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CRYPTOSPORIDIUM SP. AND *GIARDIA* SP. IN THE ST. LAWRENCE RIVER: IMPACT ON DRINKING WATER QUALITY

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of Master of Science

Department of Microbiology and Immunology, Faculty of Medicine

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ABSTRACT

This study was designed to: a) describe the occurrence and distribution of the protozoan parasites *Cryptosporidium* sp. and *Giardia* sp. in raw and treated water supplies on the St. Lawrence River; b) to find a suitable microbial indicator for the presence of *Cryptosporidium* sp. and *Giardia* sp. in raw water; c) to measure the survival capability of *Cryptosporidium parvum* in both the raw water and the treated water supplies of the St. Lawrence River, and d) to determine whether *C. parvum* oocysts aged (or stressed) in raw water are more susceptible than fresh oocysts to chlorine and monochloramine disinfection.

Twelve sampling sites were chosen on the St. Lawrence River (between Morrisburg and Lancaster, Ontario), consisting of eight raw surface water sites, and four water treatment plant effluents. Two of the plants practiced full conventional treatment of the water, including alum coagulation, rapid-sand filtration and chlorination, while the other two used a one-step chlorination process to treat the drinking water. Each site was sampled three times between July and November 1995, and a further 5 to 7 times between April and October 1996. Sampling methods for protozoan analysis followed the procedures detailed in the 19th edition of *Standard Methods for the Examination of Water and Wastewater*.[1] The results of the survey study showed that both *Cryptosporidium* and *Giardia* were ubiquitous in raw water samples from the St. Lawrence River. *Cryptosporidium* sp. oocysts were detected in 90.9% of raw water samples, while *Giardia* sp. cysts were found in 75.8% of the same samples. Mean parasite levels equaled 50.6 oocysts and 29.6 cysts per 100L, respectively. In addition, both parasites were detected on a frequent basis in treated water effluents, especially in those plants not using filtration. *Cryptosporidium* sp. and *Giardia* sp. were detected in 94.0% and 61.0% of unfiltered treated water samples, at average levels of 15.0 oocysts per

100L and 12.4 cysts per 100L. Filtered treated water samples fared considerably better: 16.7% of those were positive for *Cryptosporidium* sp. and 5.5% were positive for *Giardia* sp.

The data accumulated through the survey study were also used to calculate how efficiently the four water treatment facilities were able to reduce parasite levels through the treatment process. and to determine whether *Cryptosporidium* and *Giardia* concentrations were influenced by seasonal changes. Water treatment plant efficiencies were calculated as $\log_{10}$ reductions in parasite numbers between raw water samples and corresponding treated water samples. The two water filtration plants were able to reduce parasite levels by approximately 1 $\log_{10}$. while a maximum 0.24 $\log_{10}$ reduction was observed at the two chlorination-only plants. To test for seasonality. *Cryptosporidium* and *Giardia* levels were sorted based on the month of sample collection. and compared using a one-way ANOVA. *Cryptosporidium* demonstrated significant seasonality: levels in the spring (April and May) and in the autumn (October and November) were significantly higher than those found in the summer months. *Giardia* concentrations did not vary significantly with the seasons.

In addition to the protozoan analysis. raw water samples were analyzed for nine different microbial indicators. These included *Aeromonas* sp.. *Clostridium perfringens* (vegetative cells and spores). *Enterococcus* sp.. *Pseudomonas* sp.. total and fecal coliforms. total heterotrophic bacteria (or HPC) and somatic coliphage b. The Spearman rank order correlation was used to measure the strength of association between protozoan parasite levels and microbial indicator concentrations. *Cryptosporidium* sp. was significantly correlated to *Enterococcus* sp. ($r=-0.338$. $P=0.02$) and with HPC's ($r=0.272$. $P=0.036$). *Giardia* sp. was significantly associated with *Cryptosporidium* sp. in raw water samples ($r=0.68$. $P<0.01$). but not with any of the microbial indicators of water quality.

Further experiments were designed to determine the survival potential of *Cryptosporidium parvum* over time in both treated water effluents and in raw water. An *in situ* model was developed, using dialysis cassettes as flow-through survival chambers which kept the oocysts contained, while still allowing them to be in constant contact with the water medium. The chambers were floated in overflow tanks, which were connected to either a treated water or a raw water tap, located inside water treatment plants on the St. Lawrence River. On sampling days, cassettes were removed from the tank and the relative viability of *C. parvum* oocysts in each sample was assessed by *in vitro* excystation and total oocyst counts.

For the survival experiments in treated water, four separate trials were conducted, once in each season of the year. The maximum reduction in *C. parvum* viability was 2 $\log_{10}$ after four days of exposure, while the minimum reduction observed was 0.6 $\log_{10}$ after the same time period. *C. parvum* decay seemed to be correlated to free chlorine levels in the treated water effluent ($r=0.685$, $P=0.074$), but not to the water temperature ($r=0.071$, $P=0.843$).

Three separate trials of the *in situ* survival study in raw water were conducted, all at approximately the same water temperature (0.1 to 2.0°C). The first trial demonstrated that *C. parvum* oocysts were able to survive in river water for up to seven weeks (0.86 $\log_{10}$ reduction after that time). In the subsequent trials oocysts were exposed to both raw water and raw water that had been filtered through a 0.22μm membrane in order to remove or reduce the microbial populations naturally present in the water of St. Lawrence River. *C. parvum* oocysts decayed significantly more rapidly in natural raw water than in filtered raw

water, suggesting that one or more of the microbes in the St. Lawrence River has a detrimental effect on oocyst survival.

Cryptosporidium parvum oocysts that had been aged in raw water for 14 to 18 days were subjected to chlorine or monochloramine disinfection (10 mg/L for 5 hours) in order to test the hypothesis that aged or stressed oocysts would be more susceptible to chemical inactivation than fresh oocysts. Aged oocysts exposed to chlorine were not more susceptible than the controls, and in two of three trials they were, in fact, more resistant to inactivation than the fresh oocysts. Monochloramine did not inactivate either the aged or the fresh *C. parvum* oocysts.

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GENERAL INTRODUCTION

Cryptosporidium was first described in mice by E.E. Tyzzer[2] in 1907, however, this protozoan parasite was not recognized as a pathogen until 1955, when it was pinpointed as the cause of an outbreak of diarrheal illness in a flock of turkeys.[3] The first human cases of cryptosporidiosis were reported in 1976,[4] and for a while the parasite was thought to be an opportunistic pathogen, causing a prolonged, potentially life-threatening illness in immunocompromised patients.[5] Since that time, it has been learned that *Cryptosporidium* can infect healthy, immunocompetent individuals as well, producing a short-term, self-limiting gastrointestinal illness, characterized by profuse watery diarrhea.[6]

Cryptosporidium is now considered to be an "emerging" pathogen, responsible for thousands of infections (and a number of deaths) yearly in both industrialized and third-world nations.[7] It is now known that this parasite is ubiquitous in environmental waters, such as rivers and lakes,[8-10] and that it is highly resistant to normal water disinfection practices.[11-13] In addition, its small size (4 to 6µm) occasionally allows it to escape the filters designed to remove such organisms during water treatment. As such, waterborne outbreaks of cryptosporidiosis associated with drinking water have been occurring on a frequent basis in the United States, the United Kingdom and in Canada.

In 1993, over 400,000 residents of Milwaukee, Wisconsin, were thought to be infected (and over one hundred deaths occurred) when *Cryptosporidium* escaped the water treatment filtration and disinfection process and reached consumers' homes through their tap water.[14] Similar smaller-scale outbreaks have occurred in Canada in association with both filtered water supplies (e.g. Kitchener-Waterloo, Ontario, 1993, 200 residents affected[15]) and

unfiltered water supplies (e.g. Collingwood. Ontario. 39 confirmed cases and approximately 80 suspected cases[16]; and Kelowna. B.C.. over 15.000 cases reported[17]).

Despite these outbreaks and the knowledge that *Cryptosporidium* is present even in pristine watersheds. no legislation exists in Canada requiring water treatment facilities to monitor for *Cryptosporidium*. either in source waters or in finished drinking water. In addition. relatively few scientific survey studies have been conducted in Canadian watersheds. particularly in Ontario.

The focus of this project was the waterborne occurrence and survival of *Cryptosporidium* sp. (and *Giardia* sp.. to a lesser extent) in the surface water of the St. Lawrence River in Ontario. and also in the drinking water supplies of the treatment facilities that draw on this river for their source waters.

REVIEW OF THE LITERATURE

Cryptosporidium has traditionally been thought to cause infection in humans from direct contact with infected animals, but with the advent of the methodology to test for the presence of this parasite in environmental waters, it has been discovered that it is ubiquitous in surface waters such as rivers and lakes. This, coupled with the occurrence of several outbreaks of cryptosporidiosis in water treatment systems employing filtration,[18-22] has sparked an interest in the role of *Cryptosporidium* as a waterborne disease agent, and in the development of methods to prevent further outbreaks associated with filtered drinking water.

TAXONOMY OF *CRYPTOSPORIDIUM*

Cryptosporidium is a protozoan parasite, distinguished from other protozoa by its morphology and life cycle.[23] The complete classification is as follows:

Phylum - *Apicomplexa*
Class - *Sporozoasida*
Subclass - *Coccidiasina*
Order - *Eucoccidiorida*
Suborder - *Eimeriorina*
Family - *Cryptosporidiidae*
Genus - *Cryptosporidium*

Cryptosporidium species belong to the phylum *Apicomplexa*, along with other sporozoan parasites, due to the presence of an apical complex, and a lack of cilia and flagella.[24] The method of locomotion (gliding, undulation, body flexion) of the invasive form of *Cryptosporidium*,[5] as well as the occurrence of both sexual and asexual reproduction in the life cycle,[24] place it in the class *Sporozoasida*. It is further placed in the subclass *Coccidiasina* because its life cycle includes merogony, gametogeny and sporogony.[5]

Cryptosporidium species are members of the order *Eucoccidiorida* because of the development of merozoites within meronts in the process of merogony.[24] The independent development of macrogametocytes (female gametes) and microgametocytes (male gametes) places *Cryptosporidium* in the suborder *Eimeriorina*.[5,24] Finally, it belongs to the family *Cryptosporidiidae* due to several factors: it is monoxenous (only one host needed for life cycle), it develops just under the surface membrane of the host cell,[24] it has an oocyst without sporocysts and four sporozoites, and has microgametes without flagella.[5]

Originally, *Cryptosporidium* species were named on the basis of host specificity.[11] However, cross-transmission studies with organisms recovered from different hosts have shown that species of *Cryptosporidium* demonstrate little or no host specificity. On this basis, Tzipori et al[25] have suggested that *Cryptosporidium* may be a single-species genus. Levine, on the other hand, consolidated the 20 named species of *Cryptosporidium* into four designations, which correspond to isolates from mammals, birds, fish and reptiles.[26]

More recently, Upton and Current[27] concluded that only two species were responsible for infections in mammals: *C. parvum* (in humans and cattle) and *C. muris* (in mice and cattle). It also appears that two species can infect birds: *C. meleagridis* and *C. baileyi*.[28] It is believed that *C. parvum* is the predominant species responsible for infecting humans and animals.[11] This species inhabits the intestinal tract of mammals and has an oocyst that measures 4 to 6µm in diameter.[23]

LIFE CYCLE

The life cycle of *Cryptosporidium* can be divided into six major events: excystation (release of sporozoites). merogony (asexual multiplication within host cells). gametogeny (formation of macro- and microgametes). fertilization. oocyst wall formation and sporogony (sporozoite formation)[5] (Figure 1).

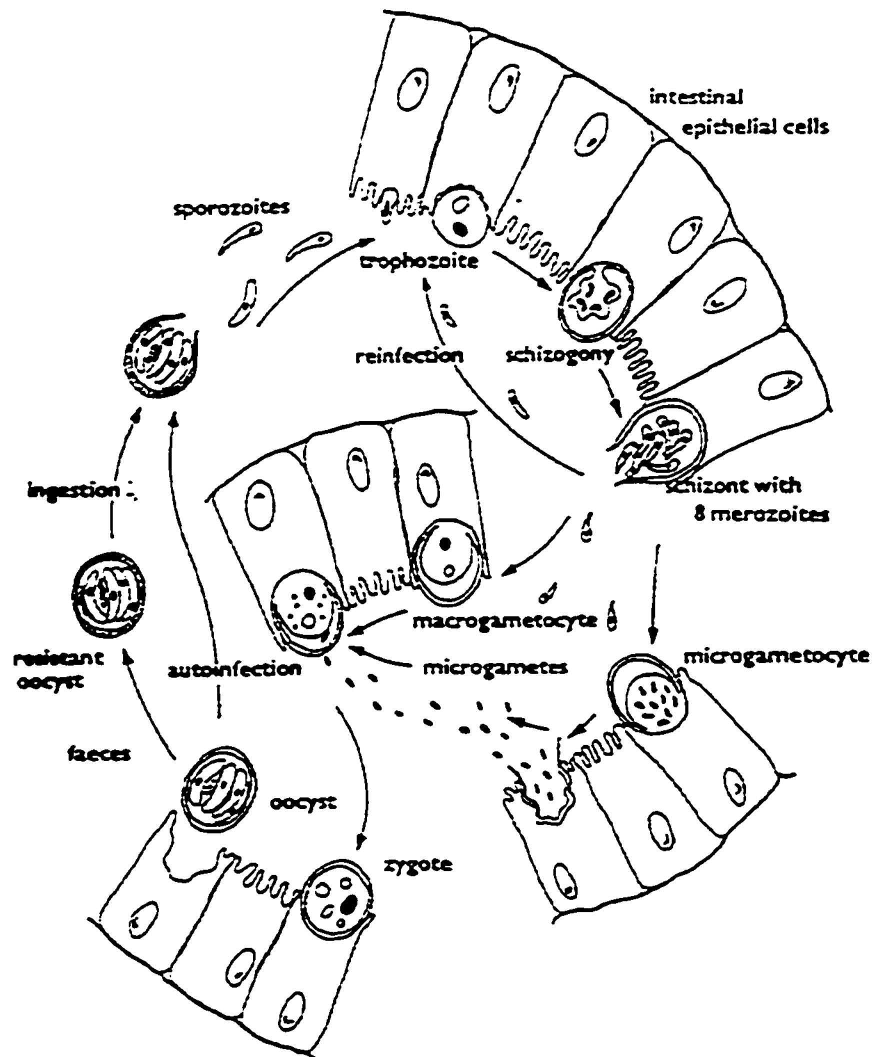


Figure 1: Life Cycle of *Cryptosporidium* sp.
(From Smith. H.V. and J.B. Rose. 1990. Parasitology Today. 6: 8-12.)[29]

Excystation (*in vivo*) occurs in the lumen of the gastrointestinal tract following ingestion of oocysts by the host. The oocyst walls of *Cryptosporidium parvum* have a suture at one pole, which partially dissolves during excystation, forming an opening through which the sporozoites escape.[5]

Merogony (asexual multiplication - referred to as schizogony in Figure 1) occurs after the sporozoites have invaded the intestinal epithelial cells. The sporozoite differentiates into a trophozoite, which undergoes asexual multiplication. Two types of meronts (or schizonts) result - type I or type II meronts. Type I meronts develop into merozoites, which can infect new host cells, and begin the cycle again. The merozoites that develop from type II meronts initiate sexual multiplication (gametogony) by differentiating into macrogametocytes (female gametes) or microgametocytes (male counterpart). When they mature, the microgametocytes contain spermlike microgametes which penetrate the macrogametes. The fertilized macrogamete develops into an oocyst. The oocysts undergo sporogony within the host and upon completion of this event each oocyst contains four potentially infective sporozoites.[30]

Current[5] has reported that 80% of the oocysts formed are thick-walled, environmentally resistant forms that are passed in the feces. However, the other 20% of the oocysts formed are thin-walled, surrounded only by a single membrane. This membrane ruptures soon after the oocyst is released from the host cell and the sporozoites re-invade new host intestinal cells. The thick-walled oocysts are responsible for transmitting the infection from one host to another (through ingestion), while the thin-walled oocysts are responsible for autoinfection within the same host. These thin-walled oocysts are believed to be responsible for severe infections in hosts exposed to only a small number of oocysts.

Giardia sp. has a much simpler two-stage life cycle consisting of an environmentally resistant cyst, and a vegetative trophozoite stage.[31] As with *Cryptosporidium*, cysts are ingested by the host through contaminated water or food. Excystation is triggered by exposure to stomach acid, and two trophozoites (per cyst) are released into the small intestine. The trophozoites attach themselves to the surface of the intestinal cells, and multiply by binary fission.[32] Some eventually detach, and a cyst wall is excreted around the organism as it passes through the large intestine.[32,33] Cysts are infectious for other hosts immediately upon excretion.

EPIDEMIOLOGY OF *CRYPTOSPORIDIUM*

The main symptom of cryptosporidiosis in humans is profuse watery diarrhea. Fayer and Ungar[30] have also reported common clinical features, including abdominal pain, nausea, vomiting and low-grade fever. The duration of symptoms varies according to the immune status of the patient. The disease is self-limiting in immunocompetent patients, with diarrhea from the infection lasting between 2 to 26 days (average 12 days).[34] Immunocompromised patients, however, may experience a prolonged, life-threatening illness.[4]

A study was performed by Dupont et al[35] to determine the infective dose of *Cryptosporidium parvum* in healthy adults. The twenty-nine subjects in the study ingested *Cryptosporidium* in doses ranging from 30 to 1 million oocysts. Eighteen subjects (62%) had enteric symptoms, and seven of those (39%) developed clinical cryptosporidiosis. Infections occurred at all inoculum levels. The ID_{50} (infectious dose) was calculated to be approximately 132 oocysts, and the mean incubation period for the infection was nine days.

There appear to be several mechanisms by which *Cryptosporidium* can be transmitted. It was thought originally that zoonotic transmission, that is, transmission from animals to humans through a fecal-oral route, was the major contributor to human infection. *Cryptosporidium* has been isolated in over forty mammalian species,[11] including calves, rodents, puppies and kittens.[5] Fayer and Ungar[30] have demonstrated, using cross-transmission studies, that isolates of *Cryptosporidium* from mammals are generally infective for other mammals, and therefore are not host-specific. Person-to-person transmission has also been documented. Cryptosporidiosis outbreaks have been reported in day-care centers,[36-38] in hospital personnel,[39,40] and within households.[41,42]

The waterborne transmission of *Cryptosporidium* has been receiving the most attention of late. Several outbreaks of cryptosporidiosis have occurred in North America and in the United Kingdom, through contaminated water. These will be discussed in greater detail under the "Waterborne Outbreaks" section of this review. *Cryptosporidium* has also been associated with traveller's diarrhea,[43-45] which gives further evidence to the waterborne mode of transmission. Foodborne transmission is rare, but has been documented.[46,47]

In 1986, Fayer and Ungar[30] reviewed 36 surveys of prevalence data for human cryptosporidiosis on six continents. In Europe, prevalence was between 1 and 2%, and in North America, prevalence rates ranged from 0.6 - 4.3%. In Asia, Australia, Africa and Central and South America, prevalence rates generally began at 3 - 4% and reached 10 - 20%. The surveys suggest that *Cryptosporidium*-related infections are more widespread in less developed countries, but that infections occur worldwide. The review also indicated that *Cryptosporidium* is ranked among the top four enteric pathogens identified in surveys. Also

of interest was the finding that children had a significantly higher prevalence rate of cryptosporidiosis than adults, and that infections tended to be seasonal, with peaks in warm, wet weather.

EPIDEMIOLOGY OF *GIARDIA*

Giardiasis presents many of the same symptoms as cryptosporidiosis, and usually takes 5 to 10 days to develop after first exposure.[32] The most common symptoms include explosive, foul-smelling diarrhea, abdominal distension associated with flatulence, and nausea.[32,33] Symptoms usually last 3 to 4 days in immunocompetent individuals,[33] but may cause a long-term, debilitating disease in the immunosuppressed.[32] Several drugs are available for treatment of giardiasis, however, including metronidazole, furazolidone and paromomycin.[31]

Giardia is reported to be the most common intestinal parasite of humans in both the United States[32] and worldwide.[31] Prevalence rates vary from 2-5% in industrialized countries to 20-30% in developing countries.[33] *Giardia* infections can occur after the ingestion of as few as 10 viable cysts.[31] Transmission can occur through inadequately treated drinking water, contamination of the water by sewage, recreational water contaminated by wild animals, contamination of food (usually by an infected food handler), or directly from person-to-person (through a fecal-oral route).[31]

OCCURRENCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN THE WATER ENVIRONMENT

Several studies have been conducted documenting the occurrence of *Cryptosporidium* and *Giardia* in various aquatic environments, including surface waters (Table 1), drinking water supplies (Table 2), and in raw sewage and treated sewage effluents (Table 3). Many of the studies demonstrate that both protozoan parasites are ubiquitous in these settings.

Surface Waters and Drinking Water (Tables 1 and 2).

Rose[8] collected 90 surface water samples from Arizona, California, Colorado, Oregon, Texas and Utah, the majority of which were receiving sewage effluents. In the waters classified as reservoirs or lakes, 75% were positive for the presence of *Cryptosporidium*, at an average concentration of 91.0 oocysts/100L. The samples categorized as rivers or streams gave similar results: 77% were positive for oocysts, at average levels of 94.0 oocysts/100L. Drinking water samples were also examined. Ten of the samples were filtered water samples: no oocysts were detected in the samples from plants practicing conventional treatment (i.e. coagulation, sedimentation, rapid sand filtration, disinfection). *Cryptosporidium* was detected in one sample after treatment by direct filtration. One of two samples receiving filtration without coagulation was also positive for oocysts. *Cryptosporidium* was also detected in two of four samples of unfiltered, chlorinated drinking water.

