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STUDIES ON THE SURVIVAL AND VIABILITY TESTING OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS IN THE WATER ENVIRONMENT

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A Thesis Submitted to the School of Graduate Studies
University of Ottawa
In Partial Fulfillment of the Requirement for the Degree of Master of Science
Department of Microbiology and Immunology, Faculty of Medicine

Dedicated to my parents, Judit and Dezsö; for their continued support.

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LIST OF ABBREVIATIONS

$CaC0_3$:	Calcium carbonate
CCD:	Cooled charge couple device
CFU:	Colony forming units
ddH_20:	Double distilled water
$DiBAC_4(3)$:	bis-(1.3-dibutylbarbituric acid) trimethine oxonol
DMSO:	Dimethylsulfoxide
FACS:	Fluorescence activated cell sorting
FITC:	Fluorescein isothiocyanate
Gy:	Gray
kGy:	Kilo-Gray
HBSS:	Hank's balanced salt solution
HPC:	Heterotrophic plate count
MAb:	Monoclonal antibody
mL:	Milli-Litre
NGS:	Normal goat serum
PCR:	Polymerase chain reaction
PBS:	Phosphate buffered saline
ppm:	Parts per million (milligram per litre)
PI:	Propidium iodide

µL:	Microlitre
s.e.m.:	Standard error from the mean
TOC:	Total organic carbon
TSB:	Tryptic soy broth

ABSTRACT

Since the major outbreak of cryptosporidiosis in Milwaukee, WI, several others have been reported including those in Collingwood, ON, and in Kelowna, BC. Concentrations of *Cryptosporidium* oocysts in source waters, especially surface sources, are of great concern to the drinking water supply industry. Understanding *Cryptosporidium* survival in such waters can help to assess the treatment challenges and health risks from potential or actual sources of contamination. Several studies have suggested that oocysts can survive for weeks to months in natural or tap water. One of the objectives of this study was to assess if differences in water chemistry, biology or seasonal variations affect oocyst survival.

Three watersheds, the Grand River, the St. Lawrence River, and the Carp River, all in Ontario, Canada, were selected to assess the survival of oocysts in waters with different geography. Water samples were collected from each river at two locations, one upstream from the urban district, the other at or downstream from the urban district. Carp River water was sampled within one kilometer of the origin of the river and at its mouth, where it empties into the Ottawa River. Water was sampled from each location within each watershed during different seasons of the year and seeded with 5×10^5 *Cryptosporidium* oocysts/mL. Synthetic hard water (100 ppm as $CaCO_3$, pH 7.0) seeded with an equivalent number of oocysts served as the control for each experiment. Survival of seeded oocysts was determined by a standardized *in vitro* excystation assay combined with total oocyst counts; bacterial heterotrophic plate counts (HPC) at the start of each experiment were determined on R2A agar and counted after 5 days of incubation at room

temperature. All water samples were sent to a commercial laboratory for chemical analysis. Water samples collected in the summer were incubated at 20°C and 30°C, winter water samples were incubated at 4°C and 20°C and spring water samples were also incubated at 4°C and 20°C.

Results indicate that water chemistry does not appear to be an important factor for *in vitro* oocyst survival ($p>0.05$). Temperature, season of water sampling, river, site within a river, and the oocyst suspension all had a measurable effect for the *in vitro* survival of oocysts in natural waters. However, only temperature had a "cross-the board" effect. All other factors were important for *in vitro* oocyst survival only under specific conditions. Test waters held at 4°C enhanced oocyst survival compared to test waters at 20°C and 30°C ($p<0.05$) while test waters with higher initial HPC levels appeared to have a detrimental effect on oocyst survival.

In vitro excystation is time consuming and requires a skilled microscopist. Moreover, oocyst viability as determined by *in vitro* excystation, may not necessarily be synonymous with the ability of the oocysts to infect a host. In lieu of this, a new oocyst viability assay was investigated using flow cytometry, due to its ease and speed of sample processing. *Cryptosporidium* oocysts were double-stained, first using a monoclonal antibody specific for the oocyst wall; this antibody was conjugated to the fluorochrome Cy3. The second stain, $DiBAC_4(3)$, a new vital dye that is sensitive to changes in cellular membrane potential, was assessed for its usefulness in oocyst viability studies. Viability results obtained from flow cytometric analysis were compared to *in vitro* excystation results over three different treatments. The first treatment, aging, followed two separate oocyst cultures over time where viability was determined once per week over 5 weeks.

The second treatment used oocysts heated at 55°C over a course of 180 seconds, with 6 sampling points in total. The third treatment involved gamma irradiation of oocysts, up to an exposure of 16596 Gy.

Results indicate that flow cytometry using this double-stain technique was comparable to *in vitro* excystation while following two cultures over time, similar trends were obtained with the two techniques over gamma irradiation, while heat treatment studies led to variable results. Therefore $DiBAC_4(3)$ appears to show promise as a vital stain in oocyst studies. Additionally, flow cytometry proved to be very rapid with the potential to process hundreds of samples within a day. Also, use of the monoclonal antibody to identify oocysts within a sample using flow cytometry was shown to identify a relatively clean population of oocysts. Therefore, flow cytometry appears to be a promising technology for use in oocyst viability studies, once an adequate staining assay, possibly involving $DiBAC_4(3)$, is developed.

GENERAL INTRODUCTION

Cryptosporidium was first identified as a cause of human gastroenteritis in 1976 (99), and since then, it has been implicated in numerous outbreaks of the disease. Since 1984, *Cryptosporidium* has been implicated in diarrheal disease outbreaks associated with recreational waters (1,47,88,127), day care centres (4,132), a day camp (106), travelers (66), agricultural animal handling (80,91), hospitals (70) and contaminated water supplies (35,47,58,83,123,125). The 1993 waterborne outbreak in Milwaukee, WI affected 403,000, with over 100 deaths (24), in a community of about 800,000 (52,83). Recently, there have been two large outbreaks in Canada, one in Collingwood, ON (2), and another in Kelowna, BC, with over 13,000 people affected (111). There have been very few reports of foodborne *Cryptosporidium* infection (143); however, it was recently associated with outbreaks due to the consumption of chicken salad (12) and apple cider (89).

Because of the potential of *Cryptosporidium* oocysts to infect a very large number of people over a very short period of time through contaminated drinking water, it is crucial to understand the survival capabilities of oocysts in the water environment. This would enable water utilities to determine the type of corrective measures required if oocysts are detected in the water supply. Since very few studies have been conducted to assess oocyst survival in raw and treated tap waters (109), the first objective of this study was to assess *in vitro* survival of oocysts in waters of different chemistry over different seasons of the year, and at different sites within a river, to determine the effect of such factors on oocyst survival.

The "gold standard" of *Cryptosporidium* oocyst viability studies is ultimately animal infectivity. This procedure is expensive, very lengthy and has a high degree of biological and statistical variability. Because of this, a new, rapid technique is required that would give comparable viability (infectivity) results with animal studies, yet be simple, quick and inexpensive. Flow cytometry is a very rapid and simple method for analyzing and processing liquid samples. This technology is ideally suited for use in oocyst survival studies since it can process a large number of samples in a short period of time. Historically, flow cytometry has had limited use in *Cryptosporidium* research and has only been used as a detection (136,138,139) and enumeration tool (7); it has only recently been used for viability studies. These preliminary studies still rely on the lengthy and subjective microscopic confirmation step (61). Therefore, the second objective of this study was to develop a rapid method of oocyst viability assessment using flow cytometry that would yield results equivalent to those obtained by an acceptable viability assay over a number of different stresses.

Because animal infectivity studies are expensive and labour intensive, *in vitro* excystation was chosen as the method of viability assessment to compare with flow cytometry results. *In vitro* excystation examination is also labour intensive, yet it is inexpensive and quicker to perform. Although studies have shown that oocyst viability results obtained from the *in vitro* excystation procedure do not correspond to animal infectivity (45), it is commonly used because of the increased speed and lower cost of the procedure.

REVIEW OF THE LITERATURE

Introduction

Cryptosporidium is a protozoan that has many stages in its life cycle, all completed within a single suitable host. The infective stage, the oocyst, is quite stable outside the host and is also resistant to levels of chlorine used in drinking water treatment (23,71). This gives it considerable potential to spread through potable waters. Therefore, it is imperative that this organism's ability to survive in various water environments be examined to understand what factors may contribute to its inactivation.

Biology of *Cryptosporidium*

Cryptosporidium was first reported in 1907 by Tyzzer (133). Its taxonomical position is given in Figure 1. There are 11 species presently in the *Cryptosporidium* genus, but there is only the one genus within the family (97). Unlike *Sarcocystis, Toxoplasma, Eimeria and Isospora,* the many life cycle stages of *Cryptosporidium* are completed within a single host (113). The oocyst stage however, is of particular importance since it is the oocysts that contain and release the infective sporozoites (33).

Cryptosporidium oocysts are smaller when compared to those of other coccidian oocysts. They measure 4-6 μm in diameter (81), contain one residual body and four sporozoites which measure approximately 1 μm in diameter each (81). Once a suitable host ingests a viable oocyst, the oocyst passes through the digestive system to the intestine where it undergoes excystation. This results in the release of sporozoites into the intestinal lumen. The sporozoites then penetrate intestinal epithelial cells and pass into

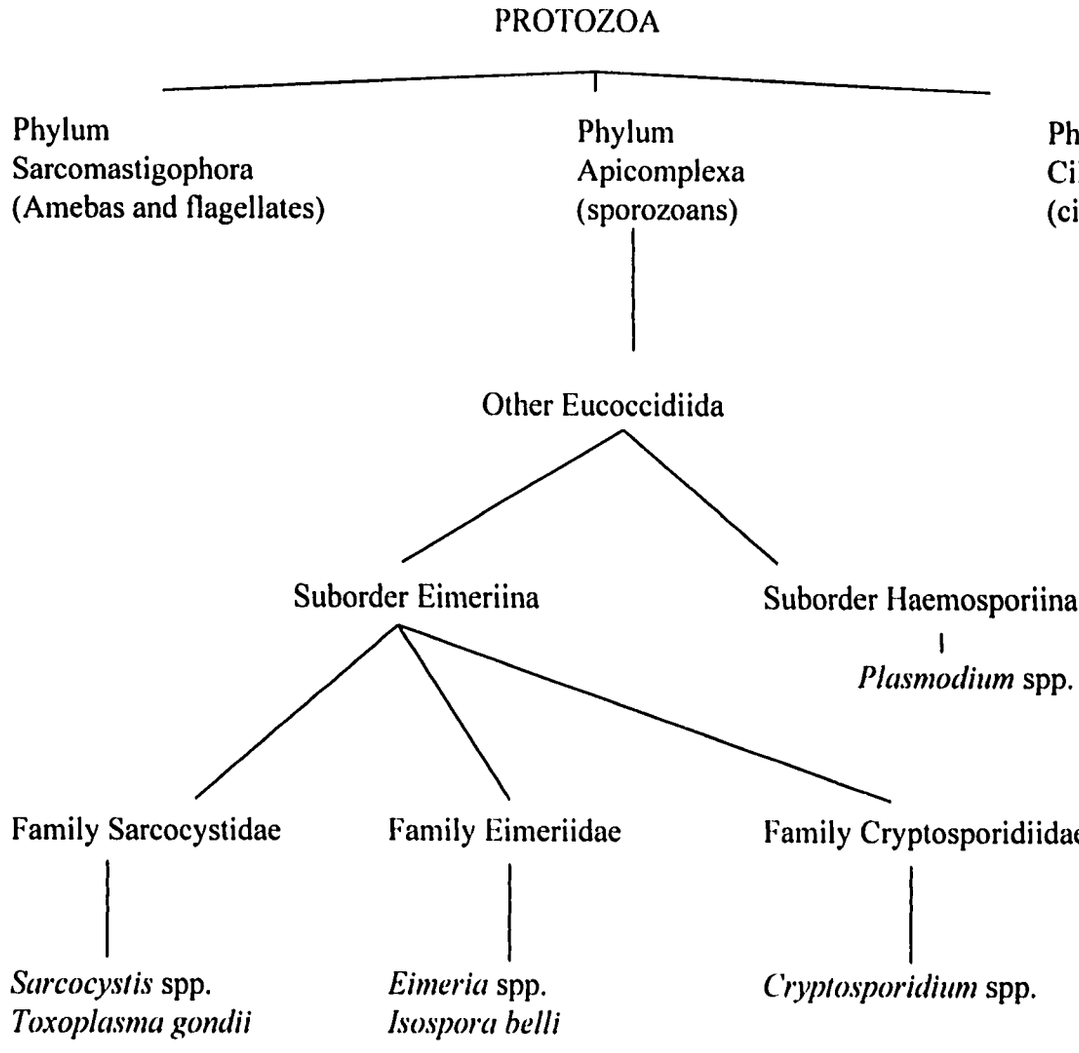


Figure 1: Taxonomic position of *Cryptosporidium*. Modified from (97)

the next stage of development (Figure 2).

