensitivity and reproducibility of in vitro cell culture infectivity assays using varying oocyst inocula of unknown ("blind") infectivity (Bukhari and LeChevallier, 2003; Bukhari et al., 2007). The optimized cell-culture-based procedure identified in this evaluation incorporated oocyst preacidification and exposure to bile salts immediately preceding inoculation onto HCT-8 monolayers, followed by incubation at 37 °C for 72 h and detection using IFA. An advantage of this CC-IFA procedure, compared with other existing procedures, is its uniqueness in taking advantage of various oocyst pretreatment triggers (i.e., acid treatment and exposure to bile). These modifications were intended to simulate the conditions oocysts encounter when ingested by a susceptible host. In addition, the assay allows identification of infectious and noninfectious oocysts (Bukhari and LeChevallier, 2003). For the latter, oocysts undergo excystation and invasion of HCT-8 cells but fail to multiply intracellularly. Where qualitative or quantitative PCR methods are used for detection of infection in the host cells, PCR-based assays cannot differentiate invasive stages from those undergoing active multiplication, which can lead to an overestimation of oocyst infectivity (Bukhari and LeChevallier, 2003). In the study performing "blind" trials using CC-IFA, it was determined that this CC-IFA procedure was highly effective for predicting the infectivity of oocysts (Figure 1.1), with a high degree of correlation ($r^2 = 0.89$) between estimated number and actual number of infectious oocysts.

No reliable in vitro viability assays for *Giardia* cysts exist at present.

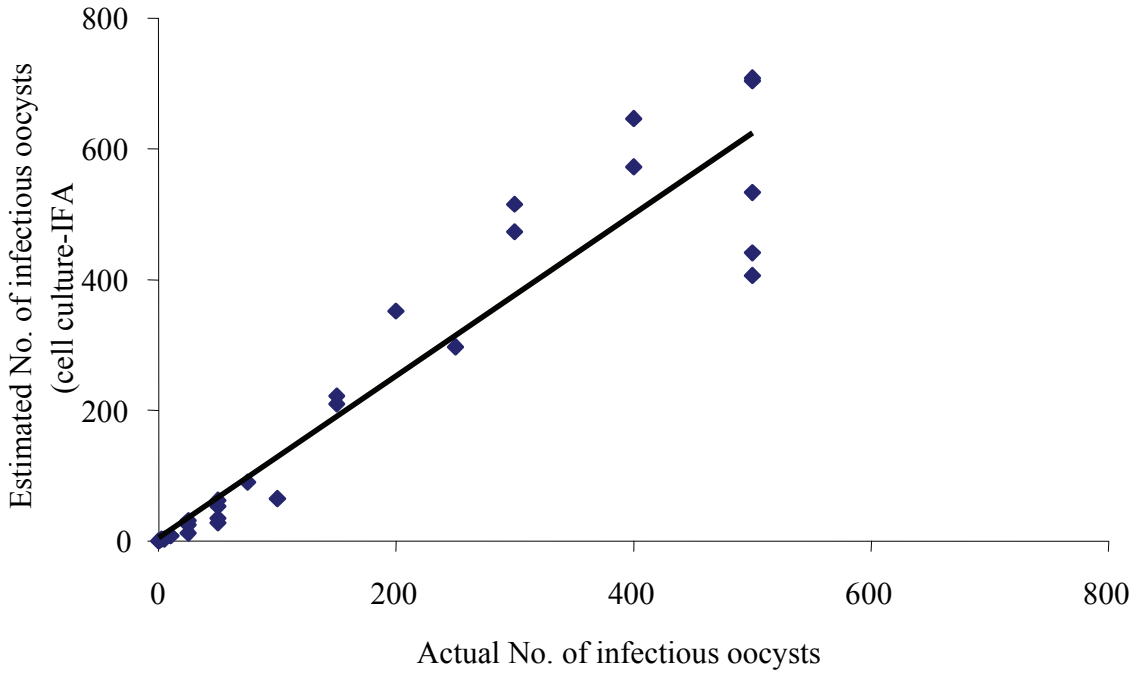


Figure 1.1. *C. parvum* infectivity using CC-IFA.

1.2.2 *Cryptosporidium* Species Identification

The genus *Cryptosporidium* includes an unknown number of species and genotypes, most of which are not infectious to humans. At present, *C. hominis* and *C. parvum* are considered responsible for the vast majority of human infections. Other species, such as *C. meleagridis*, play a minor role (Leoni et al., 2006; McLauchlin et al., 2000). Genotyping of oocysts from over 2400 human clinical specimens indicated that 98% of the infections arose from *C parvum* and *C. hominis*. Small proportions of samples were positive for other genotypes, such as *C. meleagridis* (0.9%), *C. felis* (0.2%), *C. andersoni* (0.1%), *C. canis* (0.04%), *C. suis* (0.04%), and the cervine genotype (0.04%) (Leoni et al., 2006). In another study, which examined over 7500 human clinical samples, *C. parvum* and/or *C. hominis* were detected in 96% of the samples. Less frequently detected species included *C. meleagridis* (1%), *C. felis* (0.75%), cervine genotype (0.05%), *C. canis* (0.05%), horse genotype (0.01%), and skunk genotype (0.01%) (Chalmers et al., 2009). Where water- and food-borne outbreaks of human cryptosporidiosis have been characterized, most have been caused by *C. parvum* and *C. hominis*. The only known exception was the Northamptonshire, England, outbreak, which is thought to have been caused by the rabbit genotype, now named *C. cuniculus* (Chalmers et al., 2009).

In addition to oocyst concentrations and infectivity, integration of genotyping information into MRA can provide more accurate calculation of risks of human disease associated with exposure to oocysts present in wastewater and reuse effluents. Although it would be ideal to characterize the three species known to cause the majority of human disease (*C. hominis*, *C. parvum*, and *C.*

meleagridis), logistically it would be sufficient to detect the two predominant species (*C. hominis* and *C. parvum*) that cause more than 96% of human infections.

Several genotyping methods are capable of discriminating between species or genotypes and can yield high-resolution genotypes for source tracking purposes (Feng et al., 2000; Spano et al., 1998; Tanriverdi et al., 2002, 2006; Tanriverdi and Widmer, 2006). When molecular analysis of environmental samples is conducted, there are concerns with environmental inhibitors. Although the use of immunomagnetic protocols for isolating oocysts from environmental sources has reduced the impact of PCR inhibitors, some genotyping methods have also been validated with oocysts extracted directly from various matrices (Sluter et al., 1997; Tanriverdi et al., 2002).

1.2.3 *Giardia* Assemblage Characterization

G. lamblia (synonymous with *G. intestinalis* and *G. duodenalis*) is a flagellated, unicellular intestinal microbe that causes diarrheal disease. It is known as a common cause of water-borne outbreaks of diarrhea in the United States and globally. There is also evidence for long-term asymptomatic disease with *Giardia,* as well as growth retardation of humans suffering chronic illness. Previously it has been shown, by the use of a number of genes, that *Giardia* contains numerous host-adapted assemblages (Table 1.1). To characterize the *Giardia* assemblages capable of human disease, investigators have used primers targeting the β-giardin gene (Caccio et al., 2002), triosephosphate isomerase gene (*tpi*) (Sulaiman et al., 2003), and glutamate dehydrogenase gene (*gdh*) (Read et al., 2004). The product of *Giardia*-specific primers (targeting all the assemblages) can be analyzed by restriction fragment length polymorphism or by sequencing. As an alternative, assemblage-specific primers (i.e., targeting assemblages A and B using the triosephosphate isomerase gene) can be used (Amar et al., 2002) to determine the presence of assemblages capable of causing human disease.

Table 1.1. Genotypic Variability of *Giardia lamblia*

Proposed Designation	Nash Group	Mayrhofer Assemblage	Hosts
Genotype A-1	1	A (group 1)	Human, beaver, cat, lemur, sheep, calf, dog, chinchilla, alpaca, horse, pig, cow
Genotype A-2	2	A (group 2)	Human, beaver
Genotype B	3	B (groups 3 and 4)	Human, beaver, guinea pig, dog, monkey
		C	Dog
		D	Dog
		E (or A for livestock)	Cow, sheep, alpaca, goat, pig
		F	Cat
		G	Rat

1.3 Project Goals

The objective of this project was to accrue information to better understand the occurrence of *Cryptosporidium* in reuse effluents from plants employing various upstream treatment processes (secondary clarification, cloth filtration, sand filtration, and MBRs). This information would be pertinent to development of future risk assessment models for human cryptosporidiosis that could arise from exposure to reuse effluents. To ensure that these data would facilitate development of robust risk assessment models, key parameters for inclusion in the model needed to be organism concentration, infectivity, and species.

The project was divided into specific tasks, with the initial phases focusing on standardization of sampling and infectivity procedures. The most appropriate methods were selected for assessing *Cryptosporidium* oocyst occurrence, concentration, infectivity, and genotyping from 14 geographically and climatically diverse wastewater treatment plants.

The five tasks specified in the original solicitation are summarized here:

- Task 1 will focus on laboratory-based experiments to characterize performance of various *Cryptosporidium* methods; specifically, standardization of CC-IFA for assessing infectivity of *C. hominis* and *C. parvum* oocysts, comparison between monoclonal and polyclonal IFA detection of infection, and evaluation of two procedures to determine genotyping accuracy.

- Task 2a will determine *Cryptosporidium* oocyst occurrence, concentration, infectivity, and genotype for >350 samples taken from 14 geographically and climatically diverse wastewater treatment plants. Task 2b will examine two sampling procedures to select the most appropriate method.

- Task 3 will conduct bench-scale disinfection studies using various wastewater matrices (using both *C. hominis* and *C. parvum* oocysts) to evaluate the efficiency of wastewater disinfection processes. Bench-scale data will be compared to full-scale oocyst disinfection data collected by sampling in Task 2.

- Task 4 will assemble and summarize data in a format that will facilitate future MRAs using both static and dynamic models.

- Task 5 will be the final preparation of a report in compliance with WateReuse requirements and will provide practical guidance and cost–benefit analyses for various wastewater treatment processes.

Based on the initial study findings, a supplemental project was conducted to characterize assemblages of *Giardia* cysts enumerated on glass slides using Method 1623. Molecular characterization was evaluated using three different target genes, and these assemblage characterization data have also be incorporated into this report.

Chapter 2

Materials and Methods

2.1 Sampling Procedures

To identify the most appropriate sampling procedure for reuse effluents, preliminary studies examined two sampling procedures (Portable Continuous Flow Centrifuge and Envirochek HV), which have been previously approved under the U.S. EPA Performance-Based Measurement Systems (PBMS) for sampling raw surface waters for compliance (LT2ESTWR) monitoring for *Cryptosporidium* and *Giardia.*

2.1.1 Portable Continuous-Flow Centrifuge

A portable continuous-flow centrifuge (PCFC), manufactured by Haemonetics, Inc. (Braintree, MA), and extensively modified and optimized by Zuckerman et al. (1999) for collection of pathogens from various environmental matrices (raw water, finished water, and wastewater effluents), was evaluated for reuse effluents. The currently available PCFC (Figure 2.1) can achieve a maximum centrifugal force of 4300*g*. The basic operational principles have been described earlier (Zuckerman and Tzipori, 2006; Zuckerman et al., 1999). Briefly, disposable inlet tubing is used to pump samples (i.e., secondary/tertiary effluents) into a disposable centrifuge bowl (Figure 2.1), with outlet tubing carrying the supernatant from the bowl to a drain.

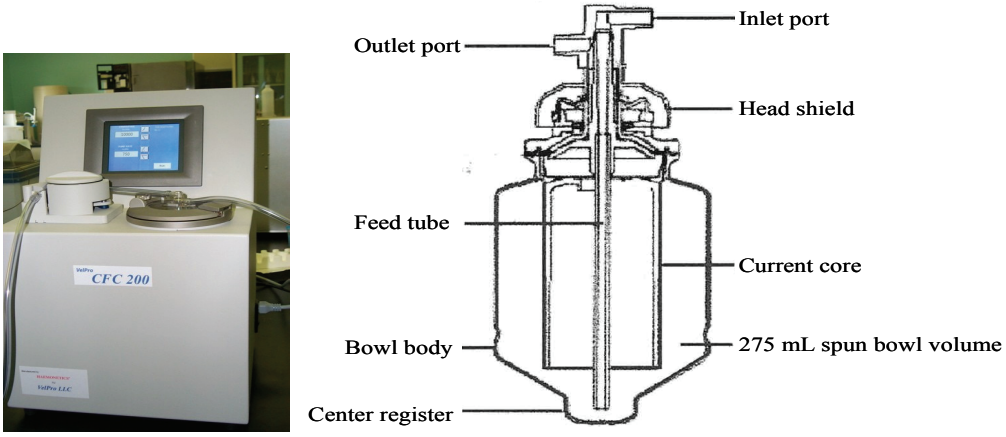


Figure 2.1. Portable continuous-flow centrifuge.

After sampling, flow from the inlet port is terminated, whereas the PCFC continues centrifugation (~10 s) of the sample to reduce the residual sample volume to <250 mL. The bowls containing sample concentrates can be shipped for analysis. As new bowls and tubing are used for collection of each sample, this avoids contamination issues.

In the evaluation phase, flow rates of 500 mL/min were used to concentrate 10-L effluent samples into individual disposable bowls at a centrifugation speed of 3400*g*. The protozoa were manually eluted from bowls by adding elution buffer, vigorously shaking the bowls for 5 min with an automatic wrist shaker, and transferring the eluant into conical centrifuge tubes.

2.1.2 Envirochek HV Filters

The Envirochek HV filters use a 1.0-µm nominal pore size membrane (hydrophilic polyethersulfone) enclosed in a plastic capsule and allow sample collection using flow rates ranging from 2 to 4 L/min. These filters are one of several U.S. EPA-approved (Methods 1622 and 1623) devices for collection of *Cryptosporidium* oocysts from water samples. The sampling/elution procedures have been described in detail previously (U.S. EPA, 2005). Briefly, a 120-mL volume of Laureth-12 buffer was added to the capsule, and each filter was placed on a mechanical shaker (Wrist Action Shaker, Labline Model 3589, Fisher Scientific) and shaken with the inlet valve in the 12 o'clock position for 5 min. The eluant was transferred to a 250-mL conical centrifuge tube, and a further 250 mL of elution buffer was added to the capsule, which was shaken again with the inlet position at 3 o'clock for 5 min. The eluant (approximately 250 mL) from the second shaking step was pooled with the eluant generated from the first wash.

2.1.2.1 Source of Oocysts for Spiking Experiments

A known number of *C. parvum* oocysts *(*100 ± 2.5) were flow-sorted and enumerated at the Wisconsin State Laboratory of Hygiene, Madison (WSLH). These oocysts were delivered in 10 mL volumes of reagent grade water containing 0.01% Tween 20. Similarly, the *C. hominis* oocysts (isolate TU502), which were produced in neonatal piglets at Tufts and were sent to WSLH, were also flow-sorted. Each sorted subsample contained 99 ± 2.5 *C. hominis* oocysts in 3 mL of reagent grade water containing 0.01% Tween 20.

2.1.2.2 Spiking Experiments

A known number of flow-sorted *C. parvum* or *C. hominis* oocysts were added to 10-L secondary effluent samples in individual sterile, disposable plastic cubitainers. The tube containing the spike dose was rinsed with 2 mL of 0.01% Tween 20, which was added to the cubitainer. This was followed by two 2-mL rinses with reagent grade water. Each rinse was shaken vigorously for 30 s before being decanted into the cubitainer containing the spiked sample.

The oocyst suspensions were stirred continuously while being concentrated by the PCFC or by filtration using Envirochek HV filters. Samples were injected into the PCFC bowl (Haemonetics 625-B bowl) at a flow rate of 500 mL/min while being centrifuged at 11,000 rpm. Filtration through the Envirochek HV capsules was performed at a flow rate of 1500 mL/min.

2.2 Concentration and Isolation of Protozoa

Eluates from the PCFC or the filtration procedure were concentrated to 10 mL by centrifugation at 1500*g* for 15 min. Specific capture of *Cryptosporidium* oocysts and *Giardia* cysts was achieved using the Dynal® IMS (Dynabeads® G/C combo IMS kits, Dynal A.S. Oslo, Norway), according to the manufacturer's instructions, with a few exceptions. Briefly, each 10-mL volume of the sample was placed in a screw-cap Leighton tube, and 1 mL of 10× SL–buffer A, 1 mL of 10× SL–buffer B, 100 µL of the anti-*Cryptosporidium* bead conjugates, and 100 µL of the anti-*Giardia* bead conjugates were added. Each sample was allowed to rotate through 360° for 1 h (at room temperature), and the Leighton tube was placed in a magnetic particle concentrator 1 (MPC-1) to separate the bead–oocyst complex from the contaminating debris. The beads were resuspended in 1 mL of 1× SL–buffer A, transferred into an Eppendorf tube, and separated by using an MPC-M, and the supernatant was removed and discarded. The beads were resuspended in 200 µL of Hanks's balanced salt solution (HBSS) with bromophenol blue (0.001%), and 50%

of the sample (100 µL) was shipped to Tufts for infectivity and genotype determination. The remaining 100 µL of the sample concentrate, representing one-half of the original sample volume, was subjected to dissociation by 0.1 N HCl, and in turn, the neutralization procedure was performed on the microscope slide, using 10 µL of 1 N NaOH. Each sample concentrate on the microscope slide was dried (42 °C) and labeled with combined anti-*Cryptosporidium*/anti-*Giardia* monoclonal antibodies, which had previously been conjugated to fluorescein isothiocyanate (FITC).

2.3 Epifluorescence Microscopy

Cryptosporidium oocysts and *Giardia* cysts were visualized and enumerated by LT2 certified analysts using an Olympus fluorescence microscope. The microscope was equipped with a blue filter block (excitation, 490 nm; emission, 510 nm) for visualization of oocysts/cysts labeled with FITC at 200-fold magnification. Confirmation of oocysts/cysts was achieved at a magnification of ×400 using a UV filter block (excitation, 400 nm; emission, 420 nm) for visualization of 4′,6-diamidino-2-phenylindole (DAPI)-stained organisms, and internal morphology of oocysts/cysts was observed using Nomarski differential interference contrast (DIC) microscopy.

2.4 *Cryptosporidium* Infectivity Using Cell Culture Immunofluorescence Assay

Cryptosporidium oocyst infectivity was determined by the CC-IFA method with monolayers of the human ileocecal adenocarcinoma (HCT-8; ATCC CCL-244) cell lines and by use of the procedures described by Bukhari et al. (2007). Briefly, 25% of the environmental sample concentrate (approximately equivalent to a volume of 2.5 L) containing *Cryptosporidium* oocysts was diluted in an equal volume of acidified (pH 2.0) HBSS (AHBSS) followed by incubation (37 °C for 1 h). Postincubation, the AHBSS was neutralized with growth medium containing bovine bile (1%) to yield a final bile concentration of 0.05%. Individual wells were inoculated with 100 µL of pretreated samples. Each 96-well plate was covered and incubated in 5% CO_2 humidified incubator at 37 °C for 72 h. After incubation of oocyst-inoculated monolayers, each 96-well plate was examined by bright-field microscopy (magnification ×100) to assess the structural integrity of the monolayers. Following this, 200 µL of phosphate-buffered saline (PBS) (pH 7.4) was added to inoculated wells with the aid of a multichannel pipette. The PBS was aspirated gently, care being taken to avoid damage to the monolayers, and the wash step was repeated three more times. The monolayers were examined again by bright-field microscopy (magnification, ×100), and cell loss (if any) was recorded.

2.4.1 Immunofluorescence-Based Detection

Each monolayer was fixed by adding 100 µL of 3.7% paraformaldehyde (prepared in PBS) and incubated in the 96-well plate at room temperature for 15 min. Excess paraformaldehyde was removed, and the monolayers were washed twice with PBS (pH 7.4). Monolayers were exposed to chilled methanol (−20 to −25 °C) for 10 min, and the excess methanol was removed by gently inverting the 96-well plate onto a clean paper towel for 1 min. The methanol-fixed cell monolayers were washed once with 200 µL of PBS, and 50 µL of appropriately diluted immunostaining reagent (Sporo-Glo A600, diluted 1:10 in PBS; Waterborne Inc., New Orleans, LA) was added to each monolayer and incubated (at 37 °C for 60 min) in a humid chamber. The stained cell monolayers were washed five times with PBS. The excess PBS was removed with a multichannel pipette, and then 50 µL of prewarmed (37 °C) mounting medium, containing 2% 1,4–diazabicyclo[2.2.2]octane (DABCO), was added to each well. IFA-based enumeration of

infectious clusters (Bukhari and LeChevallier, 2003; Bukhari et al., 2007) was performed using an inverted fluorescence microscope (Olympus, IX-70). All microplates were wrapped in foil and stored at 4 °C until completion of the experiments.

2.4.2 Optimization of Detection Procedures

HCT-8 monolayers were blocked either with 2% bovine serum albumin (BSA) in PBS, 10% fetal bovine serum (FBS) in PBS, or a 1:1000 dilution of normal goat serum (NGS) in PBS. The 1H3 monoclonal antibody (immunoglobulin M [IgM]) was diluted 1:4 in NGS/PBS, and secondary anti-mouse IgM Alexa 488 conjugate was diluted 500-fold in NGS/PBS. 2E5 antibody (IgG) was diluted 2-fold in NGS/PBS, and polyclonal antiserum was diluted 1000–fold in PBS. All antibody solutions were centrifuged at 14,000*g* for 1 min to precipitate any particulates that could potentially interfere with immunofluorescence. Monolayers were exposed to 75 µL of primary antibody for 30 min. Unreacted antibody was removed with one wash using PBS, and 75 µL of the secondary (Alexa 488) conjugated antibody was added at a 1000-fold dilution for 30 min. The plates were washed once with PBS and air-dried. Parasite fluorescence was quantified with an inverted epifluorescence microscope fitted with a camera and image analysis software (Theodos et al., 1998). Four images were captured per well, and the average parasite fluorescence reading was calculated from 12 readings acquired for each set of three replicate wells (4 readings per well).

2.4.3 Establishing Sensitivity of CC-IFA

C. parvum oocysts (TU114 isolate; 7 days old) were visualized on a forward versus side scatterplot on a FACSCalibur cell sorter (Figure 2.2) and sorted in exclusion mode to deliver three individual doses of 1000 oocysts into approximately 1 mL volumes of PBS. Each sample of sorted oocysts was concentrated to 100 µL by centrifugation, and the oocysts were activated by addition of 100 µL AHBSS, followed by incubation at 37 °C for 1 h. Thereafter, 750 µL of Roswell Park Memorial Institute (RPMI) cell culture medium was added together with 50 µL of bile, for a total volume of 1 mL.

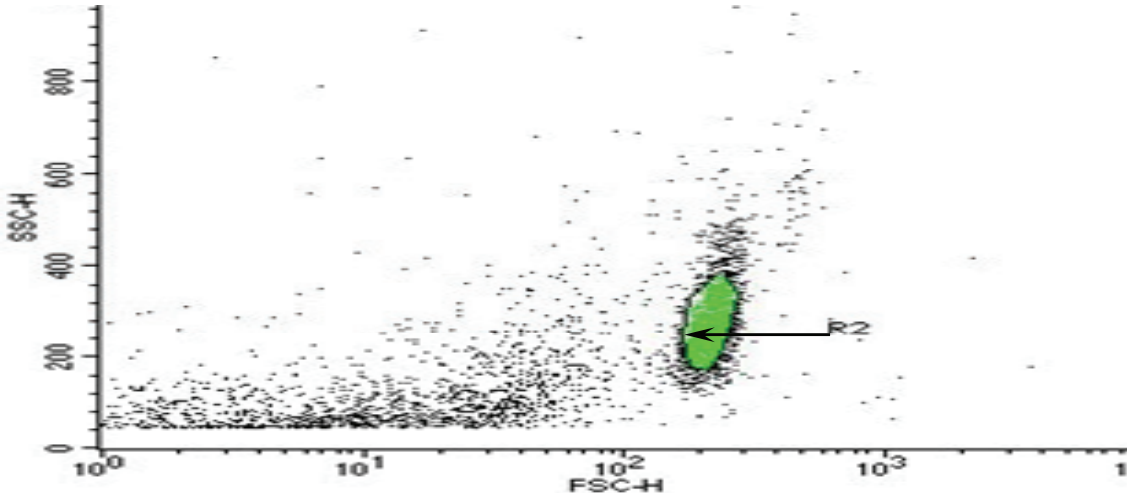


Figure 2.2. Scatterplot of purified oocysts.
(Events in the R2 region were sorted.)

Spike doses containing 10, 5, and 1 oocysts were prepared as follows: to 100 µL of the activated oocyst suspension, 900 µL of RPMI medium was added to generate a 100 oocyst/mL suspension.

One hundred microliters of this suspension, corresponding to a dose of 10 oocysts, was added to each cell monolayer. Triplicate wells were infected for each suspension. Fifty microliters of each suspension, corresponding to 5 oocysts, was added to each of three wells, and three additional wells were inoculated with 100 µL of a 10-fold dilution of the activated oocyst suspension to obtain a dose of 1 oocyst per well.

In addition to the TU114 isolate of *C. parvum* (Tanriverdi and Widmer, 2006), two more isolates (one designated MD [Okhuysen et al., 2002] and the other TUM1 [unpublished data]) were used to measure the assay sensitivity. Two of these isolates (TU114 and MD) were propagated in mice, and the third isolate (TUM1) was propagated in a calf.

The inoculated HCT-8 monolayers were incubated at 37 °C for 72 h. The cells were methanol-fixed, and the intracellular parasites were labeled with anti-*Cryptosporidium* polyclonal antibody as described in Section 2.4.

2.4.4 Bench-Scale Disinfection

2.4.4.1 Chlorine

Prior to disinfection, all glassware and stir bars were rendered chlorine demand–free by filling the bottles with chlorine solution overnight, and then rinsing with deionized (DI) water before use. Typically a stock solution of BDH® sodium hypochlorite (6%) was diluted in DI water to generate a 13.5 mg/L working solution, which was diluted to 1 mg/L to prepare chlorine demand–free glassware (100-mL glass beakers).

Individual chlorine demand–free glass beakers containing approximately 80 mL of chlorine solution (mean concentration up to 10 mg/L) were placed on magnetic stir plates and covered with parafilm. A known number of *Cryptosporidium* oocysts (~1.0×10^5 to 2.5×10^5) were added to each beaker and mixed by continuous stirring at room temperature. Immediately following oocyst addition, a 20 mL subsample was removed to determine the initial chlorine concentration by using the *N,N*-diethyl-*p*-phenylenediamine (DPD) free chlorine method using AccuVac ampoules (Hach Co., Loveland, CO). This measurement allowed direct determination of the chlorine demand exerted by the oocyst suspension. Residual chlorine concentrations were also taken 10 and 15 min postexposure. After designated exposure times, the chlorination experiments were terminated by the addition of 10 µL of a 0.08% sodium thiosulfate solution.

Cryptosporidium oocysts were exposed to stock chlorine solutions in a manner that was consistent for both the control (demand-free DI water) and the matrix samples. The CT (concentration [C] multiplied by exposure time [T]) values of 90 mg/mL, 450 mg/mL, and 600 mg/mL were examined. At each CT value, duplicates were run with fresh *Cryptosporidium* oocysts (1 week old) and aged oocysts (4 months old).