LeChevallier et al[9] examined the source waters of 66 surface water treatment plants in fourteen U.S. states and one Canadian province for *Giardia* and *Cryptosporidium*. *Giardia* cysts were detected in 81% of the raw water samples, at densities of 4.0 to 6.6 x 10^3

Table 1: Occurrence of *Cryptosporidium* and *Giardia* in Surface Waters in North America

Group	Location	# of samples	% positive samples: Crypto.	Crypto. oocysts/100L	% positive samples: Giardia	Giardia cysts/100L
Madore et al[48]	Arizona	6	100%	2.0×10^{2} - 5.8×10^{5}	-	-
Rose[8]	Western U.S.	90	77%	91.0-94.0	-	-
Rose et al[49]	Western U.S.	21	90%	80.0 - 2.9×10^{3}	57%	35-120
Ongerth and Stibbs[50]	Washington /California	11	100%	2.0×10^{2}-1.12×10^{4}	-	-
LeChevallier et al[9]	14 states/ 1 province	85	87%	7.0 - 4.8×10^{4}	81%	4.0 - 6.6×10^{3}
Rose et al[51]	17 states	257	55%	43	16%	3
Wallis[52]	Canada	113	11%	N/A	19%	N/A
Roach et al[53]	Yukon	11/22	14%	N/A	32%	N/A
Ongerth et al[54]	Washington	5	N/A	-	N/A	0.2-3.0
LeChevallier and Norton[10]	15 states/ 2 provinces	262	51.5%	6.5 - 6.5×10^{3}	45%	2.0 - 4.4×10^{3}
Wallis et al[55]	Canada	1173	4.5%	~0.5	20.9%	2.0-230
Isaac-Renton et al[56]	B.C.	153	-	-	68%	0.02-100
Hansen and Ongerth[57]	Washington	35	97%	20.0 - 6.5×10^{3}	-	-
Chauret et al[58]	Eastern Ontario	41	78.8%	~20	75%	~8

cysts/100L. *Cryptosporidium* oocysts were detected in 87% of the samples, at concentrations ranging from 7.0 to 4.8 $\times 10^4$ oocysts/100L. Overall, *Cryptosporidium* or *Giardia* was detected in 97% of the samples. Once again, higher parasite densities were found in the source waters receiving sewage or industrial effluents. In a parallel study, the same group[18] also studied *Giardia* and *Cryptosporidium* occurrence in filtered drinking water supplies. They examined the same 66 surface water treatment plants used in the surface water study[9] to evaluate how compliance with the Surface Water Treatment Rule (SWTR) would control the occurrence of *Giardia* and *Cryptosporidium* in drinking water. The SWTR stipulates that systems are required to remove and/or inactivate 99.9% of *Giardia* cysts, to ensure that at least a 0.2 mg/L disinfectant residual enters the distribution system at all times, and that filtered water turbidities be less than or equal to 0.5 NTU (nephelometric turbidity units) in 95% of the measurements taken every month. The study detected *Giardia* cysts in 17% of the 83 samples taken, at an average concentration of 4.45 cysts/100L, and found *Cryptosporidium* oocysts in 26.8% of the samples, at levels of 1.52 oocysts/100L. Of the sites that tested positive for *Giardia* or *Cryptosporidium*, 78% were in compliance with the turbidity regulations of the SWTR. Overall, it was found that removal of turbidity in the treatment process was not a statistically significant predictor of the removal of *Giardia*, but was significantly correlated with the removal of *Cryptosporidium*

Rose et al[51] conducted another study in 1991 which surveyed potable water supplies in seventeen U.S. states for *Cryptosporidium* and *Giardia*. They compared the occurrence of the two parasites in 257 water samples. *Cryptosporidium* oocysts were found in 55% of the surface water samples, at average concentrations of 43 oocysts/100L; *Giardia* cysts were

Table 2: Occurrence of *Cryptosporidium* and *Giardia* in Drinking Water in North America

Water Type	Group	Location	# of samples	% positive: Crypto.	Crypto. oocysts/ 100L	% positive: Giardia	Giardia cysts/100L
Unfiltered	Rose[8]	Western U.S.	4	50%	0.6	-	-
Filtered	Rose[8]	Western U.S.	10	20%	0.1	-	-
Unfiltered	Rose et al[51]	17 states	6	33%	0.20	-	-
Filtered	Rose et al[51]	17 states	17	11.8%	0.04	-	-
Filtered	LeChevallier et al[18]	14 states/ 1 province	83	27%	1.52	17%	4.45
Both	Wallis[52]	Canada	112	7%	N/A	14%	N/A
Unfiltered	Roach et al[53]	Yukon	42	17%	0-0.5	5%	0-1.4
Filtered	LeChevallier and Norton[10]	15 states/ 2 provinces	262	13.4%	0.29-57	4.6%	0.98-9.0
Both	Wallis et al[55]	Canada	423	3.6%	N/A	18.2%	N/A
Unfiltered	Isaac-Renton[56]	British Columbia	91	-	-	59%	0.04-7.8
Filtered	Chauret et al[58]	Ottawa	12	0%	-	0%	-

detected in 16% of the same samples, at average levels of 3 cysts/100L. The parasites were more frequently recovered in waters receiving sewage and agricultural discharges. The same study also looked at parasite levels in 36 drinking water samples. *Cryptosporidium* oocysts were detected in 17% of the samples, at levels ranging from 0.5 to 1.7 oocysts/100L. No *Giardia* cysts were detected.

An extensive survey of Canadian water supplies, conducted by Wallis et al.[55] involved the collection of 1,760 raw water, treated water and raw sewage samples from 72 municipalities across the country to determine *Cryptosporidium* and *Giardia* prevalence. Of the 72 communities chosen, 58 treated their drinking water by chlorination alone. *Cryptosporidium* oocysts were detected in 53 of 1,173 (4.5%) surface water samples and in 10 of 423 (3.6%) drinking water samples, while 21% of the surface water samples and 18.2% of drinking water samples were positive for *Giardia* cysts. The authors reported finding *Giardia* cysts more frequently and in higher concentrations in samples collected in the spring and fall.

Overall, these studies show that the occurrence of *Giardia* and *Cryptosporidium* is widespread in environmental waters, and that there is a risk of waterborne outbreaks if the water is not treated properly. At the same time, the study by LeChevallier et al[18] demonstrates that compliance with the SWTR does not necessarily ensure that filtered water will be free of *Cryptosporidium* or *Giardia*.

Sewage Waters (Table 3). Rose[8] also looked at the concentration of *Cryptosporidium* oocysts in raw and treated sewage samples. Of eleven raw sewage samples, 91% were positive for the presence of oocysts, with an average concentration of 28.4 oocysts/L.

Table 3: Occurrence of *Cryptosporidium* and *Giardia* in Sewage Waters in North America

Water Type	Group	Location	# of samples	% positive samples: Crypto.	Crypto. oocysts/L	% positive samples: Giardia	Giardia cysts/L
Raw	Madore et al[48]	Arizona	4	100%	5.18×10^3	-	-
Treated	Madore et al[48]	Arizona	9	100%	1.3×10^3	-	-
Raw	Rose[8]	Western U.S.	11	91%	28.4	-	-
Treated	Rose[8]	Western U.S.	22	91%	17.0	-	-
Raw	Rose et al[49]	Western U.S.	N/A	N/A	5.2×10^3	N/A	51.0
Treated	Rose et al[49]	Western U.S.	N/A	100%	1.4×10^3	40%	1.3
Raw	Roach et al[53]	Yukon	N/A	N/A	0 - 74.0	N/A	26 - 3.0×10^3
Raw	Wallis et al[55]	Canada	164	6.1%	1.0-120	72.6%	$<10^3$ - 8.8×10^4

Twenty-two treated sewage samples were collected; once again 91% of the samples were positive for *Cryptosporidium* oocysts, and the average concentration was calculated at 17.0 oocysts/L. A more comprehensive study was done by Rose et al[49] in the same year, which also looked at the levels of *Giardia* cysts in raw and treated sewage samples along with *Cryptosporidium* oocysts in the western United States. *Giardia* levels averaged 51.0 cysts/L in raw sewage samples, and were detected only in 40% of the treated effluents at average levels of 1.3 cysts/L. *Cryptosporidium*, on the other hand, was found at levels of 5.2×10^3 oocysts/L (on average) in raw sewage samples, and was detected in all treated wastewater samples at an average concentration of 1.4×10^3 oocysts/L. Based on this data, the group hypothesized that *Cryptosporidium* may be removed less efficiently by secondary sewage treatment processes than *Giardia*.

Wallis et al[55] surveyed raw sewage samples in 72 Canadian communities. *Cryptosporidium* oocysts were detected in 10 of 164 (6.1%) raw sewage samples, however *Giardia* cysts were found in 119 (72.6%) of the same 164 samples. Levels were not reported.

WATERBORNE OUTBREAKS

Since the mid-1980's, waterborne outbreaks of cryptosporidiosis have been documented in association with filtered and unfiltered water supplies, untreated water sources, contaminated wells and swimming pools. Outbreaks have been reported in the U.S., the U.K., and in Canada (Table 4). The first waterborne outbreak of giardiasis occurred in 1965, and since that time the parasite has been responsible for more than 80 waterborne disease outbreaks.[32-59] The causes of waterborne outbreaks of cryptosporidiosis (and

Table 4 - List of Reported Waterborne Outbreaks of Cryptosporidiosis

Water Type	Location	Year	Estimated # Infected
Unfiltered	Texas[20]	1984	> 500
Unfiltered	Sheffield[21]	1986	62
Unfiltered	New Mexico[60]	1986	78
Filtered	Georgia[61]	1987	13.000
Pool	Doncaster.U.K.[62]	1988	67
Pool	Los Angeles[63]	1988	44
Filtered	Swindon/Oxfordshire[64]	1988-89	516
Filtered	Isle of Thanet[65]	1990-91	47
Pool	British Columbia[66]	1990-91	> 85
Filtered	South London[67]	1991	44
Filtered	Oregon (Jackson Cty.)[22]	1992	43
Pool	Oregon (Lane Cty.)[68]	1992	55
Unfiltered	Warrington. U.K.[69]	1992-93	47
Filtered	Milwaukee[14]	1993	403.000
Filtered	Kitchener. ON[15]	1993	200
Pool	Oshkosh. WI[70]	1993	> 50
Pool	Dane County. WI[71]	1993	> 85
Filtered	Nevada (Clark Cty.)[72]	1994	78
Unfiltered	Collingwood. ON[16]	1996	>40
Unfiltered	Kelowna. B.C.[17]	1996	~15.000

giardiasis) are usually attributed to three factors: mechanical problems at the water treatment plant resulting in sub-optimal filtration or disinfection (and a corresponding rise in turbidity): heavy rainfall increasing agricultural runoff (parasite-containing feces) into source waters: and simple failure of the normal treatment process to remove oocysts.

Interestingly, waterborne outbreaks of cryptosporidiosis have also been documented in association with swimming pools.[62,63,66,68,70,71] These outbreaks have been associated mainly with fecal accidents occurring in the pool water, but have also occurred due to plumbing problems which allowed sewage from the main sewer to enter into the circulating pool water.

The first major outbreak of cryptosporidiosis documented in the United States occurred in July 1984 in Braun Station, a suburb of San Antonio, Texas.[20] Treatment of the water supply consisted solely of chlorination. When an outbreak of diarrhea occurred among the residents of Braun Station, stool specimens were tested for potential bacterial pathogens, such as *Salmonella*, *Campylobacter*, *Yersinia*, and also for *Giardia* and *Cryptosporidium*. Oocysts were detected in the stool samples of 47 of 79 residents who became ill in July, which led to the conclusion that *Cryptosporidium* was responsible for the outbreak. A random telephone survey in the community indicated that the incidence of diarrheal disease was at least 12 times higher in Braun Station than in neighbouring communities with a different water supply. The outbreak was traced to the fecal contamination of an artesian well which served as the community's water source. Tests showed that dye introduced into the sewage system found its way into the well water, although the exact site of contamination was not found.

Another waterborne outbreak of cryptosporidiosis affected an estimated 13,000 of 64,900 residents of Carrollton, Georgia in 1987.[61] This was the first reported contamination of a filtered water system. The water system drew water from a river that ran through surrounding pasture land. Water treatment consisted of coagulation, rapid mixing, mechanical flocculation, sedimentation and sand filtration. *Cryptosporidium* oocysts were found in the stools of 58 of 147 (39%) patients with gastrointestinal illness and were also detected in samples of treated drinking water. A telephone survey indicated that 61% of household members exposed to the public water supply experienced gastrointestinal illness, compared to 21% of those not exposed. The drinking water met all state and federal regulatory agency quality standards, and was within the limits for turbidity, coliforms and residual chlorine. It was determined later that sub-optimal flocculation and filtration probably allowed the parasites to pass into the drinking water supply. At the time of the outbreak, the mechanical agitators had been removed from the flocculation basins in anticipation of the arrival of new ones, which may have impaired the removal of particulates. The filters were also occasionally restarted without being backflushed, which may have released dirt and microorganisms from the filter beds into the treated water. Other possible contributors included a recent sewage overflow, and a low level *Cryptosporidium* infection in cattle on farms along the river which served as a raw water source.

The largest outbreak documented to date occurred in Milwaukee, Wisconsin in the spring of 1993.[14] An estimated 403,000 people were infected by an outbreak of acute watery diarrhea, which was later found to be caused by *Cryptosporidium* oocysts that passed through the filtration system of one of the city's water treatment plants. A review of the plant's

records showed a substantial increase in the turbidity of the treated water for a period of approximately two weeks, although other water quality parameters, such as coliform levels, were within the limits set by the state. At the time, monitors designed for the continuous measurement of turbidity were not in operation. There was no evidence of breakdown in either the flocculators or the filters, yet *Cryptosporidium* oocysts that entered the system from the raw water source were not removed by the coagulation and filtration processes. Possible sources of oocysts include increased runoff from area cattle farms, due to spring rains, slaughter houses and human sewage.

In Clark County, Nevada, 78 cases of cryptosporidiosis were identified in the first quarter of 1994.[72] Sixty-one of those affected were HIV-infected adults. The state-of-the-art water treatment plant produced water whose quality was better than that required by federal standards. During the time of the outbreak, the plant reported no elevated turbidity levels or coliform counts, and no oocysts were found in either the source water or the finished water during the time of the study. However, after the study period, presumptive oocysts were detected in the source water, the filter backwash and in finished water samples.

The frequency of reported outbreaks of cryptosporidiosis in Canada has been increasing in the past three years. Two hundred laboratory-confirmed cases were reported in the Kitchener-Waterloo area in April 1993.[15] *Cryptosporidium* was detected in the raw water source (Grand River) at levels ranging between not detected to 2.1×10^3 oocysts per 100L. As well, low concentrations of *Cryptosporidium* were found in two of the ground water supplies. Levels in the treated water were approximately 0.7 oocysts/100L. The water treatment plant involved was a one year-old state-of-the-art plant, and although it was shut

down during the outbreak, no significant problems were found with the plant equipment or with the turbidity levels in the treated water.

Two similar outbreaks occurred in 1996 in association with unfiltered drinking water. In Collingwood, Ontario, the drinking water was blamed for at least 39 confirmed cases of cryptosporidiosis in March 1996, along with 80 further suspected cases, mostly in elderly residents of that city.[16] A large-scale outbreak occurred later in the summer in Kelowna, B.C., infecting an estimated 15,000 residents.[17] This incident was the largest of five outbreaks of cryptosporidiosis that occurred in British Columbia in the summer of 1996.[73]

METHODS FOR THE RECOVERY OF CYSTS AND OOCYSTS FROM WATER SAMPLES

a) Standard Method

Since *Cryptosporidium* and *Giardia* do not occur in large numbers in environmental waters, large volumes of water (i.e. 150 to 1,000L) must be filtered through a cartridge filter with a nominal porosity of 1 µm. Oocysts and cysts are retained on the filter fibers, and are recovered by backflushing the filter with a detergent solution. The resulting elution fluid is centrifuged to concentrate the parasites. A flotation step is used to separate the cysts and oocysts from debris and particulate matter. The concentrated sample is then passed through a membrane filter, stained with fluorescent antibodies, and mounted on a microscope slide. Cysts and oocysts can then be identified by their apple-green fluorescence pattern and characteristic size and shape. This is the method described in *Standard Methods for the*

Examination of Water and Wastewater.[1] which was developed from various studies and experiments conducted over the past ten years.

Spaulding et al[74] first used membrane filters in 1983 to quantify *Giardia* cysts in feces. They developed a method of fixing and staining the cysts on 5μm membrane filters. which could then be mounted on microscope slides and counted using a light microscope. They stated at the time that "...the precision of the method with its good reproducibility. indicates that it may be a valuable tool for further studies with *Giardia* spp. and other similar organisms."

In 1985. Sauch[75] developed a method which used indirect immunofluorescence and phase-contrast microscopy to identify *Giardia* cysts in raw and treated water. *Giardia* specific antiserum and fluorescein conjugate were applied to cysts on cellulose acetate membrane filters. The filters were placed on microscope slides and then examined using an epifluorescence microscope. The *Giardia* cysts fluoresced bright green under the ultraviolet light. and could be located even in samples contaminated with other microorganisms and debris. The identity of the cysts could be further confirmed using phase-contrast microscopy.

The use of polypropylene cartridge filters for the detection of *Cryptosporidium* in water was first developed by Musial et al.[76] The purpose of their experiment was to develop a method for the detection and concentration of *Cryptosporidium* in water. to assess its occurrence in the environment and the potential for waterborne transmission. The study also determined the optimal conditions for concentration. filter elution. filter porosity and detection. much of which is now used in the standard method. Of note. the authors found that eluent containing 0.1% Tween 80 gave higher recovery of oocysts than did eluent

without Tween 80. The detergent is chaotropic - it disrupts hydrophobic interactions, indicating that such interactions may be a major factor in oocyst attachment to filter fibers.

In 1987, using the tools provided by the four studies mentioned above, Rose et al[77] evaluated the method for the detection of *Cryptosporidium* in wastewater and freshwater. This consisted of filtration with polypropylene cartridge filters, elution with Tween 80/detergent, concentration by Sheather's sucrose flotation and detection on membrane filters. They found that overall recovery efficiencies averaged approximately 50%. Based on the results of this preliminary study, *Cryptosporidium* could now be considered a waterborne parasite.

In 1988, Rose et al[49] conducted a study to evaluate the *Giardia* and *Cryptosporidium* recovery procedure, with the goal of improving protozoan recovery from water samples. The procedure was evaluated at the following steps: sample collection, filter elution, sample concentration and clarification, and parasite detection. At the elution step, they found that 3 replicate washings of the filter material (after backflushing), with 900 mL of eluent each time, was optimal, resulting in a 58% recovery of oocysts. For the clarification step, the group tested six different gradient solutions, and found no statistical difference among the media in terms of parasite recovery. Finally, the group also tested various membrane filters for parasite recovery. It was found that cellulose nitrate or triacetate filters performed well, and that filters with a pore size of 1.2µm gave 100% retention of both *Cryptosporidium* oocysts and *Giardia* cysts.

In 1989, Rose et al[78] evaluated the immunofluorescence techniques for detecting oocysts and cysts in environmental samples. The purpose of the study was to evaluate

membrane filtration techniques and various antibodies used for detection. Three monoclonal antibodies for *Cryptosporidium parvum* were examined. It was found that each antibody detected oocysts equally well after storage, exposure to bleach and in environmental samples. Two monoclonal antibodies and one polyclonal antibody for *Giardia lamblia* were compared. Significantly greater counts were obtained with the polyclonal antibody in seeded samples, but no difference was detected among the three antibodies in environmental wastewater samples.

In 1990, LeChevallier et al[79] compared the immunofluorescence (IFA) techniques for detecting *Cryptosporidium* and *Giardia* in water with the older zinc-sulfate flotation method. They found that the IFA method detected approximately twelve times more *Giardia* cysts in surface water samples than the zinc-sulfate method (74.1% vs. 5.9% recovery). Recovery of *Cryptosporidium* oocysts averaged 41% by the IFA technique, however they were not detectable using the zinc-sulfate method. At the same time, the group developed a method for the simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples using a monoclonal antibody cocktail for both *Cryptosporidium* and *Giardia*.

The latest study evaluating the standard method was conducted by LeChevallier et al in 1995.[80] The purpose of the study was to examine the procedures used for water sample collection, filter elution, concentration and clarification, to determine where cyst and oocyst losses occurred, and to suggest modifications to the procedure that would improve recovery efficiency. The results showed that major losses occurred during the centrifugation and clarification steps - losses were as high as 30% for each centrifugation step. As a result of the study, the authors suggested the following modifications: limiting the number of

centrifugation steps but concentrating at higher centrifugation speeds (7.000 to 10.000 x g) to minimize cyst and oocyst losses. and using the maximum specific gravity Percoll-sucrose gradient that still permits sample clarification. The study demonstrated that the recovery of oocysts was substantially reduced at a specific gravity of 1.10. but improved to 100% recovery at a specific gravity of 1.15.