Casemore et al. (28) and Current et al. (33) have described in detail the life cycle and morphology of *Cryptosporidium.* It must be noted however, that at completion of the developmental stages indicated in Figure 2, fresh oocysts are produced. Oocysts therefore enter the environment by being shed in the feces, and hence, cryptosporidial infection occurs via the fecal-oral route.

Survival and Inactivation of *Cryptosporidium*

Because of the health risks associated with cryptosporidial infection, much time and effort have been spent on research to determine how *Cryptosporidium* oocysts are inactivated. The oocysts are highly resistant to a number of environmental pressures, such as temperature (109), differing aquatic environments (109), UV radiation (82), and mechanical abrasion such as with sand (102). They are also very resistant to chemical treatment (23,39,40,59,71). Table 1 outlines the current state of knowledge with regard to oocyst survival and inactivation. It appears that oocysts are most susceptible to inactivation through heating, while showing varying levels of resistance to other methods of decontamination. It must be noted that studies that have looked at oocyst survival in natural conditions (e.g. water, freezing), found that a portion of the oocysts present still remained viable at the end of the treatment (41,109,134). This implies that in the natural environment, there is inactivation of oocysts, but a portion still retains the potential to be infective and hence cause illness.

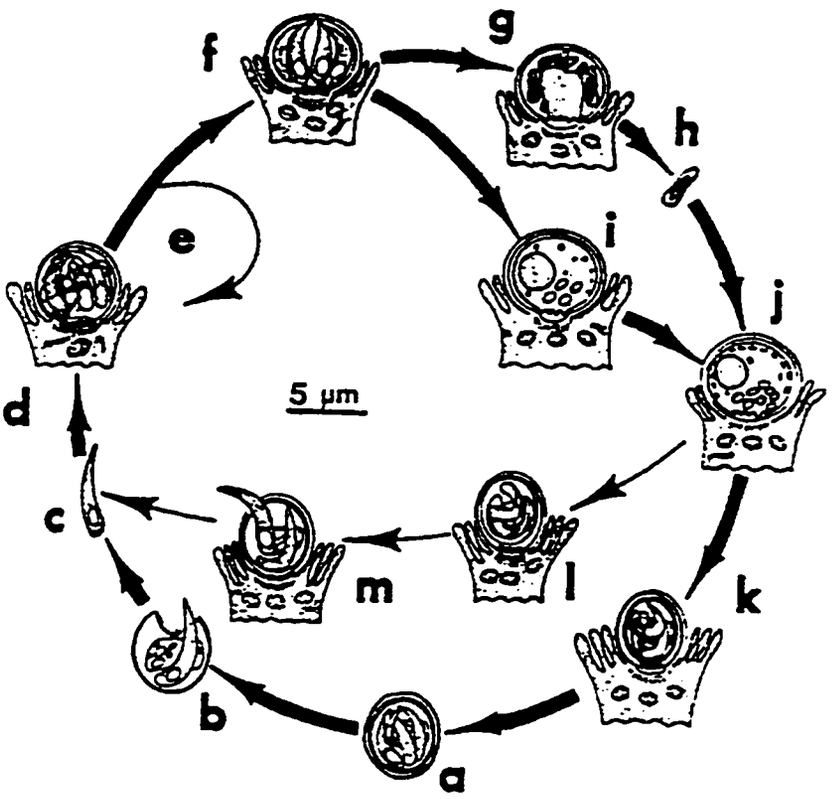


Figure 2: The life cycle of human and calf isolates of *Cryptosporidium parvum* in experimentally infected mice. (a) Sporulated oocyst in feces; (b) excystation in intestine; (c) free sporozoite in intestine prior to penetration into the microvillous region of an ileal enterocyte; (d) type I meront (6 or 7 merozoites); (e) recycling of type I merozoite; (f) type II meront (4 merozoites); (g) microgamont with approximately 16 microgametes; (h) microgamete fertilizes macrogamete (i) to form (j). Approximately 80% of the zygotes form thick-walled oocysts (k) which sporulate within the host cell. Almost all thick-walled oocysts pass unaltered in the feces and are the resistant forms that transmit the infection to another host. About 20% of the zygotes do not form an oocyst wall; their sporozoites are surrounded by a unit membrane (l). Sporozoites within autoinfective, thin-walled oocysts (l) are released into the intestinal lumen (m) and reinitiate the endogenous cycle (at c). Taken from (33).

Table 1: Oocyst Survival and Inactivation

Treatment	Level of Inactivation	Method of Viability Assessment	Reference
Heating and Freezing			
Heating above 65°C	complete	infectivity[1]	(134)
Heating at 64.2°C for 2 minutes	complete	infectivity	(38)
Heating at 72.4°C for 1 minute	complete	infectivity	(38)
Heating at 45°C for 5 minutes	complete	infectivity	(5)
Heating at 55°C for 4 minutes	complete	infectivity	
Pasteurization (71.7°C for 15, 10 and 5 s)	complete	infectivity	(57)
Freezing below 0°C for >30 minutes	complete	infectivity	(134)
Freezing at -22°C for 32 days	incomplete	infectivity	(109)
Freezing at -15° for 168 hr, -20°C for 24 hr,-70°C for 1 hr	complete	infectivity	(41)
Freezing at -10°C for 8, 24 and 168 hr, -15°C for 8 and 24 hr and -20°C for 1, 3 and 5 hr	incomplete	infectivity	(41)
Survival in Water			
Tap water (176 days)	incomplete	vital dye[2]	(109)
Natural water (coast of Scotland) (176 days)	incomplete	vital dye	(109)
River water (176 days)	incomplete	vital dye	(109)
Mechanical Abrasion			
Shaking oocysts with sand for 5 minutes	50% viable	vital dye	(102)
Shaking oocysts with sand for 90 minutes	0.3% viable	vital dye	(102)
Mechanical abrasion with sand for up to 2 hours	complete	vital dye	(102)

[1]infectivity of mice
[2](DAPI/PI)

Treatment	Level of Inactivation	Method of Viability Assessment	Reference
Irradiation			
UV irradiation at 15,000 mw/sec for 2.5 hours	complete	infectivity	(82)
UV irradiation at 8748 mW scm^{-2}	<1% viable	vital dye, *in vitro* excystation	(22)
Pulsed light (1 joule/cm^2 in 1 flash treatments)	<0.1-0.0001% viable	infectivity	(8)
Chemical Treatment			
3% Cresylic acid (18 hr exposure)	incomplete	oocyst shedding[3]	(23)
2-5% Hypochlorite solution	incomplete	oocyst shedding	(23)
10% Formaldehyde	complete	oocyst shedding	(23)
5% Benzylkonum chloride	incomplete	oocyst shedding	(23)
5-10% Ammonia	complete	oocyst shedding	(23)
0.02M Sodium Hydroxide	incomplete	oocyst shedding	(23)
1-4% Iodophore	incomplete	oocyst shedding	(23)
1% "Virkon" (per-oxygen)	incomplete	*in vitro* excystation	(59)
6% "Sactimed" (quaternary ammonium)	incomplete	*in vitro* excystation	(59)
2% "Cidex" (glutaraldehyde)	incomplete	*in vitro* excystation	(59)
"Phoraid" (0.034% iodine)	incomplete	*in vitro* excystation	(59)
5% "Pentapon DC1" (beta-ene)	complete	*in vitro* excystation	(59)
5% "Pentapon HDY (beta-ene)	incomplete	*in vitro* excystation	(59)
"Clorox" (5.25% aqueous sodium hypochlorite) for 120 min. (21°C)	incomplete	infectivity	(39)

[3]infected rats

Treatment	Level of Inactivation	Method of Viability Assessment	Reference
80 ppm chlorine treatment for 90 minutes	10% viable	*in vitro* excystation and infectivity	(71)
1 ppm ozone for 5 minutes	10% viable	*in vitro* excystation and infectivity	(71)
1.3 ppm chlorine dioxide for 1 hour	10% viable	*in vitro* excystation and infectivity	(71)
80 ppm monochloramine for 90 minutes	10% viable	*in vitro* excystation and infectivity	(71)
Saturated atmosphere (21-23°C for 24 hr) of:			
ammonia	complete	infectivity	(40)
carbon monoxide	incomplete	infectivity	(40)
ethylene oxide	complete	infectivity	(40)
formaldehyde	incomplete	infectivity	(40)
methyl bromide	complete	infectivity	(40)
Sequential chemical treatment:			(44)
ozone followed by monochloramine	<1% viable	infectivity	(44)
chlorine dioxide followed by chlorine	<1% viable	infectivity	(44)
chlorine followed by monochloramine	<10% viable	infectivity	(44)

Of major concern is the finding that *Cryptosporidium* oocysts are very resistant to chlorine (23,39,71). Therefore, any water treatment facility that uses chlorine as a disinfectant retains the risk of distributing viable oocysts in the public water supplies due to incomplete inactivation of the oocysts. *Cryptosporidium* oocysts however, are inactivated by ozone (45,71,104). Although they are more resistant to ozone than are *Giardia* cysts, inactivation by ozone requires a fairly low concentration of the chemical for a short period of time to achieve disinfection of the oocysts (Table 1) (71). Chlorine

dioxide and monochloramine were also found to be effective at inactivating oocysts at the concentrations and exposure times detailed in Table 1. This suggests that for rapid disinfection, ozone alone or a sequential use of disinfectants (44) is required to obtain the desired anticryptosporidial effect.

Another method of inactivating *Cryptosporidium* oocysts is to release the sporozoites from within the oocyst into the water environment by mechanical abrasion. Shaking oocysts with sand for 5 minutes can decrease the number of viable oocysts to 50% and increasing shaking time to 90 minutes decreases viability to 0.3% (Table 1) (102). Parker and Smith (102) note that although this experimental shaking of *Cryptosporidium* oocysts with sand is a more severe treatment than occurs to oocysts while passing through rapid sand filters of water treatment facilities, they stress that the collision that occurs between the oocysts and the sand of the filters makes the oocysts more susceptible to subsequent chemical disinfection, thus requiring lower concentrations of each of the disinfectants compared to using the disinfectants separately, to kill the oocysts. Also, any sporozoites released from oocysts rupture shortly after release into water and are therefore no longer viable (33).

Predation of bacteria by protozoans and other microscopic water organisms has been examined previously and reports show that protozoans feed extensively on and therefore have serious impact on the survival and numbers of resident bacteria (10,14,54,86,117,141). However, there is little information on the converse. Very few studies address the possibility of other microorganisms, particularly bacteria, seriously affecting the survival of protozoans such as *Cryptosporidium* in their natural environment. One recent study has found that *Serratia marcesens* is a very effective

predator of *Cryptosporidium* and kills oocysts by their chitinolytic activity (144). Another study suggests that zooplankton may be an important predator of protozoans in a fresh water ecosystem (25). This relationship has also been observed by Stoecker and Mcdowell-Capuzzo (131). Therefore, if we are to gain a better understanding of the factors that affect *Cryptosporidium* oocyst survival in the environment, the microbial ecology of the water environment must also be studied.

Occurrence of *Cryptosporidium* in the Environment

The occurrence of *Cryptosporidium* in animals and humans is evidently worldwide, having been reported in all continents (81). Its occurrence in the environment, however, seems to be related to areas that have had contact with either human or animal feces. The presence of *Cryptosporidium* in the environment is mainly related to water. Table 2 outlines the surveys reported in the literature to determine the concentrations of oocysts found in natural water systems.

Rose (114) and LeChevallier et al. (76) have found that waters receiving sewage effluent have higher *Cryptosporidium* concentrations than those that do not receive such effluents. Also, waters receiving run off from agricultural lands where cattle are present, have with up to 184 times greater concentrations (114) of oocysts than in those that do not (56,100,114). It has also been shown that *Cryptosporidium* oocysts have a continuous presence in water sources and do not just appear intermittently (56).

Graczyk et al. (51) have recently proposed that birds may also be a carrier for *Cryptosporidium* oocysts. They found that ducks (*Anas platyrhynchos*) orally inoculated with infectious oocysts (non-avian strain) did not support oocyst development, but did

excrete infective, inoculum-derived oocysts in the feces, 7 days post inoculation. The significance of this is that birds, particularly waterfowl, that ingest infective oocysts, can spread these oocysts to many different geographical locations and contaminate water sheds, possibly explaining why even in so-called pristine water systems, *Cryptosporidium* oocysts can be found. Therefore, waterfowl must also be taken into consideration when determining the sources of oocyst contamination.