For matrix disinfection, stock chlorine solution was diluted in either chlorine demand–free water (control) or effluent from a plant employing tertiary sand filtration (matrix).

2.4.4.2 UV Light

Bench-scale UV disinfection experiments were conducted using a collimated beam unit, as described previously (Bukhari et al., 1999). UV fluence was verified using biodosimetry with MS2 bacteriophage, according to the U.S. EPA's *UV Disinfection Guidance Manual* (U.S. EPA, 2006).

2.4.4.3 Ozone

To measure the effectiveness of CC-IFA to determine the disinfection level of oocysts exposed to ozone, parallel experiments were conducted with *C. parvum* (isolate MD) and *C. hominis* (isolate TU728) oocysts. Bench-scale disinfection experiments were performed using various CT values of ozone. The ozone was generated by feeding a corona-discharge ozone generation system with compressed air to produce ozone gas, which was then bubbled through a 1 L sidearm glass flask containing DI water. Prior to disinfection experiments, all glassware was rendered ozone demand–free by immersion into a 3–5 mg/L ozone solution overnight. Following this, glassware was rinsed several times with ozone demand–free DI water, and oocysts disinfection experiments were conducted for both *C. parvum* and *C. hominis* using 1.8 mg/L · min (3 min exposure) or 3.0 mg/L · min (5 min exposure).

Ozone concentrations before, during, and after disinfection were measured using the indigo trisulfonate method with AccuVac ampoules (Hach Co., Loveland, CO). Individual ozone demand–free glass beakers containing approximately 80 mL of ozonated water were placed onto magnetic stir plates and covered with parafilm. Following determination of the initial ozone concentration, a known number of *Cryptosporidium* oocysts (~2×10^6) were added to each beaker and mixed by continuous stirring. Immediately following oocyst addition, a 20 mL subsample was removed to determine the ozone concentration and to calculate the ozone demand exerted by the oocyst suspension. Following predefined exposure times, a further 20 mL subsample was removed for determination of residual ozone concentrations. Simultaneously, the disinfection process was terminated by adding 50 µL of sodium thiosulfate to the oocyst suspensions. CC-IFA was used to determine infectivity of ozone-exposed microorganisms. Use of process control organisms (subjected to all the experimental manipulations except exposure to the ozone) enabled determination of *Cryptosporidium* inactivation.

2.5 Description of Utilities and Sampling Strategy

Cryptosporidium oocyst occurrence, infectivity, and genotyping and *Giardia* cyst occurrence were determined on a monthly basis for 14 utilities over a 1-year period. The locations of the utilities surveyed are presented in Figure 2.3. Of these facilities, eight used secondary clarification (SC, DM, SF, GL, AR, EH, SP, and CV), two had systems of tertiary sand filtration (BH and HS), three used MBRs (SL, MN, and AM), and one system (VR) employed cloth filtration. The treatment characteristics of these utilities have been summarized in Table 2.1. For each participating utility, the samples collected were handled as shown in Figure 2.4. The American Water (AW) research laboratory in Delran dispatched sampling kits (a cooler containing two Gelman Envirochek filters, ice packs, disposal sampling tubing, and sample chain-of-custody sheets) to each utility a week prior to the sampling event. All utilities were provided with a written sampling protocol (Appendix A) and phone-based instructions on the sample collection procedures. In addition, utilities were asked to review the U.S. EPA website (http://www.epa.gov/safewater/lt2/training/index.html) for sampling instructions for unpressurized facilities.

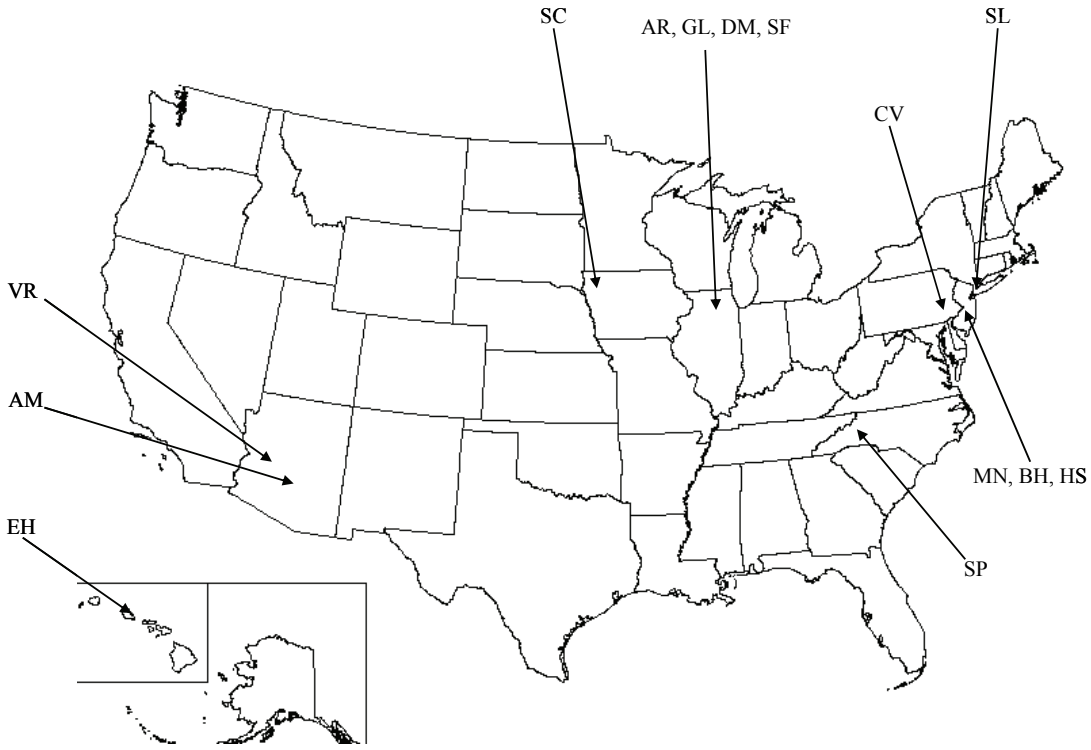


Figure 2.3. Geographic distribution of participating utilities.

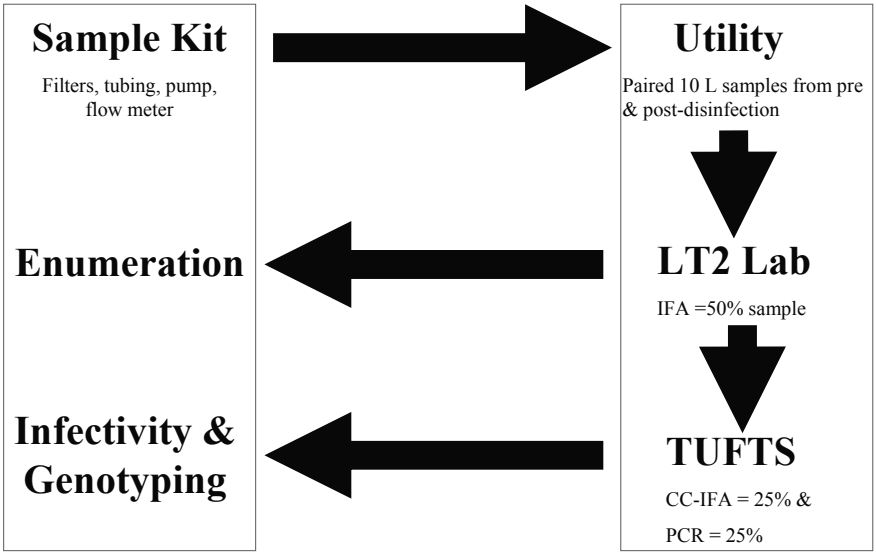


Figure 2.4. Overview of sampling and analyses for environmental samples.

Each utility used a graduated 10 L carboy to collect 10 L samples and filtered these samples through Envirochek HV filters, using flow rates of <2 L/min (LPM). The filtered samples were packed on ice in a cooler and sent to the LT2-certified AW central lab in Belleville, IL, where samples were eluted and *Cryptosporidium* and *Giardia* organisms were isolated according to

procedures described in U.S. EPA Method 1623. Immediately following IMS, one-half of the IMS concentrate from each sample was sent to Tufts for subsequent cell culture infectivity analysis and genotyping. The remaining half was subjected to acid dissociation at the LT2 certified lab (AW Belleville lab), and the oocysts and cysts were enumerated by IFA as described in Section 2.3.

2.5.1 Cell Culture Infectivity of Environmental Samples

At Tufts, each sample was concentrated by centrifugation, washed once with PBS, and resuspended in 100 µL of PBS. A 100 µL volume of AHBSS was added to each sample, followed by incubation at 37 °C for 1 h to dissociate oocysts from the IMS beads and simultaneously pretreat organisms for enhanced in vitro excystation. Following acidification, samples were placed in a magnetic rack to separate the IMS beads from the dissociated organisms. The supernatant containing the organisms was separated from the IMS beads, and for each sample, the supernatant was split into two equal 100 µL portions.

One 100 µL portion of each sample was subjected to CC-IFA analyses, as described in Section 2.4. The second 100 µL portion of each sample was subjected to DNA extraction and PCR, as described in Section 2.5.2.

2.5.2 Molecular Analyses of *Cryptosporidium*

Following IMS, a subsample of each concentrate was subjected to DNA extraction for subsequent *Cryptosporidium* genotype characterization. Typically a 100 µL aliquot of the acidified IMS concentrate was diluted with 1 mL PBS, the organism suspension was concentrated by centrifugation, and the 1 mL supernatant was removed. The remaining 100 µL residual was resuspended and freeze–thawed three times by cycling between freezing (−80 °C) and warm (37 °C) temperatures. DNA was extracted with HighPure Template purification columns (Roche Diagnostics) and eluted in 15 µL of elution buffer. A 1-µL aliquot of DNA solution from each sample was added to an individual 50 µL PCR volume. Initially a seminested PCR protocol, targeting the rRNA gene and using the published primers cry4/cry20 followed by cry4/cry2 (Carraway et al., 1996), was used. Later the protocol was switched to use primers targeting the *Cryptosporidium* oocyst wall protein (COWP). It was reasoned that targeting this gene may generate better results because the oocyst wall is unique to *Cryptosporidium* oocysts. A nested PCR protocol was used with cry15/FS2 primers (Spano et al., 1998) for the primary reaction and COWP NF (CAATCWGACACAGCTCC) and COWP NR (CAGACAGGTTGRGTTGG; unpublished data) primers for the secondary reaction. One mixed position was included in each nested primer to accommodate sequence polymorphism within *C. parvum*. PCR products were analyzed on 2% agarose gels, and the ethidium bromide-stained DNA bands were visualized under UV light.

2.5.3 Molecular Analyses of *Giardia*

Because molecular characterization of *Giardia* cysts was a project extension, the analyses were performed later than the *Cryptosporidium* analyses described in Section 2.5.2. Unlike the *Cryptosporidium* analyses, which were performed directly on a subsample of the IMS concentrate, the *Giardia* DNA was recovered from glass slides previously used to perform IFA-based enumeration of *Cryptosporidium* and *Giardia* according to Method 1623.

2.5.3.1 Recovery of Giardia *Cysts from Glass Slides*

A known number of *Giardia* cysts (5–100) were fixed onto glass slides and IFA labeled according to Method 1623. Following IFA-based enumeration, each slide was placed on a clean paper towel and wiped first with a soft tissue (moistened with 10% bleach) and then with an alcohol wipe. A sterile cotton swab moistened with nonacetone nail polish remover was used to swab the edges of the coverslip to soften the nail varnish. A sterile scalpel was used to cut the softened nail varnish around the coverslip, and a corner of the coverslip was raised from the slide surface. The coverslip was placed (face up) on a clean tissue. Residual mounting medium was washed off the slide well by gently applying 50 µL of molecular grade water (MGW) to the edge of the slide well and rotating the slide to roll the droplet around the well. The slide was tilted, and a sterile cotton swab was placed at the edge of the well to aspirate the liquid. MGW (25 µL) was added to the slide, and the slide was scraped with a sterile loop in two directions perpendicular to each other. The slide was rotated 90° and scraped again in two directions perpendicular to each other. The loop was placed on a support such that it did not come in contact with any surface. To aspirate the detached cysts, a pipette tip was rinsed with 0.01% Tween 20 (2 times) and placed at the edge of the well while the slide was tilted toward the pipette tip. The aspirated sample was transferred into a labeled 1.5 mL microcentrifuge tube. The second scraping regime was performed on the same slide with the addition of a second 25 µL aliquot of MGW. This suspension was combined with the first wash in the centrifuge tube. The tubes containing the suspended cysts were stored at −20 °C until further processing. To verify that cysts were removed by the extraction procedure, the glass slides were IFA-stained again and examined using florescence microscopy.

2.5.3.2 DNA Extraction

Cysts suspended in MGW were subjected to eight freeze/thaw cycles. DNA was then isolated using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The purified DNA was obtained from two 50 µL elutions and stored at −20 °C until PCR amplification.

2.5.3.3 PCR Amplification

The amplification of the β-giardin gene was performed using a nested PCR protocol. For the primary PCR assay, a 753 bp fragment was amplified using previously described primers G7 and G759 (Caccio et al., 2002). Primary PCR cycling conditions were as follows: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, followed by 1 cycle of 72 °C for 10 min. The sequential nested PCR amplified a 511 bp fragment using the forward primer (5′-GAACGAACGAGATCGAGGTCCG-3′) and reverse primer (5′-CTCGACGAGCTTCGTGTT-3′), both previously described (Lalle et al., 2005). Nested PCR cycling conditions consisted of 1 cycle of 95 °C for 15 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by 1 cycle of 72 °C for 10 min. The nested PCR primers were designed to amplify regions of the β-giardin gene representing assemblages A, B, and E.

The amplification of the glutamate dehydrogenase gene (*gdh*) was performed as a seminested PCR using primers previously described (Read et al., 2004), which produced a 432 bp fragment. PCR cycling conditions for the primary and nested reactions were as follows: 1 cycle of 95 °C for 15 min, 50 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 45 s, followed by 1 cycle of 72 °C for 10 min. The primers allowed for the amplification of DNA from all assemblages.

The amplification of the triosephosphate isomerase gene was performed using a nested PCR protocol with primers previously described (Sulaiman et al., 2003), which allowed for the amplification of DNA from all assemblages. The primary PCR primers (AL3543 and AL3546) amplified a 605 bp fragment prior to the nested PCR primers, which amplified a 530 bp fragment. PCR conditions for the primary and nested reactions were as follows: 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min; and 1 cycle of 72 °C for 10 min.

All PCRs were performed on a LightCycler 480 II (Roche Diagnostics, Mannheim, Germany). For all primary reactions, the PCR mix consisted of 1 unit of 2X Perfecta SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD), 10 pmol of each primer, and 10 µL of purified DNA in a final reaction volume of 20 µL. In all nested reactions, 1 µL of the primary PCR product was diluted 10-fold in MGW with all other components of the reaction remaining at concentrations identical to those of the primary reaction. PCR products were visualized on 1% ethidium bromide-stained agarose gels. In each round of PCR, negative (MGW) and positive (DNA from *G. lamblia*, H3 isolate, Waterborne, New Orleans, LA) controls were included.

2.5.3.4 DNA Sequencing

PCR products were purified using ExoSAP-IT (USB Products, Affymetrix, Cleveland, OH). Sequencing of PCR products was performed at Genewiz Inc. (South Plainfield, NJ) using an ABI 3730xl DNA Analyzer and appropriate internal primers. Sequences were aligned with published reference sequences (Thompson et al., 1994).

Table 2.1. Treatment Characteristics of Utilities Surveyed for *Cryptosporidium* and *Giardia*

Utility ID	DM	EH	SC	SP	AR	CV	GB	SF	HS	BH	SL	AN	MT	VR
Type	Domestic	Domestic/ industrial	Domestic/ industrial (animal rendering)	Domestic	Domestic	Domestic	Domestic	Domestic/ industrial	Domestic	Domestic	Domestic	Domestic	Domestic	Domestic
Treatment	Activated sludge, diffused aeration and nitrification	Activated sludge plus tertiary filtration	Activated sludge	Oxidation ditch, activated sludge, and nitrification	Activated sludge plus Eimco carrousel	Activated sludge, diffused aeration, and nitrification	Activated sludge, diffused aeration, nitrification, and phosphorus removal	Activated sludge, diffused aeration, and nitrification	Lagoons plus sand filtration	SBR plus sand filtration	MBR Ozone/UV	MBR–zenon UF membrane	Domestic/ MBR	Activated sludge, MLE + 10 µm polyester cloth filters
Coagulant Addition	No	No	No	No	No	Yes (alum)	No	No	Yes (alum)	Yes	Yes (alum)	No	No	No
Tertiary Filter	No	Yes	No	No	Yes	No	No	No	Yes	Yes	N/A	N/A	N/A	Yes
Ammonia	Low	Low	High/seasonal	Low	Low	Low	Intermediate	Intermediate	Intermediate	Low	Low	Intermediate	Low	Low
Nitrate	Low	Low	High/seasonal	Low	High	High	Low	Low	Low	Low	Low	Intermediate	Low	Low
Disinfection Type	Chlorine	Chlorine	Chlorine	Chlorine	UV	UV	UV	UV	Chlorine	UV	UV/ozone/RO	UV	UV	Chlorine
Flow (MGD)	0.6	3.85	0.35	0.35	0.063	4.2	0.3	0.5	0.15	16	0.012	1.8	0.24	0.2
Type of Reuse	Land application	Land application	None	None	Land application	Irrigation	Land application	Land application	Irrigation	Groundwater recharge	Cooling tower or irrigation	Irrigation	None	Irrigation/ landscape

Chapter 3

Results

3.1 Evaluation of Sample Collection Procedures

To facilitate determination of health risks associated with *Cryptosporidium* oocysts discharged in wastewater effluents from utilities as shown in Table 2.1, the most efficient and robust sample collection methods need to be deployed. Among the various considerations, ease of use, cost, and field applicability are desirable features of a sampling device. The Pall-Gelman Envirochek filters meet most of these specifications; however, they still remain costly and may experience clogging in matrices with relatively high turbidity, such as wastewaters. An alternative, relatively inexpensive procedure, using a portable continuous-flow centrifuge (PCFC), has also been approved for *Cryptosporidium* sampling using Method 1622/1623 (U.S. EPA, 2005). Haemonetics has constructed two types of PCFC machines from standard components of a blood separation system. One unit is a manually operated machine (CFC 200) and consists of a centrifuge (Magstar, MN) capable of running from 1 to 12,000 rpm (maximum relative centrifugal force 4000) and a peristaltic pump that runs from 0 to 1000 mL/min. The second is an automated machine (CFC 100A) housing a centrifuge and peristaltic pump as well as pneumatic valves controlled by a computer system to allow automated concentration and elution of protozoa. A standard Haemonetics 625–B bowl, developed for concentration of *Cryptosporidium* and *Giardia* species, can be used in both machines. As the PCFC relies on centrifugation rather than filtration, it was postulated in the original proposal that it may be better suited for wastewater applications.

Prior to the extensive sampling campaign for this study, a comparison of the performance of the automated PCFC machines with that of the Pall-Gelman Envirochek HV filters was conducted. Data comparing the PCFC and the Envirochek HV for recovering *C. parvum* oocysts from secondary effluents are shown in Table 3.1.

Table 3.1. *Cryptosporidium* Recovery from 10-L Secondary Effluent Samples

Method	*C. parvum* Spike Dose (Mean ± SD)	*C. parvum* % Recovery (Mean ± SD)	*C. hominis* Spike Dose (Mean ± SD)	*C. hominis* % Recovery (Mean ± SD)
CFC 100A	100 ± 2.5	27.4 ± 9.6 (*n* = 8)	99 ± 2.5	28.9 ± 11.5 (*n* = 22)
Envirochek HV	100 ± 2.5	18.1 ± 9.3 (*n* = 8)	99 ± 2.5	33.6 ± 11.5 (*n* = 22)

Notes: Turbidity of samples: *C. parvum* spike, 0.6 nephelometric turbidity unit (NTU); *C. hominis* spike, 0.7–1.0 NTU.

For *C. parvum* the mean oocyst recovery with PCFC (27.4%) was higher than that with the Envirochek filters (18.1%), as shown in Table 3.1. In contrast, mean oocyst recovery was lower for PCFC (28.9%) than for Envirochek HV filters (33.7%) when spiking was conducted with *C. hominis* oocysts (Table 3.1). Statistical analysis using a *t*-test showed no significant differences between the two sample collection methods ($P > 0.05$) when *C. parvum* or *C. hominis* oocysts were used. For the latter, mean turbidity was slightly higher for the samples tested with the Envirochek filter, but the difference was not statistically significant (rank sum test, $P = 0.42$).

3.2 Standardization of CC-IFA Procedure

Preliminary experiments were performed to standardize the cell culture IFA procedures between AW and Tufts. Using flow-sorted *C. parvum* oocysts (isolate MD), CC-IFA was performed in parallel at both AW and Tufts with unknown inocula of *C. parvum* oocysts. Also, to ensure that the method for determining infectious clusters was standardized between the two laboratories, "blind" trials were conducted in which analysts independently enumerated clusters on pre-prepared IFA plates. These preliminary studies were extended to include evaluation of three different antibodies to optimize detection of infectious clusters in HCT-8 monolayers. These antibodies were the commercially available Sporo-Glo (Waterborne Inc., New Orleans, LA), which consists of a fluorescein-labeled rat anti-*C. parvum* sporozoite polyclonal antibody; the 2E5-IgG monoclonal antisporozoite (provided by Abhineet Sheoran and conjugated at Tufts with AlexaFluor 488) and the rabbit antisporozoite polyclonal (Tufts polyclonal) antibodies, also provided by Abhineet Sheoran. Both the Sporo-Glo and 2E5-IgG antibodies directly label sporozoites, merozoites, and all other intracellular reproductive stages. In contrast, the rabbit antisporozoite polyclonal antibodies were used in combination with a fluorescence-labeled secondary antibody (goat anti-rabbit IgG). The effects of three blocking agents (BSA, FBS, and NGS) on immunofluorescence were also examined, using the two monoclonal antibodies (1H3 and 2E5) and a single polyclonal antibody (Tufts polyclonal), and spiking trials were also conducted to measure the sensitivity of the CC-IFA procedure.

3.2.1 Standardizing Infectious Cluster Determination Between AW and Tufts

Interlaboratory quality assurance/quality control (QA/QC) was performed to assess the criteria used for cluster determination between AW and Tufts. A set of IFA-labeled plates were prepared and enumerated at AW and then were sent to Tufts for repeat enumeration of infectious clusters. The data from Tufts are presented in Figure 3.1. No significant differences were observed between the cluster counts from the two labs ($P = 0.82$, $t = 0.23$, df = 10). Where Tufts used flow-sorted oocysts to develop dose–response curves (Figure 3.2), a high correlation was observed between oocyst numbers (inoculum) and infectious clusters ($R^2 = 0.98$), which corroborated previous findings (Bukhari et al., 2007). The linear regression equation for Figure 3.2 was determined as $y = 0.1165x + 1.9568$ and was rearranged to $x = (y - 1.9568)/0.1165$ to calculate the unknown number of infectious oocysts (x) from a known value (y) of infectious clusters.

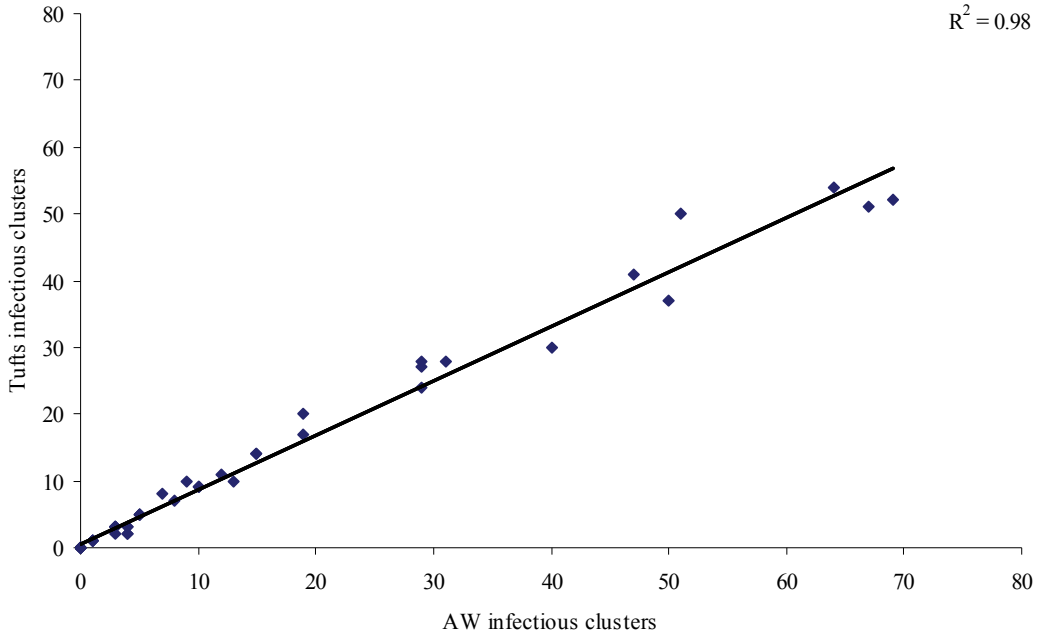


Figure 3.1. Comparison of infectious cluster counts between AW and Tufts.

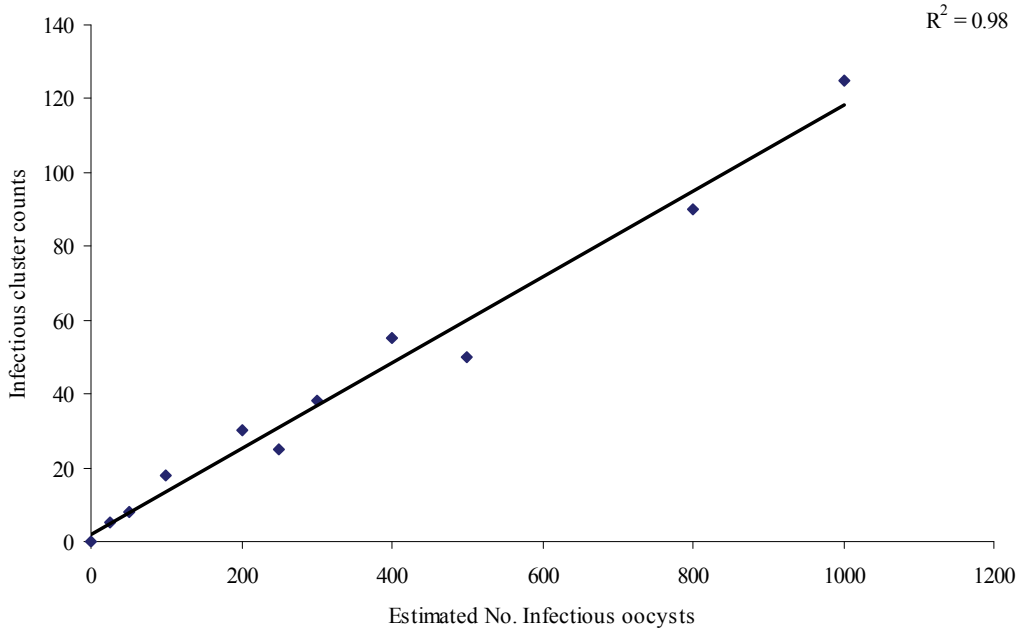
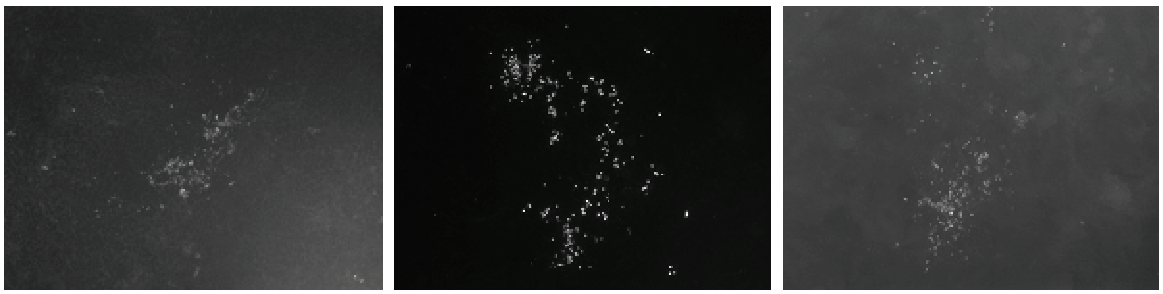


Figure 3.2. Dose–response curve for the CC-IFA procedure.