An interesting survey was conducted by Clancy et al[81] which compared the abilities of 12 commercial laboratories to recover a known concentration of *Cryptosporidium* oocysts and *Giardia* cysts from spiked cartridge filters. Cyst recoveries ranged from 0.8 to 22.3 percent (average 9.1%): oocyst recoveries ranged from 1.3 to 5.5 percent (average 2.8%). The authors suggest that the poor recovery rates are not necessarily an indication of laboratory capability. but that the results are seriously limited by the methodology. It has been suggested previously that the standard method vastly underestimates the number of *Cryptosporidium* oocysts in water.[82]

Proposed improvements to the standard method include the use of DAPI[83,84] in combination with monoclonal antibodies to enhance the identification of *Cryptosporidium* by increasing the numbers of internal features detectable by immunofluorescence (DAPI highlights the sporozoites). and the addition of goat serum[85] to the membrane filters. which serves to block nonspecific fluorescence associated with algal species.

b) Alternatives for the Recovery of *Cryptosporidium* and *Giardia* from Water Samples

1. Calcium Carbonate Flocculation

This method. first reported in 1993.[86] uses calcium carbonate crystals to trap oocysts in water samples. Oocysts are concentrated into a stable residue. which is then dissolved in

sulphamic acid. Samples can be concentrated further by centrifugation, and are then examined by immunofluorescence microscopy or flow cytometry. The authors report recovery efficiencies of 76%, 73.7% and 75.6% for seeded samples of deionized, tap and river water samples respectively. Further studies have reported that the flocculation method gave better recovery rates (72.9% for tap water; 41.9% for river water) than both cartridge and membrane filtration techniques.[87] The same three methods were also compared by Shepherd and Jones[88] and once again flocculation gave the best results, with recovery values of 73.6% for tap water and 71.3% for river water.

2. Flow Cytometry

The use of flow cytometry has also been investigated in detecting *Cryptosporidium* in water samples. Vesey et al[89] analyzed seeded raw and treated water samples and oocyst-contaminated treated sewage samples by flow cytometry as well as by fluorescence microscopy. While flow cytometry was successful at detecting oocysts in all samples tested, and was quicker and easier than microscopy, the method lacked sensitivity, with a detection limit of 1,000 oocysts/L. The same group investigated the use of flow cytometry in combination with cell sorting (FCCS) to try to improve sensitivity by purifying the oocysts from the contaminating debris.[90] The sorted particles could then be screened by microscopy. Using FCCS prior to microscopy proved to be faster and more sensitive than direct fluorescence microscopy. When equal volumes of a sample were examined, 62 of 156 environmental samples were positive by FCCS, but only 4 were positive by direct microscopy.

3. Enzyme Immunoassays

Chapman et al[91] developed an enzyme immunoassay (EIA) using a monoclonal antibody to *Cryptosporidium* oocysts for detection in fecal and environmental samples. Screening of 494 fecal samples and 81 environmental samples showed that the EIA was at least as sensitive as microscopy, but had the added advantage that it was less subjective. Problems with false positive results demonstrated the need for further refinement of this technique.

Siddons et al[92] evaluated a commercial ELISA kit for the detection of oocysts in fecal and environmental samples. They examined 435 fecal samples, 10 concentrated reservoir samples and 6 seeded tap water samples using the kit, and compared the results with conventional microscopy. Once again EIA seemed to be at least as sensitive as microscopy and of superior specificity. Further advantages included ease of use, rapid results and less subjectivity than microscopy.

4. Polymerase Chain Reaction

While PCR methods have proven to be rapid and sensitive in the detection of *Cryptosporidium* and *Giardia* in water samples, the disadvantage remains in the fact that it cannot discriminate between live and dead oocysts.

Johnson et al[93] developed a PCR method for the rapid detection of *Cryptosporidium* in water samples. The procedure demonstrated good specificity, sensitivity and reproducibility when used with small volume samples, however sensitivity was shown to decrease up to 1,000-fold with concentrates derived from 100-400L water samples. This

interference could be alleviated by separating the targets from the environmental debris by either flow cytometry or magnetic antibody capture techniques.

Mayer and Palmer[94] developed a nested PCR method for the detection of oocysts in wastewater samples. This procedure allowed for the detection of as few as 100 oocysts/L. however. PCR results and immunofluorescence results did not always correlate. Discrepancies were thought to be due to cross-reactions with non-target organisms in the immunofluorescence method. or due to the inhibitory effects of substances in sewage samples on PCR enzymes.

Rochelle et al[95] described the development of a multiplex PCR method for the simultaneous detection of both *Cryptosporidium* and *Giardia* in water samples. They used primers targeting the 18S rRNA gene in *C. parvum* and the heat shock protein gene of *Giardia*. This procedure was able to detect 1 cyst/oocyst in purified preparations and 50 cysts/oocysts in seeded environmental samples.

Two groups have been successful in their attempts to solve the viability problem. Wagner-Wiening and Kimmig[96] incorporated an excystation step before DNA extraction in order to distinguish between live and dead oocysts. The sensitivity of this method. however. was dependent on the number of viable sporozoites present in the sample. and therefore required a second round of amplification using a nested PCR protocol in order to detect low numbers of sporozoites in environmental samples. Stinear et al[97] developed a reverse transcriptase-PCR (RT-PCR) method which is sensitive enough to detect a single viable oocyst in environmental samples. Synthesis of *C. parvum* heat shock protein mRNA (produced by viable oocysts only) was induced by heat stress. and was isolated by binding to

oligo(dT)$_{25}$-coated magnetic beads. The mRNA was then amplified by a *C. parvum*-specific RT-PCR procedure.

Mahbubani et al[98] used PCR both for the specific detection of *Giardia* spp. and to distinguish between live and dead cysts. The giardin gene proved to be a specific and sensitive target for PCR amplification. Cyst viability was distinguished by measuring the amounts of giardin mRNA produced before and after the induction of excystation. During excystation, live cysts produce significant amounts of mRNA, whereas dead cells cannot carry out transcription.

5. Cell Culture

Eleven different continuous cell lines have been used (most successfully) for the *in vitro* cultivation of *Cryptosporidium parvum*.[99] Upton et al[99] compared the ability of all eleven to support the growth and development of *C. parvum*. They found that the HCT-8 cell line (human ileocecal adenocarcinoma) was able to support twice as much parasite growth as any other cell line tested. The same group reported a rapid and reproducible cell culture method[100] using MDBK cells. Whole oocysts were pretreated with 10% bleach and added to the cell monolayers. Incubation at 37°C for 2 hours allowed the oocysts to excyst, and the freed sporozoites had immediate access to the host cells, effectively reducing the amount of time normally needed to purify sporozoite fractions, and preventing the exposure of free sporozoites to potentially harmful environmental conditions.

Rochelle et al[101] have developed a method that combines *in vitro* cell culture of *Cryptosporidium* (using CaCo-2 cells) with PCR. The procedure is both species-specific for

C. parvum and able to enumerate viable, infectious oocysts. The method was tested on purified oocyst preparations, but shows promise for concentrated drinking water samples.[102]

Viability and Disinfection

The measurement of the viability of *Cryptosporidium* sp. oocysts can be assessed in three different ways: animal infectivity studies (usually a mouse model), *in vitro* excystation and vital dye staining. While animal infection studies are considered the "gold standard" of viability assays, the disadvantages lie in the fact that these studies are expensive and lengthy to perform, and not quantitative unless large numbers of animals are used. While *in vitro* excystation is less time-consuming and less costly to perform, some researchers have demonstrated that this assay actually underestimates oocyst inactivation in comparison to animal infectivity studies.[126,127] All three methods have been used in the following studies to determine oocyst viability following chemical and physical inactivation.

Fayer and Leek[103] studied the conditions that are necessary for the *in vitro* excystation of *Cryptosporidium* oocysts. They found that oocysts could excyst in water and saline solutions in the absence of reducing conditions and digestive enzymes, and that the percent excystation was greater at 37°C than at 20°C. They concluded that temperature-activated sporozoites can initiate their own liberation even in the absence of excystation medium.

Speer and Reduker[104] examined the effects of temperature, tap water, excysting fluid and storage on the excystation capabilities of *C. parvum* oocysts. They found that young oocysts (14 weeks old) will only excyst at 37°C in excystation fluid, contradicting the previous group's findings.[103] Older oocysts (32-43 weeks old) apparently deteriorate with

age and become susceptible to hypotonic environments, eventually causing them to burst and release nonviable sporozoites that soon lyse.

a) Chemical Disinfection

Peeters et al[105] studied the effect of ozone and chlorine dioxide on the viability of *C. parvum* oocysts using a mouse infectivity model. Both chemicals reduced the infectivity of oocysts significantly, however this effect was dependent on the number of oocysts in solution prior to disinfection. For example, 1.1 mg/L of ozone was sufficient to disinfect 10^4 oocysts/mL after 6 minutes contact time. However, it took 2.25 mg/L of ozone and 8 minutes contact time to completely disinfect 5 x 10^5 oocysts/mL. Chlorine dioxide (0.4 mg/L) significantly reduced the viability of *C. parvum* oocysts after 15 minutes contact, however some oocysts remained viable after 30 minutes of contact.

Similarly, Korich et al[12] tested the effects of ozone and chlorine dioxide, as well as chlorine and monochloramine, on the survival of *C. parvum* oocysts in order to estimate the concentrations and exposure times necessary for disinfection. The results showed that ozone and chlorine dioxide were more effective at inactivation than the other disinfectants. The estimated CT (concentration x time in minutes) value of ozone for 99% inactivation was 5-10. The value for chlorine dioxide was 78, while chlorine and monochloramine were both calculated to be 7200.

Fayer[13] undertook an experiment to determine the concentration of sodium hypochlorite and exposure time necessary to render *C. parvum* oocysts noninfectious. Oocysts were exposed to 1.31%, 2.62% or 5.25% (undiluted) commercial bleach for either 10, 30, 60 or 120 minutes, and then administered to mice. Although the treatments

significantly reduced the number of infected intestinal epithelial cells, all mice developed *C. parvum* infections.

Fayer et al[106] also examined the efficiency of gaseous forms of disinfectants on *C. parvum* viability. Oocysts were exposed to a saturated atmosphere of ammonia, carbon monoxide, ethylene oxide, formaldehyde or methyl bromide gas for 24 hours, and then inoculated into mice. Carbon monoxide did not inactivate the oocysts, and formaldehyde was marginally effective. No mice given oocysts exposed to ammonia, ethylene oxide or methyl bromide were found to be infected. The results show that low molecular-weight gases may be used as potential disinfectants for *C. parvum*.

b) Inactivation by Heating and Freezing

Fayer[107] studied the effect of high temperatures on the viability of *C. parvum*, using a mouse infectivity assay. Vials of oocysts were held in the heated block of a thermal DNA cycler for either 1 minute or 5 minutes at set temperatures ranging from 60°C to 100°C, then inoculated into mice. Results showed that oocysts remained infectious when exposed to temperatures up to 67.5°C for 1 minute, but were inactivated at 72.4°C or more. Infectivity was also lost when the temperature was held at 64.2°C for more than 2 minutes.

Anderson[108] investigated the effects of moist heat on the inactivation of *Cryptosporidium* oocysts, using inocula such as calf feces, ileal scrapings and cecal contents. The results showed that oocysts could be inactivated in all three inocula by warming to 55°C for 15 to 20 minutes, suggesting that pasteurization conditions are sufficient to render *Cryptosporidium* oocysts noninfectious.

Harp et al[109] exposed *C. parvum* oocysts to the high-temperature, short-time conditions typical of commercial pasteurization in order to determine the effects on oocyst infectivity. Oocysts were suspended in milk and water, heated to 71.7°C for 5, 10 or 15 second intervals, and then inoculated into mice. No mice receiving heat-treated oocysts (all time intervals) were infected with *C. parvum*, while 100% of control mice receiving nonpasteurized oocysts became infected.

Fayer and Nerad[110] studied the effect of low temperatures on *C. parvum* viability. Oocysts were frozen at -20°C, -15°C and -10°C for different time intervals and then thawed and inoculated into mice. Oocysts remained viable when frozen at -20°C for up to 5 hours, but lost infectivity after 24 and 168 hours. At -15°C, oocysts remained infectious for up to 24 hours, but were inactivated after 168 hours. All mice that received oocysts frozen at -10°C for 8, 24 and 168 hours developed a cryptosporidial infection.

TREATMENT

More than 100 antiparasitic, antimicrobial and antidiarrheal agents have been tested against *Cryptosporidium*, without much success.[7,11] Spiramycin, a macrolide antibiotic, has shown some promise in immunocompromised patients with severe cryptosporidiosis.[111,112] In one study, five of ten AIDS patients had complete recovery from diarrheal symptoms within one week of the commencement of treatment with spiramycin,[111] although three of the patients continued to shed oocysts in the stool.

Paromomycin, an aminoglycoside antibiotic, has also been reported to have some success at resolving diarrheal symptoms due to cryptosporidiosis.[113,114] Five of five AIDS patients in one study showed symptomatic improvement after receiving oral paromomycin.

and three of the five also stopped shedding oocysts during treatment.[113] A second, larger study (that included control patients receiving a placebo) showed that both oocyst excretion and stool frequency decreased significantly in those patients receiving paromomycin.[114]

Recently, Arrowood et al[115] evaluated five different dinitroaniline herbicide compounds (trifluralin, profluralin, nitralin, pendimethalin and fluchloralin) against cultured *Cryptosporidium parvum*. *C. parvum* development was completely suppressed in cell culture by four of the five compounds, all of which demonstrated little or no toxicity for the host cells.

While these agents have shown some promise in the treatment of cryptosporidiosis, larger trials that include both immunocompromised and immunocompetent patients are needed to determine the true efficacy of these compounds. For now, supportive therapy, such as oral and parenteral rehydration, is the only intervention available for cryptosporidiosis infection.[5] This type of treatment is often required by both immunodeficient and immunocompetent patients, especially children.

CONCLUSION

Cryptosporidium sp. and *Giardia* sp. are commonly detected even in the most pristine watersheds, and have on many occasions breached the water treatment process to enter into drinking water supplies, causing acute and even life-threatening disease in immunocompromised individuals. These factors, along with the fact that both parasites have proven resistant to chemical disinfection, demonstrate the importance of further characterizing the occurrence of these protozoa in watersheds used as sources of drinking water. This information will be of use to water treatment plant operators to predict peaks of

contamination and, in so doing, attempt to prevent outbreak situations, until more adequate chemical or physical barriers are put into place to combat these resistant organisms.

STATEMENT OF OBJECTIVES

1. To conduct a survey of the raw and treated water supplies on the St. Lawrence River, in the Cornwall, Ontario, area, for the occurrence and spatial and temporal distribution of *Cryptosporidium* sp. and *Giardia* sp. (Chapter 1)
2. To find a suitable microbial indicator of water quality for *Cryptosporidium* sp. and *Giardia* sp. in raw water (Chapter 1)
3. To assess the *in situ* survival ability of *Cryptosporidium parvum* in treated water effluents (Chapter 2)
4. To assess the *in situ* survival ability of *Cryptosporidium parvum* in raw water from the St. Lawrence River (Chapter 2)
5. To assess the affect of chlorine and monochloramine on aged and fresh *Cryptosporidium parvum* oocysts.

CHAPTER 1:

SURVEY STUDY OF THE ST. LAWRENCE RIVER FOR *CRYPTOSPORIDIUM* SP. AND *GIARDIA* SP.

INTRODUCTION

Many studies on the occurrence and distribution of *Cryptosporidium* and *Giardia* in water systems have been conducted in the United States over the past decade. However. relatively few similar studies have been undertaken for Canadian watersheds. Only one study has monitored parasite levels in surface and drinking water supplies across Canada.[55] while others have focused on raw and treated water supplies in British Columbia.[56,116] and in the Ottawa. Ontario. region.[58]

Outbreaks of cryptosporidiosis have been occurring more and more frequently in association with drinking water supplies in Canada. For example. in March 1996. an outbreak occurred in Collingwood. Ontario. that affected more than 100 residents.[16] Furthermore. it was reported that five separate outbreaks of cryptosporidiosis occurred in British Columbia in the summer of 1996.[17,73] In light of these situations. survey studies of watersheds become relevant to track levels of *Cryptosporidium* and *Giardia* in both raw water and drinking water. and may be of use in predicting peaks of contamination and possible outbreak situations.

A survey study of *Cryptosporidium* sp. and *Giardia* sp. in the St. Lawrence River. in and around the Cornwall. Ontario. area. was undertaken for this project. The presence of urban and rural communities in close proximity. with both primary and secondary (full conventional) water treatment facilities. as well as the strong environmental awareness and interest of the local politicians and citizens. made this area of the St. Lawrence River an ideal candidate for a survey study of this nature. Parasite levels were monitored on a weekly basis in surface raw water samples and in filtered and unfiltered drinking water samples. The

study was conducted over the course of the spring, summer and autumn seasons in 1995 and 1996.

Efficiency of parasite removal in water treatment plants.

The Surface Water Treatment Rule (SWTR),[117] introduced by the U.S. Environmental Protection Agency in 1989, was intended to reduce the annual risk of acquiring a waterborne *Giardia* sp. infection to less than one in ten thousand (or 10^{-4}) per person. The rule states that utilities using filtration and chemical disinfection are required to remove and/or inactivate at least 99.9% (3 $\log_{10}$) of *Giardia* cysts in finished drinking water. However, this requirement also assumes that cyst levels in raw water are not greater than 7 cysts per 100L.[9] Based on extensive data collected from raw and finished water from 72 surface water treatment plants in the U.S. and Canada,[10] it was determined that the average plant would actually have to achieve a 5.52 $\log_{10}$ reduction of *Giardia*, and a 4.68 $\log_{10}$ reduction of *Cryptosporidium* in order to meet the SWTR goals.

Only two survey studies have attempted to calculate water treatment plant efficiency in removing *Cryptosporidium* and *Giardia* from finished water. Rose[8] compared oocyst levels in source water to levels in finished water, and determined that a 91% reduction (approximately 1 $\log_{10}$) had been achieved. However, the data collected were limited, and the author suggested that these results should be interpreted with caution. LeChevallier et al[18] used data collected in raw and treated water samples from 66 surface water filtration plants to calculate treatment efficiency in removing *Giardia* cysts from finished water. Most of the plants studied achieved a 2.0 to 2.5 $\log_{10}$ reduction of cysts.

During the course of the survey study of the St. Lawrence River, treated water samples and surface water samples (as close to the water treatment plant intake as possible)

were collected consecutively, in an attempt to calculate the reductions of parasites through the treatment process.

Seasonality of *Cryptosporidium* and *Giardia* in raw water samples.

A review of prevalence data from around the world[30] revealed that cryptosporidiosis infections seemed to occur on a seasonal basis, usually during the wettest months of the year. In North America and the United Kingdom,[118] this peak of incidence occurs in the spring or late fall, when rainfall and/or spring runoff is at its highest.

In the same vein, researchers have attempted to demonstrate that the concentration of oocysts in environmental waters also varies according to a seasonal pattern. Hansen and Ongerth[57] collected water samples from two rivers in Washington State from June until September. They found that oocyst concentrations were highest in June and subsequently decreased throughout the summer. Since samples in June were collected following significant rainfall, the authors speculated that *Cryptosporidium* levels were influenced by post-rainfall runoff. A study of raw and treated water supplies across Canada[55] noted that while cysts and oocysts were found throughout the year, the parasites were detected more frequently in the late winter-early spring and in the fall, when water temperatures were at their coldest. Similarly, a survey conducted in two British Columbia watersheds, focusing on *Giardia* cysts,[116] showed that mean cyst levels were highest during the winter months.

The raw water data collected during the survey study of the St. Lawrence River were also analyzed to determine whether *Cryptosporidium* oocyst and *Giardia* cyst concentrations followed a seasonal pattern.

Correlation of Protozoa with Microbial Indicators of Water Quality.

The approved standard method for detecting *Cryptosporidium* and *Giardia* in environmental waters[1] has many drawbacks: it is technically complex, labour-intensive, time-consuming and expensive, and still provides a low recovery efficiency. These factors have led researchers to search for a suitable microbial indicator that would reliably predict the presence or absence of these parasites in both raw and treated water.

The ideal indicator for either parasite would have the following characteristics: present only when the parasite is present, and absent when the parasite is absent; easily and rapidly detected; equally resistant to environmental conditions and disinfection as the parasites; and preferably non-pathogenic.

This task has not proven easy. Results have differed widely from study to study. In 1988, Rose et al[119] attempted to correlate *Cryptosporidium* and *Giardia* with total and fecal coliforms in a Western U.S. watershed, without success. This led to the conclusion that "Neither... are reliable predictors for the absence of enteric protozoa in the study watershed". A second attempt was made by Rose et al[51] in 1991, this time using fecal streptococcus (now known as *Enterococcus*) and heterotrophic plate counts (HPC) in addition to total and fecal coliforms. The conclusion this time was "... the bacterial indicator system used to assess microbial water quality may be inadequate for the determination of parasitological water quality". LeChevallier et al[9] used total and fecal coliforms as indicators in a study of watersheds in 14 U.S. states and 1 Canadian province. This group was successful, finding significant correlations between *Giardia* and both bacterial indicators at the 99% confidence level. They suggested that the discrepancy from previous results "...may be due to

differences in the type of water samples analyzed". In other words, these correlations may be dependent on the watershed under study.

Since total and fecal coliforms had failed as indicators in two out of three studies, the next step was to look at more environmentally resistant microorganisms. Payment and Franco[120] used *Clostridium perfringens* and somatic coliphages to test their suitability as indicators through the drinking water treatment process. They found that *Clostridium* (both vegetative cells and spores) was significantly correlated to both *Cryptosporidium* and *Giardia* in raw water.