Table 2: Occurrence of *Cryptosporidium* Oocysts in Natural Waters

Location	**Concentration (oocysts/L)**	**Reference**
Yukon, Canada	0-0.005	(108)
Ottawa and Rideau Rivers, Ontario, Canada	<0.001-2.25	(30)
British Columbia, Canada	0.005-3	(100)
United States (fresh water)	2-5800	(114)
United States (potable water)	<1-44	(116)
United States (surface water)	<1-5800	(84)
United States (Northwest)	0-0.03	(55)
United States (Hawaii)	0-0.22	(63)
United States (Central to Northeastern)	0.07-484	(76)
U.S. Virgin Islands (Cistern Water)	0.01-0.70	(32)
Scotland (untreated water)	0.006-2.3	(124)

The levels of *Cryptosporidium* oocysts in raw and treated waters peak during certain months of the year (124). It was found that in raw surface waters, oocyst levels peaked in the months of July and February, while in treated waters the oocyst levels were highest in August and November (124). The peak of oocysts in July in raw water did not correspond with any increase in oocyst levels in treated water while the increased oocyst levels in treated water in November was mirrored by an increase in oocyst concentrations in raw water (124). This finding may indicate that oocysts are not able to survive in

warm, summer temperatures. Conversely, oocysts appear to be hardier in colder waters and can thus survive for longer periods. This is evidenced by the fact that in November of the study period, while there was an increase in oocyst levels in raw waters, there was a corresponding increase in oocyst levels in treated waters. This may in part be due to a lowering of efficiency of the water treatment process in colder weather.

An increase in oocyst levels has also been shown to occur within seasons, such that periods of higher rainfall (spring), hence more run-off into water systems, have higher levels of oocysts than periods with lower rainfall (56). As was mentioned previously, the possible sources of oocyst contamination of these water systems could be due to the presence of dairy cattle, wild animals and human activity (56,114). This has interesting implications in determining the risk due to infection associated with *Cryptosporidium*. There seems to be a seasonal peak in oocyst levels, yet there also seems to be a seasonal difference in survival capabilities of *Cryptosporidium*. Therefore these, and probably many other factors must be taken into consideration when assessing the risk of infection.

Because the presence of *Cryptosporidium* in a water supply is a potential health risk, it would be very beneficial to be able to correlate its concentration in water with levels of water quality indicators, such as total coliforms, particle counts, turbidity and pH (115). In one study, it was found that *Cryptosporidium* levels could be correlated to some of these variables; however, the most important variable seemed to be the level of watershed protection on the water system as defined by the number and type of pollution being received by the water system (76); the higher the level of protection, the lower the levels of *Cryptosporidium* oocysts (56,76). However, a useful prediction model could not

be determined on the basis of these parameters (75). The correlation of these variables to *Cryptosporidium* levels was explained to be just a matter of increasing or decreasing water quality (75,76).

Cryptosporidiosis and the Epidemiology of *Cryptosporidium* Infection

The ability of *Cryptosporidium* oocysts to remain viable under various environmental conditions poses significant health risks, with infection resulting in cryptosporidiosis. *Cryptosporidium* infection may be totally asymptomatic or result in loose, watery, self-limiting diarrhea in immunocompetent individuals. Infection in an immunocompromised individual may result in prolonged and profuse diarrhea (72), with a mortality rate as high as 50% (96). Additionally, respiratory infections have been known to occur in immonodeficient individuals (33). *Cryptosporidium parvum* appears to be the principal species causing disease in humans (33).

Cross-species transmission studies of *Cryptosporidium* show that oocysts shed from humans and cattle are able to infect a wide variety of animals whereas avian isolates could not cause infection in mammals (135). In immunocompetent individuals the incubation period averages 7.2 days while the duration of illness averages 12.2 days. Over 90% of both symptomatic days **and** oocyst-positive stool days occur between the 7th and 28th day of infection (65). Additionally, infected individuals with diarrhea shed more oocysts than infected individuals without diarrhea (29).

The minimal infective dose of *Cryptosporidium* was thought to be small (15), ranging from 10-50 oocysts in a primate model (90). A recent study involving healthy

volunteers has shown the ID_{50} (infective dose to infect 50% of the population) to be 132 oocysts (37) with the particular strain of *Cryptosporidium* used.

Person-to-person transmission has been shown to occur in environments such as daycare centres (4,132) and hospital settings (70). Food-associated cryptosporidiosis involving apple cider (89), fresh vegetables (92), raw milk (143) and chicken salad (12) has also been documented. Zoonotic transmission also occurs relatively frequently through contact with farm animals (91,129), but it is contamination of drinking water supplies that poses a significant risk for communities because of the ability to infect large numbers of people over a short period of time.

Cryptosporidiosis due to contaminated drinking water has occurred on numerous occasions over the past several years. Table 3 lists the outbreaks reported in the literature since 1984. The most dramatic outbreak however, was the one in Milwaukee, WI, USA. On this occasion, an estimated 403,000 people contracted cryptosporidiosis because of oocysts passing through the city's water treatment system and into the public drinking water supply (83). It is interesting to note that a survey of 66 surface water treatment plants in the US and Canada (not at the same time as the Milwaukee incident) showed that 27% of the finished drinking water samples were positive for *Cryptosporidium* oocysts, yet there were no outbreaks of cryptosporidiosis reported during the length of the survey (77). If the infective dose of *Cryptosporidium* oocysts is relatively low, why weren't there any reports of cryptosporidiosis when over one quarter of the drinking water supplies surveyed was contaminated with oocysts? A good risk assessment model must be developed to answer such a question.

Table 3: Reported Outbreaks of Cryptosporidiosis Where Water was Implicated

Year	No. of People Affected	Type of Contamination	Location	Water Filtered?	Reference
1984	34% of residents	contaminated artesian well	Braun Station, TX, USA	no	(35)
1987	13,000	unknown	Carrollton, GA, USA	yes	(58)
1988	27	broken fireclay pipe; contaminated finished water reservoir	Ayrshire, UK	?	(123)
1988 -89	516	heavy rainfall	Swindon and Oxfordshire, UK	yes	(107)
1990 -91	47	increased rainfall	Isle of Thanet, UK	?	(67)
1991	44	unknown	South London, England	yes	(85)
1992	125	heavy rainfall, deteriorating water quality, a slow sand filter brought back into service	Bradford, England	yes	(9)
1993	200 1 death	unknown	Kitchener-Waterloo, ON, Canada	yes	(50)
1993	403,000, 104 deaths	snow melt, heavy rains, farm runoff	Milwaukee, WI, USA	yes	(52,83) (18)
1994	78	unknown	Nevada, USA	yes	(49)
1996	157	increased farmland runoff	Collingwood, ON, Canada	no	(2)
1996	13,000	unknown	Kelowna, BC, Canada	no	(111)

Drinking water however, is not the only vehicle of waterborne cryptosporidiosis. Contact with contaminated public swimming/wave pools (88,127) and also contaminated recreational surface waters (47) has resulted in a number of outbreaks of cryptosporidiosis.

There does not appear to be a difference in the rates of infection among males and females of the human population (26). Cryptosporidial infection does however, have an age distribution. It has been noted that young children have a higher rate of infection than the general population, possibly due to greater opportunity for fecal-oral transmission or because of higher susceptibility to infection (42,93,122). A recent study (72) has confirmed this finding. This study also showed that young children attending daycare centres had a higher seroprevalence to *Cryptosporidium parvum* than children of the same age group that did not attend daycare (72). Another interesting finding was that the prevalence of *Cryptosporidium parvum* antibodies increased after the age of fourteen years, with the authors suggesting possible oocyst exposure in this age group through sexual activity (72). It has also been suggested that cryptosporidioisis is distributed over socio-economic status such that individuals with lower socio-economic status have a greater seroprevalence of *Cryptosporidium parvum* antibodies than their higher socio-economic status counterpart (72).

A possible seasonality of cryptosporidiosis has been noted many times. During a statewide laboratory surveillance for *Cryptosporidium* infection in Oregon, it was found that not only was spring a peak period for number of positive stool specimens for *Cryptosporidium* oocysts, but so were summer and early autumn (122). Similar peaks in cryptosporidiosis were found to occur in British Columbia (93). Seasonal *Cryptosporidium* oocysts levels in natural waters have also been noted (124), with increased oocyst levels being observed during periods of higher rainfall (56).

Experimental Methods of *Cryptosporidium* Analysis and Flow Cytometry

Detection and Purification

A number of techniques have been developed to isolate and purify *Cryptosporidium* from fecal and environmental samples, and to determine viability of oocysts after their exposure to various treatments or environmental pressures.

Purification and identification of *Cryptosporidium* from feces is time-consuming, complex and frequently difficult, even for the experienced technician. Because of the large amount of fecal debris and the abundance of other microorganisms in fecal smears, various staining techniques have been developed to aid the technician in the rapid identification of *Cryptosporidium* in feces. Moreover, although techniques such as modified Ziehl-Neelsen, safranin-methylene blue, Giemsa, and phenol-auramine may detect large numbers of oocysts, they are non-specific (27). Immunofluorescence techniques have increased our ability to detect and identify *Cryptosporidium* oocysts in specimens, even when they are present in low numbers (48).

Most immunofluorescence techniques use a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody that is either used directly by being specific for the cryptosporidial oocyst wall (114) or used indirectly by being specific for a primary, non-labeled antibody specific for the oocyst wall (137). The sample is incubated with the labeled monoclonal antibody on a microscope slide and examined by fluorescence microscopy. Oocysts will fluoresce bright green and have an oval shape with a diameter of approximately 6 μm (48). This method is quick and easy to use and does not require a highly experienced technician.

Purification of oocysts from fecal material is also time consuming and requires an experienced technician. Most purification procedures involve a Sheather's sugar flotation to remove fecal debris, followed by a Percoll-sucrose purification step (34). This procedure yields good numbers of *Cryptosporidium* oocysts relatively free of debris. However, it was recently found that the method by which oocysts are concentrated may affect the viability of the resulting, purified culture. Bukhari and Smith (17) have found that a water-ether method of oocyst concentration not only yielded the highest number of oocysts, but also the highest number of viable oocysts over all methods tested. This method may not necessarily be a less-harsh purification procedure, but may only be selectively concentrating healthy oocysts.

Immunofluorescence detection of *Cryptosporidium* oocysts using FITC-conjugated monoclonal antibodies can also be used to detect oocysts in environmental samples. Rose et al. (114) developed a technique using a series of filters to concentrate oocysts from environmental samples. The final polycarbonate membrane filter (pore size 1.2 μm) is then stained with a direct FITC-conjugated monoclonal antibody and then observed by fluorescence microscopy. Stained oocysts will fluoresce bright green and can be easily counted. This number can then be readily related to the volume of sample water passed through the initial filter. This method was slightly modified by Musial et al. (95) by the use of a polypropylene cartridge filter through which the sample water is passed. The filter is then eluted, shredded and subjected to a series of flotations to purify the oocysts. In the final steps, the oocysts are stained with FITC-conjugated monoclonal antibodies and are observed by fluorescence microscopy.

One drawback to these processes is the loss of oocysts on the series of filters and during the concentration and flotation processes required to purify the oocysts. Recovery efficiencies have been found to range from 9.5% (101) to 59 % (114) with losses up to 30% occurring at each centrifugation step (78). It has been shown that 1-30% of oocysts still remain in the filter cartridge after the shredding, squeezing and elution process, or the oocysts are damaged beyond recognition because of this process (137). These filtration techniques therefore underestimate the true number of oocysts present in water samples.

Another problem with these techniques is the loss of antibody epitopes from the oocyst wall due to repeated centrifugations and sonications. Vesey and Slade (137) found that the percent recovery as detected by immunofluorescence decreases with increased centrifugation speeds and increased sonication times. They found that staining these same samples with auramine-phenol (27) revealed the oocysts were still present. Another problem with FITC-conjugated monoclonal antibodies is that non-specific binding has been observed, resulting in objects of similar size and shape to oocysts fluorescing. The use of another fluorogenic stain can overcome this problem. DAPI (4',6-diaminidino-2-phenylindole) can be used to stain the nuclear material within oocyst (sporozoite DNA), thus a positive oocyst identification would require a green fluorescing oocyst wall as well as sporozoite nuclear material fluorescing bright blue (53). Although staining procedures have advanced to the point where positive identification of oocysts in samples can be a relatively straight forward process, the present-day techniques used to determine the number of oocysts present in water samples all underestimate the true number present due to oocyst loss at various steps or loss of antibody epitopes, and this must be taken into consideration when analyzing such data.