2E5 monoclonal Tufts polyclonal Sporo–glo

Figure 3.3. Representative images of *C. parvum* foci in HCT–8 monolayers.
Intracellular parasites were detected with three different antibodies using the AW protocol (Bukhari et al., 2007). 2E5 and Sporo-Glo were conjugated directly to the fluorochrome. The Tufts polyclonal antibody was labeled with a secondary Alexa 488-labeled antibody.

3.2.2 Evaluating the Performances of Various Antibodies

Infectious foci were visible with all three antibodies but differed in their signal/noise ratios. The antisporozoite (2E5-IgG) monoclonal antibodies presented very weak signals that faded rapidly (usually within 24 h of staining). It is possible that this occurred because of poor conjugation of the antibody with the AlexaFluor 488 fluorochrome. The Tufts polyclonal antibodies rendered very well-defined (crisp) signals with little or no background, indicating high binding specificity. The Sporo-Glo provided an intermediate signal with much higher background than observed with the Tufts polyclonal (Figure 3.3).

3.2.3 Effects of Blocking Agents on Immunofluorescence

Results from the blocking experiments are presented in Figure 3.4. The mean signals ± standard deviations obtained with 1H3, 2E5, and the polyclonal antibody were 117 ± 77.8, 126 ± 79.6, and 215 ± 68.5, respectively. An equivalent number of measurements were obtained from monolayers mock infected with heat-inactivated oocysts, and the signal/background ratios were calculated by dividing the mean signal from infected wells by the mean background from the heat-treated organisms. Although the polyclonal antibody produced the highest fluorescence in the infected monolayers (“signal”), this antibody also produced the highest background in mock-infected monolayers (“noise”). As a result, the polyclonal antibodies produced a lower signal/noise ratio, as shown in Figure 3.4. Based on signal/noise ratios, the least variability was seen with NGS and then with fetal calf serum (FCS) (Figure 3.4).

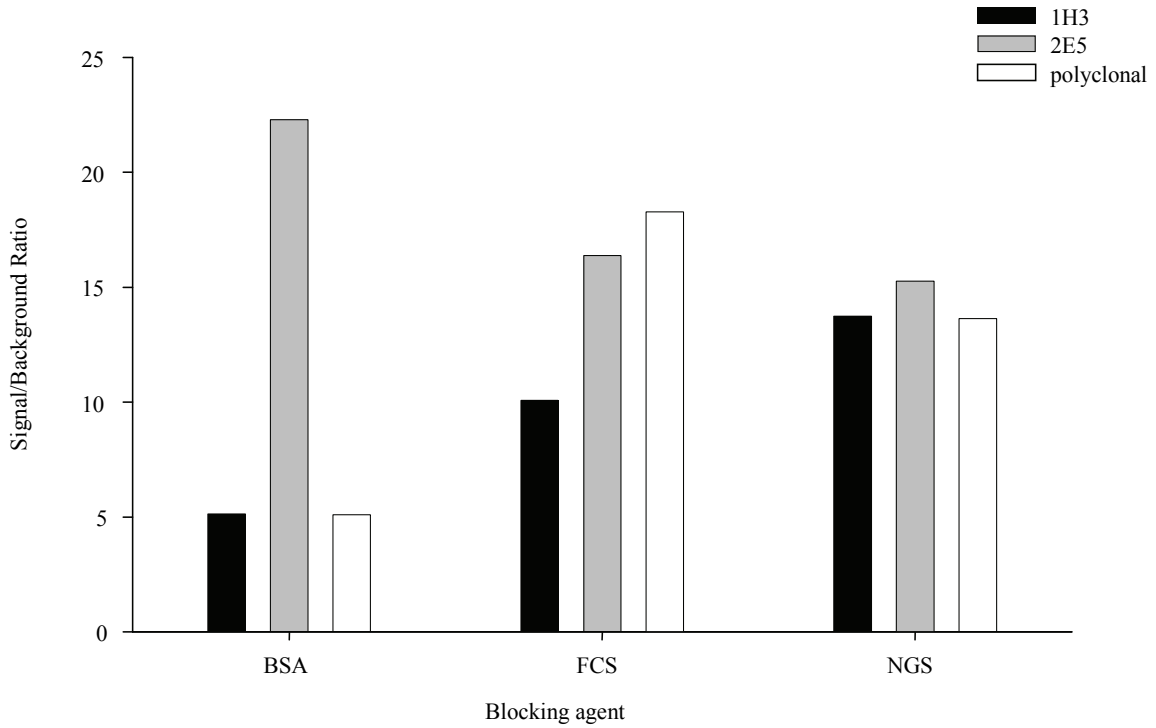


Figure 3.4. Effect of blocking agents on the signal and background of *C. parvum*-infected monolayers.

Whereas the Tufts polyclonal produced a brighter signal than the commercially available Sporo-Glo, the lot-to-lot variability of the experimental Tufts polyclonal could not be guaranteed for this study. As a result, it was considered most appropriate for the monitoring phase of the project to continue relying on the commercially available polyclonal (Sporo-Glo) for detection of endogenous stages of *Cryptosporidium* in cell culture.

3.2.4 Establishing Sensitivity of CC-IFA Procedure

Assuming one infectious oocyst can generate a single focus of infection in HCT-8 monolayers, the theoretical limit of detection for the CC-IFA would be a single oocyst. The actual sensitivity was measured using flow-sorted oocyst inocula of 10, 5, and 1 oocysts/well with oocysts aged ≤15 days or >36 days (Table 3.2). For oocysts aged ≤15 days, an inoculum of 10 oocysts revealed 100% (9 of 9) positive samples with 2.4 ± 1.7 infectious clusters per well. Reducing the inoculum size to 5 oocysts yielded 1.2 ±1.1 infectious clusters per well in 77.7% (7 of 9) of the positive samples. Administration of a single oocyst yielded no CC-IFA positive samples.

When oocysts aged ≥36 days were used, an inoculum of 10 oocysts revealed 66.7% (6 of 9) positive samples with 0.9 ± 0.8 infectious clusters per well. Reducing the inoculum size to 5 oocysts yielded 0.2 ± 0.4 infectious clusters per well in 22% (2 of 9) of the positive samples. Again, administration of a single oocyst yielded no CC-IFA positive samples.

Using the assumption that all the oocysts ≤15 days were infectious, data in Table 3.2 indicate that the oocyst infectivity threshold was 24.4%. This indicates that theoretically >4 oocysts would be required to generate one focus of infection with the CC-IFA procedure. However, using an inoculum of 5 oocysts yielded a 77.7% positive rate. Based on these data, approximately 22% of

the samples with an inoculum of 5 infectious oocysts may be false negative by CC-IFA. Although several isolates were examined, environmental samples will likely have a greater diversity in species and isolates of *Cryptosporidium*. These variations can also have further impacts on the CC-IFA sensitivity.

Table 3.2. Enumeration of *C. parvum* Infectious Foci in HCT-8 Monolayers Inoculated with Flow-Sorted Oocysts from Three Isolates

	Oocyst Dose									
Isolate	10			5			1			Oocyst Age (Days)
TU114	4	3	1	0	1	0	0	0	0	7
TU114	1	2	1	1	1	1	0	0	0	15
MD	2	2	6	3	3	1	0	0	0	15
MD	0	2	0	0	0	0	0	0	0	36
MD	0	2	1	0	0	0	0	0	0	36
TUM1	1	1	1	0	1	1	0	0	0	84
Infectivity Threshold[a]	24.4% (≤ 15 days) 8.9% (≥36 days) **16.7% (mean)**			24.4% (≤15 days) 4.4% (≥36 days) **14.4% (mean)**						

[a] (Mean no. of foci/original oocyst inoculum) * 100.

3.3 Bench-Scale Disinfection Experiments

Previously *Cryptosporidium* disinfection experiments have typically relied on the neonatal mouse infectivity assay. Performance of the CC-IFA procedure described by Bukhari et al. (2007) has not been examined following oocyst disinfection. In the United States, chlorine continues to be the most popular choice for disinfection. A survey conducted recently indicated that 75% of publicly owned treatment works (POTW) employ chlorine-based disinfectants (Leong et al., 2008). Based on the Pomona virus studies in the 1970s (SDLAC, 1977), the chlorine CT values recommended by California Title 22 are 450 mg-min/L with a modal contact time of 90 min for wastewater effluents. Although chlorine can be effective for bacteria and viruses, it has little effect on protozoan parasites such as *Cryptosporidium.* Previous disinfection experiments have shown that chlorine CT values between 6000 and 7200 mg-min L^{-1} yield marginal (i.e., 1.0–1.7 log) inactivation (Korich et al., 1990; Venczel et al., 1997). Not only is chlorine ineffective for inactivating *Cryptosporidium*, but also its use has various other operational, security, and water quality problems. For example, matrices with high organic content exert significant chlorine demands, in turn reducing availability of free chlorine for microbial inactivation. Microbes that

are shielded by particles or that fail to receive an adequate disinfectant dose may simply be injured and proceed to repair the incurred damage. Microbial regrowth has been noted in reuse systems (Jjemba et al., 2009). Chlorination of organic compounds can also produce potentially carcinogenic disinfection byproducts (i.e., trihalomethanes and haloacetic acids), which can present short- and long-term human health impacts. As a result, there is an increasing trend toward utilization of alternative disinfectants. It is estimated that 21% of POTWs are currently employing UV disinfection, with almost 40% of these systems switching between 2001 and 2005. Substantially more conversions are expected in the next 5 years (Leong et al., 2008). UV light is highly effective against chlorine-resistant protozoa such as *Cryptosporidium* (e.g., 3 to >4 log inactivation with doses of 5–10 mJ cm^{-2}) (Bukhari et al., 1999, 2004). A 4 log inactivation of various bacteria (*Bacillus subtilis*, *Escherichia coli* O157:H7, and *Legionella pneumophila*) requires UV doses between 2 mJ · cm^{-2} and <8 mJ · cm^{-2} (Marshall et al., 2003; Yaun et al., 2003). Considerably higher UV doses (i.e., 27 mJ · cm^{-2} to 36 mJ · cm^{-2}) have been necessary to achieve 4 log reductions in viruses such as caliciviruses, polioviruses 1, and coxsackieviruses (Husman et al., 2004; Tree et al., 2005). Certain viruses (i.e., adenoviruses) that are highly susceptible to free chlorine (e.g., 4 log inactivation with a CT of 0.22 mg · min/L [Durance et al., 2005]) exhibit considerable resistance to UV disinfection, with 4 log inactivation requiring doses of >100 mJ · cm^{-2} (Ballester and Malley, 2004; Meng and Gerba, 1996). In addition, effective delivery of the targeted UV dose requires matrices with high UV transmission, and particle presence can shield microbes, thereby protecting them from UV light, and leading to a "tailing" phenomenon as reported for *Giardia* cysts (Craik et al., 2000). Further, UV disinfection is instantaneous, and no disinfectant residuals are maintained in the treated samples, which may lead to microbial regrowth issues when treated effluents are distributed for reuse applications.

In contrast to chlorine and UV, ozone is rarely used as a primary disinfectant. This may be due partly to the cost of producing ozone and also to its highly reactive nature, which can make it difficult to maintain adequate disinfectant residuals. In addition, ozone oxidation processes may break down large organic molecules to smaller, readily metabolizable, compounds (i.e., assimilable organic carbons) that could contribute to microbial regrowth. Despite this, ozone can be useful to manage odor, enhance coagulation, and improve the UV transmission of the effluent. The residual ozone may also contribute to simultaneous disinfection of microbes.

As no disinfectant is a "silver bullet," it seems reasonable that different wastewater systems would employ different disinfectants, depending on their end treatment goals. This means that *Cryptosporidium* oocysts in treated wastewater effluents may be exposed to a variety of disinfectants. How this impacts the ability of the CC-IFA procedure to reliably measure the treated oocysts infectivity is unknown. The mechanism of oocyst inactivation can impact infectivity measurements with different assays (Bukhari et al., 2000). For example, oxidative disinfectants may initially disrupt the structural integrity of an organism, whereas UV inactivation impacts DNA first. To ensure that the CC-IFA provides reliable infectivity data for oocysts recovered from various wastewater effluents following different physical removal or disinfection processes, the assay performance was evaluated in bench-scale disinfection experiments using chlorine, UV light, and ozone.

3.3.1 Chlorine

Chlorine-disinfected samples were pretreated for infection of HCT-8 monolayers, as described previously. Oocysts treated with sodium thiosulfate alone (10 µL of 0.08% thiosulfate solution) and subjected to infectivity indicated no deleterious impacts of the examined sodium thiosulfate concentration on HCT-8 monolayers. Results of the chlorination experiments are presented in

Figure 3.5 and indicate 1 log or lower inactivation of oocysts, with the mean levels being <0.5 log.

Oocyst CT values of 450 mg · min/mL and 600 mg · min/mL indicated substantial differences between fresh and aged oocysts, with aged oocysts showing marginally higher levels of inactivation.

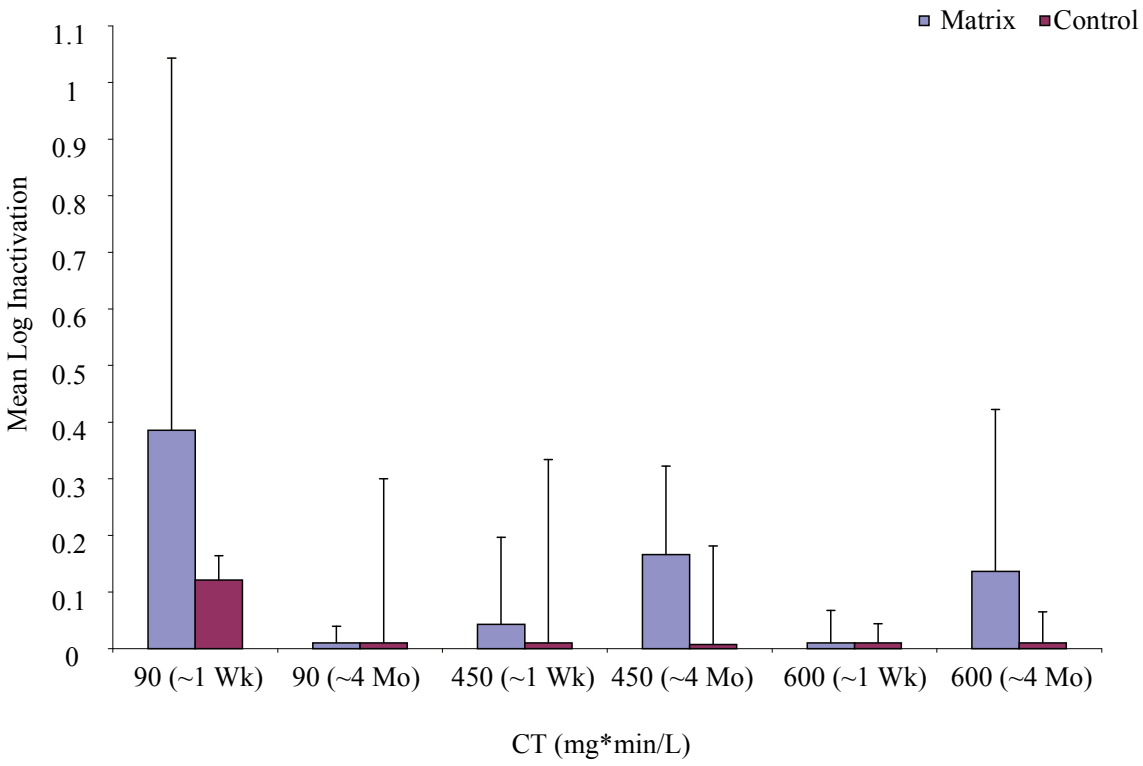


Figure 3.5. Inactivation of *C. parvum* oocysts following chlorination.
Note: Wk = weeks; Mo = months.

3.3.2 UV Light

In the last decade, numerous UV disinfection studies have been conducted; however, only a limited number of studies (Bukhari et al., 2004) have examined CC-IFA for measuring *Cryptosporidium* oocyst inactivation following UV exposure. Bench-scale data of this nature will help to establish the usefulness of CC-IFA as a tool for collecting infectivity information, which may then be used in future MRA for oocysts derived from reuse matrices.

Using a range of UV fluence, oocyst inactivation levels of >1.52 log were noted at 1 mJ · cm^{-2} and exceeded 4.5 log with a fluence of 20 mJ · cm^{-2} (Table 3.3). These data further support that CC-IFA data can reliably measure oocyst inactivation following UV disinfection. Also the disinfection data obtained with this cell culture assay were in agreement with data from previous UV disinfection studies using mouse infectivity assays (Bukhari et al., 1999).

Table 3.3. Log Inactivation of *C. parvum* Oocysts Following Exposure to Various UV Doses

Target Inoculum	1 mJ · cm^{-2}	5 mJ · cm^{-2}	10 mJ · cm^{-2}	20 mJ · cm^{-2}
50,000	TNTC	>4.61	>4.69	>4.69
10,000	>1.67	>4.0	NA	NA
5000	>1.80	>3.69	NA	NA
5000	>1.92	>3.69	NA	NA
1000	>1.89	>3.0	NA	NA
500	>1.73	>2.69	NA	NA
50	>1.69	>1.69	NA	NA
Mean Log Inactivation	>1.78 ± 0.11	>4.61	>4.69	>4.69

Note: TNTC = too numerous to count; NA, not applicable; $n = 6$.

3.3.3 Ozone

Ozonation experiment results for oocysts suspended in DI water and a 30% matrix from a plant employing tertiary sand filtration have been presented in Figure 3.6. The original intent was to use undiluted effluent samples for the ozonation experiments; however, the matrix demand for ozone was high (>10 mg/L), making it impractical to meet these high ozone demands in the bench-scale experiments. As a result, various matrix dilutions (50%, 40%, 30%, 20%, etc.) were examined to determine the highest matrix concentration that allowed maintaining adequate ozone residuals to conduct disinfection experiments. These preliminary matrix titration experiments determined that the use of 30% matrix samples (i.e., 30 mL of matrix and 70 mL of ozone demand–free water) allowed adequate ozone residuals for conducting bench-scale disinfection experiments.

An applied ozone dose of 1.0 to 1.5 mg/L in DI water revealed residual ozone concentrations of 0.77–1.2 mg/L following addition of the oocysts. In contrast, an almost fivefold increase in the applied ozone dose (4.0–5.7 mg/L) was required in 30% matrix water to generate similar residual ozone concentrations (0.85–0.88 mg/L) following oocyst addition. To facilitate comparison of *Cryptosporidium* disinfection for oocysts suspended in DI water versus those suspended in a 30% matrix, ozone CT values were used as a standardized approach to account for effects of matrix and other compounding factors.

Figure 3.6 presents the *Cryptosporidium* inactivation data following ozonation. Except for several outliers (data points highlighted with circles), increasing CT values increased oocyst inactivation. DI water and a 30% matrix revealed similar inactivation at CT values between 5 and 10 mg · min/L. Increasing CT values from 10 to 30 mg · min/L showed increasing oocyst inactivation levels in the 30% matrix samples. These data indicate that low levels of ozone (CT values <10 mg · min/L) can cause 1 to 1.5 log inactivation of *Cryptosporidium*. However, because of high matrix demands for ozone, effluents other than those from systems employing MBRs are unlikely to be candidates for ozonation during final disinfection. For MBR effluents that also use UV

disinfection downstream, ozonation may be particularly beneficial in helping to improve UV transmittance, in turn enhancing the effectiveness of UV disinfection.

In addition to measuring the effectiveness of CC-IFA for determining infectivity of *C. parvum* oocysts exposed to chlorine, UV light, and ozone, additional ozonation experiments were conducted to determine whether differences existed between *C. parvum* (isolate MD) and *C. hominis* (isolate TU728) infectivity when examined via CC-IFA. The CT values for both species were either 1.8 mg · min/L (3 min exposure) or 3.0 mg · min/L (5 min exposure).

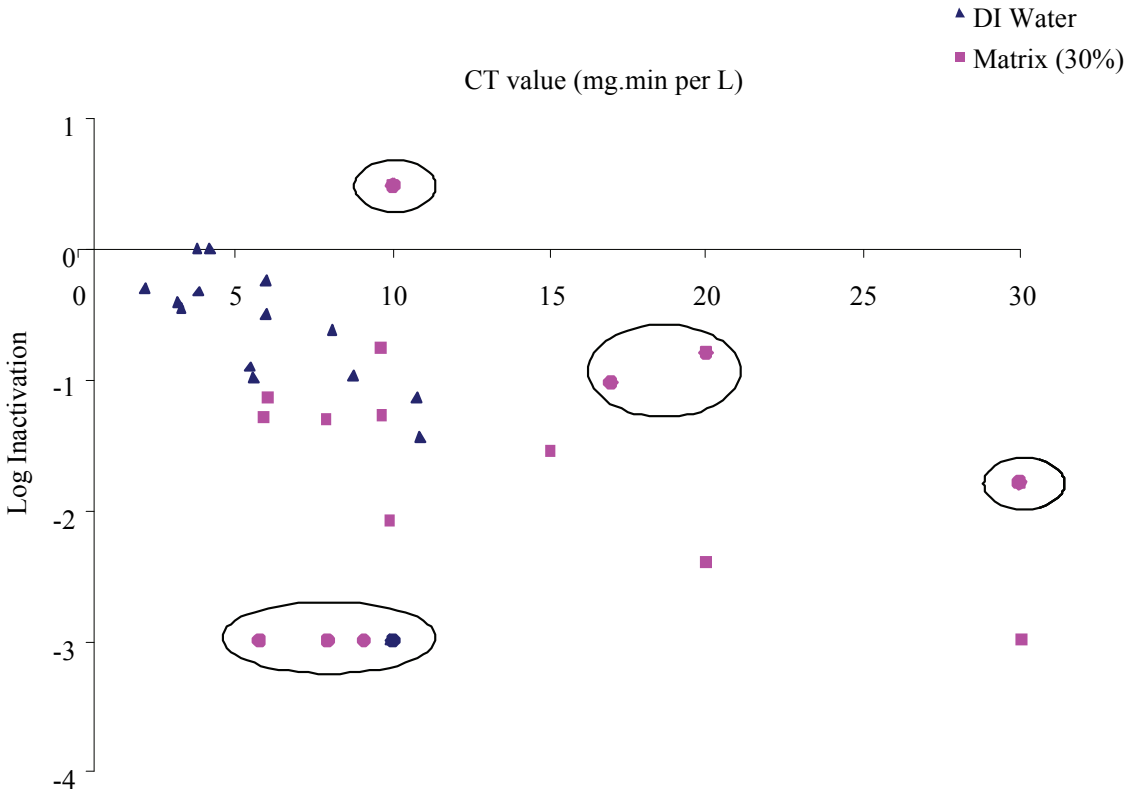


Figure 3.6. Inactivation of *C. parvum* oocysts following ozonation.

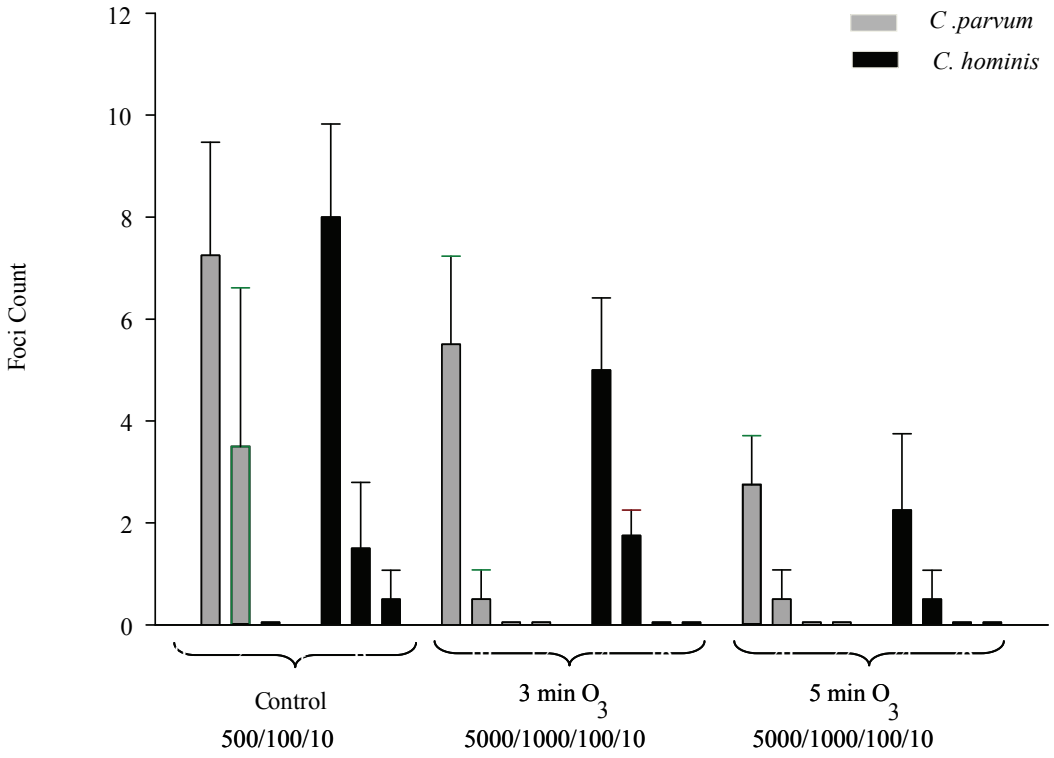


Figure 3.7. Comparing cell culture infectivity for C. *parvum* and C. *hominis* oocysts.

No appreciable differences in the infectivity of *C. parvum* and *C. hominis* oocysts either in the untreated controls (500 inocula) or after treatment with ozone for 3 or 5 min were observed (Figure 3.7).

Oocyst inactivation following a 3-min ozone exposure with an inoculum size of 5000 oocysts per well yielded a mean number of foci of 5.5 ± 1.73 standard deviations for *C. parvum* oocysts and 5 ± 1.41 for *C. hominis* oocysts. Extrapolation of the number of foci indicated approximately 250 infectious oocysts that were inoculated per monolayer in each suspension of 5000 oocysts.

Log inactivation at 3 min exposure was calculated as follows:

(log original inoculum) – (log infectious oocysts) = (log 5000) – (log 250) =

3.69 log – 2.39 log = 1.3 log.