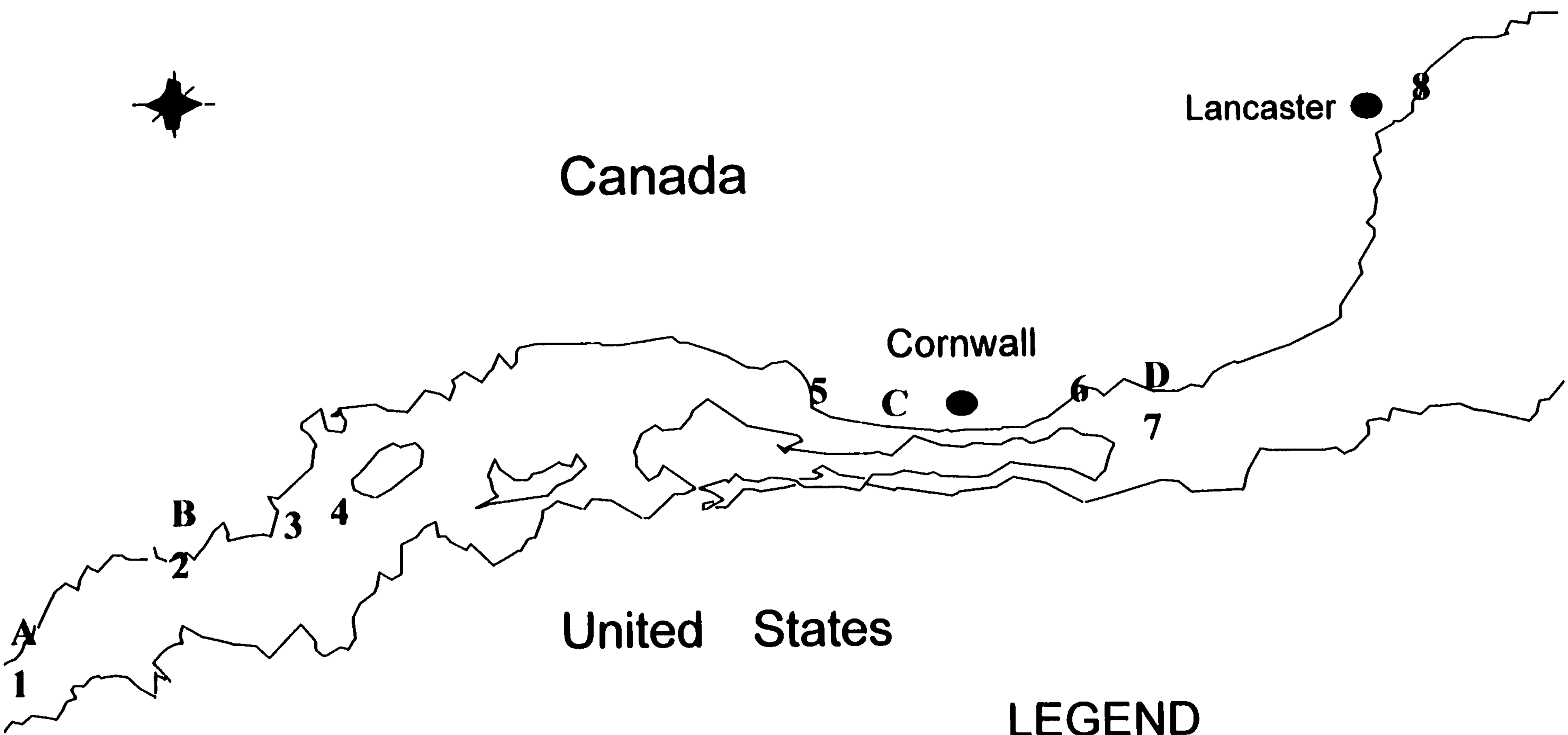
The research conducted in this laboratory has focused on the St. Lawrence River, and has used a wide variety of potential microbial indicators, including total and fecal coliforms, *Enterococcus* sp., *Clostridium perfringens* (vegetative cells and spores), *Aeromonas* sp., total heterophic counts, *Pseudomonas* sp., and somatic coliphage b. The objective was to determine if any of these indicators could be correlated to either *Cryptosporidium* sp. or *Giardia* sp., and to decide whether the goal of finding a universal indicator is at all feasible.

MATERIALS AND METHODS

Study area. The St. Lawrence River is approximately 1,200 km long, issuing from Lake Ontario and flowing northeast to the Gulf of St. Lawrence. The St. Lawrence Valley is an agricultural region, with both crop and dairy farms. The area near Cornwall, Ontario, is also known for its many industrial plants, particularly the pulp and paper mill. Water samples were collected from eight raw water sites on the St. Lawrence River, along an 80 km stretch between Morrisburg, Ontario and Lancaster, Ontario (refer to Figure 2 for a map of the sampling site locations). Four additional sites were located inside water treatment plants - the water sample collected was the finished treated water effluent from the plant. Two of these plants practiced full conventional (secondary) treatment of the incoming surface water, including alum coagulation, rapid-sand filtration and chlorination, while the other two plants used a one-step chlorination (primary) process to treat the drinking water. Each site was sampled three times in 1995 (between July and November), and five to seven times in 1996 (from April to October). A total of 102 samples were collected over the course of the survey study.

Sample collection. Surface water samples were collected from the shore of the river using a gasoline-powered pump. Between 200 and 500L of raw or treated water were filtered through a ten-inch, 1µm porosity wound fiber cartridge filter (Parker Filtration, Lebanon, IN). The filters were then placed in Ziploc bags and returned to the laboratory on icepacks. The filters were stored at 4°C until processed, which occurred within 48 hours of sample collection.

Figure 2: Map of the St. Lawrence River indicating the location of the sampling sites used in the survey study


Canada
Lancaster
8
Cornwall
5
C
6
D
7
B
2
3
4
A
1
United States
km
0
5
10
LEGEND
1-8: Surface Water Sites
A,B: Chlorination Plants
C,D: Filtration Plants

Protozoan assay. Procedures for protozoan parasite analysis followed those detailed in the 19th edition of *Standard Methods for the Examination of Water and Wastewater*.[1] The cartridge filters were first backflushed with two liters of 0.25% (v/v) Tween 80 solution (J.T. Baker Chemical Company, Phillipsburg, N.J.) in order to detach parasites from the filter fibers. The filter was then cut in half and the fibers shredded. The fiber strands were homogenized in the original Tween 80 eluent, using a stomacher homogenizer (Stomacher 3500, Seward Ltd., London, U.K.). The eluent was collected and concentrated by centrifugation (7,500 rpm for 15 min.: Beckman Instruments, Inc., model J2-21M, JA-10 rotor). The resulting pellets were pooled and concentrated further (9,000 rpm for 35 min.: Beckman, model J2-21M, JA-20 rotor), until all pellets could be pooled into one 50 mL conical centrifuge tube. A final centrifugation step (3,000 rpm for 40 minutes, IEC centrifuge, Universal Model, swinging-bucket rotor) was conducted to pack the pellet down to a 10 mL volume. The concentrated samples were then clarified by Percoll-Sucrose sedimentation. The clarified samples were filtered through 0.45μm cellulose acetate membrane filters (Sartorius Inc., Bohemia, NY) and stained with a FITC-labeled monoclonal antibody cocktail for both *Cryptosporidium* and *Giardia* (Aqua-Glo™, Waterborne Inc., New Orleans, LA). The membrane filters were then mounted on microscope slides. The slides were sealed with clear nail polish and stored at -20°C. Slides were scanned using an epifluorescence microscope (Leitz, Laborlux K: 450–490nm) under 250x magnification. Parasites were initially identified by their apple-green fluorescence. Once a potential cyst or oocyst was spotted, a higher magnification (400x) was used to confirm the identity of the

parasites. using characteristic shape and size. as well as the presence of a suture line in the case of *Cryptosporidium* oocysts. as identifying features.

Quality control. Separate equipment for treated water samples and raw water samples was used throughout the sample collection and filter processing procedures to reduce the likelihood of cross-contamination. After use. all equipment was soaked in diluted bleach for 15 minutes. rinsed with deionized water. then soaked in 10% sodium thiosulfate solution for 15 minutes. and finally soaked and scrubbed in 7X cleaning solution (ICN Biomedicals Inc.. Aurora. OH) before a final rinse with deionized water. The equipment was then dried thoroughly prior to reuse.

Positive and negative control slides were run in parallel to the sample slides every two weeks. Negative controls consisted of diluted Tween 80 solution. which was concentrated. clarified and stained along with the sample slides. Positive controls were prepared by spiking a two-liter volume of Tween 80 solution with a known concentration of *Cryptosporidium* oocysts and *Giardia* cysts (~1.000 to 2.000 parasites). and then processed along with the environmental samples. The positive controls were also used to calculate the efficiency of the standard method. Recovery values for *Cryptosporidium* averaged 16.1% in raw water (range: 1.6 - 40.6%) and 16.8% in treated water (range: 1.0 - 85.0%). Values for *Giardia* averaged 20.1% in raw water (range: 4.4 - 31.9%) and 20.2% in treated water (range: 1.8 - 55.0%).

Additionally. split samples of the source water were run in order to check the consistency of the method using identical samples. The results showed no major discrepancies in parasite concentrations between identical samples.

Bacterial enumeration. Grab samples for enumeration of bacterial indicators were collected at the same time that filtered samples were obtained for parasite analysis. Samples were returned to the lab on ice and were stored at 4°C prior to analysis. which occurred within 24 hours of collection. Enumerations were performed by filtering 100 mL of the water samples (or 10 mL volumes of the diluted sample) through a 0.22μm membrane filter (Millipore Corp., Bedford, Mass.), and placing them on the appropriate agar medium. Total coliforms were enumerated on MT7 agar; fecal coliforms on mFC agar; *Enterococcus* sp. on KF agar; *Pseudomonas* sp. on Pseudomonas P agar; heterotrophic plate counts on R2A agar (All from Difco Laboratories, Detroit, Michigan). *Aeromonas* sp. was enumerated on modified Ryan agar (Oxoid, Basingstoke, U.K.). *Clostridium perfringens* levels were determined by the method of Payment and Franco.[120] All plates were incubated at 37°C, except for the fecal coliform plates (44.5°C) and the HPC plates (room temperature). A complete list of agars used, incubation temperatures and incubation periods is included in Appendix A.

Other analyses. Somatic coliphage b concentrations were determined as described in *Standard Methods for the Examination of Water and Wastewater*[1], with the exception that the plates were incubated overnight at room temperature.

Statistical analyses. *Cryptosporidium* sp. and *Giardia* sp. counts, as well as microbial indicator concentrations, were $\log_{10}$-transformed for analysis. All statistical analyses were performed using SigmaStat® software (Jandel Corporation, San Rafael, CA). The Spearman rank order correlation was used to determine the relationship between protozoan parasites

and microbial indicators. This test allows the determination of the strength of association between variables without specifying which variable is dependent or independent.

In order to test the seasonality of *Cryptosporidium* and *Giardia* in raw water, it first had to be shown that there were no site-to-site differences in parasite concentrations among the eight raw water sampling sites. The raw water data was separated into categories based on the site the sample was collected from, and a one-way analysis of variance (ANOVA) was performed. The data was then re-organized into categories based on the month in which samples were collected. (Note: in order to have approximately equal group sizes, data collected in April and May was combined into one group, and data collected in October and November was combined into one group). A second one-way ANOVA was performed to test for the seasonality of both parasites, and the Tukey test (pairwise multiple comparison procedure) was used *post hoc* to determine which months were significantly different from one another.

RESULTS

Survey study. The results of the survey study are reported in condensed format in Table 5. while the extended version (including concentrations of both protozoa and microbial indicators for every sample collected) can be found in Appendix B. *Cryptosporidium* sp. was detected in 60 of 66 (90.9%) of raw water samples. at a mean level of 50.6 oocysts/100L. Levels ranged from 3.3 to 1.200 oocysts per 100L. *Giardia* sp. was found in 75.8% of raw water samples. ranging from 1.7 to 490 cysts per 100L. The average concentration was 29.6 cysts per 100L.

Both parasites were found with similar frequency in the unfiltered treated water samples. although concentrations were somewhat lower than in the raw water samples. *Cryptosporidium* sp. was found in 94% of unfiltered samples. at a mean level of 15.0 per 100L. while *Giardia* sp. was detected in 61% of the samples at a concentration of 12.4 cysts per 100L. Filtration seemed to be much more effective at reducing parasites concentrations in finished drinking water. Only 3 of 18 (16.7%) filtered treated water samples were positive for *Cryptosporidium* sp.. and 1 of 18 (5.5%) was positive for *Giardia* sp. cysts. Parasite levels were usually below the detection limit in these samples. however on one occasion. the *Cryptosporidium* oocyst count was surprisingly high. at a level of 320 oocysts per 100L.

Efficiency of parasite removal in water treatment plants.

Water samples were collected from four water treatment plants on the St. Lawrence River. Two of these plants used full conventional treatment. including rapid sand filtration and chlorine disinfection. while the two smaller plants used a one-step chlorination process to treat the drinking water.

Table 5: *Cryptosporidium* sp. and *Giardia* sp. in the St. Lawrence River: Results of the Survey Study (1995-96 data)

Water Type	Parasite	Mean levels (per 100L)	Range (per 100L)	% Positive Samples
Raw water	*Cryptosporidium*	50.6	3.3-1200	90.9% (60/66)
	Giardia	29.6	1.7-490	75.8% (50/66)
Treated (unfiltered)	*Cryptosporidium*	15.0	1.3-30.0	94.0% (17/18)
	Giardia	12.4	<2.3-30.0	61.0% (11/18)
Treated (filtered)	*Cryptosporidium*	N/A	<0.4-320	16.7% (3/18)
	Giardia	N/A	<0.4-3.3	5.5% (1/18)

The point of interest was to compare how efficiently both types of water treatment plants reduced both *Cryptosporidium* and *Giardia*. On each day that a treated water sample was collected, a matching raw water sample was collected from the river outside the water treatment plant. While it was not possible to sample directly from the plants' raw water intake pipes, an effort was made to get as close to the intake pipe as possible. The concentrations of parasites in paired raw and treated water samples were determined, and log_{10} reductions for both *Cryptosporidium* and *Giardia* were calculated. When no cysts or oocysts were detected, the value of the limit of detection was used for the calculations. Mean log_{10} reductions were then tabulated for each water treatment plant (Table 6). The plants employing filtration (Plants A and B) were more successful at reducing the parasites, with reductions approaching or exceeding 1 log_{10}. However, those using chlorination (Plants C and D) showed very little parasite reduction. Plant C demonstrated almost no reduction at all (0.07 and 0.06 log_{10} reduction for *Cryptosporidium* and *Giardia*, respectively), while Plant D fared only a little better (0.24 and 0.13 log_{10}, respectively).

Seasonality of *Cryptosporidium* and *Giardia* in raw water samples.

The raw water *Cryptosporidium* and *Giardia* data was first separated into groups according to the sampling sites. A one-way ANOVA was performed in order to determine whether there were any differences in parasite levels among the eight sampling sites. No significant differences were found. The data was then sorted into groups according to the month in which the sample was collected. A second one-way ANOVA was performed to determine whether any seasonal differences in parasite concentrations could be detected. *Cryptosporidium* demonstrated significant seasonality (Figure 3). Oocyst levels in April and

Table 6: Log_{10} reductions of *Cryptosporidium* sp. and *Giardia* sp. through Water Treatment Plants on the St. Lawrence River

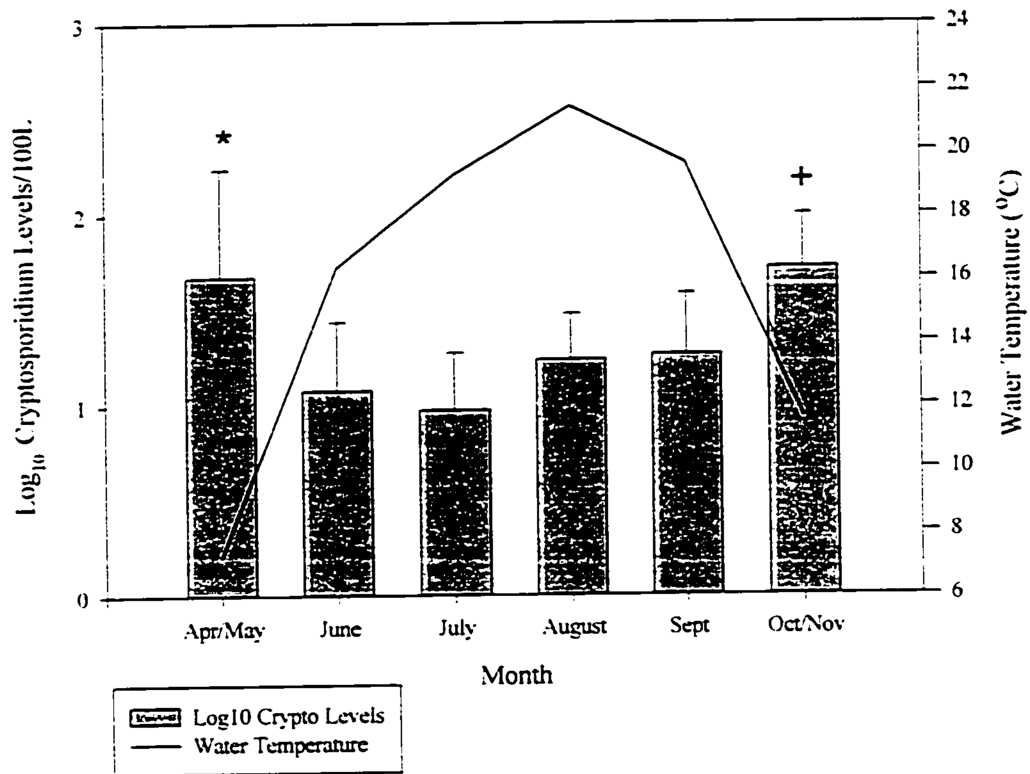
Plant	Filtration	n	*Cryptosporidium* log_{10} reduction	*Giardia* log_{10} reduction
Plant A	Yes	9	>0.93	>0.80
Plant B	Yes	8	>1.38	>1.31
Plant C	No	9	0.07	0.06
Plant D	No	8	0.24	0.13

May (pooled data) were significantly higher than levels in June and July ($P<0.05$), and oocyst levels in October and November (pooled data) were significantly higher than those in June, July and August ($P<0.05$). As water temperatures increased over the course of the summer, oocyst levels decreased. *Giardia* concentrations in raw water did show a trend towards seasonality, however the differences between months were not significant (Figure 4).

Correlations of Protozoa with Microbial Indicators of Water Quality.

In addition to the protozoa, several other microbial indicators of water quality were monitored and enumerated in the same water samples. With the exception of *Pseudomonas* sp. and somatic coliphage b, all the microbial indicators were detected on a frequent basis in raw water samples, with levels ranging from <1 to 10^7 per 100mL. The concentrations of these microorganisms in water were compared to the protozoan concentrations. The Spearman rank order correlation was used to measure the strength of association between these pairs of variables. The levels of *Cryptosporidium* sp. were significantly correlated to the levels of *Giardia* sp. ($r=0.68$, $P=<0.01$) in raw water samples (Table 7a,b). Significant associations between *Cryptosporidium* sp. and *Enterococcus* sp. ($r=-0.338$, $P=0.02$), and *Cryptosporidium* sp. and heterotrophic plate counts (HPC) ($r=0.272$, $P=0.036$) were also detected (Table 7a). *Giardia* sp. concentrations were not significantly correlated with any of the chosen microbial indicators (Table 7b).