Because of the time and expertise required to concentrate and microscopically examine environmental samples to detect and count oocysts, researchers have recently turned to flow cytometry to determine if this technology can be a useful tool in *Cryptosporidium* analysis. Flow cytometry works by analyzing the emission characteristics of a sample passing through a beam of single wavelength laser light (cell by cell). Samples analyzed by their autofluorescence characteristics are discriminated by their forward scatter emissions (an indication of size) and side scatter emissions (an indication of granularity). Depending on the make of the flow cytometer, there can be a different number of filters that can be employed to analyze different emission characteristics of the sample. These different emission characteristics can be added to a sample with the addition of fluorochromes. Fluorochromes can be added as a marker of viability (DAPI), or many other physiological conditions of the cell, or it can be added conjugated to a monoclonal antibody specific to a point of interest within the sample. Once the fluorochrome is added, the sample can be analyzed according to the non-fluorescing (non-stained) and fluorescing (stained) portions of the sample. Depending on the make of the flow cytometer, multiple fluorochromes may be used in parallel. These characteristics can then be used to give vital information with regards to the components of interest identified within the sample (121). For example, if one wanted to quantify the number of dead organisms within a culture, one may want to incubate the culture with a viability marker, such as propidium iodide (PI), and analyze by flow cytometry. All events (cells) that are PI positive using the appropriate filter are dead and are easily quantified.

Vesey et al. (139) attempted to analyze raw water concentrates stained with FITC-conjugated monoclonal antibody specific for the *Cryptosporidium* oocyst walls, and realized that although flow cytometry was very quick and could process a very large number of organisms, there was a great deal of autofluorescence occurring from other organisms of the same size in the water sample. In subsequent studies, oocysts in environmental samples were first purified using fluorescence activated cell sorting (FACS) and were then labeled with FITC-conjugated monoclonal antibodies. These labeled oocysts were subsequently analyzed by flow cytometry (138). This study found that FACS followed by flow cytometry was more sensitive than direct epifluorescence microscopy, since flow cytometry detected oocysts in 92 of 325 samples whereas oocysts were detected in only 12 of 325 samples using epifluorescence microscopy (138). This technique has also been used to analyze sewage effluents with similar results (136). More recently, flow cytometry has been used not only to detect, but also to enumerate the number of *Cryptosporidium* oocysts in a stool specimen using again, FITC-conjugated monoclonal antibodies to label the oocysts within the sample (7).

Flow cytometry incorporating cooled charge couple device (CCD) has recently been evaluated as a means of detecting *Cryptosporidium* oocysts. Oocysts were labeled with an FITC-conjugated monoclonal antibody, stained with PI and DAPI and then analyzed and sorted by FACS (20). The authors found that due to the large amount of contaminating debris, visual inspection of the sorted sample by epifluorescence microscopy was necessary to confirm the presence of oocysts. However, when CCD was used on the sorted oocysts, a three dimensional visualization of single oocysts was possible thereby making microscopy unnecessary (20).

Enzyme immunoassays have also been developed for the detect of *Cryptosporidium* oocysts, but mainly for examining stools (69,98). It has been found that although enzyme immunoassays are sensitive and specific (98), the reagents and hands-on technician time required to perform the tests make other methods, such as immunofluorescence assays (IFA) preferable (69).

A final method of oocyst detection and recovery that has been developed recently involves filtration of the water sample through a cellulose acetate membrane filter to capture the oocysts. The filter is subsequently dissolved in acetone and the fluid is concentrated and resuspended. Oocysts are labeled with an FITC-conjugated monoclonal antibody and then viewed under epifluorescence microscopy. This technique is very inexpensive and has a stated recovery rate of 70.5% using seeded samples (3). Because the method widely in use today of concentrating oocysts from environmental and tap water samples can have recoveries ranging from 9.5% (101) to 59 % (114), this membrane filter-dissolution method appears promising.

Determination of Oocyst Viability

There are three commonly used procedures for determining the viability of oocysts. The first procedure is based on the premise that oocysts that are capable of excysting are probably capable of infection. Thus, an *in vitro* excystation technique was developed to simulate gut conditions that promote oocyst excystation. *In vitro* excystation is a somewhat lengthy procedure that first involves counting an original, untreated purified oocyst sample. The oocysts are then subjected to an acid treatment and then to an excystation medium consisting of bile salts. Next, the oocysts are examined microscopically and the number of shells, partially excysted and full oocysts are counted.

Percent excystation can then be calculated relative to the original sample (21,45,110). This figure is considered synonymous with percent viability and is thought to be closely related to the infectivity of oocysts. However, when compared to animal infectivity models, Finch et al. (45) have noted that excystation "consistently underestimated inactivation when compared with animal infectivity ($P \leq 0.05$). As inactivation increased, the difference between excystation and infectivity also increased." This has implications in that damaged oocysts may excyst but may be unable to infect hosts. Also, because different isolates of *Cryptosporidium* oocysts have different *in vitro* excystation efficiencies (21,110), viability and infectivity numbers derived from *in vitro* excystation experiments must be approached with caution.

The second procedure involves direct oral inoculation of oocysts into neonatal mice (5,45,71). The mice are sacrificed 5-7 days after inoculation, and the intestines are removed for microscopic examination to determine if infection has occurred as implied by the presence of various life cycle stages of *Cryptosporidium* (5,45,46,71). This method is not always practical because it is lengthy, costly and large numbers of animals are required to yield quantitative results. Also, infectivity of neonatal mice may or may not be similar to infection of other animals, thus again, numbers derived from mouse infectivity experiments must also be considered carefully. However, this method is considered the "gold standard".

The third method for oocyst viability determination uses dyes which can readily penetrate dead cells (60), including oocyst walls but not viable cells (21). For example, oocysts that will take up DAPI but exclude PI will readily excyst under a suitable

excystation protocol showing a very strong correlation between the DAPI/PI method and *in vitro* excystation (21). Experiments carried out with *Giardia* cysts revealed that all cysts positive for PI uptake never produced *Giardia* infection in test animals and thus cells positive for PI are dead and no longer capable of infection (119). Recently, newer vital stains, all available from the commercial supplier Molecular Probes (Eugene, OR) have been evaluated for their usefulness as *Cryptosporidium* oocyst viability indicators. The kit, Live/Dead BacLight®, relies on the differential permeability of the two dyes provided in the test kit into live cells to reveal viability, while the single stain SYTO9® stains dead cells. It was found that a modified Live/Dead BacLight® kit was the best of the viability kits tested, while SYTO9® showed viability results similar to infectivity studies in animals (11). A recent report has found that the use of both acridine orange and bis-benzimide, both fluorochromes, can differentiate between viable and non-viable *Cryptosporidium* sporozoites released into the surrounding medium (16), but their usefulness at determining *oocyst* viability has yet to be determined.

A cooled charge couple device (CCD) has recently been adapted for *Cryptosporidium* viability studies. This device detects very low-level light emissions. When the CCD is coupled with a fluorescence microscope, *Cryptosporidium* oocysts stained with fluorogenic dyes can be readily processed and imaged using support software. So far, 27 vital stains have been assessed for their usefulness in *Cryptosporidium* viability assays using CCD (19). However, the software involved in processing CCD samples is not readily available, and is therefore not an option for most laboratories.

An electrical spectrum method of viability assessment has also been developed. Electro-rotation involves the use of a dielectrophoretic electrode apparatus that, when the frequency of the applied electric field is varied, untreated (viable), autoclaved and ozone treated (non-viable) oocysts can be easily distinguished (6).

Oocyst staining techniques using vital stains hold promise for oocyst viability analysis using flow cytometry. Oocysts double-stained with FITC-conjugated monoclonal antibodies and a single vital stain such as PI could be analyzed by flow cytometry and the portion of the oocyst population (FITC-labeled) that is PI positive should be the non-viable portion of the oocyst population. Flow cytometry has already been successfully used in viability studies of other microorganisms such as bacteria (36,68,94) and in bacterial analysis in food and beverage industries (74).

In addition to the previous methods, polymerase chain reaction (PCR) has recently been developed as not only a detection methodology for *Cryptosporidium* oocysts in suspension, using *Cryptosporidium parvum* specific primers (64,73,79,87,112), but is very rapidly being developed as a sensitive viability assessment assay. PCR was first introduced into the literature as an oocyst viability assessment procedure in 1994 (43), improved in 1995 (140), but the method ultimately relied on *in vitro* excystation as the final viability test. Fortunately, there have been numerous improvements on the procedure such that the present technique, reverse transcription-PCR, is now sensitive enough to detect a single viable oocyst in an environmental water concentrate (130). The improvement on the technique is a shift in focus from *in vitro* excystation as the viability marker to monitoring the production of a heat shock protein mRNA (*Hsp70*) by the oocyst; only viable oocysts will transcribe the heat shock mRNA

after a heat treatment. Therefore, this procedure relies on the physiological state of the oocyst whose product is directly screened, rather than interpreting the results of an *in vitro* excystation assay. The procedure is economical, straightforward and can provide results within one day (130). However, molecular techniques are sophisticated and require highly experienced technicians to carry out protocols, so a need still exists for an inexpensive, simple and rapid viability assay that does not require a high level of training to be able to perform. Additionally, molecular techniques are not readily applicable to large samples, such as would occur in the water treatment industry.

Conclusion

Already, much is known about the morphology, biology, occurrence, epidemiology and survival of *Cryptosporidium*, but the state of knowledge is far from complete. Through *Cryptosporidium* oocyst viability and survival studies, we can further advance our understanding about the factors influencing oocyst survival in natural waters and hence determine the risk of infection due to the presence of oocysts in these environments. Rapid, accurate, easy and inexpensive techniques must be developed to identify *Cryptosporidium* oocysts in natural, potable and recreational waters, and also in laboratory specimens. Because the infective dose is thought to be relatively low, it is imperative that a thorough understanding of oocyst transmission and survival capabilities is achieved in order to properly assess the risk associated with the presence of oocysts in waters, to disinfect contaminated waters, and to aid in the prevention of further spread of this highly infectious and transmissible protozoan.

OBJECTIVES

In vitro Cryptosporidium Oocyst Survival in Natural Waters

Despite numerous surveys over the past several years documenting the distribution and the concentration of *Cryptosporidium* oocysts in natural waters (30,32,55,63,76,84,100,108,114,116,124), there has been little research on the survival of *Cryptosporidium* oocysts in natural waters. Robertson et al. (109) found that *Cryptosporidium* oocysts can survive in natural or tap waters for months, but no significant research has been done to further the knowledge of oocyst survival capabilities and factors affecting inactivation. This knowledge is crucial if health officials are to determine the risk to a population if oocysts are found in the water supply. Water utilities must also have this knowledge if they are to apply the proper disinfection strategy to the drinking water supply if oocysts are found in the water source. Therefore, a portion of this study was designed to achieve the following objectives:

1. assess the *in vitro* survival of *Cryptosporidium* oocysts in three different watersheds.
2. assess the effect of incubation temperature on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters.
3. assess the effect of season of water sampling on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters.
4. assess the effect of different locations within a single river on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters.

5. assess the effect of water chemistry and heterotrophic bacteria on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters.

Development of a Rapid *Cryptosporidium* Oocyst Viability Assay Using Flow Cytometry

The "gold standard" for viability assessment of *Cryptosporidium* oocysts is mouse infectivity. This procedure is very expensive and time consuming. Because of this, other techniques, such as *in vitro* excystation, have been developed to act as surrogates for the animal infectivity assay. However, techniques such as *in vitro* excystation are often criticized for their lack of correspondence to animal infectivity assays (45), and therefore a real need exists for a viability assessment assay that is rapid, inexpensive and yields viability results similar to animal infectivity. Therefore, the second portion of this study was designed to achieve the following objectives:

1. define a flow cytometric protocol to be assessed as a potential oocyst viability assay.
2. compare viability results obtained with the new flow cytometric assay with *in vitro* excystation[4] over different oocyst stresses to determine its applicability as a new assay.

[4] Animal infectivity was not chosen as the comparison assay due to its cost and time frame required for experimental work.

GENERAL MATERIALS AND METHODS

Purification of *Cryptosporidium* Oocysts from Bovine Feces

Oocysts were purified from calf feces obtained from Dr. Bruce Anderson (Animal and Vet. Sciences, Univ. of Idaho, Caldwell, Idaho), by a modified Sheather's sugar flotation, followed by a Percoll-sucrose purification (34). A Sheather's solution (500g sucrose, 9 mL phenol (1 g/15 mL H_20) and 320 mL H_20), 1X Alsever's solution (20.5 g glucose, 8.0 g trisodium citrate, 4.2 g sodium chloride and 1 L H_20) and 10X Alsever's solutions (41.0 g glucose, 16.0 g trisodium citrate, 8.4 g NaCl and 200 mL H_20) were prepared. A 1:2 and 1:4 dilution of the Sheather's was made resulting in a specific gravity of 1.103 and 1.064, respectively, using Dulbecco's phosphate buffered saline (PBS) without $CaCl_2$, and $MgCl_2$ (GibcoBRL, Grand Island, NY) but with 1% Tween 80.