The 5 min ozone exposure, followed by administration of 5000 oocysts per well, also yielded similar infectious foci for *C. parvum* (2.75 ± 0.96) and *C. hominis* (2.25 ± 1.5) oocysts. These data indicated that there were no appreciable differences between *Cryptosporidium* species in their response to CC-IFA or ozone disinfection.

3.4 Environmental Monitoring for Protozoa

3.4.1 Sampling Campaign

Nine of 14 utilities collected 12 paired samples over the sampling period (Figure 3.8). Of the remaining 5 facilities, 2 collected 14 predisinfection and 10 postdisinfection samples, 2 collected 13 predisinfection and 10 postdisinfection samples, and 1 facility collected 14 predisinfection and 9 postdisinfection samples. A total of 333 samples were analyzed by U.S. EPA Method 1623 for *Cryptosporidium* oocysts and *Giardia* cysts. Of these samples, 85% (n = 283) were analyzed by PCR for *Cryptosporidium* genotyping, and 85.6% (n = 285) were analyzed by CC-IFA for *Cryptosporidium* infectivity. For each of these methods, the sample distribution for different treatments processes is shown in Table 3.4.

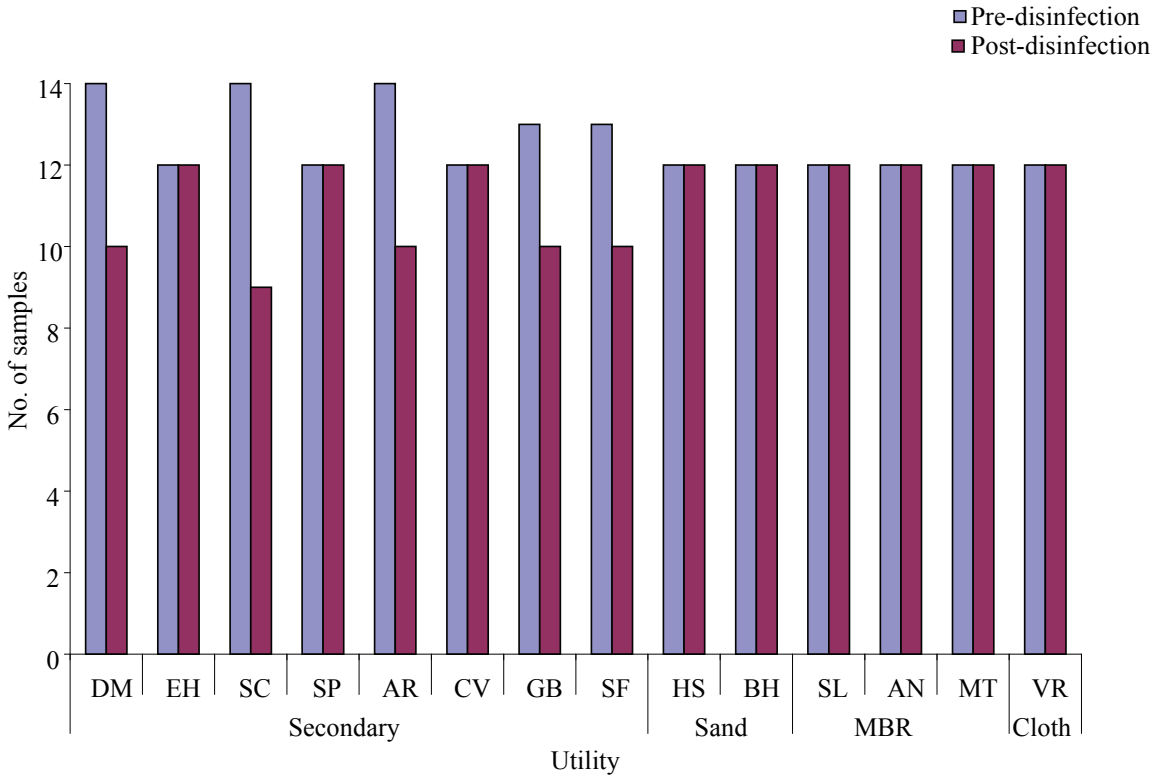


Figure 3.8. Sample distribution for the 14 utilities participating in the survey.

Table 3.4. Summary of Samples Collected from Various Reuse Facilities

			Number of Samples Analyzed		
Utility	Number of Samples Collected	Type of Treatment	Method 1623	PCR	CC-IFA
DM	24	Secondary clarification			
EH	24				
SC	23				
SP	24		189	162	163
AR	24				
CV	24				
GB	23				
SF	23				
HS	24	Tertiary sand filtration	48	44	44
BH	24				
SL	24	MBRs			
AN	24		72	56	56
MT	24				
VR	24	Cloth filtration	24	21	22
Total			**333**	**283**	**285**

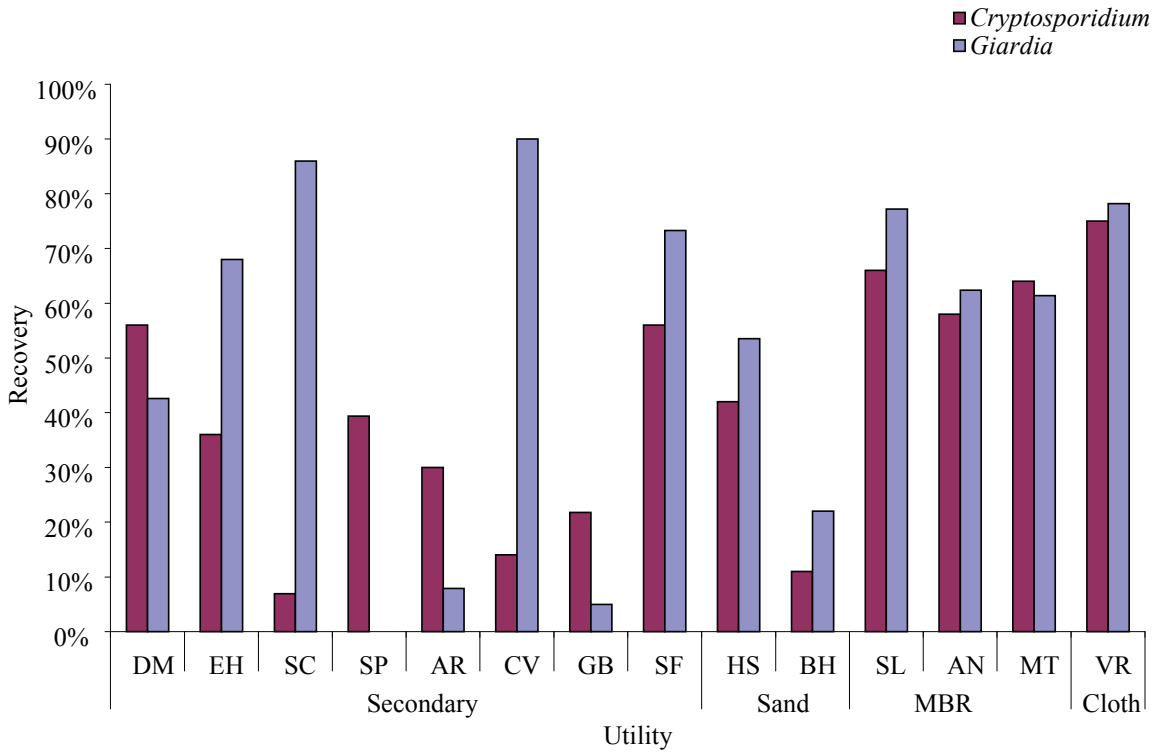


Figure 3.9. Matrix spike recoveries for *Cryptosporidium* oocysts and *Giardia* cysts.

3.4.2 Matrix Spikes

To ensure QA performance of Method 1623, grab samples (10 L) of the matrix from each utility were individually spiked with approximately 100 (n = 99–101) flow-sorted *C. parvum* oocysts and *G. lamblia* cysts. Each spiked sample was filtered through an Envirochek HV filter, eluted, and enumerated according to U.S. EPA Method 1623 to determine recovery efficiencies. Results shown in Figure 3.9 indicate that recoveries ranged from 6.9% to 75% for *Cryptosporidium* oocysts and from 7.9% to 90% for *Giardia* cysts.

Figure 3.10 shows mean recoveries for the different wastewater treatment processes. In all processes, *Giardia* recoveries were higher than *Cryptosporidium* recoveries. For both organisms, recoveries were lower following secondary clarification (n = 8) and sand filtration (n = 2) than in MBR (n = 3) effluents and cloth filtration effluents. As only a single cloth filtration plant was used in the study, recovery results for that system need to be interpreted with caution. Of the three remaining systems, the MBR systems yielded the highest recovery, which may be associated with the high effluent quality presenting little challenge for Method 1623. In contrast, sand filtration showed the lowest recoveries, which may be a limitation of the small number of systems.

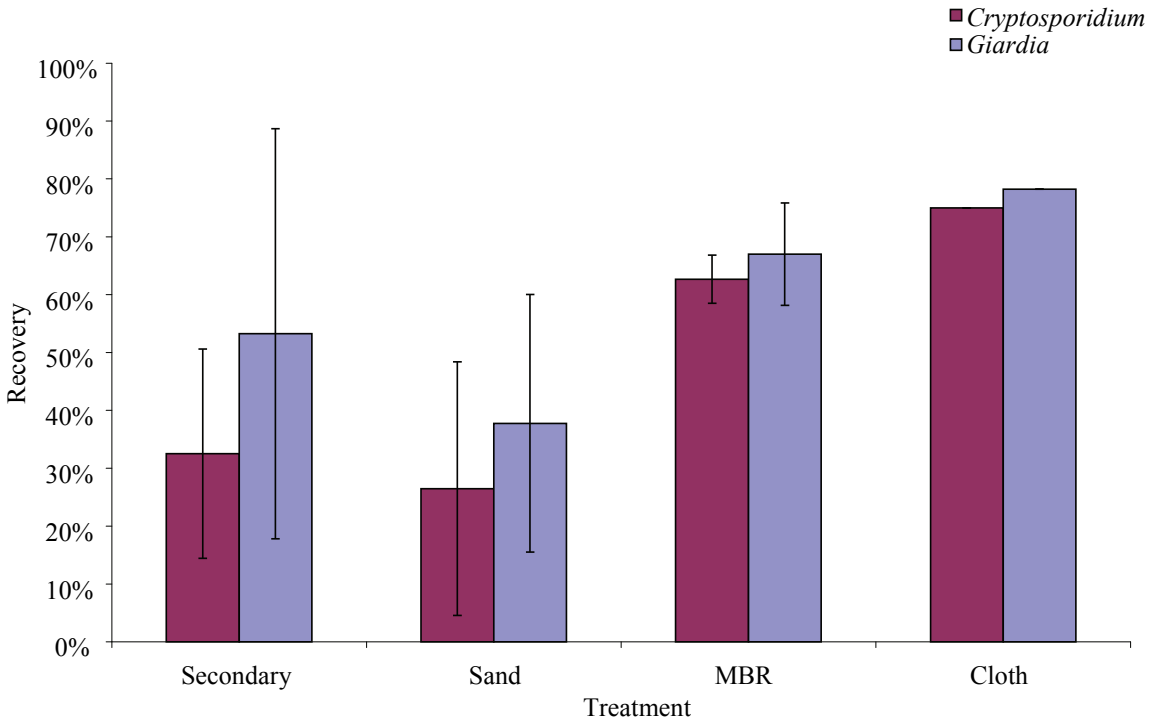


Figure 3.10. Matrix impact on *Cryptosporidium* and *Giardia* recoveries.

3.4.3 *Cryptosporidium* and *Giardia* Occurrence

Data for occurrence of *Cryptosporidium* and *Giardia* as determined by IFA are shown in Figure 3.11 and Figure 3.12, respectively. All eight plants employing secondary treatment were frequently positive for *Cryptosporidium* (20–66.7%) and *Giardia* (>83–100%). One of the two plants employing sand filtration (BH) demonstrated that 8.3% of the samples were *Cryptosporidium* oocyst positive. *Giardia* cysts were detected at both sand filtration plants, with plant BH demonstrating a higher occurrence (i.e., predisinfection, 33.3%, and postdisinfection, 50%) than plant HS (8.3% samples positive).

Of the three plants utilizing MBR treatment, only one (AN) demonstrated *Cryptosporidium*-positive samples (postdisinfection, 8.3%). Two of the three MBR plants (AN and MT) demonstrated *Giardia*-positive samples. In both of these systems, the frequency of *Giardia*-positive samples was 58.3 to 83.3%.

One plant employed cloth filtration and was positive for both *Cryptosporidium* and *Giardia*. The predisinfection samples for this plant contained substantially higher frequency of *Giardia*-positive samples (66.7%) than of *Cryptosporidium*-positive samples (33.3%); however, for both organisms 16.7% of the samples were positive postdisinfection. Overall 21.9% (73 of 333) *Cryptosporidium* and 70.1% (234 of 333) of the *Giardia* samples were positive with IFA-based enumeration using Method 1623.

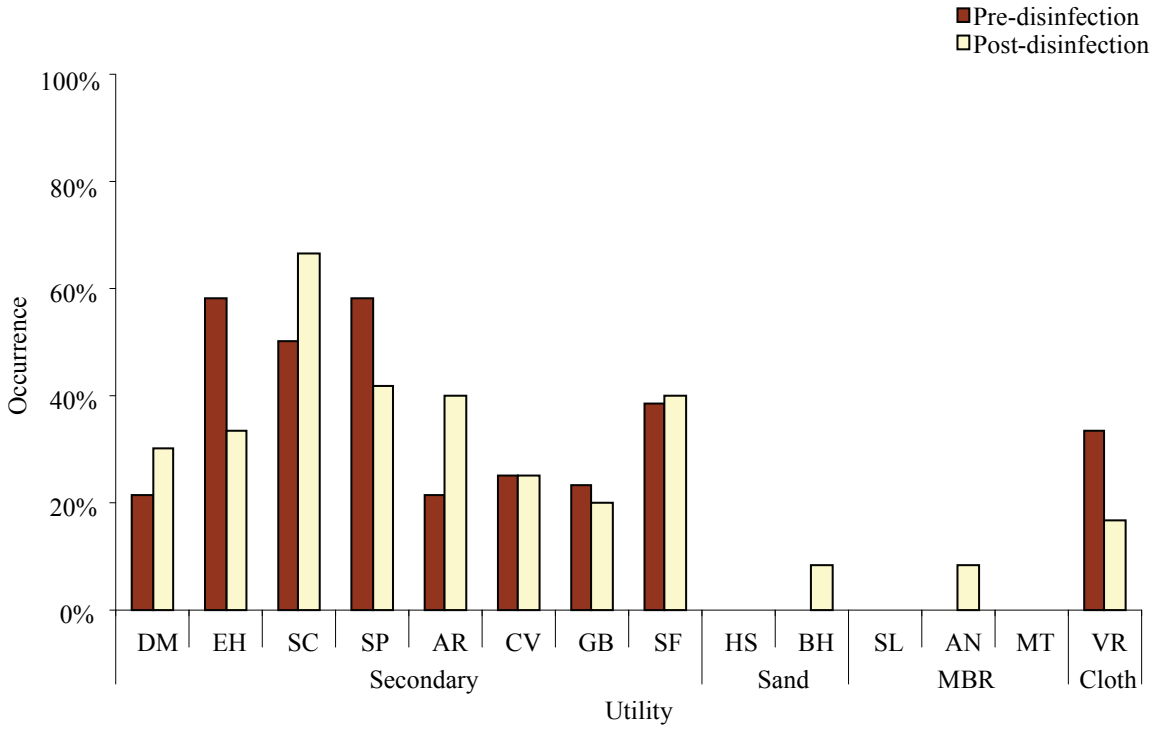


Figure 3.11. ***Cryptosporidium*** **occurrence in utilities employing different treatments.**

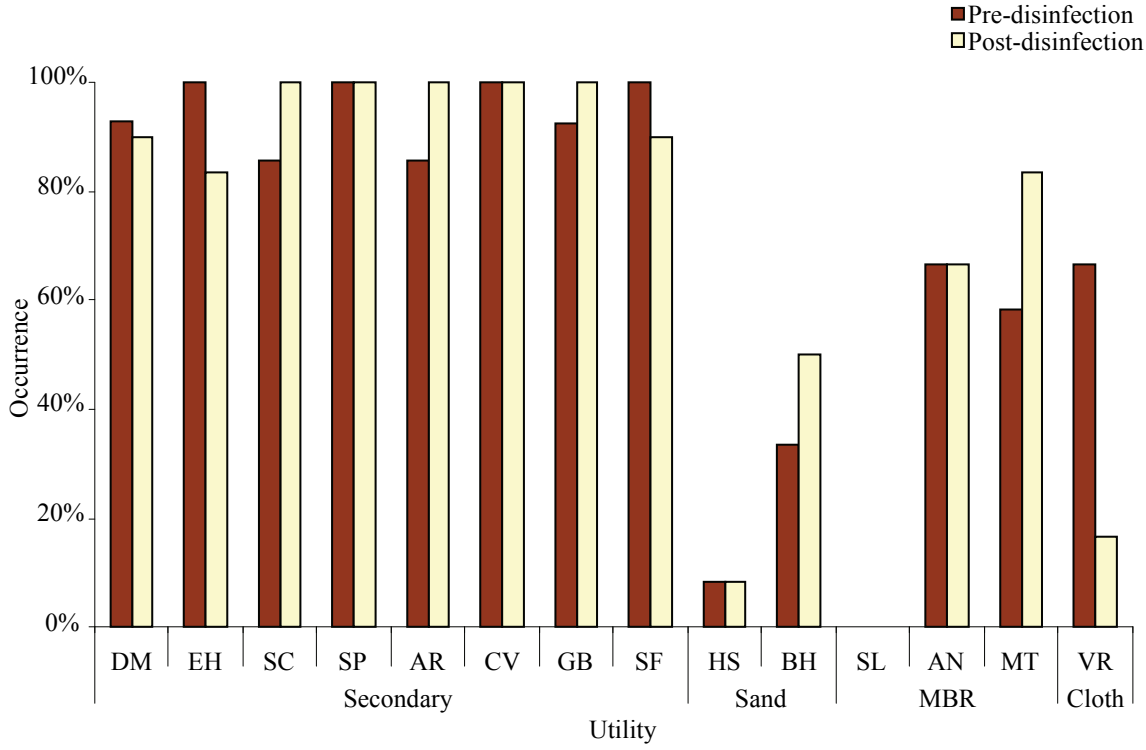


Figure 3.12. ***Giardia*** **occurrence in utilities employing different treatments.**

3.4.4 *Cryptosporidium* and *Giardia* Concentrations

Mean *Cryptosporidium* concentrations in the eight plants employing secondary clarification ranged from 0.5 oocysts/L to 11.6 oocysts/L (Figure 3.13). Mean *Giardia* concentrations for these eight plants were 2.7 cysts/L to 41.5 cysts/L (Figure 3.14). One of the two plants employing sand filtration (BH) and one of the three plants employing MBR each had a mean concentration of 0.2 *Cryptosporidium* oocysts/L (Figure 3.13). For *Giardia*, the two plants employing sand filtration indicated a mean concentration of 0.4 cysts/L (HS) and 0.9 cysts/L (BH). Two of the three MBR plants that were *Giardia*-positive contained 0.8 cysts/L (AN) and 1.5 cysts/L (MT). See Figure 3.14.

Giardia occurred more frequently and at higher concentrations in most systems. However, utility EH (secondary clarification) and VR (cloth filtration) both indicated fourfold higher mean *Cryptosporidium* oocyst concentrations than *Giardia* concentrations.

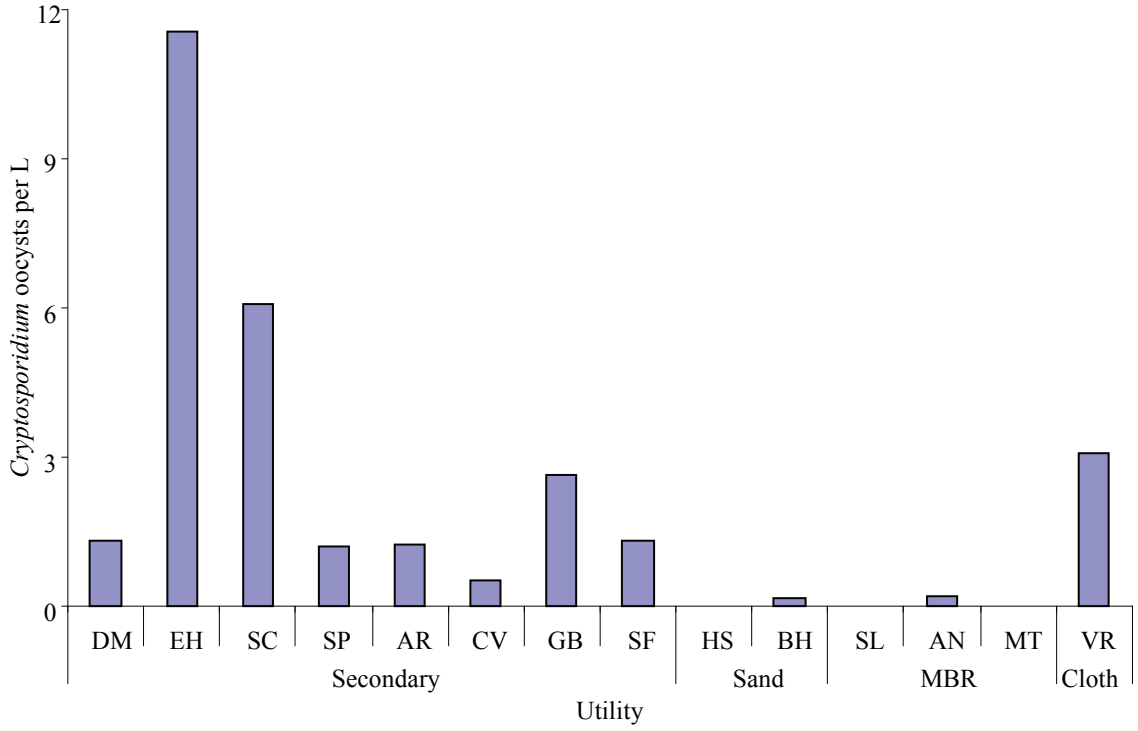


Figure 3.13. *Cryptosporidium* concentrations in utilities employing different treatments.

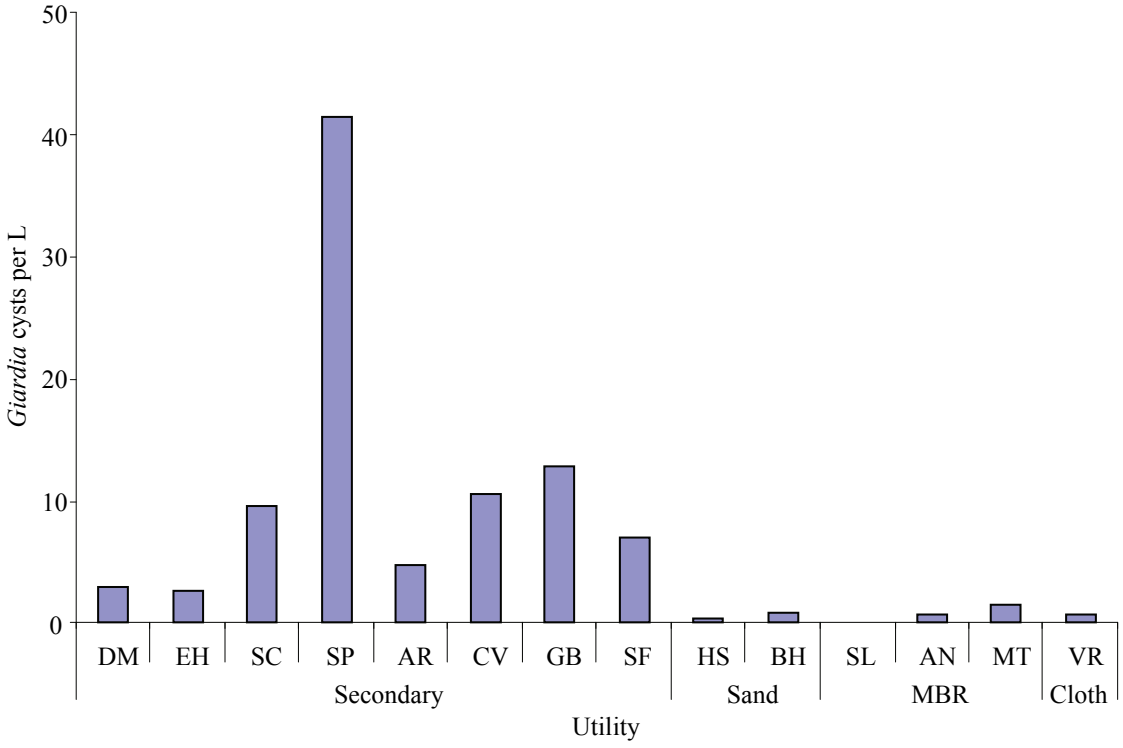


Figure 3.14. *Giardia* concentrations in utilities employing different treatments.

3.4.4.1 Protozoa Occurrence Based on Treatment Processes

Figure 3.15 shows the mean protozoa occurrence for each of the four treatment processes. For all treatments the mean frequency of positive samples was greater for *Giardia* than for *Cryptosporidium*. The differences in the occurrence between the two organisms was greatest for MBRs (~33-fold) and sand filtration (~12-fold), followed by secondary clarification (~3-fold) and then cloth filtration (~2-fold).

Figure 3.16 shows the mean concentration of protozoa for each of the four treatment processes. For three treatment processes (secondary clarification, sand filtration, and MBR) the mean concentration of *Giardia* cysts was greater than that of *Cryptosporidium* cysts. The differences in the occurrence between the two organisms were greatest for MBRs (~6-fold) and sand filtration (~4-fold) followed by secondary clarification (~3-fold).

Cloth filtration was the only treatment process for which the mean *Cryptosporidium* concentration was approximately fivefold higher than the mean *Giardia* concentration.

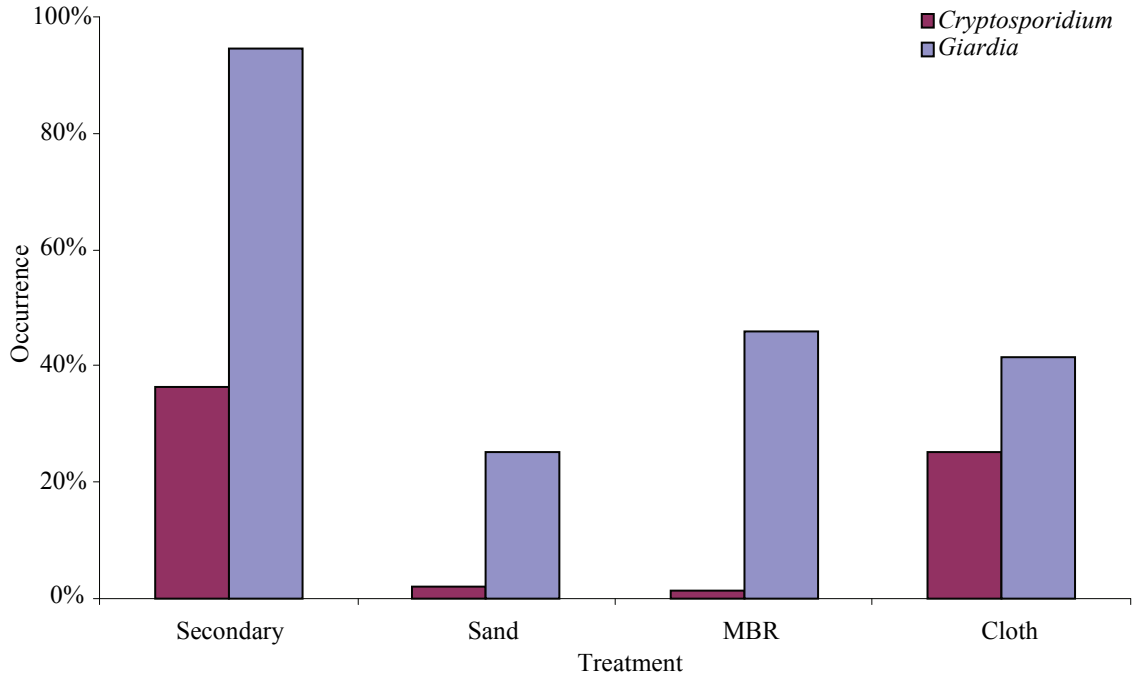


Figure 3.15. Occurrence of protozoa based on various treatments.