Figure 3: Seasonality of *Cryptosporidium* sp. in comparison with monthly water temperatures (N.B.: error bars represent the standard deviation of the mean)



* Indicates that Apr/May levels are significantly higher than June and July levels

\+ Indicates that Oct/Nov levels are significantly higher than June, July and August levels

Figure 4: Seasonality of *Giardia* sp. in comparison with monthly water temperatures

(N.B.: error bars represent the standard deviation of the mean)

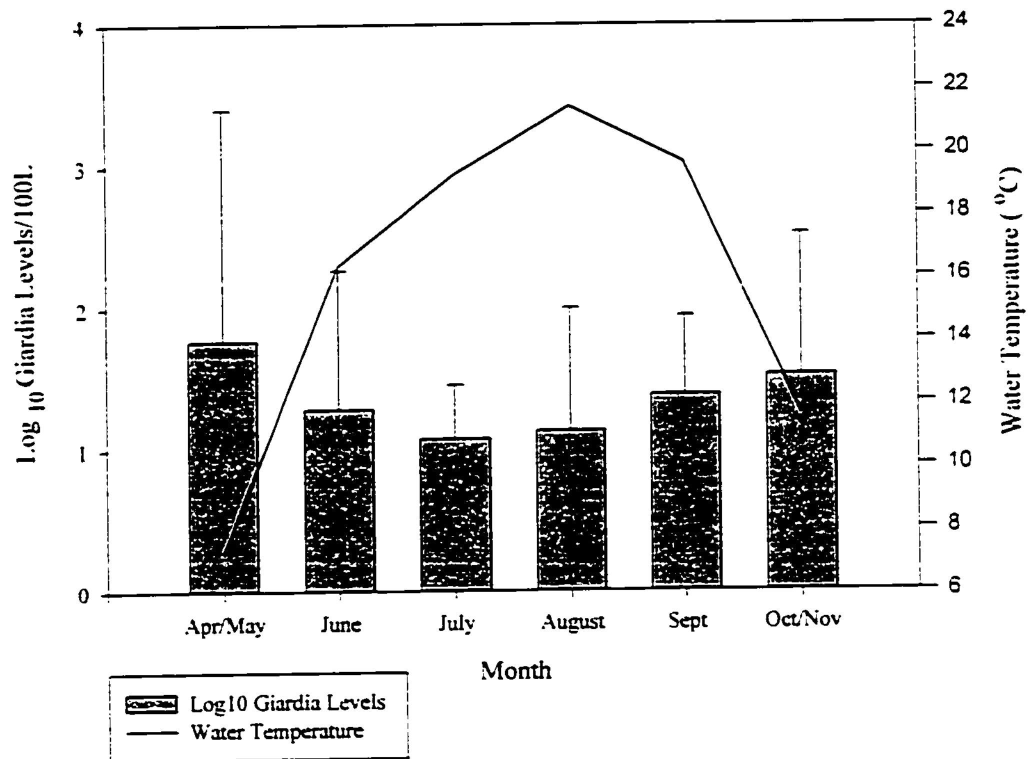


Table 7a: Spearman correlation coefficients among *Cryptosporidium* sp. oocysts and various microbial indicators of water quality enumerated in raw water samples collected from the St. Lawrence River, from July 1995 to November 1995 and April 1996 to October 1996 (n, number of samples; r, Spearman correlation coefficient; P, P value associated with correlation coefficient; NS, not statistically significant (P>0.05))

Microorganism	*Cryptosporidium* sp.		
	n	r	P
***Giardia* sp.**	**47**	**0.68**	**0.00**
Aeromonas sp.	60	-0.153	NS
Clostridium perfringens	51	0.022	NS
C. perfringens spores	34	-0.030	NS
***Enterococcus* sp.**	**47**	**-0.338**	**0.02**
Fecal coliforms	56	-0.080	NS
Total coliforms	56	-0.148	NS
HPC	**60**	**0.272**	**0.036**
Pseudomonas sp.	10	0.490	NS
Somatic coliphage b	17	-0.383	NS

Table 7b: Spearman correlation coefficients among *Giardia* sp. cysts and various microbial indicators of water quality enumerated in raw water samples collected from the St. Lawrence River, from July 1995 to November 1995 and April 1996 to October 1996 (n, number of samples; r, Spearman correlation coefficient; P, P value associated with correlation coefficient; NS, not statistically significant (P>0.05))

Microorganism	*Giardia* sp.		
	n	r	P
***Cryptosporidium* sp.**	**47**	**0.68**	**0.00**
Aeromonas sp.	49	-0.082	NS
Clostridium perfringens	42	0.076	NS
C. perfringens spores	28	0.048	NS
Enterococcus sp.	38	-0.097	NS
Fecal coliforms	46	-0.137	NS
Total coliforms	46	-0.178	NS
HPC	50	0.081	NS
Pseudomonas sp.	8	0.543	NS
Somatic coliphage b	11	-0.382	NS

DISCUSSION

Survey study. *Cryptosporidium* oocysts and *Giardia* cysts were detected frequently in raw water samples throughout the course of this survey (in 90.9% and 75.8% of all samples, respectively). These results compare favorably to other survey studies conducted in the United States and in Canada. Chauret et al[58] found that 78.8% and 75.0% of raw water samples collected from the Ottawa River were oocyst and cyst positive, respectively. LeChevallier et al[9] studied surface water samples in the U.S. and Canada and found that 87.0% were positive for *Cryptosporidium* sp., while 81% were positive for *Giardia* sp.

The high incidence of parasite positive samples suggests that this section of the St. Lawrence River is subject to a continuous source of *Cryptosporidium* and *Giardia*. While sources of parasites may include animal populations, such as cattle, wild animals or birds, it is more likely that sewage outflows from wastewater treatment plants are responsible. Larger urban centers, such as Kingston and Brockville, are located upstream of the sampling area, as well as the outflows from several smaller communities. While no attempt was made to enumerate *Cryptosporidium* and *Giardia* levels in treated sewage effluents along the St. Lawrence River, previous studies[48,49,58] have demonstrated that significant numbers of protozoa (anywhere from 10 to 10^3 parasites per liter) can escape the treatment process and return to the river in sewage effluents.

The most interesting results were obtained from the unfiltered treated drinking water samples - 94% were positive for *Cryptosporidium* sp., and 61% were positive for *Giardia* sp. Parasite levels were lower (~15.0 parasites per liter) than those found in raw water (Table 5). These results suggest that parasites entering the water treatment facility are not being eliminated completely, and are therefore reaching the consumer through tap water on a

frequent basis. As mentioned previously, the standard method does not give the investigator any indication of whether or not detected parasites are viable. In addition, the parasite levels found are not likely high enough to cause illness, and no cases of cryptosporidiosis or giardiasis were reported during the course of this investigation. One wonders whether the residents of these communities have developed an immunity to these infections due to a long-term exposure to low levels of parasites.

Both *Cryptosporidium* and *Giardia* were detected infrequently in filtered treated water samples. In those samples that were positive, levels were quite low (from 1.2 to 3.3 parasites per 100L), with one notable exception. One sample collected in September 1995 was found to contain 320 oocysts per 100L. A review of sampling and processing procedures, as well as quality control measures, found no indication that this result could have been due to cross-contamination with other samples, and the result was accepted as true. It must be stated again that the standard method does not give any indication of the potential infectivity of parasites. Dupont et al[35] determined that human cryptosporidiosis infections could be initiated following the ingestion of as few as 30 oocysts. Assuming that the parasites remained viable throughout the treatment process, consumers drinking this water may have ingested approximately 3.2 oocysts/L, which is probably not enough to become ill. However, if the numbers are adjusted for a 10 to 20% recovery efficiency, then it is possible that a sufficient number of *Cryptosporidium* oocysts (between 16 and 32 oocysts/L) were present in that particular drinking water sample to cause human illness. No cases of cryptosporidiosis were reported in the community following the collection of this sample.

Efficiency of parasite reduction in water treatment plants.

As expected, the two water treatment facilities using rapid-sand filters were more successful at reducing both *Cryptosporidium* and *Giardia* levels than the two facilities that used only chlorine disinfection. The filtration plants were able to reduce parasite concentrations through the treatment process by approximately 1 $\log_{10}$. While a 90% removal rate appears to be a decent efficiency, the U.S. Surface Water Treatment Rule states that utilities must remove and/or inactivate 3 $\log_{10}$ of *Giardia* cysts in finished drinking water. This survey study did not have the means to assess parasite inactivation, however subsequent *in situ* survival studies (described in the following chapter) demonstrated that *Cryptosporidium parvum* oocysts could survive for at least four days in treated water effluent from these same plants. It is therefore doubtful that these facilities would meet the requirements of the SWTR on a regular basis. No similar legislation concerning *Cryptosporidium* or *Giardia* exists in Canada, however.

The two chlorination-only plants did not perform efficiently in reducing parasite concentrations. One plant, in fact, was barely able to remove either parasite (~0.06 $\log_{10}$ reduction). While this finding was not unexpected, these results may be of interest or use to those public or political figures campaigning for the allocation of funds to upgrade their water treatment facilities. These results, however, must be viewed with caution. While raw water samples were collected as close to the intake pipe as possible, this pipe was located deep in the middle of the channel, hundreds of metres from the shore, and accessible only by boat. Raw water and treated water samples, while collected on the same morning, were not temporally associated as they would have been had they gone through the actual treatment process.

Seasonality of *Cryptosporidium* and *Giardia*. Studies worldwide have noted the seasonal incidence of cryptosporidial infections and outbreaks.[30] and laboratory-based experiments have observed that *Cryptosporidium* oocysts and *Giardia* cysts survive much longer in cold conditions than at higher temperatures.[107,110,121] DeRegnier et al[122] determined that *Giardia* cysts survived longer in the environmental when water temperatures were below 10°C. The survey study of the St. Lawrence River extended from mid-spring to late autumn. and included the full range of water temperatures normally experienced on the river (from 3.2°C to 23.0°C). which afforded the opportunity to determine whether cyst and oocyst levels in the river were influenced by different seasons.

Cryptosporidium sp. did demonstrate significant seasonality. Oocyst levels were highest in the spring (April/May) and in the fall (October/November). when water temperatures were at their coolest. As the river temperature increased throughout the summer. oocyst levels decreased. *Giardia* sp. levels did not demonstrate a significant seasonal pattern. While monthly *Giardia* levels did decrease during the summer. and increase again in the fall (behaving essentially as *Cryptosporidium* did). these monthly differences were either not great enough to be significant. or the large range in cyst levels within months was too great to allow differences to be significant.

These findings are of particular interest to water treatment plant personnel. who can use this information regarding seasonal patterns to be on the lookout for increased *Cryptosporidium* levels in surface water in the spring and autumn. and therefore adjust the plant operations accordingly (by increasing disinfectant concentrations. for example) to treat for expected elevated parasite levels.

Correlation of Protozoa with Microbial Indicators of Water Quality.

Most of the microbial indicators monitored were detected on a frequent basis, and in all seasons. *Cryptosporidium* sp. and *Giardia* sp. were strongly correlated with one another in raw water samples from the St. Lawrence River. This relationship has been reported in most survey studies of this type[9,51,58,119] and is an indication that both protozoa are originating from a common source (i.e. from the same sewage outflow, or as a result of parasite co-infection in an animal population).

Cryptosporidium levels were significantly inversely correlated with *Enterococcus* sp. ($r=-0.338$) in raw water samples. This association was also found by Chauret et al[58] in the Ottawa River, however the correlation was positive ($r=0.347$) in that study, rather than inverse. These opposite results may be explained by the fact that Chauret's study was conducted in the summer months (June, July and August) when the river's temperature was relatively high. This study covered three seasons, and a range of water temperatures. While *Cryptosporidium* and *Giardia* are resilient and can survive well at both cold and warm water temperatures, many of the bacteria included in this study do not behave in the same fashion. Total and fecal coliform levels, for example, increase as water temperatures rise through the summer, and then decline again in the autumn.

Cryptosporidium sp. levels were also significantly associated with heterotrophic plate count levels ($r=0.272$), a correlation which has not been demonstrated in any previous study. *Giardia* sp. levels were not significantly correlated to any of the microbial indicators used in this study. This lack of consistency with previous studies may also be due to differences in watershed factors. Every water system, whether river, lake or stream, has a different water

chemistry and is subject to different sources and levels of fecal pollution. All these factors may affect the relationships between microbial indicators and protozoa.

In addition, the standard method for detecting *Cryptosporidium* and *Giardia* in environmental water samples is largely unreliable in terms of recovery efficiency. In raw water samples, recovery efficiency averaged 16% and 20% for *Cryptosporidium* and *Giardia*, respectively. The possibility of obtaining false negatives clearly exists, and may be responsible for skewing the correlations found with microbial indicators.

It is clear that any relationships found between *Cryptosporidium* and *Giardia* and the microbial indicators of water quality are dependent on the watershed under study, and may also change with the seasons. While *Enterococcus* sp. may be a promising candidate as an indicator for *Cryptosporidium* in raw water, this relationship must be tested in many more watersheds and over the entire range of natural water temperatures before it can be declared genuine. The widely varying results obtained from study to study so far lead to the conclusion that no ideal or "universal" indicator may exist for *Cryptosporidium* and *Giardia* in environmental water. As a result, the complex and laborious standard method must be relied on until a more reliable detection method becomes available.

CHAPTER 2:

IN SITU SURVIVAL OF *CRYPTOSPORIDIUM PARVUM* IN RAW AND TREATED WATER SUPPLIES ON THE ST. LAWRENCE RIVER

INTRODUCTION

There have been very few studies documenting the survival capacity of *Cryptosporidium* in environmental waters. This is due partially to the difficulty involved in developing suitable containers that can withstand the pressures of a river or lake environment (strong currents. for example) and still allow the environmental water to reach the inside of the container. Robertson et al developed such a container in 1993.[19] The container was described as low cost and low technology. suitable for studying the transmission stages of parasites within aquatic environments. The group tested the container over a period of 47 days in three different environments - tap water. river water. and cow feces - in a survival study using *Cryptosporidium* oocysts. They found that the container allowed the oocysts to remain in contact with the water environment throughout the study. and at the same time easily allowed for repeated sampling of the oocysts to determine their viability by DAPI/PI vital staining.

This container was used by the same group to test the survival of *C. parvum* oocysts under a range of environmental pressures. including freezing. desiccation. water treatment processes. and physical environments such as tap water. river water. sea water and cow feces.[121] The viability of the oocysts was determined by an *in vitro* excystation assay and fluorogenic dye exclusion. Desiccation was found to be lethal for the oocysts. but a small proportion of oocysts survived freezing at -22°C. The authors also demonstrated that oocysts could survive for long periods of time (up to 47 days) in all of the water environments tested. Interestingly. in laboratory models of water treatment processes. it was found that if the pH. contact time and concentrations were corrected to those at which processes are normally

implemented at water treatment plants. the treatments appear to have no significant effect on oocyst viability.

A similar study was conducted with *Giardia muris* cysts.[122] Cysts were placed in glass vials covered with filter paper. and then suspended in lake. river and tap water. Studies were carried out in two different seasons (fall and winter). when water temperatures ranged from 19-27°C in the fall and 0-2°C in the winter. Of the physiochemical parameters tested. only water temperature was correlated with cyst viability. It was found that cysts exposed to water temperatures below 10°C remained viable for between 1 and 3 months.

Because *Cryptosporidium* was detected many times in treated water samples (both filtered and unfiltered) collected from water treatment plants on the St. Lawrence River. experiments were designed to determine the survival potential of this parasite over time in treated water effluents. An *in situ* survival study was designed to model conditions in the drinking water distribution system in order to determine how long (in days) *Cryptosporidium parvum* oocysts were able to survive in such an environment.

These experiments were carried out at two water treatment plants on the St. Lawrence River: the Cornwall Water Purification plant (full conventional treatment. including rapid-sand filtration and chlorine disinfection) and the Ingleside Water Treatment plant (treatment by chlorine disinfection alone). The experiment was carried out once in each season (four trials in total) in 1996 to assess the effects of water temperature and free chlorine concentration on *C. parvum* oocyst viability.

The survival ability of *C. parvum* in raw water was also of interest. An estimation of the lifespan of oocysts from their point of entry into the river water until they reach the first

surface water treatment plant downstream may be useful to plant operators to give an indication of whether oocysts entering their plants are viable or nonviable.

The same *in situ* survival study design was used to study the effects of raw water on *C. parvum* oocyst viability over a period of several weeks. Oocysts were exposed to both raw water and filtered raw water to determine whether the indigenous microbial populations in the St. Lawrence River had an antagonistic effect on their survival potential. This experiment was carried out at the Cornwall Water Purification plant (three trials in total), which had an indoor raw water tap available. At certain points during each trial, some oocysts were removed and subjected to disinfection with chlorine and monochloramine in an effort to determine whether oocysts aged (or stressed) in raw water were more susceptible to chemical inactivation than controls aged in hard water.

MATERIALS AND METHODS

***Cryptosporidium* oocyst purification.** Calf feces containing *C. parvum* oocysts were obtained from Dr. B. Anderson (University of Idaho, Caldwell, ID) and purified in our laboratory using Sheather's sugar flotation followed by isopycnic Percoll gradient centrifugation, according to Dubey et al.[123] The purified oocysts were stored at 4°C in phosphate-buffered saline (PBS, pH 7.0) containing streptomycin and penicillin (Gibco Laboratories, Grand Island, NY).

It is important to note that the viability of purified *C. parvum* stocks decreases over time, even when stored at 4°C, and therefore may become unusable after a period of two to three months. For this series of experiments, *C. parvum* stocks were used only if the total

population viability exceeded 90% (as measured by *in vitro* excystation prior to the start of the trial). Because these experiments were conducted over the period of 13 months, it was necessary to use a different *C. parvum* stock for each trial.

Survival Setup (treated water experiments). Treated water sources used in these experiments were from the Ingleside Water Treatment Plant (treatment by chlorination) and the Cornwall Water Purification Plant (treatment by filtration and chlorination), both of which draw their source water from the St. Lawrence River. The temperature and pH of the water was monitored on a daily basis, and daily chlorine levels were obtained from the respective water treatment plants. Trials 1, 2, and 3 were set up to run concurrently at both the Ingleside and Cornwall facilities. Trial 4 was conducted at the Cornwall Water Purification plant only.

Purified oocysts from the stock vials were washed in PBS (Gibco Laboratories) in order to remove the antibiotics. Oocyst preparations were inoculated into survival chambers (Slide-A-Lyzer™ dialysis cassettes, Pierce Chemical Company, Rockford, IL) at a concentration of 1.0×10^6 oocysts per cassette, along with 1.0mL of treated water. The cassettes were floated in an overflow tank through which treated water effluent flowed continuously at a rate of approximately 2 liters/minute, over a period of four to five days. Control oocysts were inoculated into screw-cap microcentrifuge tubes (5×10^5 oocysts per tube) in synthetic hard water (100 ppm as $CaCO_3$, pH 7.0, prepared and measured according to AOAC standards). The microtubes were placed in the overflow tank, however the control oocysts were not in contact with the treated water. Sufficient cassettes and control tubes

were inoculated to allow duplicate (or triplicate. Trial 4) sampling at each time point. Cassettes and controls were removed from the tank each day for 4 days. and the oocysts were returned to the lab in microtubes on ice for viability analysis.

Survival Setup (raw water experiments). The raw water source for these experiments was the St. Lawrence River (obtained from a raw water tap at the Cornwall Water Purification Plant). The filtered raw water used in these experiments was obtained by filtering an aliquot of the raw water through a 0.22μm membrane (Nalgene Company. Rochester. NY) to reduce or remove microbial populations. The temperature and pH of the water was monitored on a weekly basis.

Purified oocysts from the stock vials were washed in PBS (Gibco Laboratories) in order to remove the antibiotics. Oocyst preparations were inoculated into survival chambers (Slide-A-Lyzer™. Pierce Chemical Company) at a concentration of $1.0x10^6$ oocysts per cassette. along with 1.0mL of either raw or filtered raw water. The cassettes were floated in an overflow tank through which raw water flowed continuously at a rate of approximately 2 liters/minute. over a period of five to seven weeks. Control oocysts were inoculated into microcentrifuge tubes ($5x10^5$ oocysts per tube) in the presence of either synthetic hard water. raw water or filtered raw water. and were also placed in the overflow tank. Sufficient cassettes and control tubes were inoculated to allow duplicate sampling at each time point. Cassettes and controls were removed from the tank on a weekly basis. and the oocysts were returned to the lab in microtubes for viability analysis.

Disinfection Assay. On certain days during the raw water experiments, oocysts that had been aged in the overflow tank were subjected to chlorine or monochloramine disinfection to determine whether oocysts exposed to raw water for various lengths of time were more susceptible to inactivation than control oocysts.

A stock solution of chlorine (about 100 mg/L) was produced by diluting 1 mL of sodium hypochlorite (6% solution of commercial bleach) to 500 mL using synthetic hard water. To prepare the monochloramine stock solution, equal volumes of chlorine stock solution (Stock 1: prepared by adding 0.430 mL of sodium hypochlorite to 75 mL PBS, pH 9.5) and ammonium chlorine stock solution (Stock 2: prepared by adding 80 mg of NH_4Cl to 100 mL PBS, pH 9.5) were mixed together. Freshly prepared chlorine and monochloramine stock solutions were serially diluted and the concentrations were measured by the DPD Ferrous Titrimetric method (as detailed in *Standard Methods for the Examination of Water and Wastewater*[1]) and by spectrophotometer (515nm) to construct a standard curve. Stock solutions were then diluted in synthetic hard water (pH 7.0) to give the target concentration (10 mg/L).

One mL of either disinfectant (freshly made) was added to the aged and fresh oocyst suspensions in microtubes. The microtubes were then capped, and rotated gently at 5°C for five hours. At the end of the reaction time, any residual chlorine remaining in the microtubes was neutralized by the addition of 45 µL of 10% sodium thiosulfate. Oocyst inactivation was assessed by *in vitro* excystation and total counts for each sample.

Viability Assay. Relative viability of *Cryptosporidium parvum* oocysts was assessed by an *in vitro* excystation assay and total counts for each sample. *In vitro* excystation was performed by exposing the oocysts in each sample to an acidic trigger (Hank's Balanced Salt Solution, pH 2.0, Gibco Laboratories) for one hour at 37°C, followed by incubation for 4 hours at 37°C in an excystation medium (PBS with 0.005g/mL trypsin (Sigma Chemical Co., St. Louis, MO) and 0.015g/mL taurocholic acid (ICN Biomedicals Inc., Aurora, Ohio)). After incubation, at least 100 oocysts per sample were counted and scored as either intact oocysts, partially empty oocysts or empty oocysts using a microscope (Carl Zeiss, Germany) equipped with differential interference contrast (DIC) optics or Hoffman modulation contrast optics at 1,000X magnification. The percent excystation was calculated by dividing the sum of partially excysted and empty oocysts by the total number of oocysts counted and subtracting the baseline excystation percentage (derived by counting and scoring oocysts prior to the excystation procedure).

Total counts were determined by staining the oocysts with an FITC-labeled monoclonal antibody (Crypt-A-Glo™, Waterborne Inc., New Orleans, LA) and counting using an Improved Neubauer counting chamber, under epifluorescence microscopy (Leitz Laborlux K) at 400X magnification. The total number of viable oocysts was calculated by multiplying the percent excystation by the total count and dividing by 100. The concentration of viable oocysts at each sampling point (day t) was then expressed as a $\log_{10}$ reduction from the original concentration of viable oocysts at the start of the experiment (day 0).

Statistical analyses. For both the treated water and raw water experiments. *Cryptosporidium* survival (log_{10} reductions) for each set of experimental conditions was plotted against time on a log-linear graph (SigmaPlot®. Jandel Corporation. San Rafael. CA). First-order linear regression lines were generated for each treatment using the same program. The slopes for each line (Bx) and their respective standard errors (SE_{Bx}) were obtained from the linear regression analyses. The rate of inactivation (K*i*) was determined from the slope of each line for each treatment. as described by Segel.[124] The effect of either treated or raw water on *Cryptosporidium* survival was determined using the student t-test for comparing independent regression lines. The statistical analysis for the survival experiments is outlined with an example in Appendix C.