Ten mL of the 1:4 solution was placed in 16 50 mL conical centrifuge tubes (Fisher Scientific, Pittsburg, PA). The 1:4 solution was floated on 10 mL of the 1:2 solution. Five mL of well vortexed fecal material was carefully layered on top of the 1:4 layer followed by 30 minutes of centrifugation at 1,500 X g. Three layers were obtained after centrifugation and each layer was recovered individually, pooled and washed with dd H_20 (centrifuged at 1,500 X g for 15 minutes). The supernatant was aspirated to just above the pellet. All pellets were vortexed and pooled into one 50 mL conical tube. The tubes were rinsed with one 7.5 mL aliquot of PBS by transferring it from one tube to another until all had been washed. An equal volume of 5% (w/v) dichromate was added such that the total volume did not exceed 20 mL.

Five mL of the pooled suspension was layered on top of four new gradient tubes and centrifuged at 1,500 X g for 30 minutes. The pooling of layers and pooling of pellets was repeated as previously mentioned. The preparation was then centrifuged at 1,500 X g for 10 minutes and aspirated to just above the 5 mL mark. A 7:1:7 Percoll (Pharmacia, Pharmacia Biotech AB, Uppsala, Sweden) gradient was prepared using Percoll:10X Alsever's:1X Alsever's which was dispensed into 6 high speed centrifuge tubes. One mL of the suspension was carefully layered over the gradient and centrifuged at 22,000 X g for 30 minutes. Three bands were observed. The top orange band was aspirated and discarded. The remaining bands were recovered and pooled individually (the pellet being discarded). Each pooled layer was washed with an equal volume of PBS and then were pooled together. An equal volume of PBS with penicillin and streptomycin (GibcoBRL) was added to result in a final concentration of 500 U/mL. The suspension was stored at 4°C until used. When ready to use, 100 µL of this stock was washed in sterile ddH_20 at 13,500 X g for 10 minutes and resuspended in 1.8 mL sterile ddH_20. This was the working stock solution.

***In vitro* Excystation**

Oocyst viability was determined using an *in vitro* excystation protocol modified from Woodmansee (142). Approximately 5 x 10^4 oocysts were required for *in vitro* excystation. A 1X excystation medium was prepared using 0.005 g trypsin (Sigma Chemical Co., St. Louis, MO), 0.015 g sodium taurocholate (ICN Biomedicals Inc. Aurora, OH) in 1.0 mL PBS (GibcoBRL) and then filter sterilized with a 0.22 µm membrane filter (Nalge Company, Rochester, NY). A trigger medium of Hank's

Balanced Salt Solution (HBSS, GibcoBRL) with pH of 2.0 was also prepared. To the oocysts, 50 μL of trigger medium was added and the contents vortexed. The oocysts were then incubated at 37°C for 1 hour. When removed, 25 μL of the suspension was transferred to a slide, microscopically observed under Hoffman optics and the number of shells counted. One hundred oocysts were counted. Counts were repeated. The remaining suspension was centrifuged at 13,500 X g for 10 minutes, the supernatant aspirated and discarded and 25 μL of 1X excystation medium was added. The suspension was vortexed and placed in a 37°C incubator. After 4 hours of incubation, the oocysts were removed and observed under Hoffman modulation optics. The number of shells, partially empty oocysts and full oocysts were counted. One hundred oocysts were counted. Percent excystation (viability) was then determined by:

% excystation = (Shells + Partials after excystation) - (Shells prior to excystation)

Total *Cryptosporidium* Oocyst Counts

After a 20 μL oocyst sample was placed on an Improved Neubauer counting chamber, a cover slip was applied. The slide was then viewed under the 40X objective using Hoffman modulation optics. A total of 25 large squares was counted on the first grid and the count was repeated on the second grid. The two numbers were then averaged, giving the number of oocysts per 0.1 mm^3. The total oocyst population was then determined by:

$$\#\ oocysts/mL = \#\ oocysts/0.1mm^3 \times (1.0 \times 10^4)$$

Total *Cryptosporidium* Oocyst Counts Using Immunofluorescence Assay (IFA)

Nineteen microlitres of oocyst sample was added to a 1.5 mL microcentrifuge tube (Sarstedt, St. Laurent, Quebec). To this, 1 μL of a FITC-labeled monoclonal antibody (anti-*Cryptosporidium* oocyst mouse IgM, Waterborne, Inc., New Orleans, LA) was added, giving a final antibody concentration of 1/20, and the contents vortexed. The sample was then incubated at room temperature in the dark for 30 minutes, after which the oocysts were examined microscopically.

The 20 μL labeled oocyst sample was placed on an Improved Neubauer counting chamber, as described previously for Total *Cryptosporidium* Oocyst Counts. The slide was viewed under the 40X objective using epifluorescence microscopy, with the oocysts fluorescing as bright apple-green circles. Again, two grids were counted, counting 25 large squares per grid. The two numbers were then averaged and the number of oocysts per mL was calculated according to the previous equation.

Calculation of Log_{10} Reduction

Log_{10} reduction was calculated using the following formula:

$$\log_{10}\text{reduction} = \log_{10}(\#\text{viable oocysts}_{t} / \#\text{viable oocysts}_{t0})$$

IN VITRO CRYPTOSPORIDIUM SURVIVAL IN NATURAL WATERS

Introduction

Oocysts are ubiquitous and have a continuous presence in water sources (56). There are numerous reports on the levels of oocysts detected in various water systems (30,32,55,63,76,84,100,108,114,116,124), and an even longer list dealing with the various disinfection strategies of *Cryptosporidium* oocysts (22,23,38-41,44,57,59,71,82,102,109,134). However, little is known about the factors effecting oocyst survival in the natural environment.

Current methods for the detection of oocysts in natural waters do not allow for species identification, nor for the assessment of viability of the detected oocysts. Detected oocysts may not be infectious or even viable, and therefore may not pose any threat to human health. However, since little is known about the effects of various factors within the watershed on the viability of oocysts, all oocysts present in a water system have been assumed to be viable and hence infectious (to err on the side of caution). Knowing what the key factors involved in oocyst inactivation are in the natural setting would allow water authorities to develop more realistic risk assessment models when oocysts are detected within the drinking water source. It would also enable water authorities to develop optimal or better watershed management practices depending on the watershed's characteristics. In addition, it would allow water treatment facilities to determine the levels of disinfection required for their particular watershed, hence potentially decreasing disinfection costs.

Robertson et al. (109) have studied the effect of tap water, natural sea water and river water on the survival of *Cryptosporidium* oocysts and have found that even after 176 days of incubation, a portion of the seeded oocysts still remained viable. Is this true for all water systems? What are the environmental factors that are responsible for oocyst inactivation? The answers are presently unknown.

Until now, the effect of microorganisms on the survival of *Cryptosporidium* oocysts in natural water has not been examined. Predation of bacteria by protozoans and other microscopic water organisms has been examined previously and reports show that protozoans feed extensively on and therefore have serious impact on the survival and numbers of resident bacteria (10,14,54,86,117,141). However, there is little information on the converse. Very few studies have addressed the possibility of other microorganisms, particularly bacteria, seriously affecting the survival of protozoans such as *Cryptosporidium* oocysts in their natural environment. A recent study has found that *Serratia marcescens*, a species found in the water environment, can degrade *Cryptosproidium* oocysts by chitinolytic enzyme activity (144). Another study suggests that zooplankton may be an important predator of protozoans in a fresh water ecosystem (25). This relationship has also been observed by Stoecker and Mcdowell-Capuzzo (131). Therefore, since protozoans can be preyed upon by other resident microflora, if we are to gain a better understanding of the factors that affect *Cryptosporidium* oocyst survival in the environment, the microbial ecology of the water environment must also be studied.

MATERIALS AND METHODS

Water Sampling

Water was collected by grab sampling in two clean 1 L containers from the shores of the selected river at predetermined sites or from the raw water tap at the water treatment facility as outlined in Table 4, Figure 3, Figure 4 and Figure 5. Water was immediately sent to the laboratory and stored at 4°C until processed.

Table 4: River Sampling Sites

River	Site #1	Site #2
Grand River	Kaufman Flats, upstream from the City of Waterloo, Ontario	Raw water intake at the Mannheim Water Treatment Facility, downstream from the City of Waterloo, Ontario
St. Lawrence River	Screen House at the water treatment facility, Cornwall, Ontario	Location on the east end of Cornwall (downstream), Cornwall, Ontario
Carp River	The mouth of the Carp River as it empties into the Ottawa River at Fitzroy Harbour Provincial Park, Ontario	Within 1 km of the origin of the Carp River, Ontario