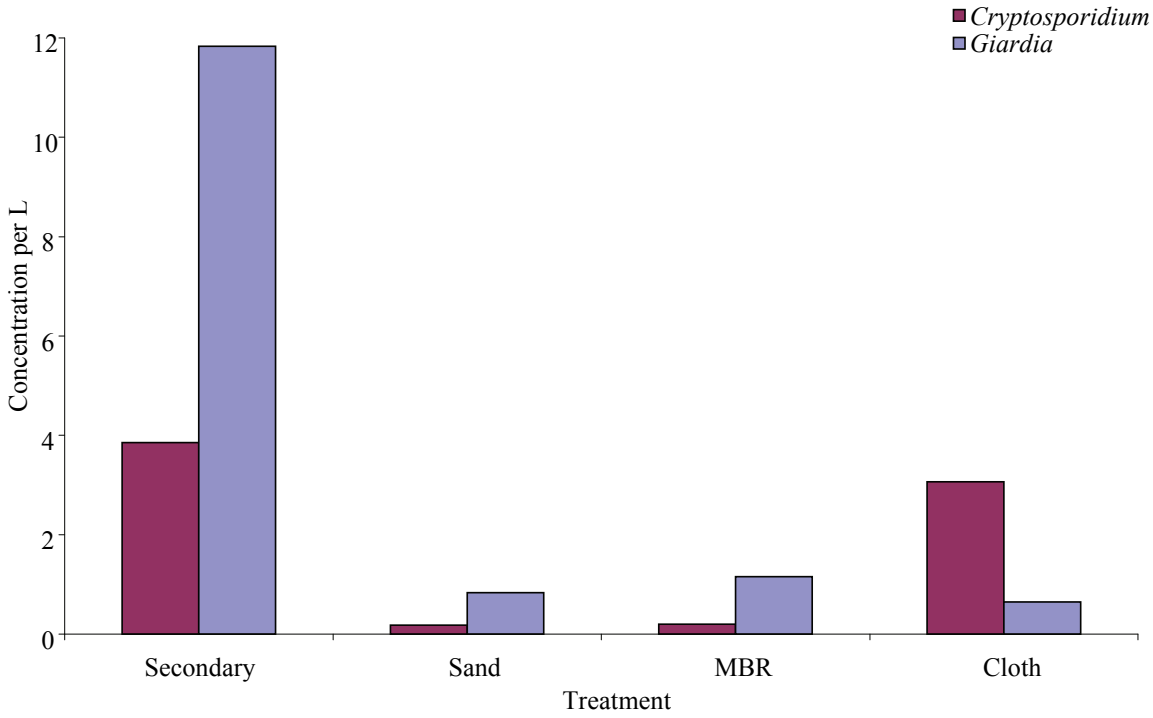


Figure 3.16. Concentration of protozoa based on various treatments.

3.4.5 Comparison of *Cryptosporidium* Occurrence Using Method 1623 and PCR

A total of 333 samples were examined for *Cryptosporidium* oocysts by IFA according to Method 1623. Approximately 85% (n = 283) of these samples were analyzed by PCR (Table 3.4). Figure 3.17 shows that no oocysts were detected by IFA or PCR in 61% (n = 173) of the samples that were screened. Among the remaining samples, the frequency of positives with Method 1623-based microscopy was 21% (n = 59), and by PCR it was 13% (n = 37). Only 5% (n = 14) of the samples were positive with both detection methods (Figure 3.17). Overall 39% (110 of 283) of the samples analyzed by either IFA or PCR were *Cryptosporidium* positive. Of the *Cryptosporidium*-positive samples, 55% were determined positive by IFA and 32% by PCR (Figure 3.18). Agreement between both detection methods was noted only for 13% of the *Cryptosporidium*-positive samples. Figure 3.19 presents comparison of mean (pre- and postdisinfection combined) oocyst occurrence using either Method 1623 or PCR. Secondary effluent samples from 7 of 8 plants and samples from the cloth filtration plant indicated higher *Cryptosporidium* occurrence with Method 1623 than by PCR. In contrast, plants employing sand filtration or MBR indicated higher *Cryptosporidium* occurrence with PCR than with Method 1623. No oocysts were detected in samples from HS, SL, or MT by IFA; however, 5.6–25% of these samples were PCR positive.

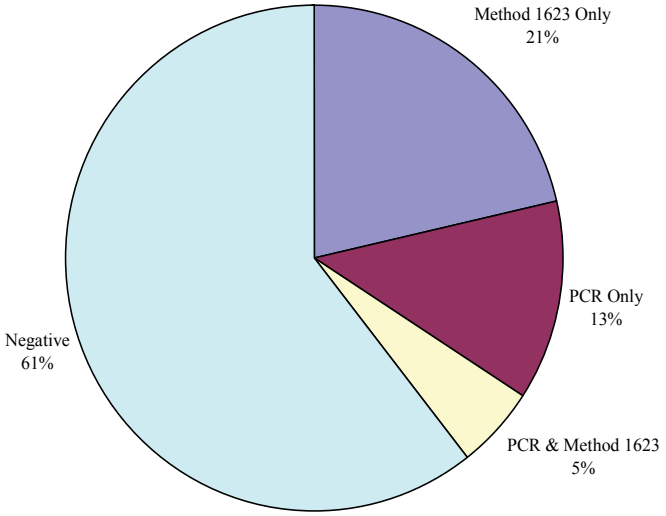


Figure 3.17. *Cryptosporidium* occurrence using two different detection methods.

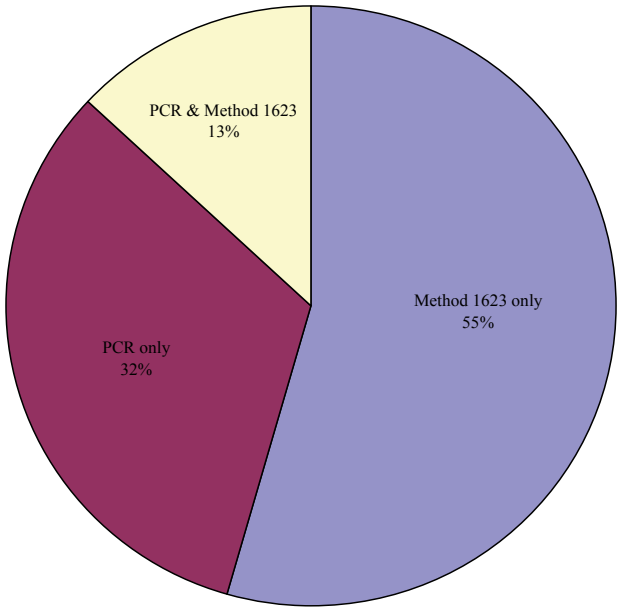


Figure 3.18. Detection frequencies using two different detection methods.

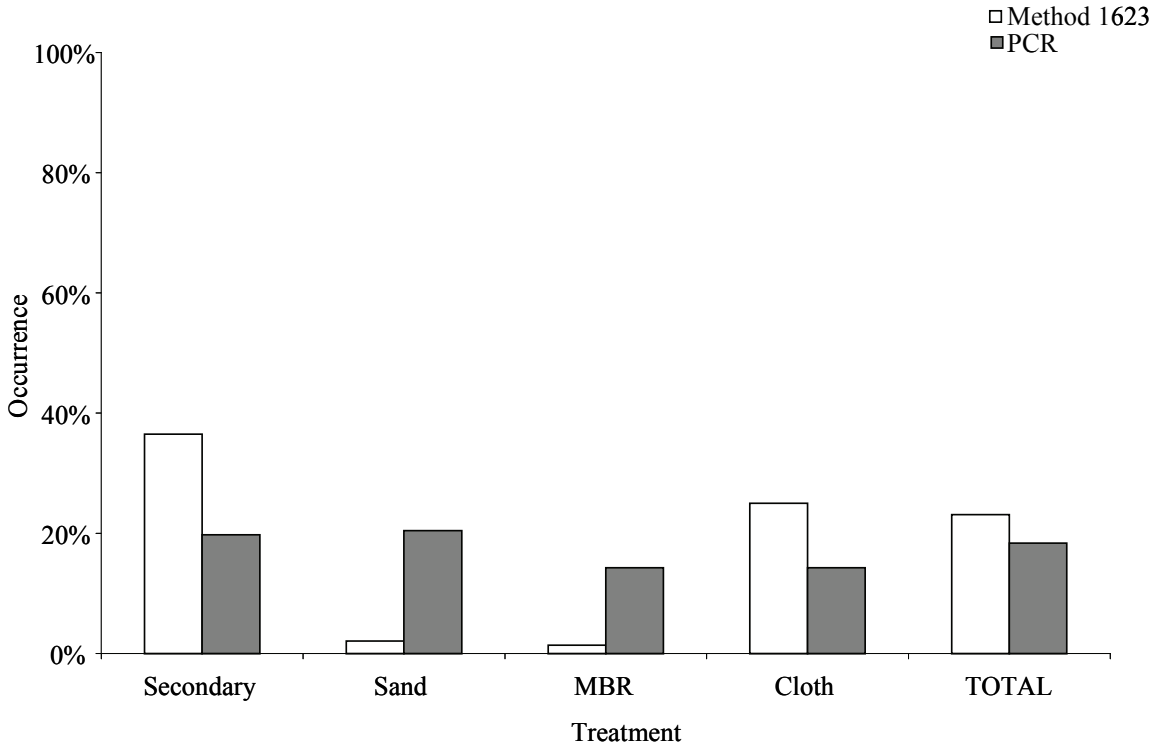


Figure 3.19. *Cryptosporidium* occurrence by two detection methods.

Cryptosporidium occurrence was lower with PCR than with Method 1623 for effluents from plants employing secondary clarification and cloth filtration. In contrast, PCR detected *Cryptosporidium* more frequently than Method 1623 for effluents following sand filtration and MBR (Figure 3.19).

3.4.6 *Giardia* Assemblage Characterization

Before *Giardia* assemblage characterization, sensitivity analyses were performed with known spike doses of cysts in DI water and using three different primer sets (β-giardin, GDH, and TPI). Results (data not shown) indicated β-giardin was >10-fold more sensitive than the other two primer sets. In addition, the frequency of PCR positives was compared to cyst concentrations in the environmental samples, as detected by IFA-based enumeration according to Method 1623 (Figure 3.20). IFA indicated absence of *Giardia* cysts in 98 samples; however, 56% of these samples were PCR positive with the β-giardin gene. In contrast, PCR with TPI gene indicated 29% positives, whereas the GDH gene indicated only 15% PCR positives for *Giardia* cysts. With increasing *Giardia* cyst concentrations, only PCR with the β-giardin gene indicated a dose-related response. However, even where >100 cysts per slide were detected, only 80% of the samples were PCR positive. For the remaining two primer sets, a substantially lower proportion (15–57%) of the IFA positive samples were PCR positive.

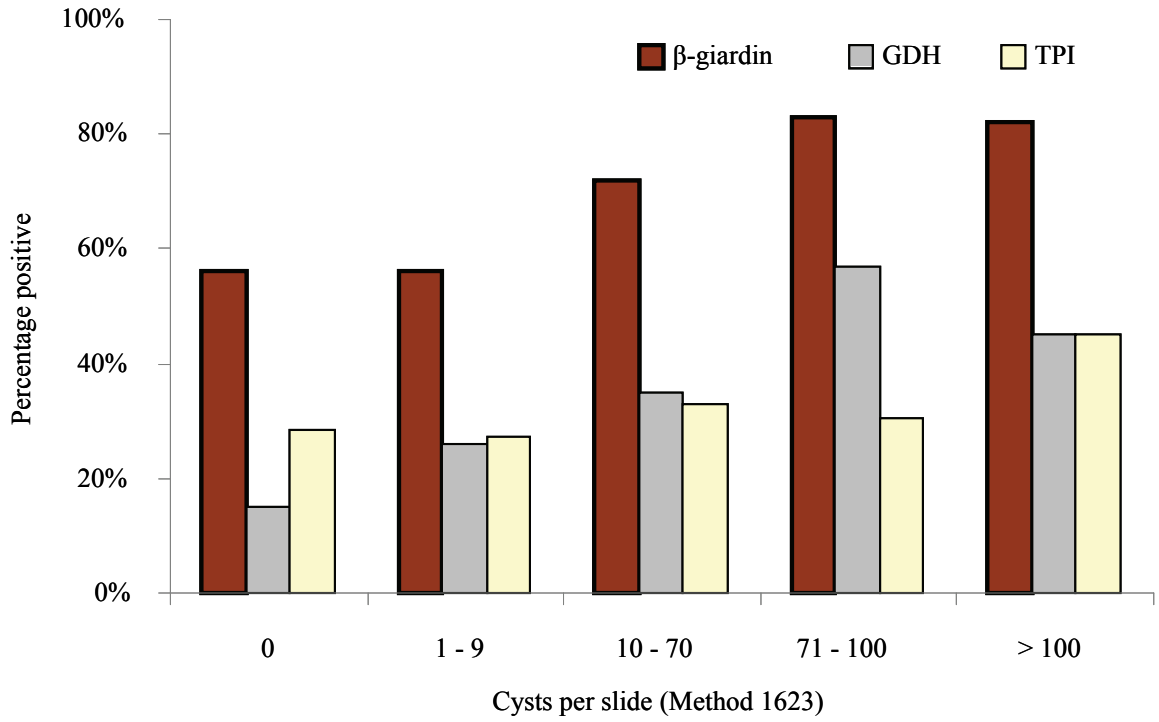


Figure 3.20. Sensitivity comparison of PCR targeting three different *Giardia* genes.

3.4.6.1 Giardia Occurrence by Four Detection Methods

Measurements of *Giardia* occurrence in each of the four different treatment processes by four detection methods are presented in Figure 3.21. Compared to IFA-based enumeration using Method 1623, PCR targeting the TPI gene consistently underestimated *Giardia* occurrence. *Giardia* occurrence as determined by the GDH gene was similar to that determined by the TPI gene for three (secondary, sand, and MBR) of four treatment processes. For effluent samples taken following cloth filtration, *Giardia* occurrence (approximately 80%) was higher with the GDH target than with the other detection methods. For both the GDH and TPI genes, occurrence for all treatment processes was approximately 30% and was a substantial underestimation of *Giardia* occurrence when compared to the occurrence measured by IFA in Method 1623 (71%). *Giardia* occurrence with the β-giardin gene was 72% in secondary effluents compared with 94% by Method 1623. In contrast to secondary effluents, the β-giardin gene yielded higher occurrence than Method 1623 in effluents following sand filtration, MBR and cloth filtration. For the β-giardin gene, occurrence for all treatment processes was approximately 65%, compared to 71% by IFA using Method 1623.

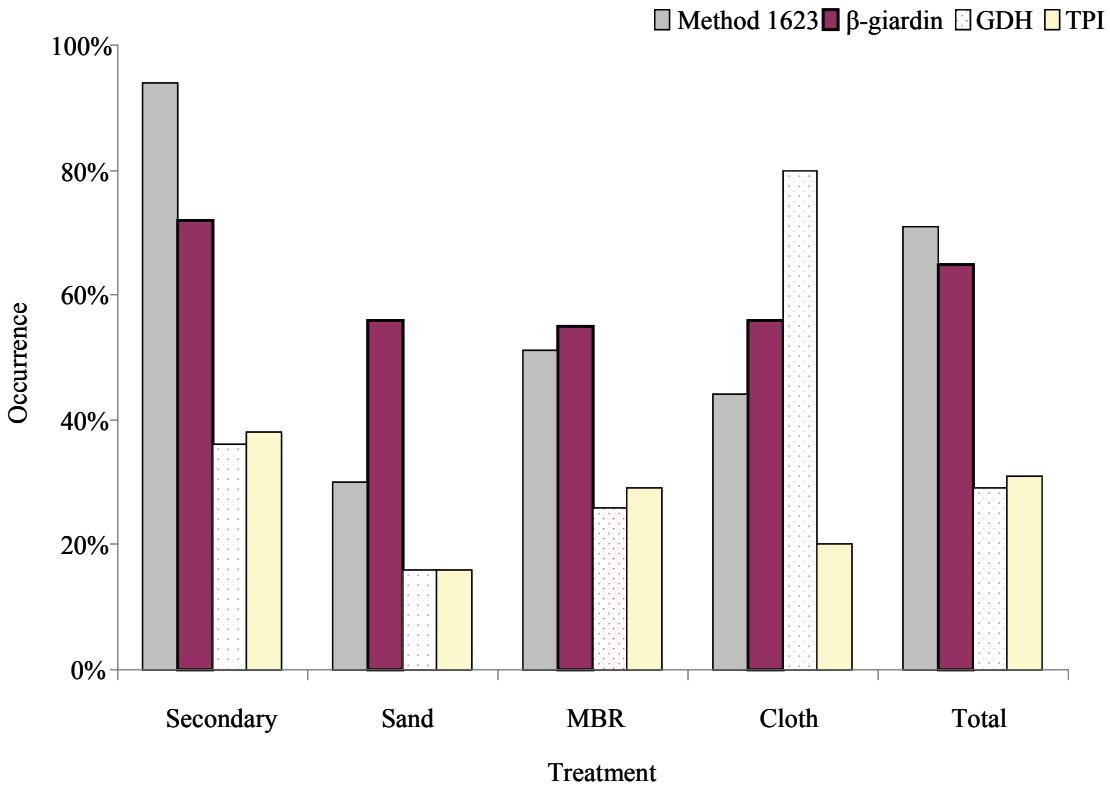


Figure 3.21. Comparison of *Giardia* occurrence by conventional and molecular procedures.

3.4.7 Infectivity of *Cryptosporidium* Recovered from Wastewater

A total of 18 samples were determined to contain infectious *Cryptosporidium* oocysts by CC-IFA, with 15 of 18 samples being derived from three plants (SC, SP, and GB) employing secondary clarification. The remaining 3 samples were recovered from a single plant (VR) employing cloth

filtration. The numbers of infectious clusters in positive samples are shown in Figure 3.22. For three locations (SC, SP, and GB), a single infectious cluster was detected predisinfection and no clusters were detected postdisinfection. In these cases the absence of infectious oocysts postdisinfection was probably due to limitations in assay sensitivity rather than due to effects of the disinfectant. In plants where >2 infectious clusters were detected prechlorination, infectious clusters were usually also detected postchlorination. In contrast, a plant (GB) using UV disinfection indicated 4 infectious clusters predisinfection; however, no infectious clusters were detected after UV disinfection (Figure 3.2).

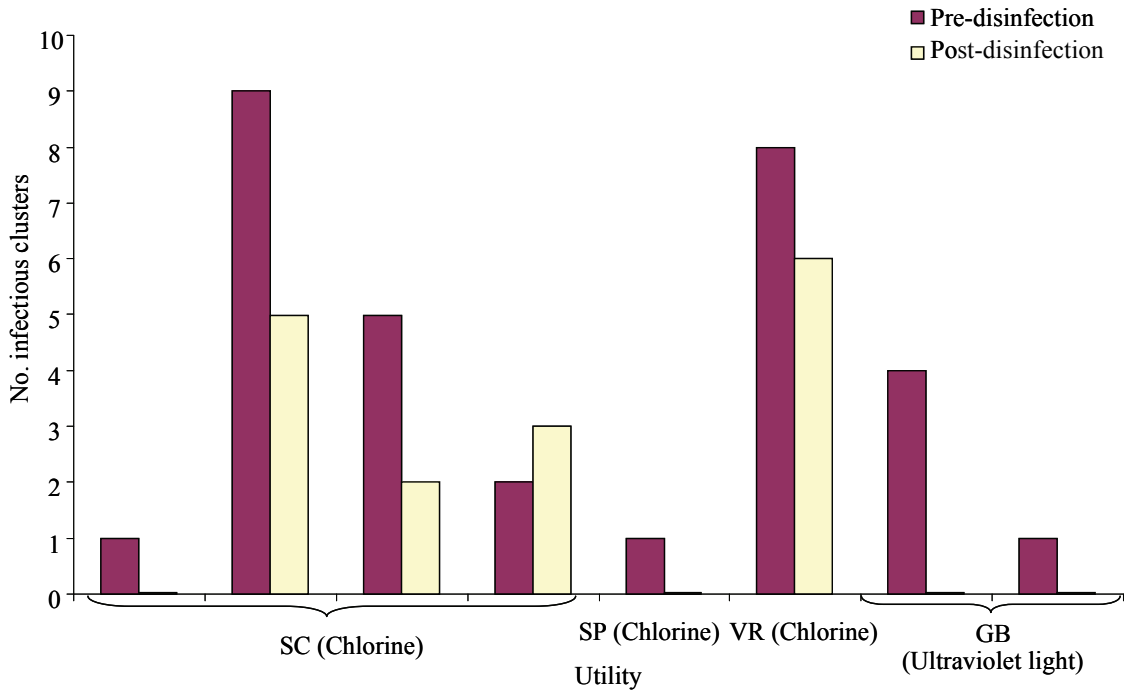


Figure 3.22. Detection of infectious oocyst clusters following disinfection.

Using the linear regression equation in Figure 3.2, the number of infectious oocysts (x) per sample was calculated using the following equation:

$$x = (y - 1.9568)/0.1165$$

For plants employing secondary clarification (SC and SP) followed by chlorination, the number of infectious oocysts per approximately 2.5 L sample ranged from <0.4 to 60.5 oocysts before disinfection to 0.4 to 26.1 oocysts after chlorination. One sample from the plant employing secondary clarification followed by UV disinfection indicated 17.5 infectious oocysts before disinfection; however, no infectious oocysts were detected after UV disinfection. A single sample from a plant using cloth filtration (VR) indicated 51.9 infectious oocysts before chlorination and 34.7 infectious oocysts after chlorination.

Applying the linear regression equation with the values for infectious foci from Table 3.6 allowed the infectivity percentage for environmentally recovered oocysts to be estimated. Except for the three highlighted samples in Table 3.6, the remaining samples with a known number of foci all indicated >100% infectivity by CC-IFA. For the three highlighted samples in Table 3.6, the calculated oocyst infectivity was 18.5% (0.8 oocysts/L by Method 1623), 1.4% (10.8 oocysts/L by Method 1623), and 43.7% (8.2 oocysts/L by Method 1623). All three samples were from the

same plant (SC), with two of these samples having been taken postchlorination. However, the lowest infectivity value (1.4%) was in a sample taken prechlorination. According to Table 2.1, SC was the only plant receiving domestic wastewater as well as discharges from an animal rendering facility.

The sensitivity of the CC-IFA procedure for oocysts recovered from the various wastewater systems was examined in Figure 3.23. Results indicated 2.6% (3 of 111) of IFA-negative samples were CC-IFA positive and 3% of samples with *Cryptosporidium* concentrations of <1.0 oocyst/L by IFA were CC-IFA positive. Collectively for oocyst concentrations between 0 and 1.0 oocysts/L, there were 2.7% positive samples (Figure 3.23). The frequency of CC-IFA positive samples increased to 27.8% when oocyst concentrations ranged between 1.1 and 6.0 oocysts/L. Only 5 samples contained concentrations between 6.1 and 10 oocysts/L, and 80% of these were CC-IFA positive. As a volume of 2.5 L was typically analyzed by CC-IFA, the number of oocysts applied per cell culture assay can be calculated by multiplying oocyst concentration by 2.5. For example, in Figure 3.29, the concentration range 6.1 to 10.0 oocysts/L was equivalent to

(6.1 * 2.5) = 15.25 oocysts up to (10.0 * 2.5) = 25 oocysts

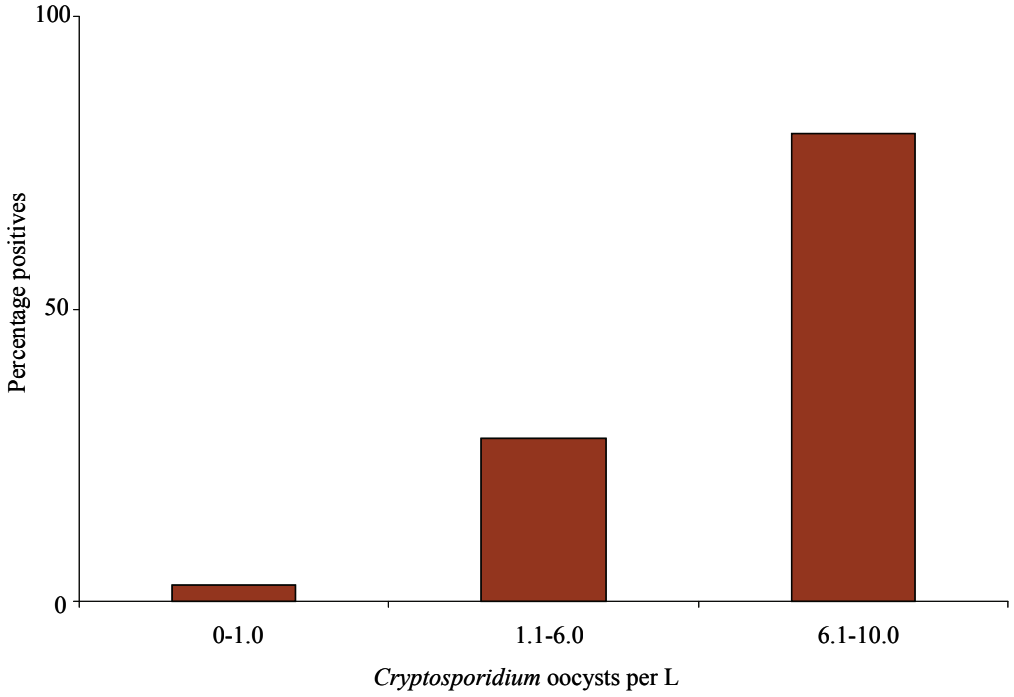


Figure 3.23. Sensitivity of CC-IFA for detection of environmentally derived oocysts.

Based on the CC-IFA sensitivity experiments in Table 3.2, it was calculated that analysis of 10 infectious oocysts by CC-IFA yielded a 100% positive rate. As the oocyst concentration range between 6.1 and 10 oocysts/L (actual inocula, 15–25 oocysts) yielded 80% positive samples by CC-IFA, this suggested that 20% of the environmental samples with the oocyst concentration range between 6.1 and 10 oocysts/L likely contained fewer than 10 infectious oocysts.