For the treated water experiments. reductions in *Cryptosporidium* viability were expressed as inactivation rates in order to determine the relationships with water temperature and free chlorine levels. The Spearman rank order correlation was used to measure the strength of association between pairs of variables. (SigmaStat®. Jandel Corporation. San Rafael. CA).

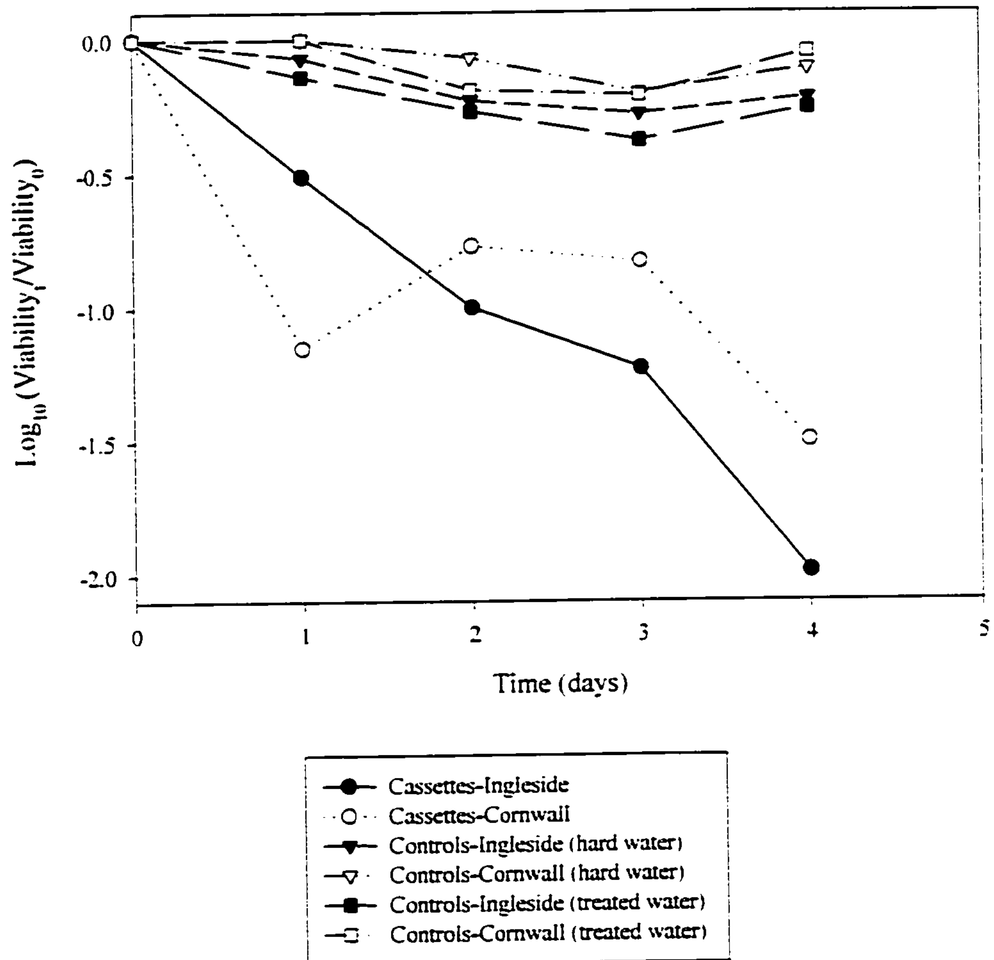
RESULTS

In situ survival in treated water

Trial 1 (Winter) - Figure 5

The reduction in *Cryptosporidium* viability after four days reached 1.99 log_{10} at the Ingleside plant and 1.50 log_{10} at the Cornwall plant. Control oocysts in treated water (at both plants) were reduced by a maximum of 0.26 log_{10}. and control oocysts in hard water were reduced by a maximum of 0.22 log_{10}.

**Figure 5: *Cryptosporidium parvum* viability in treated water
Winter Season (Water Temperature=1.2-1.5°C)**



At the Ingleside plant, oocysts continually exposed to treated water (i.e. cassette samples) showed a significant decrease in viability when compared to control oocysts both in treated water ($t=7.84$, $P<0.05$) and in hard water ($t=8.78$, $P<0.05$). Although Figure 5 shows a clear reduction in viability for the cassette oocysts at the Cornwall plant, the slope of the line was not significantly different from either set of control oocysts at the 95% confidence level.

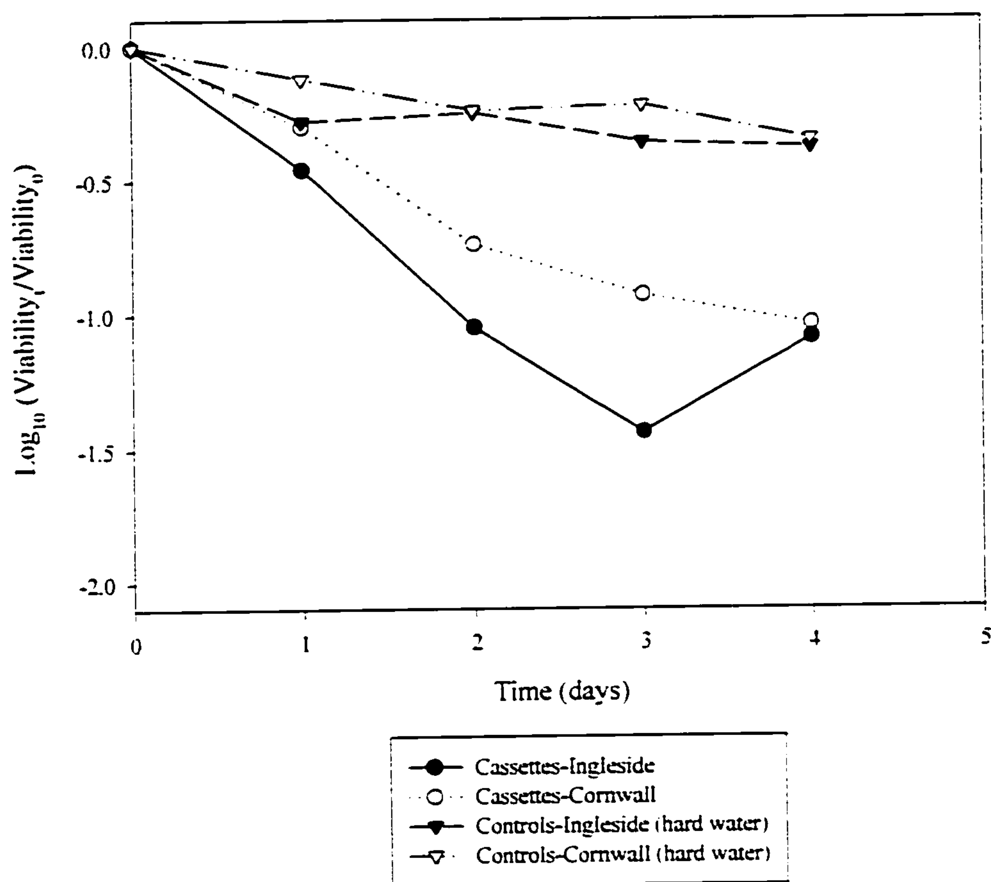
There was no significant difference between control oocysts exposed to treated water and control oocysts exposed to hard water ($t=0.30$, $P>0.05$ for Ingleside controls; $t=0.29$, $P>0.05$ for Cornwall controls), therefore the treated water controls were not included in the subsequent trials.

Trial 2 (Spring) - Figure 6

The reduction in *C. parvum* viability after four days reached 1.09 $\log_{10}$ at the Ingleside plant and 1.04 $\log_{10}$ at the Cornwall plant. Control oocysts in hard water decayed by a maximum of 0.38 $\log_{10}$.

The inactivation rate of treatment oocysts at the Cornwall plant was significantly greater than for control oocysts ($t=5.02$, $P<0.05$). Due to the large error associated with the predicted regression line for the treatment oocysts at the Ingleside plant, the difference between treatment oocysts and control oocysts was not significant at the 95% confidence level. However, this difference was significant at the 90% confidence level ($t=2.20$, $P<0.10$).

Figure 6: *Cryptosporidium parvum* viability in treated water Spring Season (Water Temperature=1.8-2.0°C)



Trial 3 (Summer) - Figure 7

After two days. *C. parvum* viability at both the Ingleside and Cornwall water treatment plants had been reduced by 1.45 $\log_{10}$. On the third day of the trial. the total oocyst numbers had returned to levels similar to those seen on Day 1 of the trial (i.e. from approximately 1.13×10^5 oocysts/cassette on Day 2 back up to 3.75×10^5 oocysts/cassette on Day 3). while the actual excystation rates remained approximately equal from Day 2 to Day 3 of the trial. On Figure 7. this is seen as an increase in viability on Day 3. up to 0.87 $\log_{10}$ for Ingleside oocysts and 0.99 $\log_{10}$ for Cornwall oocysts. Control oocysts were reduced by a maximum of 0.30 $\log_{10}$ at Ingleside and 0.16 $\log_{10}$ at Cornwall.

The error associated with the predicted regression lines for both treatment groups made it impossible to determine whether the inactivation rates were significantly different from those of the control groups.

Trial 4 (Fall) - Figure 8

Due to irregularities (i.e. viability decreasing and then increasing again) associated with the three previous trials. and since no significant differences were found in oocyst decay rates between the two plants. a decision was made to concentrate on one plant only (Cornwall) during the fourth trial. The number of replicate samplings on each day of the trial was increased from two to three. The results are presented in Figure 8.

While the regression line error problem was solved. after an initial 0.44 $\log_{10}$ reduction in *C. parvum* viability after Day 1 of the trial. decay in the treatment oocysts leveled off so that after four days. *C. parvum* viability was reduced by only 0.61 $\log_{10}$. Control oocysts decayed by 0.13 $\log_{10}$ over the same time period. Consequently. the

**Figure 7: *Cryptosporidium parvum* viability in treated water
Summer Season (Water Temperature=22.0-23.0°C)**

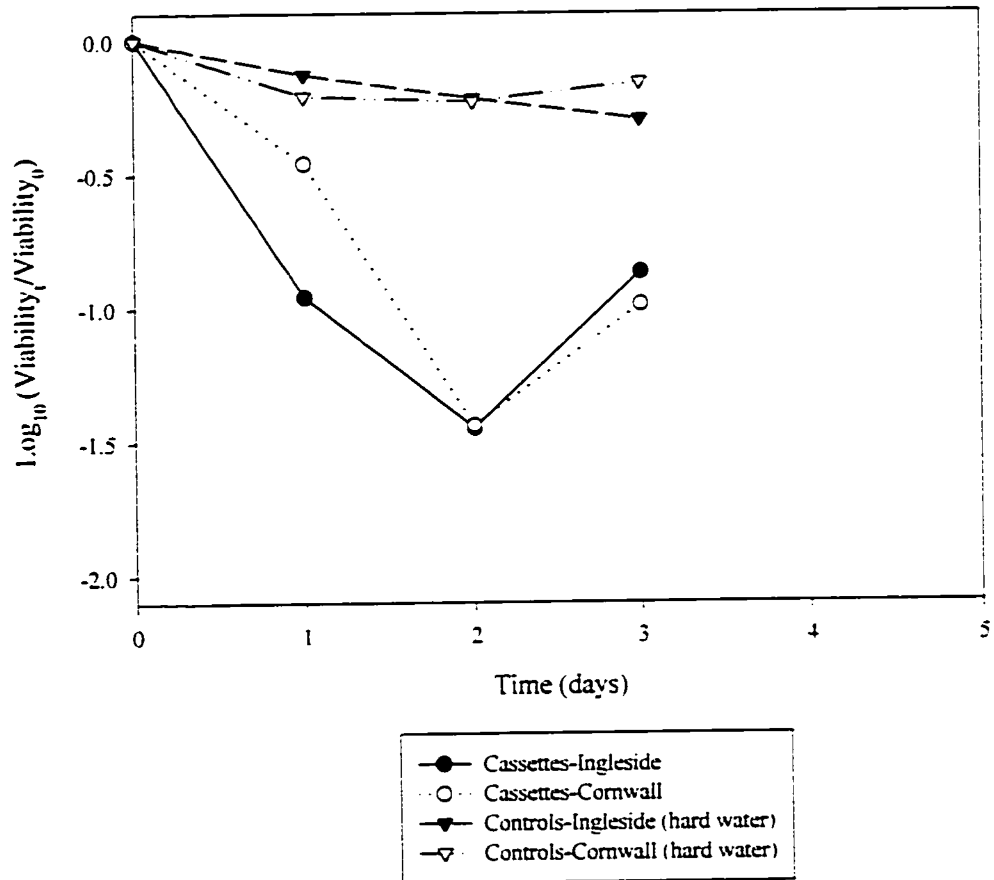
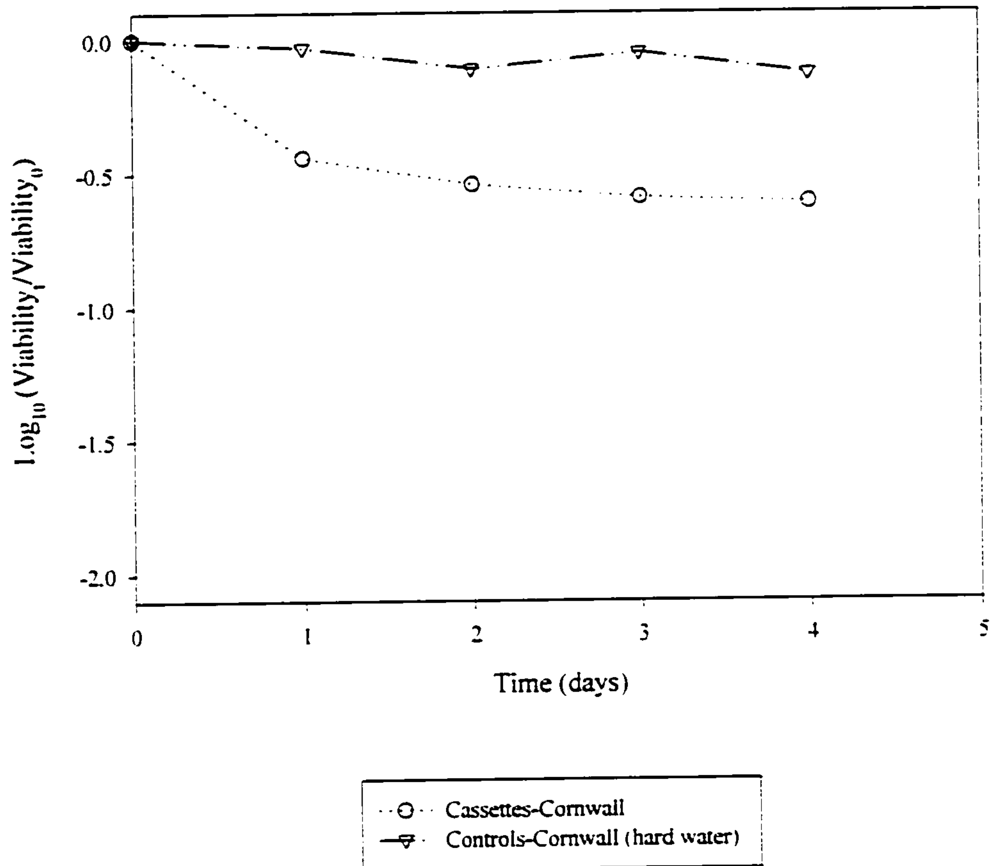


Figure 8: ***Cryptosporidium parvum*** **viability in treated water Autumn Season (Water Temperature=11.0-12.2°C)**



inactivation rate for the treatment group was not significantly different from the control group at the 95% confidence level. This difference, however, was significant at the 90% confidence level ($t=2.24$, $P<0.10$).

Spearman Correlations

The Spearman rank order correlation was used to determine whether *Cryptosporidium parvum* decay rates for all four trials were related to either water temperature or the level of chlorine in the treated water effluent. Tables 8a and 8b provide a summary of the $\log_{10}$ reductions, inactivation rates (K*i* values), water temperatures and chlorine levels recorded on each day of each trial at both water treatment facilities.

Cryptosporidium decay was significantly correlated to the free chlorine concentration in treated water, at the 90% confidence level ($r=0.685$, $P=0.0735$). As the chlorine concentration increased, *C. parvum* oocyst inactivation rates also increased. Water temperature was not correlated to *C. parvum* decay in treated water ($r=0.0714$, $P=0.843$, $n=7$).

Table 8a: Data recorded at the Ingleside Water Treatment plant during the *in situ* survival trials: log reductions in *C. parvum* viability, inactivation rates (K*i* values), water temperatures and chlorine levels

Trial #	Date	Log Reduction	K*i* value	Water Temperature (°C)	Free Chlorine Concentration (mg/L)
1	02/19	-	-	1.5	0.50
1	02/20	-	-	1.5	0.50
1	02/21	-	-	1.5	0.50
1	02/22	-	-	1.5	0.46
1	02/23	-1.99	0.470	1.5	0.45
2	03/25	-	-	2.0	0.40
2	03/26	-	-	2.0	0.40
2	03/27	-	-	1.8	0.45
2	03/28	-	-	1.8	0.45
2	03/29	-1.09	0.316	1.8	0.40
3	08/12	-	-	22.0	0.90
3	08/13	-	-	22.4	1.15
3	08/14	-	-	23.0	1.03
3	08/15	-0.87	0.31	22.0	1.23

Table 8b: Data recorded at the Cornwall Water Purification plant during the *in situ* survival trials: log reductions in *C. parvum* viability, inactivation rates (K*i* values), water temperatures and chlorine levels

Trial	Date	Log Reduction	K*i* value	Water Temperature (°C)	Free Chlorine Concentration (mg/L)
1	02/19	-	-	1.5	0.31
1	02/20	-	-	1.2	0.30
1	02/21	-	-	1.5	0.31
1	02/22	-	-	1.5	0.34
1	02/23	-1.50	0.268	1.5	0.31
2	03/25	-	-	2.0	0.31
2	03/26	-	-	1.8	0.36
2	03/27	-	-	1.8	0.32
2	03/28	-	-	1.8	0.32
2	03/29	-1.04	0.271	2.0	0.32
3	08/12	-	-	22.0	0.49
3	08/13	-	-	22.2	0.48
3	08/14	-	-	22.0	0.50
3	08/15	-0.99	0.395	22.0	0.47
4	10/28	-	-	12.2	0.32
4	10/29	-	-	12.0	0.35
4	10/30	-	-	12.0	0.32
4	10/31	-	-	11.8	0.32
4	11/01	-0.61	0.137	11.0	0.33

In situ survival in raw water

This set of experiments could only be conducted in the winter and early spring months because the source water (St. Lawrence River) used by the Cornwall Water Purification Plant is pre-chlorinated at the intake during the late spring, summer and early fall in order to control zebra mussel infestation.

Trial 1 - Figure 9

The first trial of the raw water survival experiments was useful to assess how long *C. parvum* oocysts could survive in river water. After seven weeks of exposure to raw water, *C. parvum* viability had decreased by 0.86 log_{10}. It should be noted that the reduction in viability was due mostly to a decrease in the total number of oocysts in the cassettes - 80% of the oocysts remaining in the cassettes after seven weeks were viable. Control oocysts in hard water decayed by 0.16 log_{10} after seven weeks. Comparison of the inactivation rates for both treatments showed that *C. parvum* oocysts exposed continuously to raw water decayed significantly more rapidly than the control oocysts ($t=1.90$, $P<0.10$).

A separate set of cassette and control samples were floated in the overflow tank for 14 days, and then subjected to either chlorine or monochloramine disinfection (10 mg/L for 5 hours), in order to see if *C. parvum* oocysts that were pre-aged in raw water were more susceptible to disinfection than control oocysts not exposed to raw water. The results are presented in Table 9.