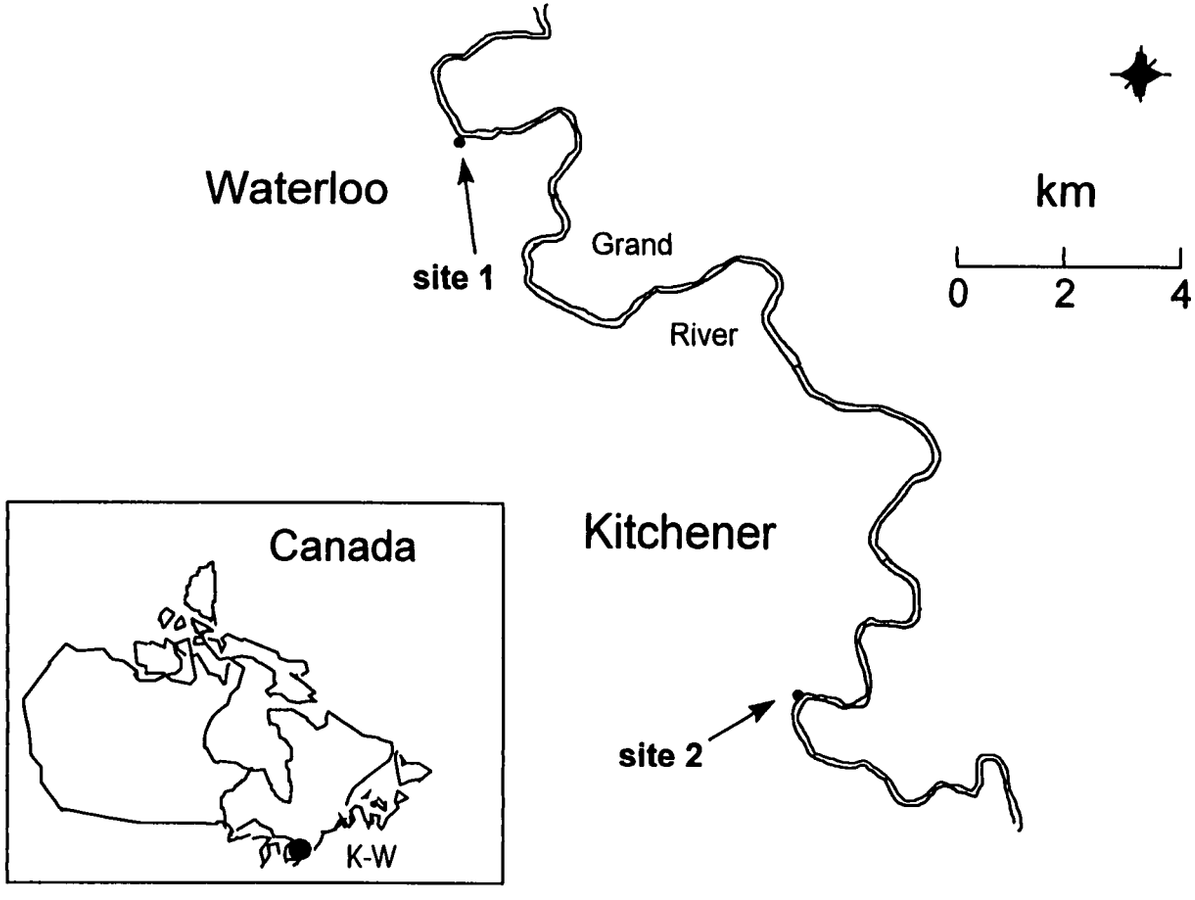


Figure 3: Grand River Sampling Locations

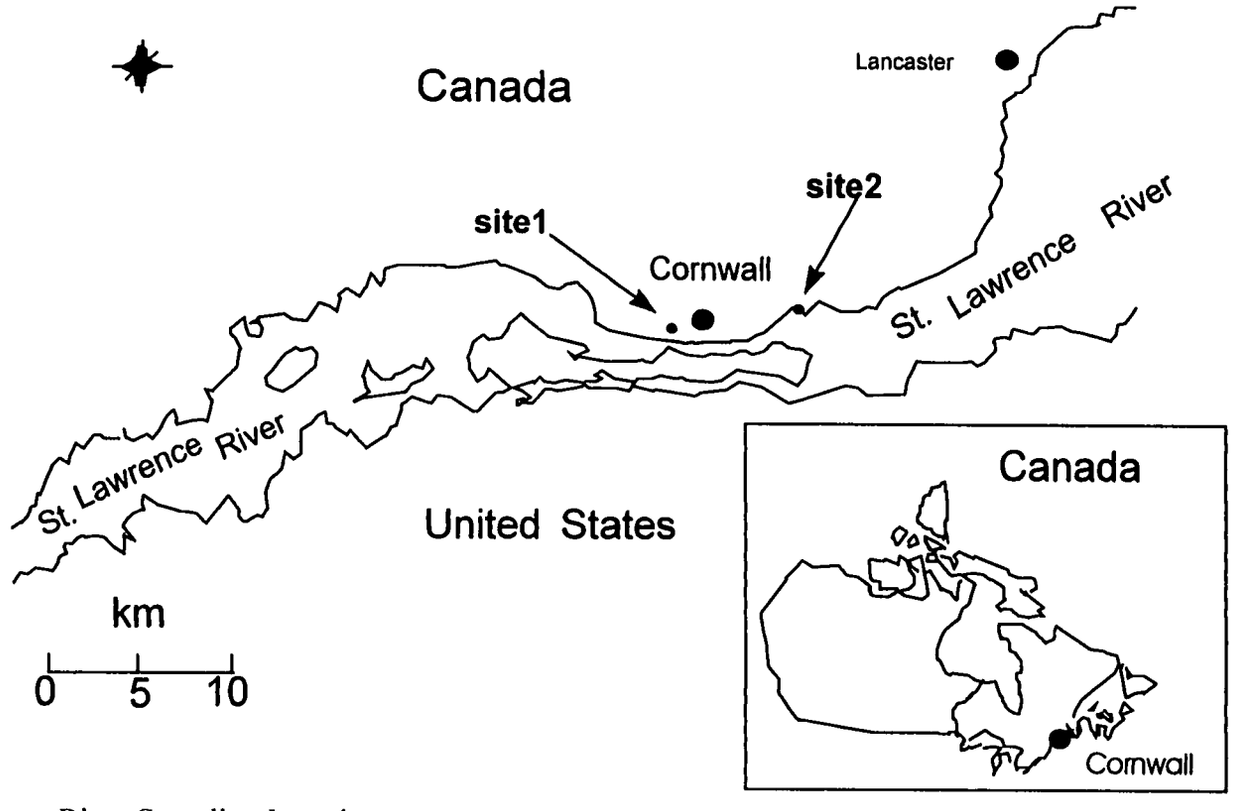


Figure 4: St. Lawrence River Sampling Locations

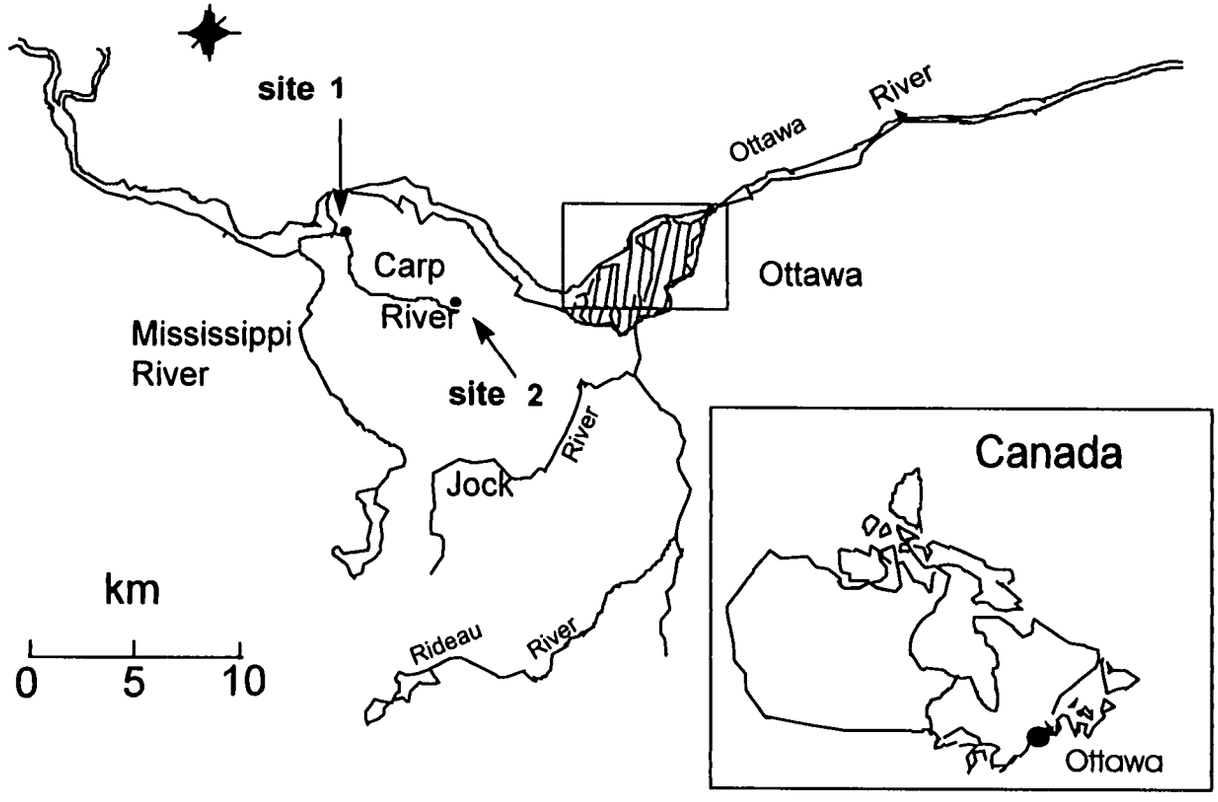


Figure 5: Carp River Sampling Locations

In vitro *Survival-Experimental Procedure*

Water from each of two river locations was plated onto R2A agar (Difco Laboratories, Detroit, MI) and incubated in the dark at room temperature for 6 days before counting, to determine the number of heterotrophic bacteria (HPC) present in the water at the start of the experiments. The pH of each sample was also determined. One mL quantities of water from the two specified locations were aliquoted into 1.5 mL sterile polypropylene screw-capped microcentrifuge tubes (Sarstedt). Duplicate samples were prepared for each sampling time point. Duplicate control samples for each sampling time point were also prepared by adding a 1 mL aliquot synthetic hard water (100 ppm as $CaCO_3$, pH 7.0) into 1.5 mL sterile polypropylene screw capped microcentrifuge tubes (Sarstedt). Both test and control tubes were inoculated with purified and washed *Cryptosporidium* oocysts at a concentration of approximately 5×10^5 oocysts/mL and the contents vortexed. Total counts on day 0 were determined by the procedure previously described. Viability on day 0 was determined by *in vitro* excystation of the *Cryptosporidium* oocyst stock multiplied by the total counts of the test and control tubes.

In vitro *Survival*

Samples were collected and processed according to the scheme shown in Table 5 using the sampling procedure described earlier at the sites previously detailed. All samples were processed within 7 days of collection, except for the Carp River samples, which were processed as follows.

Table 5: Rivers, Sampling Seasons, Incubation Temperatures and Durations

Rivers (2 sites on each)	Summer, 1995 Sampling date	Summer, 1995 Incubation temperatures and duration	Winter, 1995 Sampling date	Winter, 1995 Incubation temperatures and duration	Spring, 1996 Sampling date	Spring, 1996 Incubation temperatures and duration
Grand River	July 26	20°C-50 days 30°C-50 days	Dec. 6	4°C-100 days 20°C-50 days	May 30	4°C-100 days 20°C-50 days
St. Lawrence River	Aug. 7	20°C-50 days 30°C-50 days	Dec. 6	4°C-100 days 20°C-50 days	not done	not done
Carp River	Sept. 30	20°C-50 days 30°C-50 days	not done	not done	not done	not done

Water samples collected for the Carp River were immediately sent to a commercial laboratory upon collection (Accutest Laboratories, Nepean, Ontario) for total organic carbon (TOC) and metals analysis. Samples were plated onto R2A agar (Difco) to determine HPC at the time of collection. pH was also determined. Water samples were then stored at 4°C, in the dark for 69 days. This lag in time between collection and processing was due to time constraints processing Grand and St. Lawrence River samples. Upon processing, the water samples were again sent to a commercial laboratory (Accutest) for TOC and metals analysis. The samples were plated on to R2A agar to determine HPC at the start of the experiments. Test water samples and controls were prepared as described previously.

All samples were processed as follows. Test and control samples were incubated at temperatures outlined in Table 5. At each sampling point, *in vitro* excystation was performed in duplicate on the test and control tubes and total counts were determined. Total counts were determined by *Total Cryptosporidium Oocysts Counts* for the Summer, 1995, season and by *Total Cryptosporidium Oocyst Counts Using IFA* for the Winter, 1995, and Spring, 1996, seasons. Viability was then calculated for each sampling point.

In vitro *Survival with a Spike of* Pseudomonas fluorescens

Isolation of *Pseudomonas fluorescens* from the Carp River: Carp River water, collected on March 8, 1996, was plated onto R2A agar (Difco) and incubated at room temperature in the dark for 6 days. Colonies of various morphologies were inoculated into 150 mL Tryptic Soy Broth (TSB) (Que-Bact Laboratories Inc., Montreal, Quebec) and placed onto an orbital shaker at room temperature for 24 hours. Only one of the nine

isolated grew in TSB. A loopful of the culture was plated onto R2A agar (Difco) for a second time and grown for 6 days at room temperature, in the dark. The isolation of a single colony type was confirmed, and this isolate was identified by the Microbiology Laboratory at the General Hospital, Ottawa, Ontario, as *P. fluorescens*. These plates were kept at 4°C as the reference stock.

Water collection: Water from the Carp River was collected according to the water sampling procedure at Site #2 on July 2, 1996.

Preparation of *P. fluorescens* for use in *In vitro* Survival Studies: The isolated *P. fluorescens* was inoculated into 150 mL TSB (Que-Bact) and placed on an orbital shaker at room temperature overnight. The culture was then washed three times with sterile double distilled water (ddH_2O) at 10,000 x g, and resuspended in 150 mL of sterile ddH_2O.

Preparation of water for use in *In vitro* Survival Studies: The collected Carp River water was divided into thirds for use in the studies. One third was filtered though a 0.22 μm nylon filter unit (Nalge Company, Rochester, NY). Another third was filtered though a 47mm 2.0 μm polycarbonate membrane filter (Millipore Corporation, Bedford, MA) and collected into a sterile container. The remaining one third was left unfiltered. All water samples, including unfiltered Carp River, 0.22 μm filtered Carp River, 2.0 μm filtered Carp River, and synthetic hard water (100 ppm as $CaCO_3$, pH 7.0) were plated onto R2A agar (Difco) for HPC analysis and were sent to a commercial laboratory (Accutest) for TOC and metals analysis.

In vitro Cryptosporidium* Oocyst Survival with *P. fluorescens: One mL volumes of water (0.22 μm filtered, 2.0 μm filtered, unfiltered Carp River water and synthetic hard water) were aliquoted into 1.5 mL sterile polypropylene screw-capped microcentrifuge tubes (Sarstedt). Duplicate samples were prepared for each sampling time point, except for 0.22 μm filtered river water, for which 4 samples were prepared for each sampling time point. All tubes were inoculated with purified and washed *Cryptosporidium* oocysts at a concentration of approximately 5×10^5 oocysts/mL and the contents vortexed. The tubes that will now be referred to as "test water" were prepared by inoculating half of the 0.22 μm filtered Carp River water samples with approximately 1×10^5 cfu/mL of washed *P. fluorescens*. The remaining tubes, those that did not receive an inoculum of *P. fluorescens*, will now be referred to as "control water". Tests and controls were plated onto R2A agar (Difco) on day 0 to determine HPC at the start of the experiment. Total counts (using IFA) on day 0 were determined by selecting the last sampling time point tubes as the oocyst population to follow over time and determining counts by the procedure previously described. Viability on day 0 was determined by *in vitro* excystation of the inoculating *Cryptosporidium* oocyst stock multiplied by the total counts of the test and control tubes. HPC were determined not only upon collection of the water samples, but also at each and every sampling time during the course of the experiment. All water samples were incubated at 20°C and sampled over a 50-day period.