3.4.8 *Cryptosporidium* Species Identification Using COWP Primers

Of the 51 PCR-positive samples, sequence analysis was performed on 40 samples, and *C. hominis* DNA was detected in 9 samples taken from 6 utilities. *C. parvum* DNA was detected in 31 effluent samples from 12 utilities (Figure 3.24). DNA for both species was detected in samples from three utilities employing secondary clarification, one utility with sand filtration, and one utility with MBR. Alignment of PCR products amplified from five environmental samples has been presented relative to a control *C. hominis* isolate (TU502) in Figure 3.25. Except for sample CM90671, which showed two polymorphisms relative to *C. hominis*, the amplicons from the remaining samples indicated complete homology to *C. hominis*. For sample CM90671, polymorphism was not observed on the complementary strand, which suggests a likelihood of sequencing errors. Overall *C. parvum* was detected more frequently than *C. hominis*.

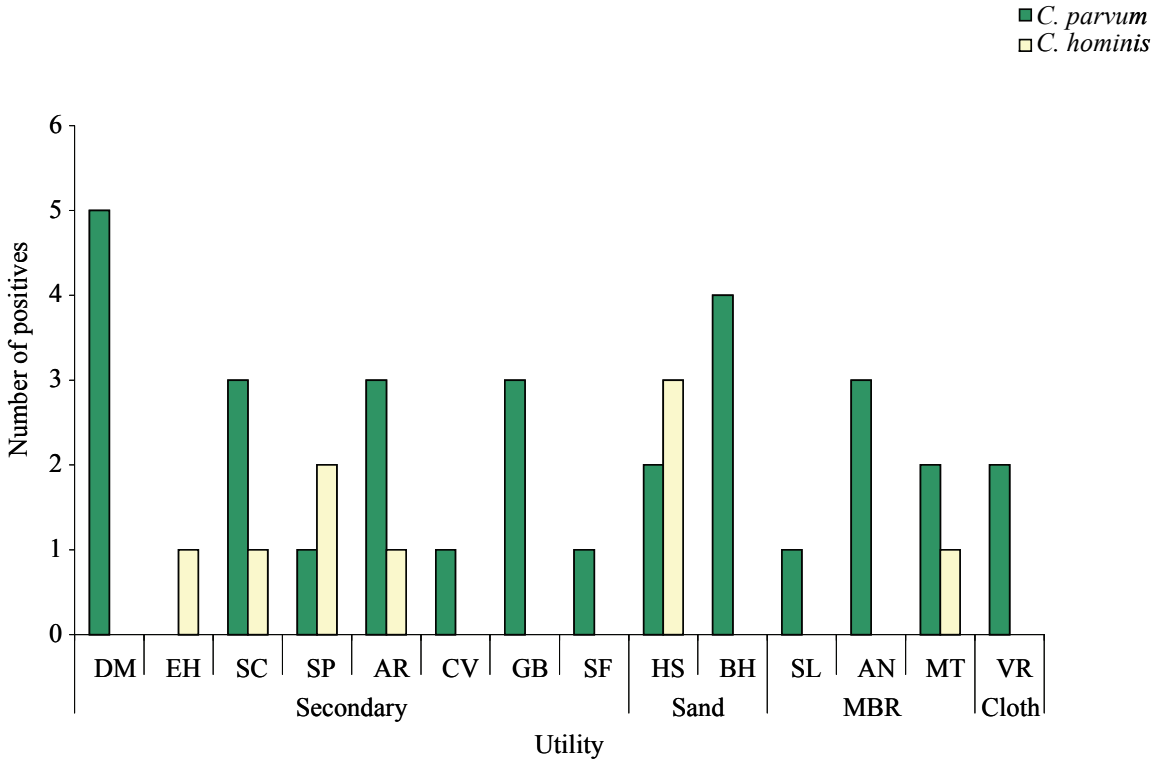


Figure 3.24. Distribution of *Cryptosporidium* species.

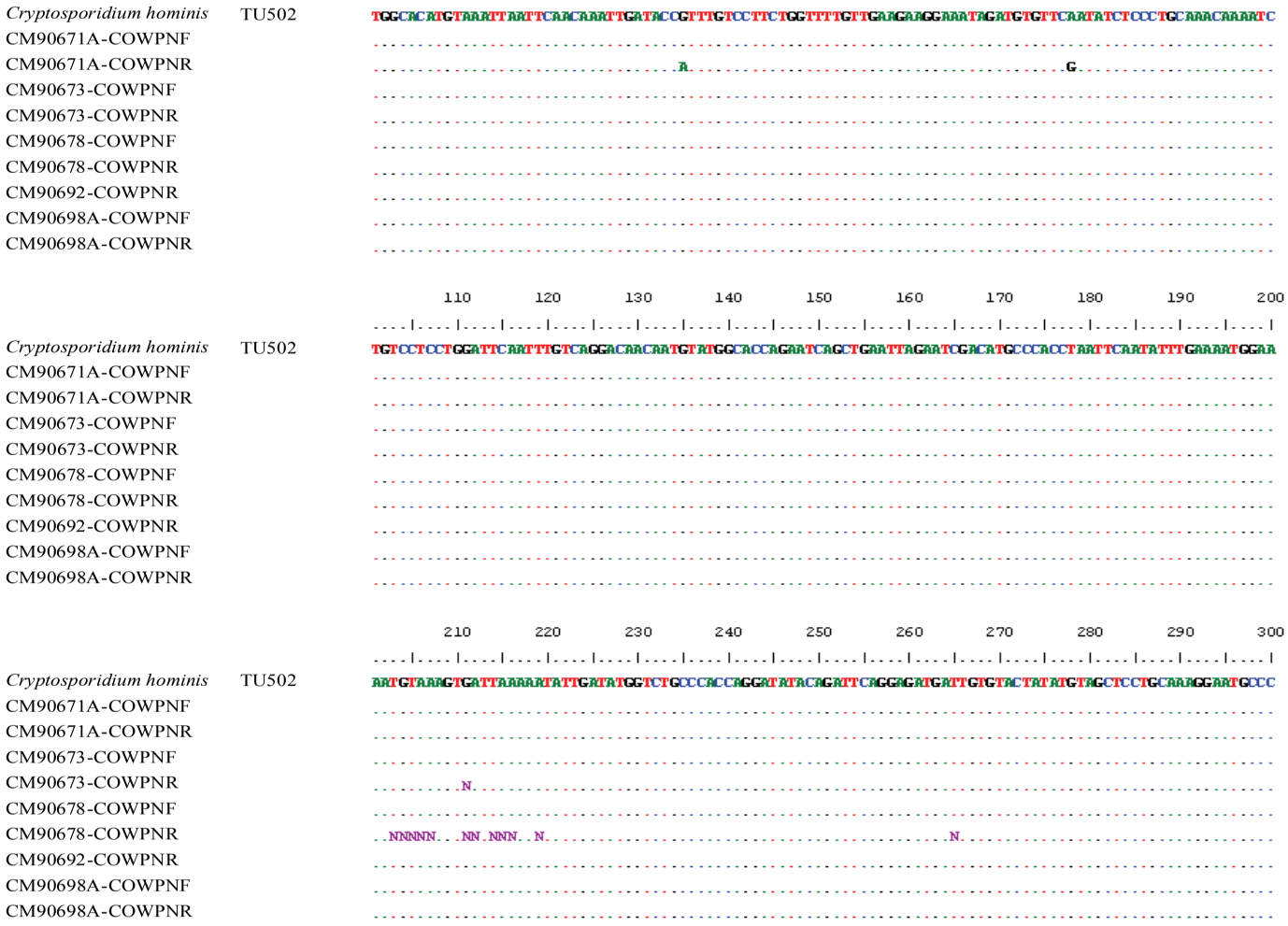


Figure 3.25 Alignment of a 300 bp fragment of COWP sequences amplified from *c. Hominis* TU502 reference sequence and five environmental samples.

3.4.9 *Giardia* Assemblage Characterization

Table 3.5 summarizes the number of samples of the various assemblages (A, B, D, and G) that were detected using the three target genes. Assemblages A and B, which can cause human infections, were detected in 100%, 97.6%, and 100% of the samples by use of the β-giardin, GDH, and TPI genes, respectively. Assemblage D (dog) and assemblage G (rodent) were detected once each, and both were identified by use of the GDH gene. Both these assemblages were found in separate plants, each of which employed secondary clarification. Overall, >99.5% of the samples taken from the wastewater effluents in this study indicated the presence of assemblages capable of causing human disease, irrespective of the targeted gene.

Table 3.5. *Giardia* Assemblage Characterization Using Three Distinct Genes

Target Gene	No. of Samples per Assemblage				Total Number of Samples
	A	B	D	G	
β-Giardin	144 (77.8)	41 (22.2)	0	0	185
GDH	54 (63.5)	29 (34.1)	1 (1.2)	1 (1.2)	85
TPI	65 (80.2)	16 (19.8)	0	0	81

Note. Values in parentheses are percentages.

3.4.10 Comparison of *Cryptosporidium* Detection Procedures

Figure 3.26 compares results obtained by Method 1623, PCR, and CC-IFA procedures. Only 6 samples were positive by all three detection methods, and 8 samples were positive by CC-IFA and PCR. Although CC-IFA and Method 1623 demonstrated the greatest agreement (14 samples) for positive samples, there were 2 samples that were positive by CC-IFA only. Even though CC-IFA optimization studies presented in Figure 3.7 indicated no appreciable differences in the infectivity of *C. parvum* and *C. hominis* oocysts using CC-IFA, all CC-IFA positive samples that were successfully sequenced contained *C. parvum* (Table 3.6).

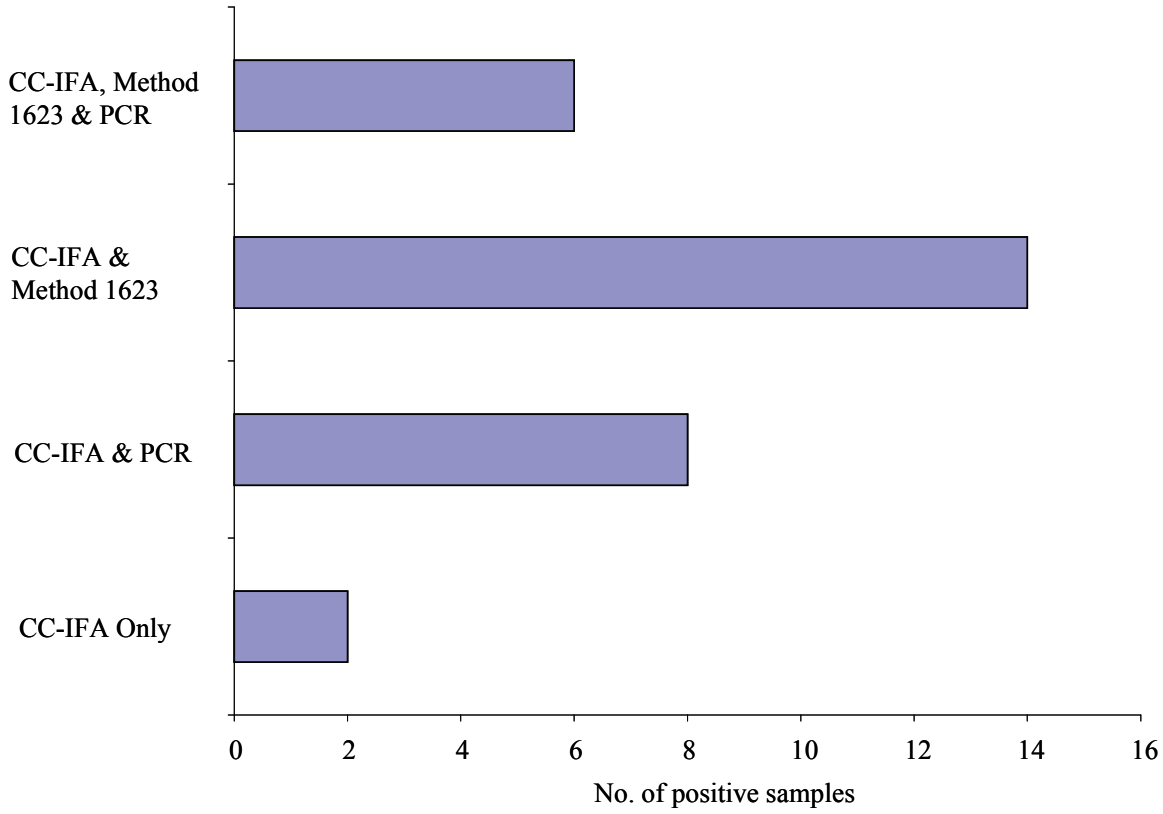


Figure 3.26. *Cryptosporidium* detection frequency using three methods.

3.4.11 Comparison of Protozoan Occurrence with Various Water Quality Parameters

Most participating utilities provided daily, weekly, or monthly (as appropriate) water quality data for their plants during the 12-month survey period. The following water quality parameters were measured: turbidity, total suspended solids (TSS), ammonia (NH_3), chlorine, fecal coliforms, dissolved oxygen, pH, and carbonaceous biochemical oxygen demand (CBOD). Not all utilities provided data for all parameters. Also, for each utility, data were not always available on every occasion when *Cryptosporidium* oocysts or *Giardia* cysts were detected. Despite these limitations, several hundred water quality data points were collected for each parameter from each facility. Various water quality data (turbidity, TSS, NH_3, fecal coliforms, chlorine) were compared with the mean concentrations for *Cryptosporidium* oocysts (Table 3.7) and *Giardia* cysts (Table 3.8). Generally, fecal coliform and TSS levels were higher in plants employing secondary clarification than other treatment processes; however, there was no clear association between either of the two protozoa and any of the monitored water quality parameters. Of the three plants using MBR (AN, MT, and SL), only SL was negative for *Cryptosporidium* oocysts and *Giardia* cysts. The effluent turbidity for SL was 0.08 NTU. AN contained 0.2 oocysts/L and demonstrated a substantially higher turbidity (i.e., 0.2 NTU) than SL. In contrast to SL, a sand filtration plant (HS) was *Cryptosporidium*-negative, despite the fact that the effluent turbidity was 35 times higher (i.e., 2.5 ± 0.9 NTU) than in SL.

Table 3.6. Detection of Infectious *Cryptosporidium* in Various Reuse Facilities

Utility	Physical Treatment	Disinfection	Sample Date	Sample Location	Method 1623 (Number of Oocysts/L)	CC-IFA	PCR
DM	Secondary	Chlorine	10/28/08	Predisinfection	1.0	Positive	Negative
			10/28/08	Postdisinfection	1.8	Positive	Negative
EH	Secondary	Chlorine	07/28/08	Predisinfection	75.4	Negative	Positive, *C. parvum*
			07/28/08	Postdisinfection	45.5	Positive	Positive
SC	Secondary	Chlorine	07/14/08	Predisinfection	0	Positive, 1 focus	Positive, *C. parvum*
			07/14/08	Postdisinfection	0	Negative	Negative
SC	Secondary	Chlorine	08/11/09	Predisinfection	9.4	Positive, 9 foci	Positive
			08/11/09	Postdisinfection	8.2	Positive, 5 foci	Positive, *C. parvum*
SC	Secondary	Chlorine	08/25/09	Predisinfection	2.4	Positive, 5 foci	Negative
			08/25/09	Postdisinfection	0.8	Positive, 2 foci	Positive, *C. parvum*
SC	Secondary	Chlorine	09/08/09	Predisinfection	10.8	Positive, 2 foci	Positive
			09/08/09	Postdisinfection	8.2	Positive, 3 foci	Negative

Table 3.6 *continued*. Detection of Infectious *Cryptosporidium* in Various Reuse Facilities

Utility	Physical Treatment	Disinfection Method	Sample Date	Sample Location	Method 1623 (Number of Oocysts/L)	CC-IFA	PCR
SP	Secondary	Chlorine	06/23/09	Predisinfection	0	Positive, 1 focus	Positive
			06/23/09	Postdisinfection	0	Negative	Negative
AR	Secondary	UV light	04/02/09	Predisinfection	0	Positive, 2 foci	Negative
			04/02/09	Postdisinfection	Not taken		
GB	Secondary	UV light	08/12/09	Predisinfection	2.8	Positive, 4 foci	Negative
			08/12/09	Postdisinfection	1.2	Negative	Positive, *C. parvum*
GB	Secondary	UV light	09/10/09	Predisinfection	3.0	Positive, 1 focus	Negative
			09/10/09	Postdisinfection	0	Negative	Negative
SF	Secondary	UV light	10/28/08	Predisinfection	6.2	Positive	Negative
			10/28/08	Postdisinfection	2.2	Negative	Negative
VR	Cloth	Chlorine	11/18/08	Predisinfection	0	Positive	Negative
			11/18/08	Postdisinfection	0	Contaminated	Negative
VR	Cloth	Chlorine	01/27/09	Predisinfection	5.6	Positive, 8 foci	Positive, *C. parvum*
			01/27/09	Postdisinfection	3.8	Positive, 6 foci	Negative

Table 3.7. Comparison of *Cryptosporidium* Populations with Various Water Quality Parameters

Utility	Number of *Cryptosporidium* Oocysts/L	Turbidity (NTU)	TSS (mg/L)	NH_3 (mg/L)	Number of Fecal Coliforms	Chlorine (mg/L)
EH	11.6		6.7 ± 4.6	3.2 ± 2.3		31.8 ± 29.6
SC	6.1		26.1 ± 14.2		9549 ± 13,454	0.17 ± 0.08
VR	3.1	0.8 ± 0.3	1.5 ± 2.2		<1	2.0 ± 1.5
GB	2.6		1.5 ± 1.2	0.5 ± 0.41	0.3 ± 1.1	UV
DM	1.3		1.8 ± 1.1	0.83 ± 0.6	2.1 ± 13.7	0.03 ± 0.01
AR	1.3		6.3 ± 4.3	1.2 ± 2.1	54.9 ± 115.6	UV
SF	1.3		5.8 ± 4.2	1.5 ± 2.1	1092 ± 5432	UV
SP	1.2		16.6 ± 8.3	0.05 ± 0.11	10.1 ± 15.0	1.5 ± 5.6
CV	0.5	5.3 ± 3.1	11.4 ± 6.7	1.1 ± 1.5	28.1 ± 47.3	0.01 ± 0.04
BH	0.2		2.1 ± 1.0	0.8 ± 1.4	21.3 ± 30.1	UV
AN	0.2	0.6 ± 0.4	4.4 ± 4.8	2.3 ± 2.6	1–2	0.03 ± 0.02
MT	0				35 ± 75	UV
HS	0	2.5 ± 0.9				–
SL	0	0.08 ± 0.08		0.05 ± 0.2		UV

Table 3.8. Comparison of *Giardia* Populations with Various Water Quality Parameters

Utility	Number of *Giardia* Cysts/L	Turbidity (NTU)	TSS (mg/L)	NH_3 (mg/L)	Number of Fecal Coliforms	Chlorine (mg/L)
SP	41.5		16.6 ± 8.3	0.05 ± 0.11	10.1 ± 15.0	1.5 ± 5.6
GB	12.9		1.5 ± 1.2	0.5 ± 0.41	0.3 ± 1.1	UV
CV	10.6	5.3 ± 3.1	11.4 ± 6.7	1.1 ± 1.5	28.1 ± 47.3	0.01 ± 0.04
SC	9.6		26.1 ± 14.2		9549 ± 13,454	0.17 ± 0.08
SF	7.1		5.8 ± 4.2	1.5 ± 2.1	1092 ± 5432	UV
AR	4.7		6.3 ± 4.3	1.2 ± 2.1	54.9 ± 115.6	UV
DM	2.9		1.8 ± 1.1	0.83 ± 0.6	2.1 ± 13.7	0.03 ± 0.01
EH	2.7		6.7 ± 4.6	3.2 ± 2.3		31.8 ± 29.6
MT	1.5				35 ± 75	UV
BH	0.9		2.1 ± 1.0	0.8 ± 1.4	21.3 ± 30.1	UV
AN	0.8	0.6 ± 0.4	4.4 ± 4.8	2.3 ± 2.6	1–2	0.03 ± 0.02
VR	0.7	0.8 ± 0.3	1.5 ± 2.2		<1	2.0 ± 1.5
HS	0.4	2.5 ± 0.9				–
SL	0	0.08 ± 0.08		0.05 ± 0.2		UV

3.5 Risk Estimates

Detailed risk assessments are outside the scope of this project; however, some preliminary estimates were attempted. For unknown environmental *Cryptosporidium*, Messner et al. (2001) used the Bayesian approach to calculate the mean infectious dose from existing human infectivity data. It was determined that a probability of infection from a single oocyst for an unknown strain was 0.028. These data have been used previously to determine the risks of infection associated with the presence of infectious oocysts in drinking water systems (Aboytes et al., 2004). Risk assessments have also been used to compare analytical methods (Method 1623 and CC-PCR) (LeChevallier et al., 2003) and UV treatment performance (LeChevallier and Hubel, 2004). Based on these previous studies, the equations for calculating the daily and annual risk of protozoan infection from drinking water are as follows:

$$\text{Daily Risk (DR)} = (1.232 \text{ L/day})\ (\text{oocysts/L})\ (\text{infection probability for unknown strain})$$

$$\text{Annual Risk (AR)} = 1 - (1 - \text{DR})^{350}$$

where 350 is used as an exponent, based on the assumption that exposure occurs for 350 days/year.

There are three main exposure pathways (ingestion, inhalation of airborne water droplets, and dermal contact, allowing access through cuts and abrasions) by which pathogens may infect humans. Pathogens derived from wastewater reuse practices may be transmitted to humans by drinking/inhalation, contact recreation, noncontact recreation, and consuming contaminated fish or shellfish. In reality, exposure to reuse water can occur over a range of conditions. Although not an exhaustive list, some possible examples of exposure include contact recreation in receiving waters, consumption of shellfish/aquaculture from receiving environment, contact with or consumption of stock grazed in the vicinity of the treatment/disposal area or on land irrigated with groundwater or river water, contact with or consumption of irrigated crops, contact with or consumption of drinking water influenced by treated effluents, and public contact during open access reuse (e.g., golf courses, parks, playgrounds, schoolyards, or residential landscape). This highlights that rather sophisticated MRAs will need to be performed to account for this wide variability in exposure routes.

The total amount of water ingested by the U.S. population has been estimated at 1.232 L/day per person (U.S. EPA, 2000). Exposure to reuse water, on the other hand, can vary depending upon the type of activities. For example, swimming can lead to consumption of up to 154 mL of water for children, with adults consuming approximately half that volume (Dufour et al., 2006). The intake due to park irrigation has been estimated to be equivalent to 0.01 to 1% of the daily intake (i.e., 0.12 mL to 12 mL) according to various studies (Cooper and Olivieri, 1998; Ottoson and Stenstrom, 2003; Sakaji and Funamizu, 1998). Soller et al. (2008) conducted 5000 simulations and determined that the median value by this exposure route was around 6 mL.

For the preliminary calculations, it was assumed that susceptible human individuals were exposed to a conservative 1 mL of reuse water. That is,

Daily Risk (DR) = (0.001 L/day) (oocysts/L) (0.028)

The exposure can be expressed as a daily exposure (for infrequent events, such as exposure to golf course irrigation) or can be annualized where exposure is frequent (e.g., indirect reuse) in unrestricted zones.

Mean AR was calculated for each treatment process and used to estimate the number of infections per 10,000 exposed humans as follows:

Number of infections per 10,000 people = (1 / mean AR) * 10,000

These results have been summarized for each of the four treatment processes examined in this study in Figure 3.27. Data indicated that direct exposure to effluents following conventional treatment (secondary clarification) presented a mean risk of approximately 600 infections per 10,000 exposed individuals. Use of cloth filtration presented a risk of approximately 70 infections per 10,000 exposed individuals. Compared to secondary clarification and cloth filtration, substantial risk reductions were noted with sand filtration (approximately 2 infections per 10,000 exposed individuals) and MBR treatment (<1 infection per 10,000 exposed individuals).

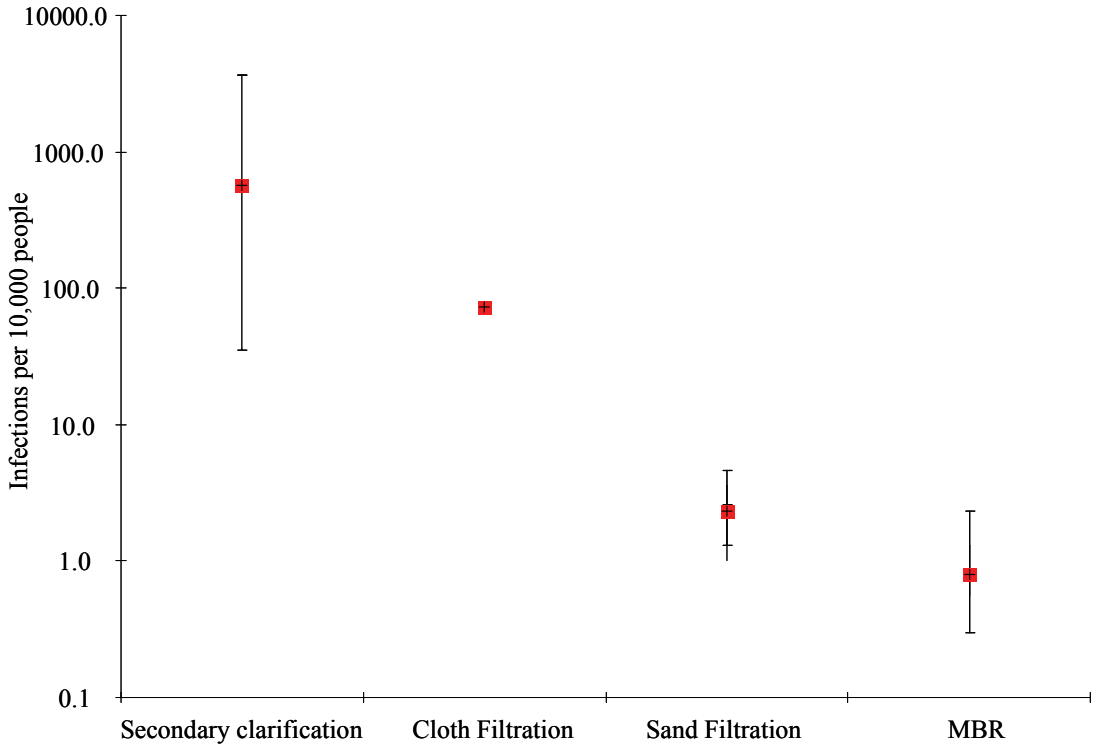


Figure 3.27. Estimate of *Cryptosporidium* infections per 10,000 exposed individuals.

Chapter 4

Discussion

A booming world population has led to greater urbanization, contributing to increased environmental pollution, sanitary challenges, and increased burdens on already limited freshwater resources. Freshwater is essential for life, and with technological advances, it is becoming feasible for water to be salvaged from what might have been deemed "unconventional" sources (i.e., seawater or wastewater) less than 20 years ago. Reclaimed water may be used to meet or supplement demand for land application, irrigation, and aquifer recharge. It may also be used for irrigation of food crops, unrestricted public access areas, toilet flushing, or cooling towers. If there is increased public exposure, the risk of disease transmission may also increase if the reuse water has not been treated adequately. Typically, wastewater treatment processes can range from secondary to tertiary treatment, including the use of advanced technologies such as MBRs in conjunction with chemical or physical disinfection. Usually, situations with the greatest potential for human exposure require the highest level of treatment and the most stringent requirements for microbial water quality. Table 4.1 summarizes some of the intended uses of reclaimed water with the likely exposure to humans and the relative level of treatment. In general, urban applications of reclaimed water where human exposure is moderate or high require the highest level of treatment and the lowest levels of microbial pathogens and indicator organisms.