Exposure to monochloramine for five hours did not have any appreciable effect on *C. parvum* viability, and oocysts aged in raw water were equally susceptible to disinfection as the control oocysts in hard water. Contrary to expectations, control oocysts were more

Figure 9: *Cryptosporidium parvum* viability in raw water Trial 1 (Water Temperature=0.1-0.5°C)

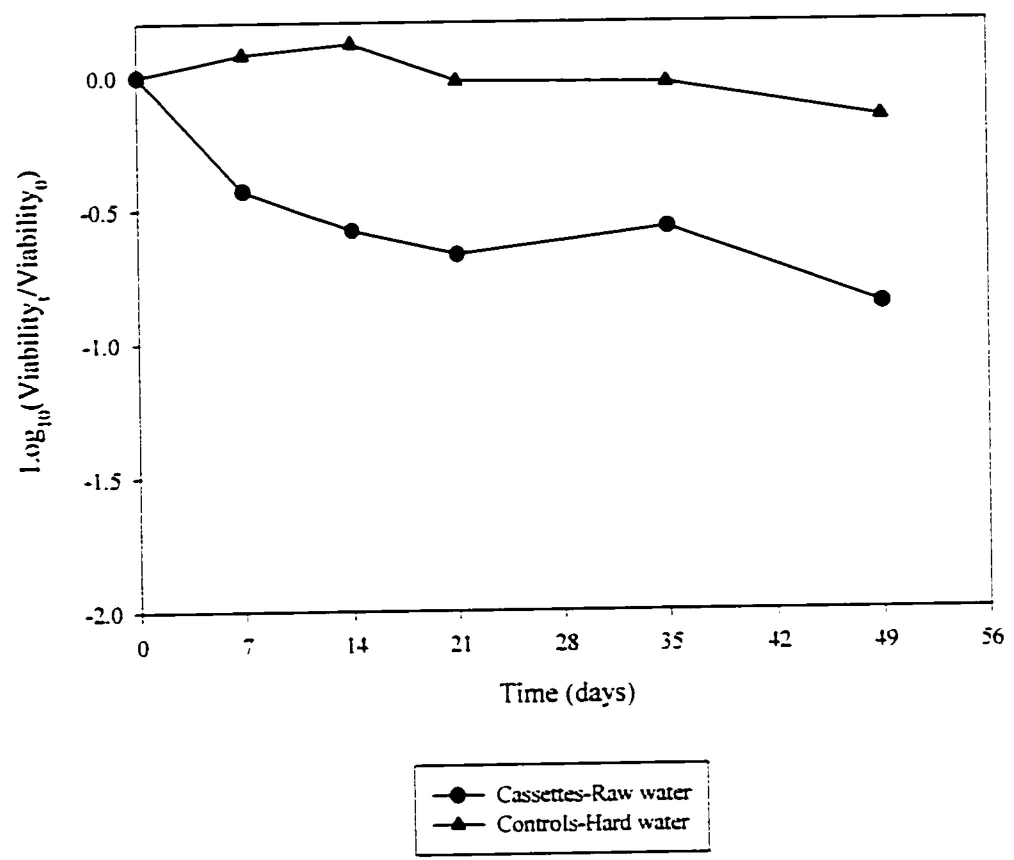


Table 9: Disinfection of aged *Cryptosporidium parvum* oocysts with 10 mg/L of chlorine or monochloramine for 5 hours at 5°C (Trials 1 to 3)

Trial Number	Aging Condition	Aging Period	Inactivation (log_{10}):	
			Chlorine	**Monochloramine**
Trial 1	Cassette (raw water)	14 days	0.05	NR
Trial 1	Control (hard water)	14 days	0.23	NR
Trial 2	Cassette (raw water)	4 days	0.25	0.08
Trial 2	Control (hard water)	4 days	0.23	0.09
Trial 2	Cassette (raw water)	18 days	0.14	NR
Trial 2	Control (hard water)	18 days	0.26	NR
Trial 3	Cassette (raw water)	4 days	0.38	NR
Trial 3	Control (hard water)	4 days	0.16	NR
Trial 3	Cassette (raw water)	18 days	0.57	0.02
Trial 3	Control (hard water)	18 days	0.32	NR

Note: NR refers to no reduction

susceptible to chlorine disinfection than were the oocysts aged in raw water for 14 days (0.23 vs. 0.05 $\log_{10}$ reduction).

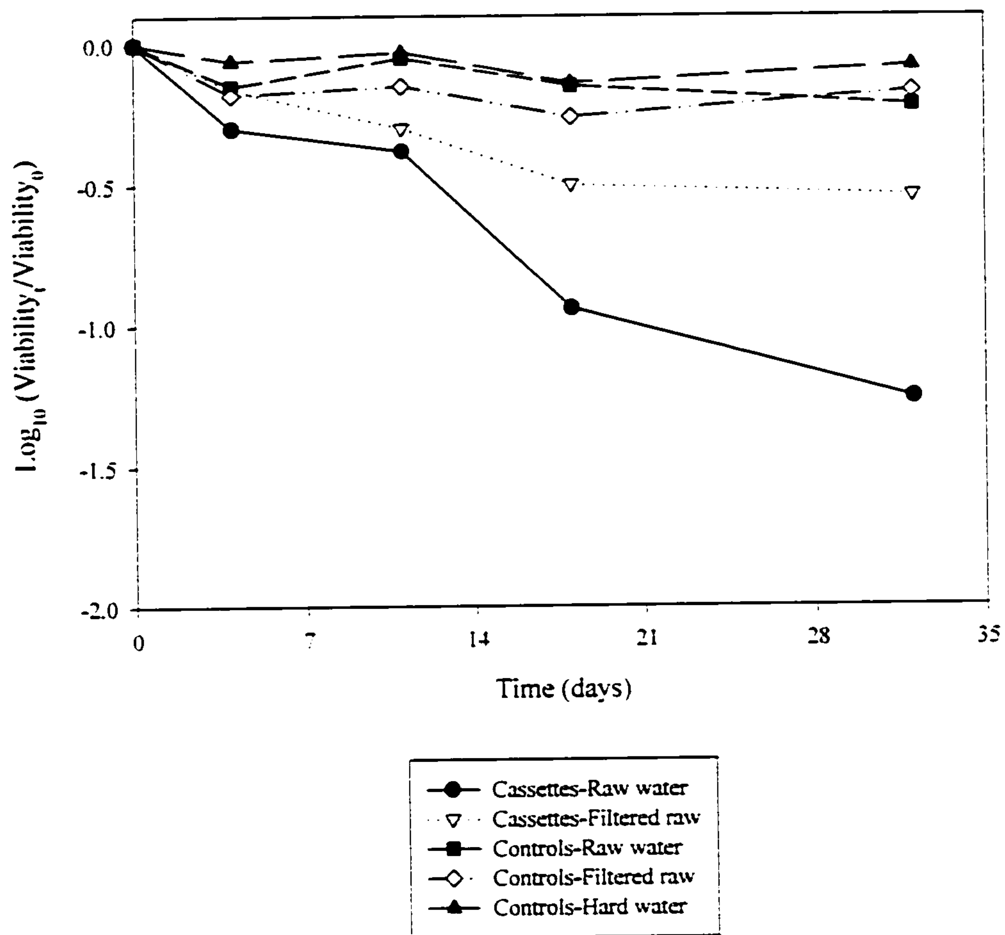
Trial 2 - Figure 10

The subsequent trials in this series were designed to determine whether the indigenous microbial flora of the St. Lawrence River had any effect on the survival potential of *C. parvum* oocysts. Treatment groups consisted of oocysts exposed to raw water and oocysts exposed to raw water that had been filtered through a 0.22µm membrane filter to reduce or eliminate microbial populations. Control oocysts were placed in raw water, filtered raw water or hard water.

After 32 days, the viability of *C. parvum* oocysts exposed to raw water had decreased by 1.26 $\log_{10}$, while those exposed to filtered raw water decreased by only 0.54 $\log_{10}$. Comparison of the inactivation rates showed that the difference between these two treatment groups was significant ($t=3.41$, $P<0.05$). Control oocysts in raw, filtered raw and hard water were reduced by 0.22 $\log_{10}$, 0.17 $\log_{10}$ and 0.08 $\log_{10}$, respectively, over the same 32 day period.

The inactivation rate for the raw water treatment group was significantly faster than the raw water control group ($t=5.69$, $P<0.05$) and the hard water control group ($t=6.41$, $P<0.05$); the inactivation rate for the filtered water treatment group was significantly faster than the hard water control group ($t=3.36$, $P<0.05$) and the filtered water control group at the 90% confidence level ($t=2.42$, $P<0.10$).

Figure 10: *Cryptosporidium parvum* viability in raw water Trial 2 (Water Temperature=0.5-2.0°C)



A separate set of cassettes and controls was again included for chlorine and monochloramine disinfection testing (see Table 9. Trial 2). For the second trial. oocysts were aged in cassettes for either four days or eighteen days. in order to gain a better understanding of the relationship between aging in raw water and chlorine inactivation detected in Trial 1.

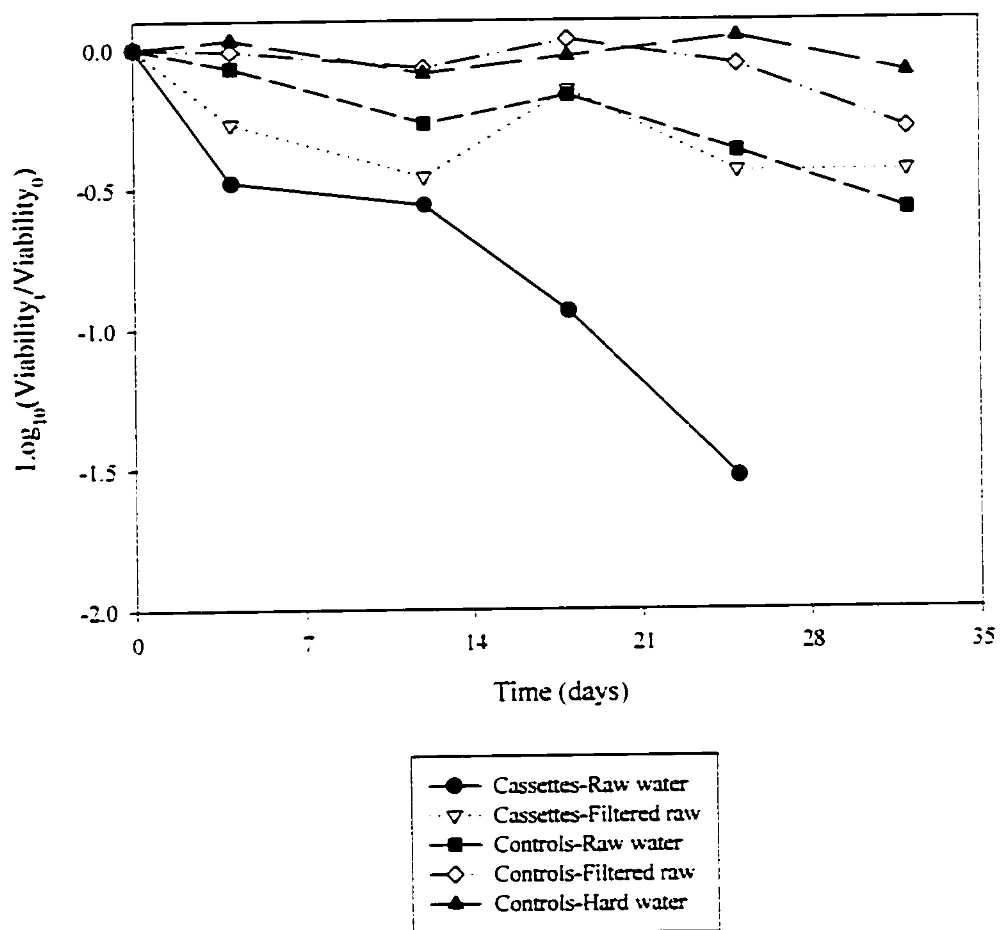
No inactivation was observed for either cassette or control oocysts following monochloramine disinfection. after both four and eighteen day aging periods. At Day 4. cassette oocysts exposed to chlorine were reduced by 0.25 $\log_{10}$. while control oocysts showed a 0.23 $\log_{10}$ inactivation. At Day 18. control oocysts were more susceptible to chlorine disinfection than oocysts aged in raw water (as in Trial 1). Cassette oocysts that were exposed to chlorine were reduced by 0.14 $\log_{10}$. while control oocysts were reduced by 0.26 $\log_{10}$.

Trial 3 - Figure 11

After 25 days. the viability of *C. parvum* oocysts exposed to raw water had been reduced by 1.53 $\log_{10}$. There was not a sufficient number of oocysts left in the cassettes at Day 32 of the trial to complete the excystation procedure. After 32 days. the viability of *C. parvum* oocysts exposed to filtered raw water had decreased by 0.44 $\log_{10}$. The differences in oocyst decay between the two treatment groups were significant ($t=4.28$. $P<0.05$). Control oocysts exposed to raw. filtered raw and hard water were reduced by 0.58 $\log_{10}$. 0.29 $\log_{10}$ and 0.09 $\log_{10}$ respectively.

The inactivation rate for the raw water treatment group was significantly faster than the raw water control group ($t=4.21$. $P<0.05$). and the hard water control group ($t=5.97$. $P<0.05$).

Figure 11: ***Cryptosporidium parvum*** **viability in raw water Trial 3 (Water Temperature=1.0-2.0°C)**



The inactivation rate for the filtered water treatment group was not significantly different from either the filtered raw water or the hard water control groups.

The results of the chlorine and monochloramine disinfection of aged oocysts are presented in Table 9 (Trial 3). Once again, monochloramine had no effect on the viability of *C. parvum* oocysts. Neither treatment oocysts nor control oocysts (on both Day 4 and Day 18) were reduced at all following exposure to 10 mg/L of monochloramine. At Day 4, cassette oocysts that were exposed to chlorine were reduced by 0.38 $\log_{10}$, while control oocysts were reduced by 0.16 $\log_{10}$. The inactivation results on Day 18 were not consistent with those observed in Trials 1 and 2, in that cassette oocysts were more susceptible to chlorine disinfection than controls (0.57 $\log_{10}$ vs. 0.32 $\log_{10}$ respectively).

DISCUSSION

Treated Water Experiments

The results were variable for each season. A maximum 2 $\log_{10}$ reduction in *C. parvum* oocyst viability was observed after four days of exposure to treated water in the winter season (T=1.5°C), while a minimum 0.6 $\log_{10}$ reduction was obtained after four days in the fall, when temperatures were approximately 10°C higher.

Reductions in *C. parvum* viability were expressed as inactivation (decay) rates (K*i* values) in order to mathematically determine the relationships between *C. parvum* decay and water temperature or free chlorine levels. The Spearman rank order correlation was used to measure the strength of association between pairs of variables. *Cryptosporidium* decay seemed to be directly related to the concentration of free chlorine in treated water effluent (as

chlorine levels increased. *Cryptosporidium* decay rates also increased), however this association fell just short of significance (r=0.685, P=0.0735).

Previous disinfection studies have demonstrated that *Cryptosporidium* oocysts are resistant to chlorination.[12,13,125] However, in these studies oocysts were exposed to an initial dose of free chlorine, which was then subject to decay over the course of the experiment. In our set of experiments, the oocysts were continuously bathed in "fresh" treated water effluent; therefore, the free chlorine levels were kept at a constant level over the four day trial period. This constant exposure to free chlorine at different levels may be responsible for the correlation with *Cryptosporidium* decay.

It was anticipated that changes in water temperature between seasons would affect oocyst viability, however, no correlation was found between *C. parvum* decay rates and treated water temperature (r=0.0714, P=0.843). This may be due to the fact that the temperature range over the course of these experiments (ranging from 1.2°C to 23.0°C between trials) was not great enough to cause a significant change in oocyst viability. It is also possible that this association was masked by the variability in the data within each separate trial.

Another factor that must be taken into consideration is the fact that different *C. parvum* stocks were used for each trial. Although each separate stock was obtained from the same source, it is possible that strain differences may exist among the batches. Unfortunately, this possibility cannot be avoided. Although many researchers have attempted it, so far *C. parvum* cannot be produced reliably as a pure culture, or in great enough quantities to be used in experiments. In addition, oocyst stocks can only be stored for

a few months at 4°C. before the population viability decreases below 90% and becomes unusable for experiments.

Taking all four trials into consideration. the greatest reduction observed in *Cryptosporidium* viability was 2 log_{10} after four days of continuous exposure to treated water effluent. These results demonstrate that *Cryptosporidium parvum* oocysts can remain viable in treated water for extended periods of time and therefore may pose a risk to the consumer if they enter into distribution systems.

Raw Water Experiments

The first trial in this series of experiments established that *Cryptosporidium parvum* oocysts are able to survive for long periods of time in raw water. After seven weeks of exposure to natural river water. oocyst viability had decreased by 0.86 log_{10}. It is also noteworthy that 80% of the oocysts remaining in the cassettes after seven weeks were viable. These results compared favorably with the study by Robertson et al.[121] who also found that *C. parvum* oocysts were able to survive for 47 days or longer in river water.

On several occasions during the first trial. bacteria were observed swarming inside and around empty oocyst shells. under the microscope. Other researchers in our laboratory had noted the same phenomenon in raw water samples. The second and third trials of this experiment were designed to investigate the role that indigenous bacterial populations in the St. Lawrence River play in the survival of *C. parvum* oocysts in this environment.

In both trials. it was demonstrated that oocysts exposed to natural raw water decayed significantly more rapidly than those exposed to raw water that had been filtered to remove

bacterial populations. While the specific culprit was not identified, it seems clear that one or more microbes present in the St. Lawrence River is a natural predator of *Cryptosporidium parvum*, and has an antagonistic effect on the parasite's survival potential in raw water.

These survival experiments have shown that *Cryptosporidium parvum* is able to survive for several weeks in raw water, and therefore oocysts entering surface water treatment plants in environmental waters have a high probability of being viable (and should be treated accordingly).

Disinfection of oocysts aged in raw water

It was hypothesized that *C. parvum* oocysts that had been aged (or stressed) in raw water would be more susceptible to disinfection with chlorine and monochloramine than controls aged for the same length of time in synthetic hard water. The results were surprising. During the first trial, oocysts were aged for two weeks (14 days) in raw water, and then subjected to chemical disinfection with 10 mg/L of either chlorine or monochloramine for five hours at 5°C. Contrary to expectations, the oocysts that had been aged in raw water were substantially more resistant to chlorine disinfection than controls (0.05 $\log_{10}$ vs. 0.23 $\log_{10}$). This phenomenon was investigated further in the second trial: aging periods of 4 days and 18 days were chosen in order to gain a better understanding of the relationship between aging in raw water and susceptibility to chlorine disinfection. After four days, the cassette and control samples were equally susceptible to chlorination. After 18 days however, the oocysts aged in raw water were again more resistant to chlorine than controls aged in hard water (0.14 $\log_{10}$ vs. 0.26 $\log_{10}$). The most likely explanation for this

observation is that after two or more weeks of exposure to raw water, the weak or damaged oocysts in the cassettes have died off, leaving the stronger, hardy oocysts, which are naturally more resistant to disinfection. The possibility also exists that a chemical or organic component of raw water is exerting a protective effect on the aged oocysts.

The third trial did not yield consistent results. This was an exact replication of the second trial. After four days, cassette oocysts were more susceptible to chlorine disinfection than controls (0.38 $\log_{10}$ vs. 0.16 $\log_{10}$). The same held true after 18 days: cassette oocysts were reduced by 0.57 $\log_{10}$, while controls were reduced by only 0.32 $\log_{10}$. It is again possible that these contradictory findings are the result of using a different *C. parvum* stock.

Monochloramine was ineffective as a disinfectant throughout the three trials. Little or no reduction in oocyst viability was observed, no matter the aging period, and no difference was detected between oocysts aged in raw water and controls aged in synthetic hard water.

These results have implications for the water treatment industry. While chlorine was much more effective as a disinfectant than monochloramine, the inactivation levels achieved were much lower than recommended to provide safe drinking water, according to the U.S. Surface Water Treatment Rule.[117] Monochloramine had no effect on *C. parvum* viability, which could be problematic in those plants using monochloramine as a disinfectant. Although more trials are needed to determine the exact relationship between aging and susceptibility to disinfection, it should not be assumed that oocysts pre-aged in environmental waters are more susceptible to chemical disinfection than fresh oocysts.

GENERAL DISCUSSION

The survey study of this project demonstrated that the protozoan parasites *Cryptosporidium* sp. and *Giardia* sp. are ubiquitous within the study area of the St. Lawrence River. In addition, to being present on a continual basis in the surface water, it was shown that these parasites can escape the water treatment process and enter drinking water supplies. This was especially true of utilities using a one-step chlorination process to treat drinking water; *Cryptosporidium* sp. oocysts were detected in 94% of these samples. Filtration plants, while much more effective at removing parasites from their finished product, were not immune to parasite positive samples. In fact, the highest concentration of *Cryptosporidium* sp. (320 oocysts/100L) detected in a treated water sample originated from a plant using rapid-sand filtration.

The standard method for detecting *Cryptosporidium* and *Giardia* in environmental waters has many drawbacks, not the least of which is the associated poor recovery efficiency. Recovery efficiencies in this study averaged 16% for *Cryptosporidium* and 20% for *Giardia*, and were similar for both raw and treated water samples. Consequently, the protozoan parasite concentrations reported here may be severely underestimating the actual levels in the river or in treated water effluents.

In addition, the standard method is complex, expensive to perform, and time-consuming. It takes approximately three to four days (depending on the number of samples collected) to run a batch of samples through the procedure, from water collection to microscopic examination of the resulting slides. While this is acceptable for a survey study of this nature, a turn-around time of a week or more is unacceptable for a water treatment facility trying to monitor parasitological water quality. By the time the results are returned,

they are of little value from a treatment point-of-view. and too late for the issuance of a boil-water advisory.

For this reason. an attempt was made to find a suitable microbial indicator for both *Cryptosporidium* sp. and *Giardia* sp. in St. Lawrence River water that could be easily and rapidly detected. Nine different microbial indicators of water quality were selected. seven of which could be enumerated within 24 hours of collection. Indicator concentrations were monitored along with protozoa levels in every raw water sample collected over the course of the survey study. The results were somewhat ambiguous - *Giardia* sp. was not significantly correlated with any of the nine microbial indicators. While *Cryptosporidium* sp. levels were significantly associated with *Enterococcus* sp. ($r=-0.338$. $P=0.02$) and heterotrophic plate count levels ($r=0.272$. $P=0.036$). these correlations were fairly weak. A lack of consistency with previous studies leads to the conclusion that any correlations detected are probably dependent on the watershed under study. and therefore the concept of a "universal" indicator for *Cryptosporidium* and *Giardia* in environmental waters is not a feasible one.

Another complaint commonly aimed at the standard method lies in the fact that it does not give any indication of whether the protozoa viewed through the microscope are viable. As mentioned previously. *Cryptosporidium* oocysts were found in 94% of the unfiltered treated water samples collected. and *Giardia* cysts were detected in 61% of the same samples. Parasites were also detected occasionally in filtered treated water samples. While these numbers are impressive. the standard method provides no way of determining whether the parasites are potentially infective for the drinking water consumer. Because this appeared to be an important issue for both the consumer and the water treatment plant

operator, a series of *in situ* survival studies were initiated to determine how long *C. parvum* could survive in both the treated water and raw water environments.