Multiple Linear Regression Analysis

Ordinary least squares regression analysis using dummy variables (118) was performed on all rivers, sites, seasons and temperatures tested. Log_{10} reduction was the

decay variable examined over time. Five models were tested to determine if incubation temperature, river, site or all factors combined had any effect on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters. Multiple linear regression analysis was performed on the resultant $\log_{10}$ reduction data over time and HPC data over time in the *In vitro Survival with a Spike of* P. fluorescens experiment.

RESULTS

All bolded values in the tables titled "Total Population Counts Over Testing Period" and "Oocyst Populations Over Time as a Percentage of Original Population" indicate those which were below the limit of detection. In the tables titled "*In vitro* Excystation Over Testing Period", bolded values indicate those that were based on only one observation instead of the mean of two observations. n/a indicates that not enough oocysts were present to determine excystation values. For assessment of total oocyst counts, since there were two distinct fields to count on a haemocytometer, and whose values were averaged to give a final count, a count of 0 on one field and 1 on another could not be averaged to give 0.5. A count of this sort implied that the sample was right *at* the limit of detection, and therefore any counts that resulted in a 0/1 were always converted to 1/1, the minimum detection limit. This number was then used in subsequent calculations.

In vitro *Survival: Summer, Winter, 1995 and Spring, 1996*

The results for all of the rivers, sites, seasons and temperatures tested are presented in the following tables (Tables 6-23) and figures (Figures 6-17). It can be noted from the tables

titled "Oocyst Populations Over Time as a Percentage of Original Population" and "*In vitro* Excystation Over Testing Period" that rapid declines in total oocyst counts is the main factor responsible for the dramatic $\log_{10}$ reductions observed in the presented figures. *In vitro* excystation does not decrease as dramatically over time as the total oocyst population does.

Table 6: Summer, 1995-Grand River Total Oocyst Population Counts Over Testing Period

	Days Incubation			
Treatment	0	7-10	27-30	48-51
Site #1 30°C Test	432500	135000	75000	**<10000**
Site #1 30°C Control	367500	242500	112500	105000
Site #2 30°C Test	460000	202500	**<12500**	**<10000**
Site #2 30°C Control	375000	282500	210000	110000
Site #1 20°C Test	382500	357500	67500	**<12500**
Site #1 20°C Control	350000	317500	157500	240000
Site #2 20°C Test	445000	317500	82500	17500
Site #2 20°C Control	390000	325000	192500	180000

Table 7: Summer, 1995-Grand River Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation			
Treatment	0	7-10	27-30	48-51
Site #1 30°C Test	100	31	17	**<2**
Site #1 30°C Control	100	66	31	29
Site #2 30°C Test	100	44	**<3**	**<2**
Site #2 30°C Control	100	75	56	29
Site #1 20°C Test	100	93	18	**<3**
Site #1 20°C Control	100	91	45	69
Site #2 20°C Test	100	71	19	4
Site #2 20°C Control	100	83	49	46

Table 8: Summer, 1995-Grand River *In vitro* Excystation Over Testing Period

	Days Incubation			
Treatment	0	7-10	27-30	48-51
Site #1 30°C Test	91.5	49.0	21.5	**20.0**
Site #1 30°C Control	91.5	67.0	32.0	16.0
Site #2 30°C Test	91.5	63.8	40.5	n/a
Site #2 30°C Control	91.5	52.0	33.0	15.5
Site #1 20°C Test	91.5	75.5	**71.0**	54.7
Site #1 20°C Control	91.5	77.5	71.5	69.0
Site #2 20°C Test	91.5	72.0	61.5	58.4
Site #2 20°C Control	91.5	67.8	64.5	60.0

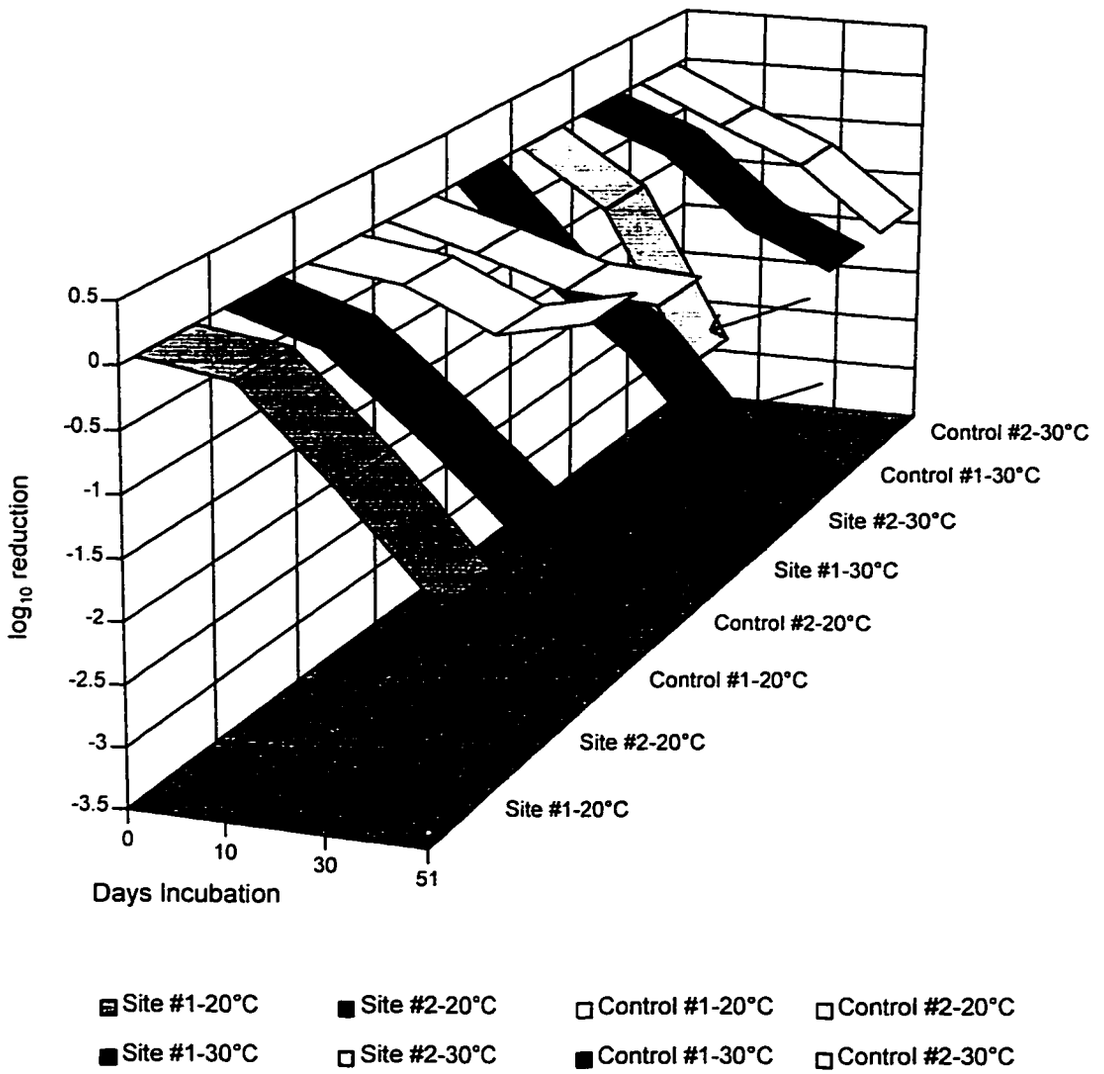


Figure 6: Grand River Log_{10} Reductions in Oocyst Viability, Summer, 1995. The arrows indicate log_{10} reductions which are less than or equal to values stated.

Table 9: Summer 1995-St. Lawrence River Total Oocyst Population Counts Over Testing Period

	Days Incubation			
Treatment	0	6-9	28-31	48-51
Site #1 30°C Test	430000	185000	10000	15000
Site #1 30°C Control	477500	290000	215000	110000
Site #2 30°C Test	410000	297500	177500	122500
Site #2 30°C Control	387500	305000	205000	170000
Site #1 20°C Test	380000	175000	**<10000**	**<10000**
Site #1 20°C Control	422500	315000	220000	160000
Site #2 20°C Test	397500	262500	**<10000**	**<32500**
Site #2 20°C Control	445000	507500	235000	212500

Table 10: Summer, 1995-St. Lawrence River Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation			
Treatment	0	6-9	28-31	48-51
Site #1 30°C Test	100	43	2	3
Site #1 30°C Control	100	61	45	23
Site #2 30°C Test	100	73	43	30
Site #2 30°C Control	100	79	53	44
Site #1 20°C Test	100	46	**<3**	**<3**
Site #1 20°C Control	100	75	52	38
Site #2 20°C Test	100	66	**<3**	**<8**
Site #2 20°C Control	100	114	53	48

Table 11: Summer, 1995-St. Lawrence River *In vitro* Excystation Over Testing Period

	Days Incubation			
Treatment	0	6-9	28-31	48-51
Site #1 30°C Test	92.3	73.5	19.0	28.4
Site #1 30°C Control	92.3	81.5	26.0	29.1
Site #2 30°C Test	92.3	74.0	18.0	31.5
Site #2 30°C Control	92.3	73.0	32.5	21.0
Site #1 20°C Test	92.3	79.0	58.0	68.0
Site #1 20°C Control	92.3	72.5	65.0	48.5
Site #2 20°C Test	92.3	78.5	59.0	**67.3**
Site #2 20°C Control	92.3	76.5	67.0	60.0

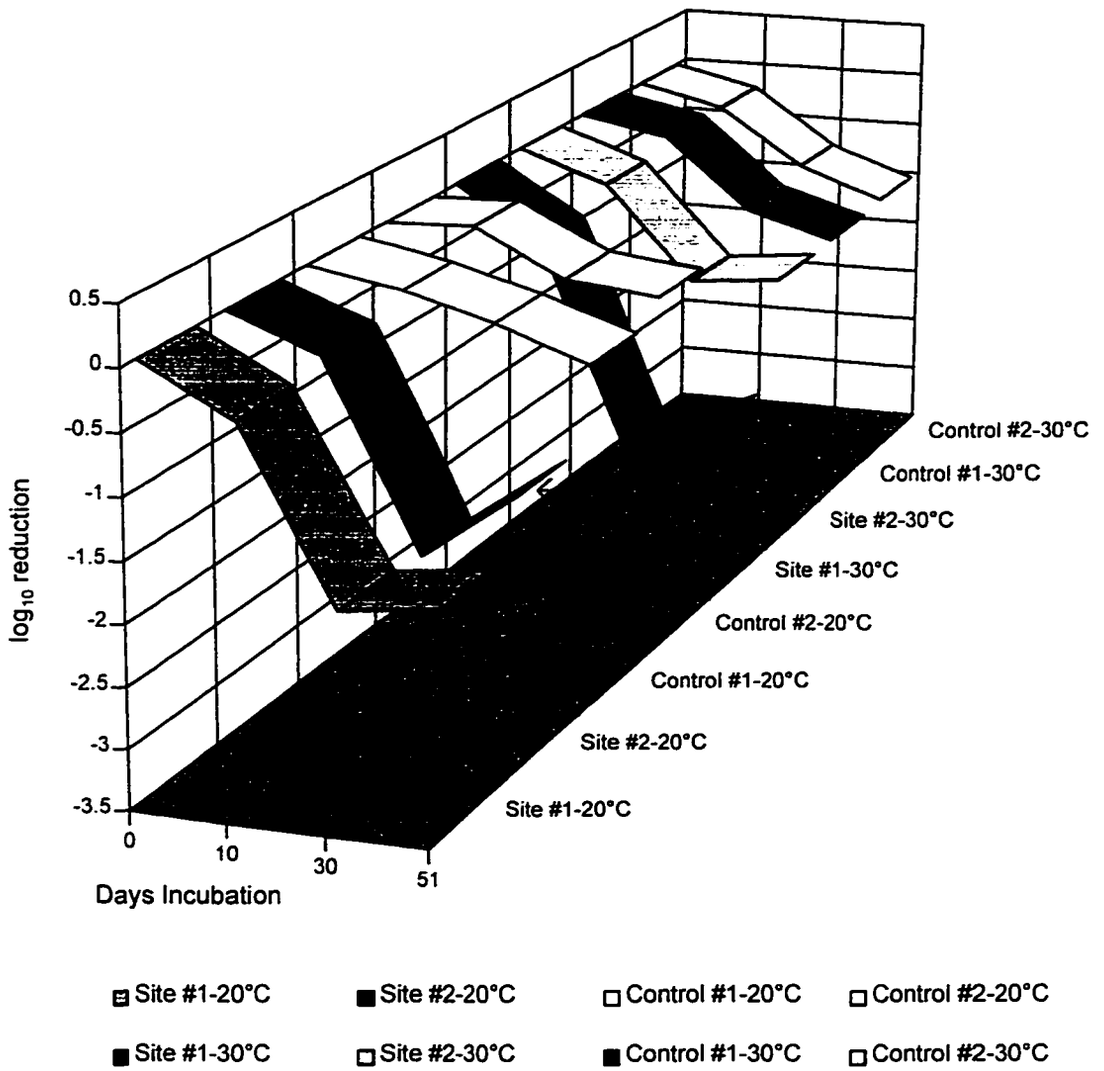


Figure 7: St. Lawrence River Log_{10} Reductions in Oocyst Viability, Summer, 1995. The arrows indicate log_{10} reductions which are less than or equal to values stated.