In a survey of 1600 sites (parks, school yards, and playgrounds) in which reclaimed water was used, there was no apparent increase in human disease occurrence compared to sites irrigated with potable water (Crook, 2005). Despite these observations, it is important to measure the effectiveness of treatment regularly, which can be achieved by measuring certain physical and chemical parameters as surrogates. For example, total nitrogen concentrations of ≤10 mg/L, turbidity of ≤2 NTU, total suspended solids (TSS) of ≤5 mg/L, biochemical oxygen demand (BOD) concentrations of ≤45 ppm, total organic carbon (TOC) of <0.5 mg/L, carbonaceous BOD (CBOD) concentrations of 60 mg/day, and residual chlorine concentrations of >1 mg/L are reflective of high-quality effluents.

In addition, understanding the occurrence, distribution, concentration, identity, and behavior of microorganisms and their potential nutrients in reuse water (Jjemba et al., 2010; Narasimhan et al., 2005) can be useful for calculating the human disease risks associated with various reuse practices. Currently, microbiological monitoring to meet regulatory compliance typically includes testing for total coliform, fecal coliform, or *E. coli*. For example, a survey of 425 reuse facilities indicated that 95% of the facilities monitored coliforms on a daily or weekly basis (Jjemba et al., 2010). Coliform measurements can provide a useful indication of treatment efficiency, and their analytical procedures are generally simple and inexpensive; however, these data are usually insufficient for developing robust MRAs for environmentally hardy organisms such as protozoan parasites (i.e., *Cryptosporidium* and *Giardia*). Outbreaks of human cryptosporidiosis in the United States, United Kingdom, Japan, Australia, etc., have occurred following consumption of drinking water meeting regulatory standards.

Table 4.1. Treatment Goals for Various Reuse Water Applications

Use Category	Intended Use	Exposure to Humans	Treatment Level
Urban Reuse	Irrigation of parks	High	High
	Irrigation of highway medians	Low	Moderate
	Irrigation of golf courses and lawns (e.g., residential, schools, business parks, etc.)	High	High
	Commercial uses such as vehicle washing, window washing, etc	High	High
	Fire protection	High	Moderate
	Dust control	Moderate	Moderate
	Street sweeping	Moderate	High
	Toilet and urinal flushing	Low	High
Periurban Reuse	Groundwater recharge	Moderate	High
	Augmenting potable supplies	High	High
Agricultural Reuse	Irrigation of farmland (pasture) and animal watering	Low (for nondairy); moderate (for dairy)	Moderate
	Irrigation of farmland (nonedible crops)	Moderate (for surface); high (for spray)	Moderate (for surface); high (for spray)
	Irrigation of farmland (edible crops)	High	High
	Irrigation of fiber, seed, forage crops	Low	Low
Industrial Reuse	Cooling towers	Moderate	High
	Boiler makeup water	Low	Moderate
	Industrial process water	Moderate	Moderate
Environmental and Recreational	Creating, restoring, and/or enhancing wetlands	Low	Low
	Recreational and aesthetic impoundments	High	High

Source: Modified from Jjemba et al. (2010).

Cryptosporidium and *Giardia* are intestinal parasites, and infections with these organisms result in discharge of environmentally robust transmissive stages, via feces, into wastewater systems. Previous monitoring studies of wastewater influents and effluents, at various stages of the treatment train, have shown that the organisms occur frequently in wastewater influents (DiBenedetto et al., 2005; Gennaccaro et al., 2003; Karim and LeChevallier, 2005; Kfir et al., 1994; Madore et al., 1987; Mayer and Palmer, 1996; Ottoson et al., 2006; Villacorta et al., 1992). Although various stages of the treatment train have been shown to demonstrate removal of these organisms, the monitoring data also verify that oocysts/cysts continue to pass into the final effluents. In the United States, the majority of wastewater effluents that employ final disinfection rely on chlorine. Whereas chlorination may be adequate to control bacterial and viral pathogens in treated effluents, it has little impact on *Cryptosporidium* oocysts. Understanding the risks of human cryptosporidiosis associated with treated wastewater effluents is a significant challenge for the reuse industry. Although the relevance of this route of disease transmission may have been recognized for the last two decades, methods for addressing this question have become available only recently.

4.1 Laboratory-Based Experimentation

In the present study, the methods used for collection, isolation, detection, infectivity, and genotype determination either were well characterized or had been validated by single or multiple laboratory testing. Despite this, initial tweaking of sample collection procedures and standardization of cell culture infectivity procedures between the collaborating laboratories were performed before commencing the field monitoring phase. For sampling, it was originally postulated that the PCFC might be cheaper and perhaps more effective than filtration using the Envirochek HV filters. One reason was that the filters might be prone to clogging issues when sampling wastewaters with varying turbidity/particle content. Using the worst-case scenario matrix (secondary effluent), preliminary evaluations established that both procedures readily enabled collection of 10 L samples. Spiking studies with *C. parvum* and *C. hominis* oocysts also demonstrated similar performances of the two procedures. The Envirochek HV filters are typically two- to threefold more expensive than the disposable bowls used in the PCFC; however, there were financial/logistical issues with using the PCFC in this project. Each PCFC unit retails for >$5000. With 14 participating utilities, each requiring a dedicated unit, the total capital expenditure (>$70,000) for this project was cost-prohibitive. Additionally, the U.S. EPA has made available sampling instructions and instructional videos for using the Envirochek filters on their website. This made it convenient for samplers to be quickly and effectively trained with the Envirochek filters but not with the PCFC. To further assist sampling, the project team also provided phone support and a standard operating procedure for sampling unpressurized systems (Appendix 1).

Preliminary evaluations were also conducted to ensure that the CC-IFA routinely used at AW (Bukhari et al., 2007) could be transferred successfully to Tufts. Furthermore, three experimental antibodies (two monoclonal antibodies and one polyclonal antibody) developed at Tufts and three blocking agents were also examined to improve the visual acuity for detection of infectious foci. No significant differences were observed in cluster enumeration between AW and Tufts, which verified that CC-IFA was a robust procedure for oocyst infectivity measurements. The CC-IFA optimization steps (i.e., antibodies or blocking agent testing) demonstrated marginal improvements, but the results were not conclusive. It was suspected that some of the inconsistencies observed in the performance of the experimental antibodies might have been attributed to the quality of their fluorochrome conjugation. However, it was outside the scope of this project to delve further into these issues or to conduct an investigation of the additional CC-IFA optimization steps. Nonetheless, future

testing of this nature could help improve the assay sensitivity and specificity and possibly also lead to assay automation. To ensure reliability of the monitoring data, it is necessary to ensure consumables are thoroughly quality controlled. As the antibodies are a critical component of the CC-IFA procedure and facilitate detection of infectious clusters, it was considered prudent to continue using the commercially available polyclonal antibody rather than substituting with experimental antibodies that showed only marginal improvements.

For environmentally derived oocysts, a positive infectivity signal with the CC-IFA is likely to be influenced by a number of factors. Even with recently voided oocysts, not all oocysts may be infectious (Bukhari et al., 1997). There may be in vitro infectivity or cell line susceptibility differences between *Cryptosporidium* isolates or species. The sampling and isolation procedures upstream of the CC-IFA may also impact oocyst infectivity. These parameters can individually or collectively contribute to false negatives. To better understand the sensitivity of the CC-IFA procedure, accurately enumerated oocyst inocula were prepared by flow cytometry and revealed that a single oocyst (≤15 days of age) was insufficient to generate a positive signal. Five to 10 oocysts were required for a consistent positive signal. This does not mean the CC-IFA procedure would not be adequately sensitive for monitoring wastewater effluents. First, the CC-IFA sensitivity threshold identified here could be an artifact of oocyst delivery into wells of the microtiter plates. Second, most wastewater surveys conducted previously have used the ICR method, which is considerably less efficient than Method 1623. Using Method 1623, with its enhanced performance, increases the probability of recovering sufficient infectious oocysts to generate a CC-IFA positive signal. Despite this, it is important for future risk assessment to recognize that the absence of infectious oocysts in environmental samples may be due to methodological limitations.

In addition to considering the sensitivity of CC-IFA, it is important to establish whether this procedure can accurately measure the infectivity of oocysts that have been previously exposed to disinfectants. Usually performance of in vitro viability or infectivity assays is calibrated against mouse infectivity; however, animal experimentation was outside the scope of this project. Nonetheless, limited disinfection experiments were conducted with chlorine, UV light, or ozone to ensure that the levels of inactivation measured by CC-IFA were not grossly different from those expected from previous disinfection studies in which mouse infectivity had been used to measure oocyst inactivation.

For chlorination, it was noted that oocyst inactivation was higher in matrix samples than in the respective control samples subjected to the same CT values. This may be due to the matrix demand for disinfectant being nonuniform, resulting in oocysts being exposed to higher than assumed chlorine concentrations. Alternatively, other matrix-associated factors (including the presence of indigenous oxidants) may have had a synergistic effect on oocyst inactivation. Despite these subtleties, chlorine CT values between 90 and 600 mg · min/L yielded mean inactivation levels of <0.4 log. These low levels of inactivation are probably approaching the sensitivity thresholds of the CC-IFA and did not reveal dose-related responsiveness to increasing chlorine CT values. This is not surprising, as previous chlorine disinfection studies measuring oocyst inactivation with mouse infectivity reported that CT values of at least 7000 were required to inactivate 2 log units of *C. parvum* (Korich et al., 1990). The CC-IFA used for the analysis of chlorine-treated oocysts supports this and other previously published reports (Lisle and Rose, 1995; MacKenzie et al., 1995; Venczel et al., 1997). More importantly, these data indicate that the CC-IFA procedure can be used to measure infectivity of oocysts disinfected with chlorine. Bench-scale UV disinfection and ozonation also verified that CC-IFA was a suitable alternative to mouse infectivity for measuring infectivity. With the latter, it was also established that the cell line was equally

susceptible to *C. parvum* and to *C. hominis*. This was important, as *C. hominis* infects humans only and could be the predominant species in wastewaters. Based on these limited bench-scale disinfection data, it was established that infectivity determination with the CC-IFA was accurate and the data generated with this assay would be of relevance in MRA.

4.2 Environmental Monitoring

An intensive environmental monitoring phase was launched for 14 geographically dispersed utilities over a 12-month period, with 8 employing secondary clarification, 2 using sand filtration, 3 employing MBR, and 1 using cloth filtration. Sampling was designed to measure concentrations, infectivity, and genotype information after physical removal (i.e., predisinfection) and immediately postdisinfection. Method 1623 was used for sampling and specific organism isolation using IMS, followed by IFA-based detection and enumeration. Because Method 1623 can also provide information on the presence and concentration of *Giardia* cysts, it was deemed of interest to collect that information also. During the monitoring phase, a 10 L matrix sample was collected once for each utility and was spiked with a known number of oocysts/cysts. Comparing organism numbers in the spiked filter to those in the unspiked filter allowed determination of Method 1623 performance for each utility. Between utilities, recoveries were highly variable for *Cryptosporidium* (6.9–75%) and *Giardia* (7.9–90%). According to U.S. EPA Method 1623, the matrix spike/matrix spike duplicate recoveries in raw water were 13–111% for *Cryptosporidium* and 15–118% for *Giardia* (U.S. EPA, 2005). Some of the minor difference observed at the lower end of the range may have occurred due to matrix inhibition effects arising from the wastewater samples. Mean recoveries were lower in plants employing secondary clarification or sand filtration than in plants using cloth filtration or MBRs. It is possible higher recoveries were obtained in the last two treatment processes because of their higher quality effluents facilitating better elution from the Envirochek filters and more effective capture during the IMS stage. For all treatment processes, mean *Giardia* recoveries were consistently higher than for *Cryptosporidium*. This may be one reason that occurrence of *Giardia*-positive samples (70%) was threefold higher than that of *Cryptosporidium*-positive samples (23.1%). However, when mean recoveries for all 14 systems were compared for *Giardia* cysts (55.9% ± 27.3%) and *Cryptosporidium* oocysts (45.9% ± 20.1%), the differences in method recoveries were marginal. Closer scrutiny of data in Figure 3.9 indicated several locations (i.e., AR, GB, and BH) yielded unusually low *Giardia* recoveries, suggesting that comparison of mean data values was probably skewed. Comparison of median recovery values indicated approximately one-third greater recovery of *Giardia* cysts (62%) than of *Cryptosporidium* oocysts (42%). During monitoring, it was noted that *Giardia* occurrence was four- to sixfold greater than *Cryptosporidium* occurrence following MBR and sand filtration but only threefold greater for secondary effluents. Differences in method recovery efficiency are likely to be an influencing factor; however, based on the monitoring data, this is unlikely to be the only factor responsible for the *Giardia* frequency and/or concentrations being higher than those of *Cryptosporidium* oocysts. Differences in the levels of infection within the contributing communities can be another plausible explanation, especially as *Giardia* infections can be asymptomatic or can persist even after prophylactic treatment has been completed and symptoms appear to have resolved. Previously, relatively higher occurrence of *Giardia* than of *Cryptosporidium* has been documented in the literature for surveys examining wastewater influents and/or effluents (Bukhari et al., 1997; Dungeni and Momba, 2010; Robertson et al., 2000).

Method 1623 relies on IFA for detection and morphometric identification of *Cryptosporidium* oocysts and *Giardia* cysts; however, the sensitivity and specificity of the antibodies being

used for organism detection have been a cause of concern for some time. The detection antibodies' cross-reactivity issues can create challenges during microscopy, necessitating careful analysis by highly trained microscopists. This can be tedious, time-consuming, and costly. As a result, there has been substantial interest in automated and/or specific detection of oocysts/cysts derived from environmental samples. Incorporating molecular detection procedures (i.e., PCR) has become a viable option after the development/deployment of IMS, which can specifically capture and isolate target organisms from the contaminating debris present in sample concentrates. Unfortunately for reuse matrices, utilization of the PCR procedures for *Cryptosporidium* or *Giardia* did not provide the benefits anticipated from what is deemed a substantially more sensitive procedure. Following IFA analysis, only *Cryptosporidium* oocyst occurrence was 21.9%. By PCR only, *Cryptosporidium* oocyst occurrence was 13%, and by both IFA and PCR, *Cryptosporidium* occurrence was 39%. One reason for this disparity may be that splitting sample concentrates containing small numbers of oocysts may have resulted in bias toward one assay versus another. Possible effects of uneven organism distribution become more plausible when it is considered that usually 50% of the 10 L sample concentrates were analyzed by IFA and only 25% were analyzed by PCR. Differences between the assays may also be due to inherent differences in their detection mechanisms. The IFA procedure is morphology-based, targeting the outer wall of the oocyst irrespective of whether it has internal contents. In contrast, a positive PCR signal can only be generated from oocysts containing the target DNA (i.e., intact organisms). With oocyst aging or exposure to harsh treatment processes, damage leading to generation of empty oocysts may be expected to increase. This, in turn, may be responsible for increasing disparity in the frequency of positives between IFA and PCR. Despite this postulation, internal DAPI staining confirmed the presence of contents (i.e., nuclei) in all *Cryptosporidium*-positive samples, which implies the PCR method used in this study underestimated oocyst occurrence.

Comparison of the three primer sets for *Giardia* indicated that two primer sets (GDH and TPI genes) performed poorly (*Giardia* occurrence of 29% and 31%, respectively) in contrast to IFA-based *Giardia* occurrence (70.1%). The third primer set, targeting the β-giardin gene, revealed closer correlation between IFA and PCR, and with this gene 65% of the samples were *Giardia*-positive. Agreement between the three target genes was observed for 10% of the *Giardia*-positive samples and 21% for the *Giardia*-negative samples. PCR-based occurrence demonstrated closer agreement with DAPI staining, which confirmed the presence of contents (i.e., nuclei) in 58.6% of *Giardia*-positive samples.

Data from molecular detection of *Cryptosporidium* and *Giardia* highlight that future studies need to exercise care when selecting target genes for environmental monitoring of these protozoa.

When the detection rates of protozoa by IFA and PCR were compared relative to the four treatment processes, another interesting phenomenon became apparent. PCR outperformed IFA in plants with advanced treatment and generally higher quality effluents (i.e., sand filtration or MBR). In plants using secondary treatment only, occurrence determined by IFA was approximately twice that determined by PCR. These results suggest that carryover of inhibitory compounds post-IMS concentration may have impacted the effectiveness of the PCR procedure. Future studies need to examine IMS optimization steps (i.e., increased rinses of IMS concentrate), which may help to reduce the concentration of PCR inhibitory compounds. Alternatively, the effect of inhibitors could be monitored using internal PCR controls.

Until reasons for the disparity between IFA and PCR detection procedures have been elucidated and corrective actions implemented, caution needs to be exercised when using PCR only for collecting *Cryptosporidium*/*Giardia* occurrence information in reuse matrices for risk assessment calculations. Despite this, use of molecular procedures is necessary for understanding *Cryptosporidium* species identification and *Giardia* assemblage information. However, to use species information in risk assessment, it is important to recognize that factors influencing PCR performance may also be impacting amplification of one species more than that of another species in any given reuse matrix. The facilities surveyed in this study received predominantly domestic wastewaters, and because *C. hominis* is exclusively responsible for human infections, it may be reasonable to expect a more frequent occurrence of this species than of *C. parvum*, which is a zoonotic species infecting humans and various mammals, including domestic animals. Wastewater surveys in China support this rationale and have shown that *C. hominis* was the most commonly identified species (93.7% positive samples) following direct DNA extraction from wastewater samples, amplification of small subunit rRNA genes, and restriction fragment length polymorphism (Feng et al., 2009). Similarly, a study in Japan observed *C. hominis* in 78 samples versus *C. parvum* in 16 samples (Hashimoto et al., 2006; Hirata and Hashimoto, 2006). In Milwaukee, a wastewater plant receiving contributions from humans, slaughtered farm animals, rodents, and deer was monitored for *Cryptosporidium* species. Again *C. hominis* was most frequently detected (13.4%), followed by *C. andersoni* (12.8%). The prevalence for the remaining *Cryptosporidium* species was as follows: the *Cryptosporidium* cervid genotype (3.3%), *C. parvum* (2.8%), *C. muris* (2.2%), and 0.6% for *Cryptosporidium* mouse genotype (Zhou et al., 2003). A study in Peru provided further evidence to suggest predominance of *C. hominis* in wastewaters (Cama et al., 2003), whereas a study conducted in the United Kingdom noted similar occurrence rates for *C. hominis* and *C. parvum* (Chalmers et al., 2002). In contrast to the cited literature, data in our study indicated that 31 of 40 samples contained *C. parvum* oocysts, 8 samples were positive for both species, but only 1 of 40 samples contained only *C. hominis*. Reasons for the differences between our study and previous studies can be manyfold, but more than likely arise from the relative occurrence of disease within the communities responsible for wastewater contributions. Although unlikely, it is also possible that the relative occurrence of *Cryptosporidium* species could be an artifact of the methodology. Perhaps the upstream methods (especially IMS), which use specific antibody-based capture of oocysts, may preferentially select some species over others. Alternatively, the PCR primers used to amplify the target gene for species differentiation may perform better for one species than for another. Future studies will be needed to elucidate this issue. In contrast to the *Cryptosporidium* species information, *Giardia* assemblage characterization unequivocally demonstrated that the *Giardia* cysts in the effluents from all treatment plants (including MBRs) were derived from humans, with assemblages A and B (infectious to humans) being detected in 100%, 97.6%, and 100% of the samples using β-giardin, GDH, and TPI genes, respectively. In addition, a single sample from two separate treatment plants contained the non-human-infecting *Giardia* assemblages, and both these plants employed secondary clarification.

In addition to occurrence, concentration, and species characterization, it is also important to include infectivity information in any risk assessment calculations. Using CC-IFA to monitor 14 wastewater systems indicated that 18 of 285 (6.3%) samples contained infectious oocysts, with the majority of the positives being in systems employing secondary clarification and/or chlorination. In one system, in which secondary clarification was followed by UV disinfection, no infectious oocysts were detected postdisinfection. These full-scale results continue to verify the effectiveness of UV disinfection, as indicated by both the bench-scale

UV disinfection conducted in this study and findings reported previously in the literature (Bukhari et al., 1999).

In wastewater matrices, it may be argued that the oocysts are freshly voided and are in a generally favorable environment (i.e., cold and moist conditions), making it likely that the oocysts would be highly infectious and continue to remain infectious for prolonged periods. In the majority of the samples taken from sanitary sewer systems (domestic sewage only), where infectious clusters were enumerated, the calculated infectivity values exceeded 100%. Although exceeding a 100% infectivity value is theoretically improbable, the results highlight that oocyst concentrations (determined by IFA) are very likely an underestimate of the actual occurrence and concentrations in wastewater samples. In contrast to the domestic systems, a wastewater system also receiving discharges from an animal rendering facility indicated highly variable yet considerably lower oocyst infectivity values (1.4–43.7%) in three samples. What could be the reasons for the differences? Previously, animal experimentation studies have shown that up to one-half of the oocysts shed by infected animals may not viable or infectious (Bukhari et al., 1997). Animal rendering processes (i.e., heating and drying of carcasses to separate the fat from bone and proteins of dead animals) followed by exposure of discharged oocysts to further stresses such as environmental aging, wastewater treatment processes, and disinfection will likely contribute further to a decline in oocyst infectivity.

Given that the frequency of *Cryptosporidium* positives by IFA and or PCR was approximately 40%, it was surprising to observe only 6% of the wastewater samples were CC-IFA positive. Theoretically, a single infectious oocyst has the potential to generate an infectious cluster in cell monolayers. In reality, various factors (i.e., accurate delivery of dose, excystation of the oocyst, successful invasion of the cell line, multiplication, and adequate staining with target antibodies) may influence the sensitivity threshold. Spiking studies using oocyst inocula prepared with the highly reproducible flow cytometry procedure indicated that the sensitivity threshold for CC-IFA was approximately five fresh oocysts. Perhaps this explains the low CC-IFA positive rate for infectious oocysts observed in the wastewater samples. In contrast, during environmental monitoring, 2.6% (3 of 114) of samples that were deemed negative for *Cryptosporidium* oocysts by IFA microscopy were CC-IFA positive. As it is unlikely that the sensitivity of the CC-IFA was better in environmental samples than in the laboratory-based sensitivity experiments, it provides further evidence that the U.S. EPA Method 1623-based IFA procedure is likely to be underestimating the concentration and frequency of oocyst-positive wastewater samples.

To improve the consistency of Method 1623-based enumeration and CC-IFA-based infectivity, larger sample volumes need to be analyzed in future monitoring studies. How much volume can be collected and concentrated will depend on the sampling device and effluent quality, which, in turn, will be influenced by the treatment process. Based on the mean oocyst concentrations per treatment process, at least 5 to 10 L would be needed following secondary clarification and up to 1,000 L following MBR treatment or sand filtration. Future studies should examine the usefulness of ultrafiltration devices. Recently, ultrafiltration has been shown to effectively recover *Cryptosporidium* and various other microbes following large-volume filtration (Hill et al., 2009). Despite the limited infectivity data, results from this study do indicate that plants utilizing secondary clarification often discharged infectious *Cryptosporidium* oocysts following physical treatment, and this continued to be the case postdisinfection where the final disinfectant was chlorine. In one sample, infectious oocysts predisinfection were eliminated following UV disinfection. These data further support the effectiveness of UV for inactivating chlorine resistant protozoa such as *Cryptosporidium*.

4.3 Risk Estimates

This project has collected data on *Cryptosporidium* recovery, occurrence, concentration, infectivity, and genotyping. These data provide the necessary information for future risk calculations for *Cryptosporidium* and *Giardia*. Tools such as those developed by Soller et al. (2008) may help in understanding the risks of infection following different exposure routes. Nonetheless, for illustrative purposes, some preliminary risk assessments were performed, using the assumption that exposed individuals had low-level noncontact exposure (i.e., consumption of 1 mL volume). The risk estimates indicated mean annualized risk levels of 1 infection per 90 exposed individuals following secondary clarification and 1 infection per 138 exposed individuals after cloth filtration. Although the secondary clarification information was collected from eight plants, data for the cloth filtration need to be interpreted with caution, as only a single plant was examined during this study.

Employment of more advanced treatment processes reduced the risks further. Sand filtration performed better than secondary clarification and cloth filtration, whereas the overall performance of two of three MBR systems was well in excess of the acceptable risk levels defined by the U.S. EPA (1 infection per 10,000 exposed individuals) for drinking water (Macler and Regli, 1993). For one of three MBR systems, the calculated risk was 1 infection per 8000 exposed individuals. This calculation was based on the detection of two oocysts in a single sample postdisinfection. The risk calculation was based on the assumption that the discharged oocysts were infectious. This plant actually employed UV disinfection, which is known to be highly effective for treating *Cryptosporidium*; suggesting that large proportions (i.e., 2–3 log) of the *Cryptosporidium* organisms discharged in this plant were probably noninfectious. Had oocyst infectivity information been available for this sample and been included in the risk calculations, it is very likely that the calculated risk levels for even the lowest-performing MBR systems could exceed the acceptable risk levels (i.e., 1 infection per 10,000 exposed individuals) previously defined for drinking water.

Unlike the rare occurrence of *Cryptosporidium*, the significantly larger *Giardia* cysts frequently passed through MBR and sand filtration effluents. What about their potential risks? Unlike *Cryptosporidium*, *Giardia* cysts can be inactivated by various disinfectants, including chlorine. However, there are no reliable infectivity assays for *Giardia*, which makes it difficult to estimate their infectivity potential. As a result of the method limitations, the risk assessment calculations for *Giardia* become more complex than those performed here for *Cryptosporidium*. In the future, the risk assessment tools developed by Soller et al. (2008) may allow enhanced risk calculations for both organisms.

Based on the premise that *Cryptosporidium* and *Giardia* do pass through the various wastewater treatment processes, the choice of final disinfectant will be critical in meeting any acceptable risk goals. Chlorination is ineffective for *Cryptosporidium* and marginally effective for *Giardia*. Replacing chlorination with the highly effective UV disinfection can help mitigate discharge of infectious oocysts. However, UV disinfection will be most effective in effluents with consistently high water quality. The large variation in the size of particles in the secondary effluent may lead to shielding phenomena that protect *Cryptosporidium* oocysts and *Giardia* cysts from the harmful effects of UV radiation. Also, UV disinfection does not generate a disinfectant residual, which may present microbial regrowth issues in reuse applications where the treated effluent is distributed through pipes at low velocity.

4.4 Conclusion

Using standardized methods and molecular tools, this study indicates that species of *Cryptosporidium* and *Giardia* of significance to human health can readily pass through wastewater treatment processes. A clear association between the level of treatment and the occurrence of these protozoa in the effluents was noted. Although secondary clarification processes yielded frequent positives at higher concentrations, even advanced treatment processes allowed pass-through of these protozoa. There is no doubt that physical barriers such as MBR, with their submicrometer pore size, have the potential to prevent these relatively large (5–20 μm) parasites from passing through into the effluent; however, this can only be successful if the membrane integrity is guaranteed. Continuously monitoring effluent turbidity in conjunction with various physiochemical and/or operational parameters will help provide consistency in the performance of these advanced treatment processes. Physiochemical parameters including turbidity are known to show little correlation with protozoan presence. Currently, routine turbidity measurements are taken with light-based turbidimeters. Future use of laser-based turbidimeters, which can be 1000-fold more sensitive, may provide better indication of MBR integrity issues. As the need for reuse water continues to increase, risk mitigation strategies will need to be geared to adopting a multibarrier approach incorporating regular operational maintenance, continuous monitoring, adoption of best management practices to protect water quality during storage, and distribution and employment of UV disinfection with or without chemical disinfection.