C. parvum survival in treated water effluent was tested over a four day period at two water treatment plants on the St. Lawrence River. While results varied from trial to trial, the "best" result achieved was a 2 $\log_{10}$ reduction in *C. parvum* viability after four days of exposure, and the "worst" result was a 0.6 $\log_{10}$ reduction. Bear in mind that, even in large cities, treated water does not usually stay in the drinking water distribution system for this length of time. In smaller communities, and especially during times of high demand, drinking water may only take a few hours to travel from the plant to the consumer's home. Wallis et al[55] reported that there are about 1,000 communities in Canada that treat their water by chlorine disinfection alone, and with a minimal contact time of approximately 30 minutes. In that case, these *in situ* survival trials demonstrate that much of the original *C. parvum* population can remain viable with short-term (~ 24 hours) exposure to treated water effluent. To tie back in to the survey study results, viable oocysts entering the water treatment plant are likely to survive chlorination and remain viable in this water for at least 24 hours. Consequently, oocysts getting through to the consumer through tap water can be considered potentially infective.

The final question under consideration was: are oocysts entering water treatment plants through surface water likely to be viable? A similar *in situ* survival study was initiated to determine how long fresh *C. parvum* oocysts could survive in the raw water of the St. Lawrence River. The results showed that *C. parvum* was able to survive at least four weeks and as long as seven weeks in natural river water. In addition, although total numbers of oocysts decreased significantly, 80% of the oocysts remaining after seven weeks were

considered viable by *in vitro* excystation. Therefore, depending on how far downstream the water treatment facility is located from the source of parasites (whether originating from a sewage outflow or from animals), it is conceivable that *Cryptosporidium* sp. oocysts entering the plant through surface water are viable. It was further demonstrated that *C. parvum* oocysts aged in raw water were not necessarily more susceptible to chlorine disinfection than fresh oocysts.

Potential Impact: This study has demonstrated that primary water treatment plants remove or inactivate very few (if any) parasites during the treatment process, and the majority of water samples collected from these plants during the survey study contained both *Cryptosporidium* sp. oocysts and *Giardia* sp. cysts. *In situ* survival studies demonstrated that *Cryptosporidium parvum* oocysts can remain viable for several weeks in raw water, and can also survive for several days in the plant's treated water effluent. It is therefore quite possible that the parasites detected in the treated water samples from the St. Lawrence River are infective when they reach the consumer through their tap water. In light of these findings, such communities may be at risk for a waterborne outbreak of cryptosporidiosis or giardiasis.

CONCLUDING REMARKS

The following conclusions can be drawn based on the findings of this study:

1. *Cryptosporidium* sp. and *Giardia* sp. were ubiquitous in the surface water of the St. Lawrence River, within the study area. The protozoa were also detected frequently in unfiltered drinking water supplies, and occasionally in filtered drinking water samples.
2. Primary (chlorination-only) water treatment facilities were inefficient at reducing parasite levels from finished drinking water, while secondary (filtration) plants reduced parasite concentrations by approximately 1 $\log_{10}$.
3. *Cryptosporidium* sp. showed significant seasonality, with greater concentrations detected in the spring and autumn seasons.
4. *Cryptosporidium* sp. levels were significantly correlated with *Enterococcus* sp. ($r=-0.338$, $P=0.02$), and with heterotrophic plate counts ($r=0.272$, $P=0.036$) in raw water. *Giardia* sp. was not significantly correlated with any of the nine microbial indicators of water quality that were monitored.
5. *Cryptosporidium parvum* was able to survive up to four days in treated water effluent. Oocyst viability was significantly inversely correlated with free chlorine levels in treated water, but was not affected by seasonal water temperatures.
6. *Cryptosporidium parvum* was able to survive as long as seven weeks in raw water. Microbial populations from the St. Lawrence River had a detrimental effect on oocyst survival. Oocysts aged for two or more weeks in raw water did not seem more susceptible to chlorine or monochloramine inactivation.

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APPENDIX A

Microbial Indicators of Water Quality:
List of agars used, incubation temperatures, and length of incubation

Indicator	Agar Used (Company)	Incubation Temperature	Length of Incubation
Aeromonas sp.	Aeromonas Agar Base (Oxoid)	37°C	48 hours
Clostridium perfringens	Mixed according to Payment and Franco[120]	37°C (Anaerobic)	24 hours
Enterococcus sp.	KF (Difco)	37°C	24 hours
Pseudomonas sp.	Pseudomonas P (Difco)	37°C	24 hours
Fecal coliforms	mFC (Difco)	44.5°C	24 hours
Total coliforms	MT7 (Difco)	37°C	24 hours
HPC	R2A (Difco)	21°C	5-6 days

Appendix B - Survey (1995-96)

Site	Date	Aeromonas per 100mL	Clostridium per 100mL	C.p. spores per 100mL	Fecal colif. per 100mL	Enterococcus per 100mL	Total colif. per 100mL	HPC per 100mL	Pseudom. per 100mL	Coliphage per 100mL	Crypto per 100L	Giardia per 100L
Plant A	95-08-23	<1	<1	<1	<1	<1	<1	1	N/D	<20	1.25	8.75
Plant A	95-10-04	<1	<1	<1	<1	<1	<1	<1	<1	<20	2.26	<2.26
Plant A	95-11-01	<1	<1	<1	<1	<1	0.5	3.5	<1	<20	5	5
Plant A	96-04-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	6.67	3.33
Plant A	96-05-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22.9	17.1
Plant A	96-06-20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	12.5	<12.5
Plant A	96-07-17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10	10
Plant A	96-08-14	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<20	<20
Plant A	96-09-11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10	10
Plant B	95-07-26	<10	<10	1	<10	<1	<1.0E+3	<1.0E+4	N/D	8.00E+01	20	8
Plant B	95-09-13	<1	<1	<1	<1	<1	<1	1.5	<1	<20	10	15
Plant B	95-10-19	<1	<1	<1	<1	<1	<1	34.5	<1	<20	30	30
Plant B	96-05-15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	29	19.3
Plant B	96-06-12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	30	10
Plant B	96-07-10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	20	<10
Plant B	96-08-07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	25	<25
Plant B	96-09-04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10	<10
Plant B	96-10-02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10	<10
Plant C	95-08-30	<1	N/D	N/D	<1	<1	<1	<1	<1	<20	<1.7	<1.7
Plant C	95-10-11	<1	<1	<1	<1	<1	<1	1.5	<1	<20	1.24	<1.24
Plant C	95-11-08	<1	<1	<1	<1	<1	<1	1.5	<1	<20	<0.8	<0.8
Plant C	96-05-01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<4	<4
Plant C	96-05-29	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.94	<1.94
Plant C	96-06-26	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<4.2	<4.2
Plant C	96-07-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<1.6	<1.6
Plant C	96-08-21	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<2.5	<2.5
Plant C	96-09-18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<2.5	<2.5
Plant D	95-09-22	<1	<1	<1	<1	<1	<1	6	<1	<20	320	3.33
Plant D	95-10-25	<1	<1	<1	<1	<1	<1	<1	<1	<20	<0.4	<0.4
Plant D	95-11-30	<1	<1	<1	<1	<1	<1	<1	<1	<20	<0.5	<0.5
Plant D	96-05-08	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<0.52	<0.52
Plant D	96-06-05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<0.66	<0.66
Plant D	96-07-03	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<0.4	<0.4
Plant D	96-07-31	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<2.22	<2.22
Plant D	96-08-28	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<0.6	<0.6
Plant D	96-09-25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	<0.5	<0.5

Appendix B - Survey (1995-96)

Site	Date	Aeromonas per 100mL	Clostridium per 100mL	C.p. spores per 100mL	Fecal colif. per 100mL	Enterococcus per 100mL	Total colif. per 100mL	HPC per 100mL	Pseudom. per 100mL	Coliphage per 100mL	Crypto per 100L	Giardia per 100L
Site 1	95-08-23	6.95E+03	2.5	1	<10	85	1.65E+03	1.25E+05	N/D	10	N/A	N/A
Site 1	95-10-04	6.20E+03	2	<1	53	1	49.5	8.41E+05	<1	10	30	6.67
Site 1	95-11-01	2.17E+03	1	<1	25.5	1	42.5	2.81E+05	5	<20	95	80
Site 1	96-04-24	660	2	1	5.5	1	475	3.10E+05	<1	<20	8.33	1.67
Site 1	96-05-24	540	1	1	6	<1	4	4.25E+05	<1	20	30	20
Site 1	96-06-20	4.80E+03	2	1	11	29	3.10E+02	1.71E+05	<1	<20	25	<25
Site 1	96-07-17	1.04E+04	1	<1	20	54.5	7.15E+02	4.35E+05	<1	<20	10	10
Site 1	96-08-14	N/A	1	<1	23.5	8.50E+00	1.88E+03	3.75E+05	<1	N/D	<4.0	4
Site 1	96-09-11	3.35E+03	N/D	N/D	16	3.30E+02	3.75E+02	1.92E+05	1	<20	5	10
Site 2	95-07-26	8.20E+04	16	7.5	3.46E+03	3.50E+03	2.10E+04	5.94E+06	N/D	2.40E+02	13.3	5
Site 2	95-09-13	2.23E+04	1	1	7.85E+02	9.10E+02	4.60E+04	4.35E+06	<1	30	37.5	32.5
Site 2	95-10-19	1.30E+04	5.5	1.5	30.5	18.5	1.35E+04	2.42E+06	<1	<20	200	100
Site 2	96-05-15	2017.5	1	1	4	2	35.5	9.55E+05	1	<20	90	20
Site 2	96-06-12	1.87E+03	N/D	N/D	74.5	7	1.59E+03	8.00E+04	<1	30	6.7	6.7
Site 2	96-07-10	4.80E+03	1	2	11	30	6.80E+02	1.60E+05	<1	20	5	<2.5
Site 2a	96-08-07	1.42E+04	3.5	<1	65	N/A	2.60E+02	1.37E+06	<1	40	12.5	<12.5
Site 2b	96-08-07	1.42E+04	3.5	<1	65	N/A	2.60E+02	1.37E+06	<1	40	16.7	<16.7
Site 2	96-09-04	1.39E+04	3	1	46.5	9	8.40E+02	2.43E+05	<1	<20	6.7	<6.7
Site 2	96-10-02	2.45E+04	1.5	3.5	49	2.40E+02	1.09E+04	1.55E+06	1	<20	40	10
Site 3	95-08-09	2.18E+05	27	N/D	60	260	1.62E+05	2.52E+07	N/D	<20	35	5
Site 3	95-09-06	2.00E+03	2	2	6.5	2	6.00E+02	2.00E+05	<10	<20	52.5	27.5
Site 3	95-10-04	1.11E+04	4	<1	12	4	6.90E+02	6.80E+05	<1	10	50	33.3
Site 3	96-05-15	480	2.5	1	1	<1	59	6.35E+05	<1	<20	95	40
Site 3	96-06-12	1.89E+04	N/D	N/D	34.5	19.5	5.01E+03	6.30E+05	<1	20	13.3	6.7
Site 3	96-07-10	1.71E+04	5	4	51	N/A	5.35E+03	4.15E+05	<1	<20	<12.5	12.5
Site 3	96-08-07	3.07E+04	3	<1	25	1.07E+02	5.70E+02	5.22E+05	<1	<20	16.7	<16.7
Site 4	95-08-09	1.02E+04	1.5	N/D	<10	55	1.72E+04	7.07E+06	N/D	<20	20	5
Site 4	95-09-06	4.75E+03	4.5	<1	15.5	<1	3.60E+02	1.83E+06	<10	<20	40	30
Site 4	95-10-11	1.05E+03	1.5	<1	25.5	1	5.30E+02	8.05E+04	<1	<20	70	35
Site 4	96-04-24	8.25E+02	6	2.5	2	1	57	5.18E+05	<1	<20	30	5
Site 4	96-05-24	910	<1	<1	1	<1	4	3.90E+05	<1	<20	1200	490
Site 4	96-06-20	2.59E+03	1	1	6	<1	4.50E+02	1.90E+05	<1	<20	6.7	3.3
Site 4	96-07-17	1.29E+04	1.5	<1	25.5	49.5	9.95E+02	4.09E+05	<1	<20	12.5	12.5
Site 4	96-08-14	1.25E+04	2.5	<1	16	4	9.00E+02	8.40E+05	<1	N/D	25	<12.5
Site 4	96-09-04	1.56E+04	3.5	1	28.5	27	1.07E+04	1.24E+05	<1	<20	<10	<10
Site 4	96-09-11	1.31E+04	N/D	N/D	30.5	41	1.45E+03	1.30E+05	<1	<20	20	<10

Appendix B - Survey (1995-96)

Site	Date	Aeromonas per 100mL	Clostridium per 100mL	C.p. spores per 100mL	Fecal colif. per 100mL	Enterococcus per 100mL	Total colif. per 100mL	HPC per 100mL	Pseudom. per 100mL	Coliphage per 100mL	Crypto per 100L	Giardia per 100L
Site 5	95-08-30	1	N/D	N/D	<1	<1	<1	85.5	<1	10	30	50
Site 5	95-10-11	1.13E+03	1.5	<1	26.5	7	33.5	3.44E+05	1.5	40	40	16.7
Site 5	95-11-08	9.45E+02	1	<1	46	18	52.5	1.70E+05	<1	<20	30	15
Site 5	96-05-01	130.5	2.5	<1	18.5	6.5	36	1.10E+05	<1	<20	16.7	6.7
Site 5	96-05-29	9.90E+02	3	1	23.5	<1	33	3.80E+05	<1	<20	80	20
Site 5	96-06-26	1.55E+03	1.5	1	23.5	103	29.5	1.73E+05	<1	<20	5	10
Site 5	96-07-24	8.80E+03	10.5	3	39.5	26	1.22E+03	2.88E+05	1	70	6.7	<6.7
Site 5	96-08-21	3.35E+03	1	1	27.5	22	1.70E+02	2.33E+04	1	20	5.6	<5.6
Site 5	96-09-18	7.10E+03	<1	<1	48.5	15	2.10E+02	4.11E+05	<1	<20	13.3	13.3
Site 6	95-08-16	1.07E+04	2	1	2.46E+03	1.35E+02	1.42E+04	3.10E+04	N/D	<20	31.4	10
Site 6	95-09-20	2.71E+03	5	1	20	2	2.05E+02	4.03E+05	3	<20	25	31.7
Site 6	95-10-25	1.22E+04	1	1	5.00E+02	5	NA	3.21E+05	<1	10	20	16.7
Site 6	96-05-01	1455	25.5	29	89	1.5	19	2.88E+05	<1	<20	30	10
Site 6	96-05-29	2.92E+02	13	10	140.5	<1	N/A	1.70E+06	<1	<20	25	25
Site 6a	96-06-26	9.95E+02	2	1.5	58	2	44	3.65E+04	<1	<20	5	5
Site 6b	96-06-26	9.95E+02	2	1.5	58	2	44	3.65E+04	<1	<20	5	<5
Site 6	96-07-24	4.60E+03	11.5	5	15	6.5	6.40E+02	N/A	<1	<20	10	10
Site 6	96-08-21	7.70E+03	2	1	17.5	2.61E+02	70	2.80E+05	<1	<20	12.5	<6.25
Site 6	96-09-18	6.95E+03	2	2	44.5	5	2.05E+02	3.46E+05	1	<20	13.3	<6.7
Site 7	95-09-22	5.09E+05	6.5	5	495	1.04E+02	1.48E+04	2.34E+06	<1	<20	25	35
Site 7	95-10-25	1.16E+04	1	1	2.10E+02	1	8.00E+02	2.93E+05	<1	10	85	<5
Site 7	95-11-30	94	<1	<1	<1	<1	3.5	4.15E+03	<1	<20	50	20
Site 7	96-05-08	1425	2	3	1380	1.5	255	3.44E+05	2	<20	32.5	5
Site 7	96-06-05	4.15E+03	2.5	4	2.20E+02	<1	390	1.25E+05	<1	<20	30	30
Site 7	96-07-03	2.77E+03	1	1	50	2	35	8.65E+04	<1	<20	33.3	16.7
Site 7	96-07-31	1.13E+04	4.5	5	92	19.5	4.70E+02	1.35E+05	1.5	<20	<25.0	25
Site 7	96-09-25	5.05E+03	2	<1	3.00E+02	3	5.60E+02	2.61E+05	<1	<20	13.3	13.3
Site 8	95-08-16	2.32E+04	3.5	<1	<10	80	8.00E+02	4.00E+05	N/D	20	10	6.67
Site 8	96-06-05	1.49E+04	N\A	21	10	<1	N\A	9.01E+06	<1	<20	36.5	73
Site 8	96-07-03	4.55E+03	1	2	43.5	62.5	79.75	3.14E+05	<1	<20	3.33	3.33
Site 8	96-07-31	6.35E+04	1.5	1	4	57.5	N/A	1.27E+06	<1	<20	<12.5	<12.5
Site 8	96-08-28	1.84E+04	9.5	1	75.5	64.5	5.30E+02	4.65E+05	<1	N/D	<8.3	<8.3

APPENDIX C

Student t-test for Comparing Independent Regression Lines

Linear regression analysis was used to compare *Cryptosporidium parvum* viability over time for the various treatment groups in the *in situ* survival studies involving raw water and treated water.

For each treatment group, the $\log_{10}$ reduction in *C. parvum* viability was plotted against time, using SigmaPlot® software (Jandel Corporation, San Rafael, CA). Linear regression lines were generated for each treatment using the same program. The slope of the line (B) was obtained from the regression equation, and the standard error of the slope (SE_B) was generated by the software. Once these values were obtained, the independent regression lines could be compared using the t-test, as follows:

$$t = \frac{B1 - B2}{(SE_{B1}^2 + SE_{B2}^2)^{1/2}}$$

Note: t has $(n_1 + n_2 - 4)$ degrees of freedom, where n equals the number of sampling points making up the regression line

Example:

From: *In situ* survival of *Cryptosporidium parvum* in treated water. Trial 1

Control. hard water (Ingleside) $B1 = -0.065$

$SE_{B1} = 0.0219$

Cassette sample (Ingleside): $B2 = -0.47$

$SE_{B2} = 0.0406$

$$t = \frac{-0.065 - (-0.47)}{(0.0219^2 + 0.0406^2)^{1/2}}$$

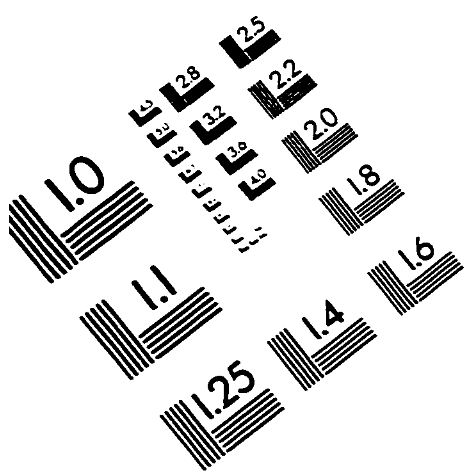
$$t = \frac{0.405}{(0.00213)^{1/2}}$$

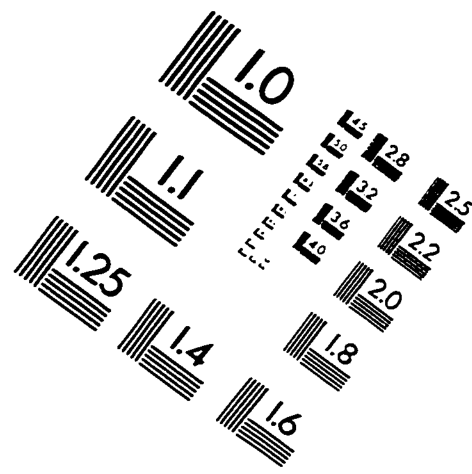
$$t = 8.78$$

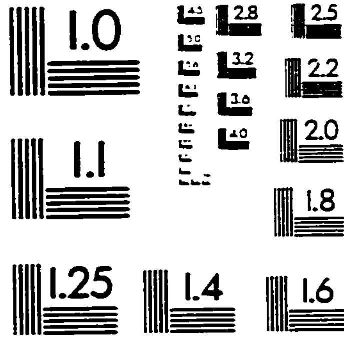
From t-tables: $t_{6\,(0.05)} = 2.45$

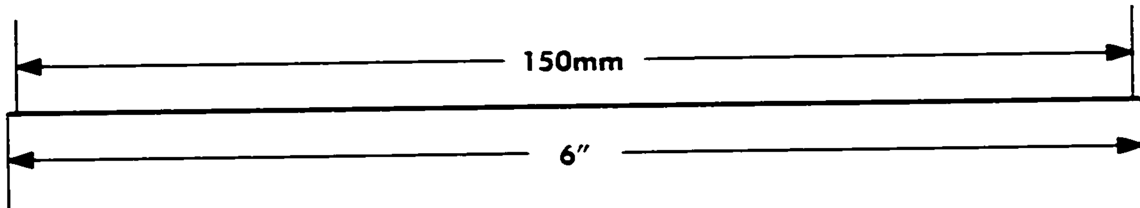
$t > t_6$ and therefore $P<0.05$

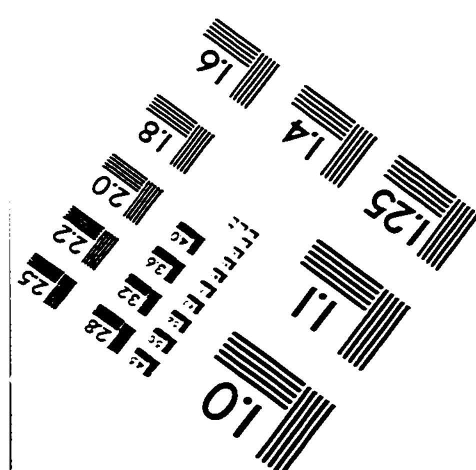
IMAGE EVALUATION
TEST TARGET (QA-3)











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