References

Aboytes, R.; DiGiovanni, G. D.; Abrams, F. A.; Rheinecker, C.; Macelroy, W.; Shaw, N.; LeChevallier, M. W. Detection of infectious *Cryptosporidium* in finished drinking water. *J. AWWA* **2004,** *96,* 88–98.

Amar, C. F.; Dear, P. H.; Pedraza-Diaz, S.; Looker, N.; Linnane, E.; McLauchlin, J. Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. *J. Clin. Microbiol*. **2002,** *40*, 446–452.

Ballester, N. A.; Malley, J. P. Sequential disinfection of adenovirus type 2 with UV-chlorine-chloramine. *J. AWWA* **2004,** *96* (10), 97–103.

Bukhari, Z.; Abrams, F.; LeChevallier, M. Using ultraviolet light for disinfection of finished water. *Water Sci. Technol.* **2004**, *50,* 173–178.

Bukhari, Z.; Hargy, T. M.; Bolton, J. R.; Dussert, B.; Clancy, J. L. Medium-pressure UV for oocyst inactivation. *J. AWWA* **1999**, *91,* 86–94.

Bukhari, Z.; Holt, D. M.; Ware, M. W.; Schaefer, F. W., III. Blind trials evaluating in vitro infectivity of *Cryptosporidium* oocysts using cell culture immunofluorescence. *Can. J. Microbiol.* **2007**, *53,* 656–663.

Bukhari, Z.; LeChevallier, M. Assessing UV reactor performance for treatment of finished water. *Water Sci. Technol.* **2003**, *47,* 179–184.

Bukhari, Z.; Smith, H. V.; Sykes, N.; Humphreys, S. W.; Paton, C. A.; Girdwood, R. W. A.; Fricker, C. R. Occurrence of *Cryptosporidium* spp oocysts and *Giardia* spp cysts in sewage influents and effluents from treatment plants in England. *Water Sci. Technol.* **1997**, *35,* 385–390.

Bukhari, Z.; Marshall, M. M.; Korich, D. G.; Fricker, C. R.; Smith, H. V.; Rosen, J.; Clancy, J. L. Comparison of *Cryptosporidium parvum* viability and infectivity assays following ozone treatment of oocysts. *Appl. Environ. Microbiol*. **2000**, *66* (7), 2972–2980.

Caccio, S. M.; De Giacomo, M.; Pozio, E. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *Int. J. Parasitol*. **2002**, *32,* 1023–1030.

Cama, V. A.; Bern, C.; Sulaiman, I. M.; Gilman, R. H.; Ticona, E.; Vivar, A.; Kawai, V.; Vargas, D.; Zhou, L.; Xiao, L. *Cryptosporidium* species and genotypes in HIV–positive patients in Lima, Peru. *J. Eukaryot. Microbiol*. **2003**, *50* (Suppl.), 531–533.

Campbell, A. T.; Robertson, L. J.; Smith, H. V. Viability of *Cryptosporidium parvum* oocysts: Correlation of in vitro excystation with inclusion/exclusion of fluorogenic vital dyes. *Appl. Environ. Microbiol.* **1992**, *58,* 3488–3493.

Carraway, M.; Tzipori, S.; Widmer, G. Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. *Appl. Environ. Microbiol.* **1996,** *62,* 712–716.

Chalmers, R. M.; Elwin, K.; Thomas, A. L.; Joynson, D. H. Infection with unusual types of *Cryptosporidium* is not restricted to immunocompromised patients. *J. Infect. Dis*. **2002**, *185,* 270–271.

Chalmers, R. M.; Robinson, G. The European rabbit (*Oryctolagus cuniculus*), a source of zoonotic cryptosporidiosis. *Zool. Public Health* **2009,** *57,* 7–8.

Cooper, R. C.; Olivieri, A. W. Infectious disease concerns in wastewater reuse. In *Wastewater Reclamation and Reuse, 10*, Asano, T., Ed.; Technomic: Lancaster, PA, 1998.

Craik, S. A.; Finch, G. R.; Bolton, J. R.; Belosevic, M. Inactivation of *Giardia muris* cysts using medium-pressure ultraviolet radiation in filtered drinking water. *Water Res.* **2000**, *34,* 4325–4332.

Crook, J. St. Petersburg, Florida, dual water system: A case study. Water conservation, reuse, and recycling. Proceedings of an Iranian-American workshop. The National Academies Press: Washington, DC, 2005.

Current, W. L.; Upton, S. J.; Haynes, T. B. The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa: Cryptosporidiidae) infecting chickens. *J. Protozool* **1986**, *33,* 289–296.

DiBenedetto, M. A.; Di Piazza, F.; Maida, C. M.; Firenze, A.; Oliveri, R. Occurrence of *Giardia* and *Cryptosporidium* in wastewater, surface water and ground water samples in Palermo (Sicily). *Ann. Ig.* **2005**, *5,* 367–375.

Di Giovanni, G. D,; Karim, M. R.; LeChevallier, M. W.; Weihe, J. R.; Abrams, F. A.; Spinner, M. L.; Boutros, S. N.; Chandler, J. S. *Overcoming Molecular Sample Processing Limitations: Quantitative PCR. 00–HHE–2b.* Copublished by Water Environment Research Foundation, Alexandria, VA, and IWA Publishing, London, 2002.

Dufour, A.; Evans, O.; Behymer, T.; Cantu, R. Water ingestion during swimming activities in a pool: A pilot study. *J. Water Health* **2006**, *4* (4), 425–430.

Dungeni, M.; Momba, M. N. B. The abundance of *Cryptosporidium* and *Giardia* spp. in treated effluents produced by four wastewater treatment plants in the Gauteng Province of South Africa. *Water SA* **2010**, *36,* 425–431.

Durance, C.; Hofmann, R.; Andrews, R. C.; Brown, M. Application of ultraviolet light for inactivation of adenovirus. *Water Environ. Fed.* **2005**, *12,* 1–12.

Fayer, R. Taxonomy and species delimitation in *Cryptosporidium*. *Exp. Parasitol*. **2009**, *124* (1), 90–97.

Fayer, R.; Ungar, B. L. P. *Cryptosporidium* spp. and cryptosporidiosis. *Microbiol. Rev*. **1986**, *50,* 458–483.

Feng, X.; Rich, S. M.; Akiyoshi, D.; Tumwine, J. K.; Kekitiinwa, A.; Nabukeera, N.; Tzipori, S.; Widmer, G. Extensive polymorphism in *Cryptosporidium parvum* identified by multilocus microsatellite analysis. *Appl. Environ. Microbiol*. **2000**, *66,* 3344–3349.

Florida DEP. http://www.dep.state.fl.us/water/reuse/activity.htm. 2010.

Gennaccaro, A. L.; McLaughlin, M. R.; Quintero-Betancourt, W.; Huffman, D. E.; Rose, J. B. Infectious *Cryptosporidium parvum* oocysts in final reclaimed effluent. *Appl. Environ. Microbiol.* **2003**, *69* (8), 4983–4984.

Hashimoto, A.; Sugimoto, H.; Morita, S.; Hirata T. Genotyping of single *Cryptosporidium* oocysts isolated from sewage and river water. *Water Sci. Technol*. **2006**, *40,* 2527–2532.

Hill, V. R.; Simmons, B. A.; Amburgey, J. E. *Progress Towards a Rapid and Simultaneous Concentration of Microbes in Drinking Water Using Ultrafiltration and*

Dielectrophoresis. Water Research Foundation Report 3171. Water RF: Denver, CO, 2009.

Hirata, T.; Hashimoto, A. Genotyping of single *Cryptosporidium* oocysts in sewage by semi-nested PCR and direct sequencing. *Water Res*. **2006**, *54,* 197–202.

Husman, A. M.; Bijkerk, P.; Lodder, W.; Van den Berg, H.; Pribil, W.; Cabaj, A.; Gehringer, P.; Sommer, R.; Duizer, E. Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength UV) and ionizing (gamma) radiation. *Appl. Environ. Microbiol*. **2004**, *70,* 2190–2240.

Jjemba, P.; Weinrich, L.; Cheng, W.; Giraldo, E.; LeChevallier, M. W. *Guidance Document on the Microbiological Quality and Biostability of Reclaimed Water following Storage and Distribution.* WateReuse Research Foundation: Alexandria, VA, 2010.

Jjemba, P. K.; Weinrich, L.; Cheng, W.; Giraldo, E.; LeChevallier, M. W. *Guidance Document on the Microbiological Quality and Biostability of Reclaimed Water following Storage and Distribution*. WateReuse Research Foundation Report WRF-05-002. WateReuse Research Foundation: Alexandria, VA, 2009.

Karim, M. R.; LeChevallier, M. W. *Microbiological Quality of Reuse Water*. American Water Works Association: Voorhees, NJ, 2005.

Kfir, R.; Hillner, C.; Du Preez, M.; Bateman, B. Studies on the prevalence of *Giardia* cysts and *Cryptosporidium* oocysts in South African water. *Water Sci. Technol.* **1994**, *31,* 435–438.

Korich, D. G.; Mead, J. R.; Madore, M. S.; Sinclair, N. A.; Sterling, C. A. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. *Appl. Environ. Microbiol*. **1990**, *56,* 1423–1428.

Lalle, M.; Pozio, E.; Capelli, G.; Bruschi, F.; Crotti, D.; Caccio, S. M. Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int. J. Parasitol*. **2005**, *35,* 207–213.

LeChevallier, M. W.; Di Giovanni, G. D.; Clancy, J. L.; Bukhari, Z.; Bukhari, S.; Rosen, S. J.; Sobrinho, J.; Frey, M. M. Comparison of method 1623 and cell culture–PCR for detection of *Cryptosporidium* spp. in source waters. *Appl. Environ. Microbiol*. **2003**, *69,* 971–979.

LeChevallier, M. W.; Hubel, R. E. *Cryptosporidium* risk analysis and UV disinfection system reliability. *IUVA News* **2004,** *6*, 9–14.

Leong, L. Y. C.; Kuo, J.; Tang, C. C. *Disinfection of Wastewater Effluent: Pros and Cons of Technologies*. Water Environment Research Foundation Report 04–HHE–4. WERF: Alexandria, VA, 2008.

Leoni, F.; Amar, C.; Nichols, G.; Pedraza–Diaz, S.; McLauchlin, J. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhea in England between 1985 and 2000. *J. Med. Microbiol.* **2006**, *55,* 703–707.

Levine, N. D. Taxonomy and review of the coccidian genus *Cryptosporidium* (Protozoan: Apicomplexa). *J. Protozool.* **1984**, *31,* 94–98.

Lisle, J. T.; Rose, J. B. *Cryptosporidium* contamination of water in the USA and UK: A mini–review. *J. Water SRT Aqua* **1995**, *44,* 103–117.

MacKenzie, W. R.; Kazmierczak, J. J.; Davis, J. P. An outbreak of cryptosporidiosis associated with a resort swimming pool. *Epidemiol. Infect*. **1995**, *115* (3), 545–553.

Macler, B. A.; Regli, S. Use of microbial risk assessment in setting US drinking water standards. *Int. J. Food Microbiol.* **1993**, *18,* 245–256.

Madore, M. S.; Rose, J. B.; Gerba, C. P.; Arrowood, M. J.; Sterling, C. R. Occurrence of *Cryptosporidium* spp. oocysts in sewage effluents and selected surface waters. *J. Parasitol*. **1987**, *73,* 702–705.

Marshall, M. M.; Hayes, S.; Moffett, J. Sterling, C. R.; Nicholson, W. L. Comparison of UV inactivation of three *Encephalitozoon* species with that of spores of two DNA repair–deficient *Bacillus subtilis* biodosimetry strains. *Appl. Environ. Microbiol*. **2003**, *69* (1), 683–685.

Mayer, C. L.; Palmer, C. J. Evaluation of PCR, nested PCR, and fluorescent antibodies for detection of *Giardia* and *Cryptosporidium* species in wastewater. *Appl. Environ. Microbiol.* **1996**, *62,* 2081–2085.

McLauchlin, J.; Amar, C.; Pedraza–Diaz, S.; Nichols, G. L. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: Results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *J. Clin. Microbiol*. **2000**, *38,* 3984–3990.

Meng, Q. S.; Gerba, C. P. Comparative inactivation of enteric adenoviruses, poliovirus, and coliphages by ultraviolet irradiation. *Water Res*. **1996**, *30,* 2665–2668.

Messner, M. J.; Chappell, C.; Okhuysen, P. C. Risk assessment for *Cryptosporidium*: A hierarchical Bayesian analysis of human dose response data. *Water Res.* **2001,** *35*, 3934–3940**.**

Messner, M. J.; Wolpert, R. L. *Cryptosporidium* and *Giardia* occurrence in ICR drinking water sources—Statistical analyses of ICR data, Chapter 19. In *Information Collection Rule Data Analysis.* AWWA Research Foundation: Denver, CO, 2002.

Muthusamy, D.; Rao, S. S.; Ramani, S.; Monica, B.; Banerjee, I.; Abraham, O. C.; Mathai, D. C.; Primrose, B.; Muliyil, J.; Wanke, C. A., Ward, H. D.; Kang, G. Multilocus genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus–infected individuals in South India. *J. Clin. Microbiol*. **2006**, *44* (2), 632–634.

Narasimhan, R.; Brereton, J.; Abbaszadegan, M.; Ryu, H.; Butterfield, P.; Thompson, K.; Werth, W. *Characterizing Microbial Water Quality in Reclaimed Water Distribution Systems.* AWWA Research Foundation: Denver, CO, 2005.

Okhuysen, P. C.; Rich, S. M.; Chappell, C. L.; Grimes, K. A.; Widmer, G.; Feng, X.; Tzipori, S. Infectivity of a *Cryptosporidium parvum* isolate of cervine origin for healthy adults and interferon-gamma knockout mice. *J. Infect. Dis.* **2002**, *185,* 1320–1325.

Ottoson, J.; Hansen, A.; Bjorlenius, B.; Norder, H.; Stestrom, T. Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. *Water Res*. **2006**, *40,* 1449.

Ottoson, J.; Stenstrom, T. A. Faecal contamination of greywater and associated microbial risks. *Water Res*. **2003**, *37* (3), 645–655.

Parker, J. F. W. Ph.D. Thesis, University of Glasgow, Scotland, 1993.

Pieniazek, N. J.; Bornay–Llinares, F. J.; Slemenda, S. B.; da Silva, A. J.; Moura, I. N.; Arrowood, M. J.; Ditrich, O.; Addiss, D. G. New *Cryptosporidium* genotypes in HIV infected persons. *Emerg. Infect. Dis*. **1999**, *5* (3), 444–449.

Read, C. M.; Monis, P. T.; Thompson, R. C. A. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infect. Genet. Evol.* **2004**, *4,* 125–130.

Robertson, L. J.; Campbell, A. T.; Smith H. V. Survival of oocysts of *Cryptosporidium parvum* under various environmental pressures. *Appl. Env. Microbiol*. **1992,** *58*, 3494–3500.

Robertson, L. J.; Campbell, A. T.; Smith, H. V. In vitro excystation of *Cryptosporidium parvum. Parasitology* **1993**, *106,* 13–19.

Robertson, L. J.; Hermansen, L.; Gjerde, B. K. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. *Appl. Environ. Microbiol*. **2000**, *72,* 5297–5303.

Rose, J. B.; Dickson, L. J.; Farrah, S. R.; Carnahan, R. P. Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. *Water Res*. **1996**, *30,* 2785–2797.

Sakaji, R.; Funamizu, N. Microbial risk assessment and its role in the development of wastewater reclamation policy. In *Wastewater Reclamation and Reuse 10;* Asano, T., Ed.; Technomic: Lancaster, PA, 1998.

Scheller, J.; Connell, K.; Shank-Givens, H.; Rodgers, C. Design, implementation and results of US EPA's 70 utility ICR laboratory spiking program. In *Information Collection Rule Data Analysis,* AWWA Research Foundation: Denver, CO, 2002, Chapter 20.

SDLAC. *Pomona Virus Study Final Report,* prepared for California State Water Resources Control Board and U.S. Environmental Protection Agency, Sanitation District of Los Angeles Country, Los Angeles, CA, 1977.

Sluter, S. D.; Tzipori, S.; Widmer, G. Parameters affecting polymerase chain reaction detection of waterborne *Cryptosporidium parvum* oocysts. *Appl. Microbiol. Biotechnol*. **1997**, *48,* 325–330.

Smith, J. J.; Gunasekera, T. S.; Barardi, C. R.; Veal, D.; Vesey, G. Determination of *Cryptosporidium parvum* oocyst viability by fluorescence in situ hybridization using a ribosomal RNA-directed probe. *J. Appl. Microbiol*. **2004**, *96,* 409–417.

Soller, J. A.; Seto, E.; Olivieri, A. W. Microbial Risk Assessment Interface Tool: User Documentation #04HHE-3. Water Environment Research Foundation: Alexandria, VA, 2008.

Spano, F.; Putignani, L.; McLauchlin, J.; Casemore, D. P.; Crisanti, A. PCR-RFLP analysis of the *Cryptosporidium* oocysts wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbol. Lett*. **1997,** *150*, 209–217.

Spano, F.; Putignani, L.; Crisanti, A.; Sallicandro, P.; Morgan, U. M.; Le Blancq, S. M.; Tchack, L.; Tzipori, S.; Widmer, G. Multilocus genotypic analysis of *Cryptosporidium parvum* isolates from different hosts and geographical origins. *J. Clin. Microbiol*. **1998**, *36,* 3255–3259.

Straub, T. M.; Pepper, I. L.; Gerba, C. P. Hazards from pathogenic microorganisms in land disposed sewage sludge. *Rev. Environ. Contam. Toxicol.* **1993**, *132,* 55–91.

Sulaiman, I. M.; Fayer, R.; Bern, C.; Gilman, R. H.; Trout, J. M.; Schantz, P. M.; Das, P.; Lal, A. A.; Xiao, L. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. *Emerg. Infect. Dis.* **2003**, *9* (11), 1444–1452.

Suwa, M.; Suzuki, Y. Control of *Cryptosporidium* with wastewater treatment to prevent its proliferation in the water cycle. *Water Sci. Technol*. **2003**, *47,* 9–45.

SWRCB. State Water Resources Control Board. http://www.waterboards.ca.gov/water_issues/programs/grants_loans/water_recycling/munirec.shtml, 2009.

Tanriverdi, S.; Markovics, A.; Arslan, M. O.; Itik, A.; Shkap, V.; Widmer, G. Emergence of distinct genotypes of *Cryptosporidium parvum* in structured host populations. *Appl. Environ. Microbiol*. **2006**, *72,* 2507–2513.

Tanriverdi, S.; Tanyeli, A.; Baslamisli, F.; Koksal, F.; Kilinc, Y.; Feng, X.; Batzer, G.; Tzipori, S.; Widmer, G. Detection and genotyping of oocysts of *Cryptosporidium parvum* by real-time PCR and melting curve analysis. *J. Clin. Microbiol*. **2002**, *40,* 3237–3244.

Tanriverdi, S.; Widmer, G. Differential evolution of repetitive sequences in *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Infect. Genet. Evol.* **2006**, *6,* 113–122.

Theodos, C. M.; Griffiths, J. K.; D'Onfro, J.; Fairfield, A.; Tzipori, S. Efficacy of nitazoxanide against *Cryptosporidium parvum* in cell culture and in animal models. *Antimicrob. Agents Chemother.* **1998,** *42,* 1959–1965.

Thompson, J. D.; Higgins, D. G.; Gibson, T. J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994,** *22,* 4673–4680.

Tree, J. A.; Adams, M. R.; Lees, D. N. Disinfection of feline calicivirus (a surrogate for Norovirus) in wastewaters. *J. Appl. Microbiol*. **2005**, *98,* 155–162.

Tyzzer, E. E. *Cryptosporidium parvum* (sp. nov), a coccidium found in the small intestine of the common mouse. *Arch. Protistenkunde* **1912**, *26,* 394–412.

Tzipori, S.; Angus, K. W.; Campbell, I.; Gray E. W. *Cryptosporidium*: Evidence for a single species genus. *Infect. Immun.* **1980**, *30,* 884–886.

U.S. EPA. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. http://www.epa.gov/nrmrl.pubs/625r04108/625r04108.pdf. U.S. Environmental Protection Agency: Washington, DC, 2005.

U.S. EPA. Estimated Per Capita Water Ingestion in the United States; EPA-822-R-00-008. U.S. Environmental Protection Agency: Washington, DC, 2000.

U.S. EPA. *Ultraviolet Disinfection Guidance Manual for the Final Long Term 2 Enhanced Surface Water Treatment Rule,* Report No. EPA 815-R-06-007 issued in final form. U.S. Environmental Protection Agency, Office of Water (4601): Washington, DC, 2006. http://www.epa.gov/safewater/disinfection/lt2/pdfs/guide_lt2_uvguidance.pdf.

Venczel, L.; Arrowood, M.; Hurd, M.; Sobsey, M. Inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores by a mixed-oxidant disinfection and by free chlorine. *Appl. Environ. Microbiol*. **1997**, *63* (4), 1598–1601.

Villacorta, I. M. M.; Ares-Mazas, M. E.; Duran-Oreiro, D.; Lorenzo-Lorenzo, M. J. Efficacy of activated sludge in removing *Cryptosporidium parvum* oocysts from sewage. *Appl. Environ. Microbiol.* **1992**, *58,* 3514–3516.

Xiao, L.; Singh, A.; Limor, J.; Graczyk, T. K.; Gradus, S.; Lal, A. Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. *Appl. Environ. Microbiol.* **2001,** *67*, 1097–1101.

Xiao, L.; Fayer, R.; Ryan, U.; Upton, S. J. *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin. Microbiol. Rev*. **2004**, *17,* 72–97.

Yaun, B. R.; Sumner, S. S.; Eifert, J. D.; Marcy, J. E. Inhibition of pathogens on fresh produce by ultraviolet energy. *Int. J. Food Microbiol*. **2003**, *90,* 1–8.

Zhou, L.; Singh, A.; Jiang, J.; Xiao, L. Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: Implications for understanding outbreak occurrence and transmission dynamics. *J. Clin. Microbiol*. **2003**, *41,* 5254–5257.

Zuckerman, U.; Armon, R.; Tzipori S.; Gold, D. Evaluation of a portable continuous flow centrifuge for concentration of *Cryptosporidium* oocysts and *Giardia* cysts from water. *J. Appl. Microbiol*. **1999**, *86,* 955–961.

Zuckerman, U.; Tzipori, S. Portable continuous flow centrifugation and Method 1623 for monitoring of waterborne protozoa from large volumes of various matrices. *J. Appl. Microbiol*. **2006**, *100,* 1391–1395.

Appendix A

STANDARD OPERATING PROCEDURE

FIELD FILTRATION FROM AN UNPRESSURIZED SOURCE

Where sampling directly from the wastewater source, ensure that the inlet tubing/hose is submerged below the surface of the source, away from walls, bottom, other pipes or surfaces, etc.

ASSEMBLING SAMPLING APPARATUS

The sampling apparatus consists of an inlet tube leading from the wastewater source to the inlet port of the filter capsule. The tubing connected to the effluent port of the filter (outlet tubing) passes through a suitable pump (capable of creating a flow of 2 L/min or 0.5 gal/min) to a flow control valve set at 0.5 gal/min and into a calibrated collection container. This container should be marked in 0.25 L increments up to 11 L. Alternatively, the volume of sample filtered may be measured by a flow totalizer or meter rather than the calibrated container.

Remember to use clamps at all tubing connections.

FLUSHING SAMPLING APPARATUS

Before beginning sample filtration, it is important to flush all relevant sampling apparatus (except the filter) with a minimum of 20 L (5.5 gal) of the intended sample. To do this, the inlet tubing is connected directly to the outlet tubing (WITHOUT the Envirochek™ sampling capsule) using a 0.5 in. barbed connecter/coupling. Following this:

Place the open end of the inlet tubing into the wastewater source. Ensure end of the tubing is not touching walls, bottom, or environmental surfaces.

Turn ON the pump to pass 20 L (or 5.5 gal) of the sample through the apparatus to flush.

Check system for leaks and take appropriate action. Turn OFF pump after completion of flush.

Target flow rate is 2 L/min. No adjustments are required where a flow control valve (Plast-o-matic #FC050B-½-PV set at 0.5 gal/min) is used.

Note that the flush was performed and make note of flush volume on the sample collection data sheet.

During flushing or directly after the system has been performed, collect a grab sample (500 mL).

This sample is sent to the laboratory for measurement of physiochemical parameters (i.e., turbidity, pH, temperature, etc).

SAMPLE FILTRATION

Remove the blue caps from the influent and effluent ports of the Envirochek capsules. Place these blue caps in a safe place, as they will be needed to seal the filter after sample collection.

Disconnect the 0.5 in. barbed connector joining the inlet and outlet tubing, and install the Envirochek HV filter in line. Make sure the inlet/outlets tubes are connected to their respective ports on the filter.

Using clamps, secure the inlet and outlet tubing to the respective sampling ports on the filter. Make sure the flow direction though the filter is correct.

Visually inspect the filter membrane and plastic housing to ensure that the integrity of the membrane or capsule has not been compromised.

Record filtration start time as well as meter readings (if used).

Turn ON the pump to begin sample flow via the inlet tubing and into the filter capsule.

Unscrew the air vent (or bleed valve) on the capsule to open. Leave open until all the residual air has been expressed and the capsule is full of the sample.

Close the air vent (or bleed valve) once all the air has been removed from the capsule.

Pump 10 L of the sample through the filter. The volume filtered can be determined by using a flow totalizer or meter or by using a calibrated collection container to measure the volume of sample exiting the effluent port of the filter.

Turn OFF the pump once the desired volume has been reached.

Loosen the outlet tubing from the filter and allow residual liquid to drain from the outlet port. Cap the outlet port of the filter capsule with the blue filter cap.

Loosen the inlet tubing from the filter. DO NOT lose any of the water from the filter inlet port, as this liquid is considered part of the sample. Cap the inlet end of the filter capsule with the blue cap.

Record filtration END time as well as meter readings (if used) or make note of the volume collected in a calibrated container. Make sure all relevant information is recorded on data forms, chain of custody form, and filter label.

Place the Envirochek™ HV filter capsule into the Whirlpak bag. Wrap with a layer of bubble wrap and place in cooler with ice packs or in a refrigerator at 1 °C to 10 °C.

Include paperwork (placed in plastic ziplock bag) with sample.

Ship filters by overnight delivery. Make sure recently frozen ice packs are used during shipment. It is important that sufficient ice packs are used to ensure that samples arrive at the laboratory at ≤20 °C, BUT NOT FROZEN.

Advancing the Science of Water Reuse and Desalination

WATEREUSE RESEARCH

1199 North Fairfax Street, Suite 410
Alexandria, VA 22314 USA
(703) 548-0880
Fax (703) 548-5085
E-mail: Foundation@WateReuse.org
www.WateReuse.org/Foundation