Table 12: Summer, 1995-Carp River Total Oocyst Population Counts Over Testing Period

	Days Incubation				
Treatment	0	5	13	30	51
Site #1 30°C Test	475000	439474	202632	121053	31579
Site #2 30°C Test	482500	378947	273684	147368	36842
30°C Control	487500	481579	357895	357895	455263
Site #1 20°C Test	430000	405263	273684	373684	89474
Site #2 20°C Test	582500	505263	471053	181579	23684
20°C Control	562500	505263	492105	318421	428947

Table 13: Summer, 1995-Carp River Total Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation				
Treatment	0	5	13	30	51
Site #1 30°C Test	100	93	43	25	7
Site #2 30°C Test	100	79	57	30	8
30°C Control	100	99	73	73	93
Site #1 20°C Test	100	94	64	87	21
Site #2 20°C Test	100	87	81	31	4
20°C Control	100	90	87	57	76

Table 14: Summer, 1995-Carp River *In vitro* Excystation Over Testing Period

	Days Incubation				
Treatment	0	5	13	30	51
Site #1 30°C Test	73.0	75.0	66.0	38.5	40.65
Site #2 30°C Test	73.0	79.5	47.0	15.0	41.6
30°C Control	73.0	80.5	53.5	63.0	56.9
Site #1 20°C Test	73.0	73.0	77.5	73.5	45.5
Site #2 20°C Test	73.0	80.0	75.5	71.5	59.2
20°C Control	73.0	76.0	84.0	80.5	67.0

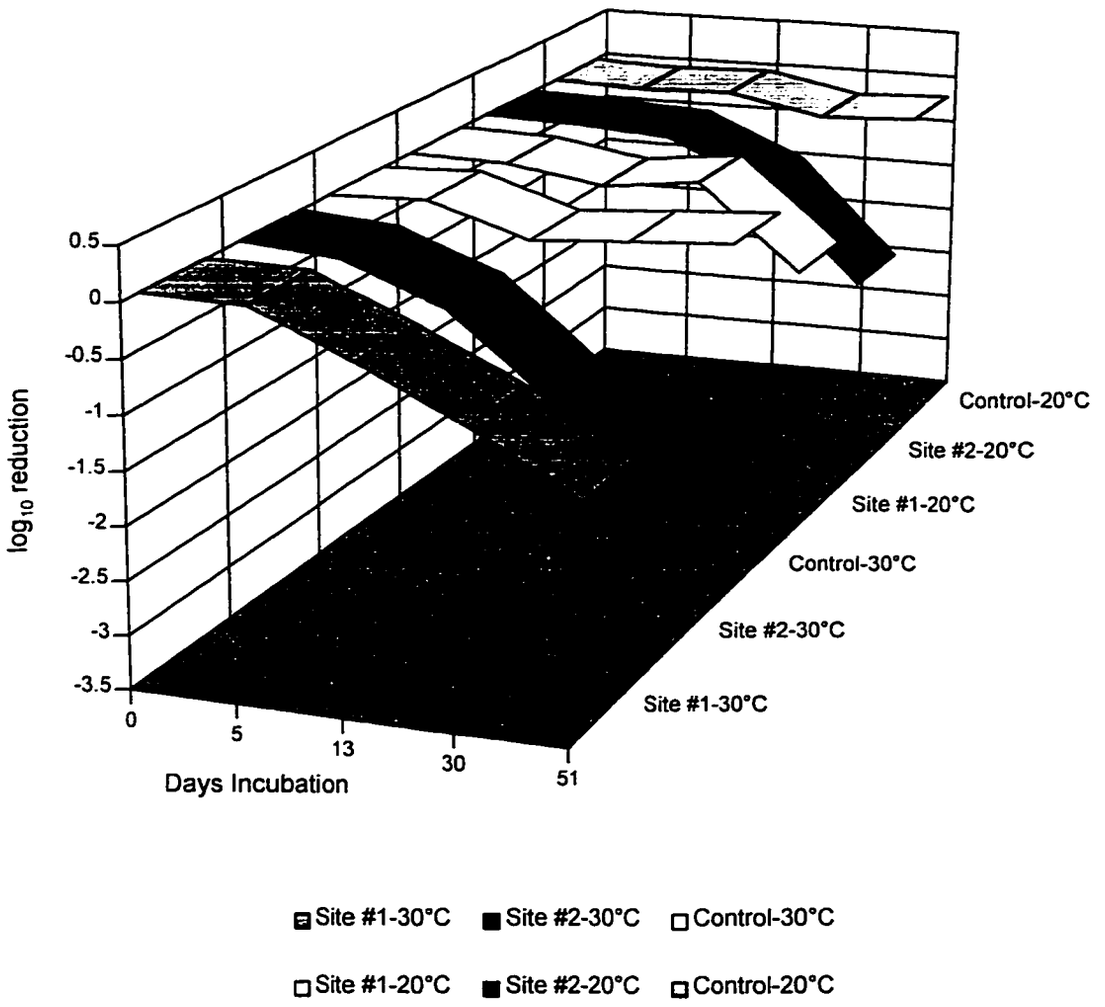


Figure 8: Carp River Log_{10} Reductions in Oocyst Viability, Summer, 1995.

Table 15: Winter, 1995-Grand River Total Oocyst Population Counts Over Testing Period

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	694737	542105	376316	289474	473684	357895	218421
Site #2 4°C	605263	513158	389474	402632	126316	205263	107895
4°C Control	486842	531579	431579	331579	321053	344737	386842
Site #1 20°C	518421	257895	223684	55263	50000	-	-
Site #2 20°C	505263	223684	284211	63158	73684	-	-
20°C Control	513158	423684	373684	244737	281579	-	-

Table 16: Winter, 1995-Grand River Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	100	78	54	42	68	52	31
Site #2 4°C	100	85	64	67	21	34	18
4°C Control	100	109	89	68	66	71	79
Site #1 20°C	100	50	43	11	10	-	-
Site #2 20°C	100	44	56	12	15	-	-
20°C Control	100	83	73	48	55	-	-

Table 17: Winter, 1995-Grand River *In vitro* Excystation Over Testing Period

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	80.8	73.0	75.6	82.0	70.0	72.5	64.0
Site #2 4°C	80.8	68.0	84.8	79.5	67.5	64.5	77.5
4°C Control	80.8	77.5	81.0	72.0	76.0	67.6	64.0
Site #1 20°C	80.8	73.5	66.0	75.0	66.0	-	-
Site #2 20°C	80.8	82.0	74.5	66.5	65.5	-	-
20°C Control	80.8	77.0	79.5	65.5	67.0	-	-

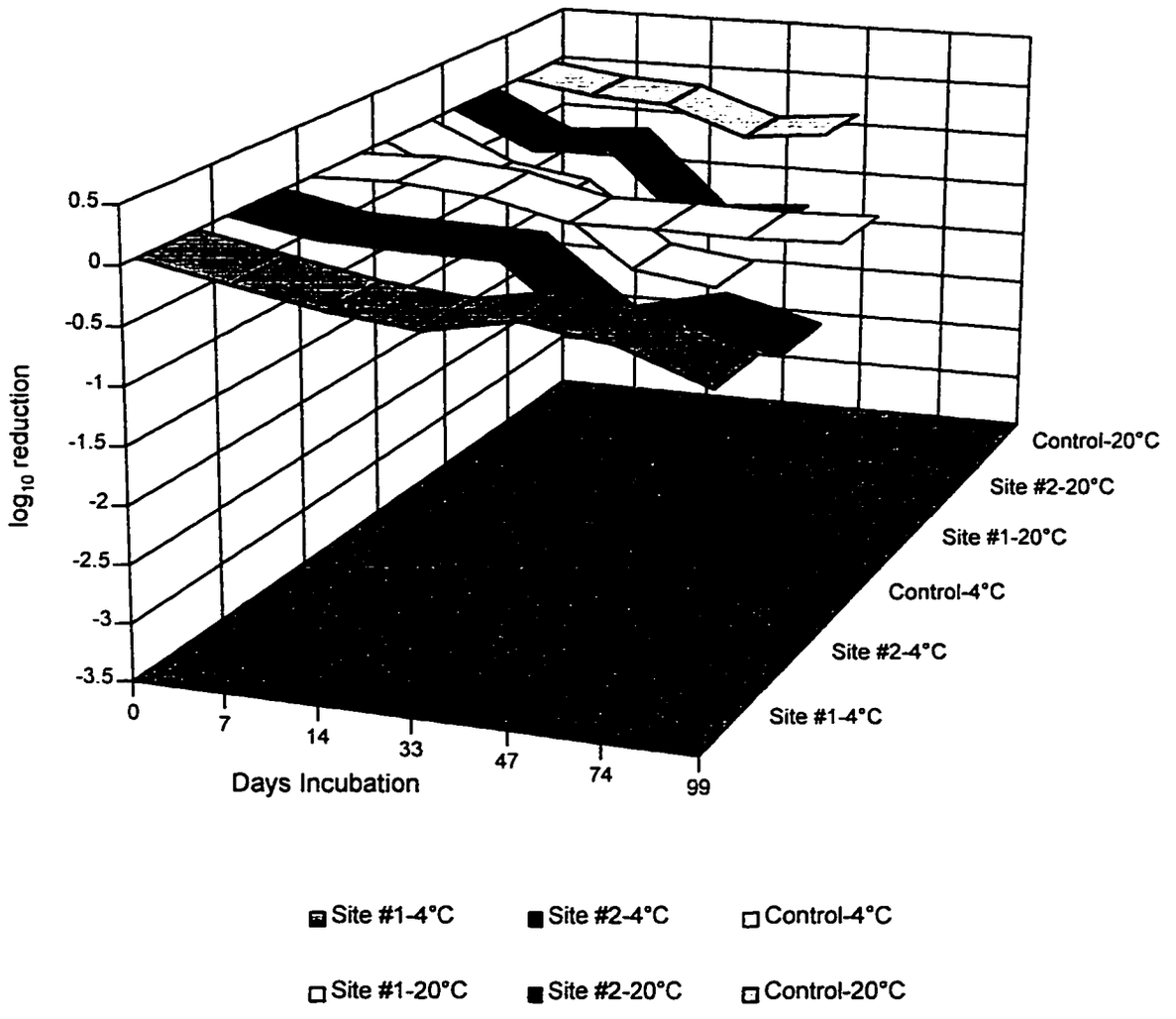


Figure 9: Grand River Log_{10} Reductions in Oocyst Viability, Winter, 1995.

Table 18: Winter, 1995-St. Lawrence River Total Oocyst Population Counts Over Testing Period

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	539474	468421	481579	663158	513158	423684	386842
Site #2 4°C	444737	473684	573684	444737	373684	405263	336842
4°C Control	486842	531579	431579	331579	321053	344737	386842
Site #1 20°C	471053	521053	265790	144737	126316	-	-
Site #2 20°C	421053	457895	142105	142105	144737	-	-
20°C Control	513158	423684	373684	244737	281579	-	-

Table 19: Winter, 1995-St. Lawrence River Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	100	87	89	123	95	79	72
Site #2 4°C	100	107	129	100	84	91	76
4°C Control	100	109	89	68	66	71	79
Site #1 20°C	100	111	56	31	27	-	-
Site #2 20°C	100	109	34	34	34	-	-
20°C Control	100	83	73	48	55	-	-

Table 20: Winter, 1995-St. Lawrence River *In vitro* Excystation Over Testing Period

	Days Incubation						
Treatment	0	6-7	13-14	32-33	46-47	74	99
Site #1 4°C	80.8	75.5	64.2	65.5	70.3	65.0	87.0
Site #2 4°C	80.8	78.0	72.2	79.5	67.0	68.5	79.9
4°C Control	80.8	77.5	81.0	72.0	76.0	67.6	64.0
Site #1 20°C	80.8	78.0	79.0	83.5	71.5	-	-
Site #2 20°C	80.8	89.0	65.0	71.0	71.5	-	-
20°C Control	80.8	77.0	79.5	65.5	67.0	-	-

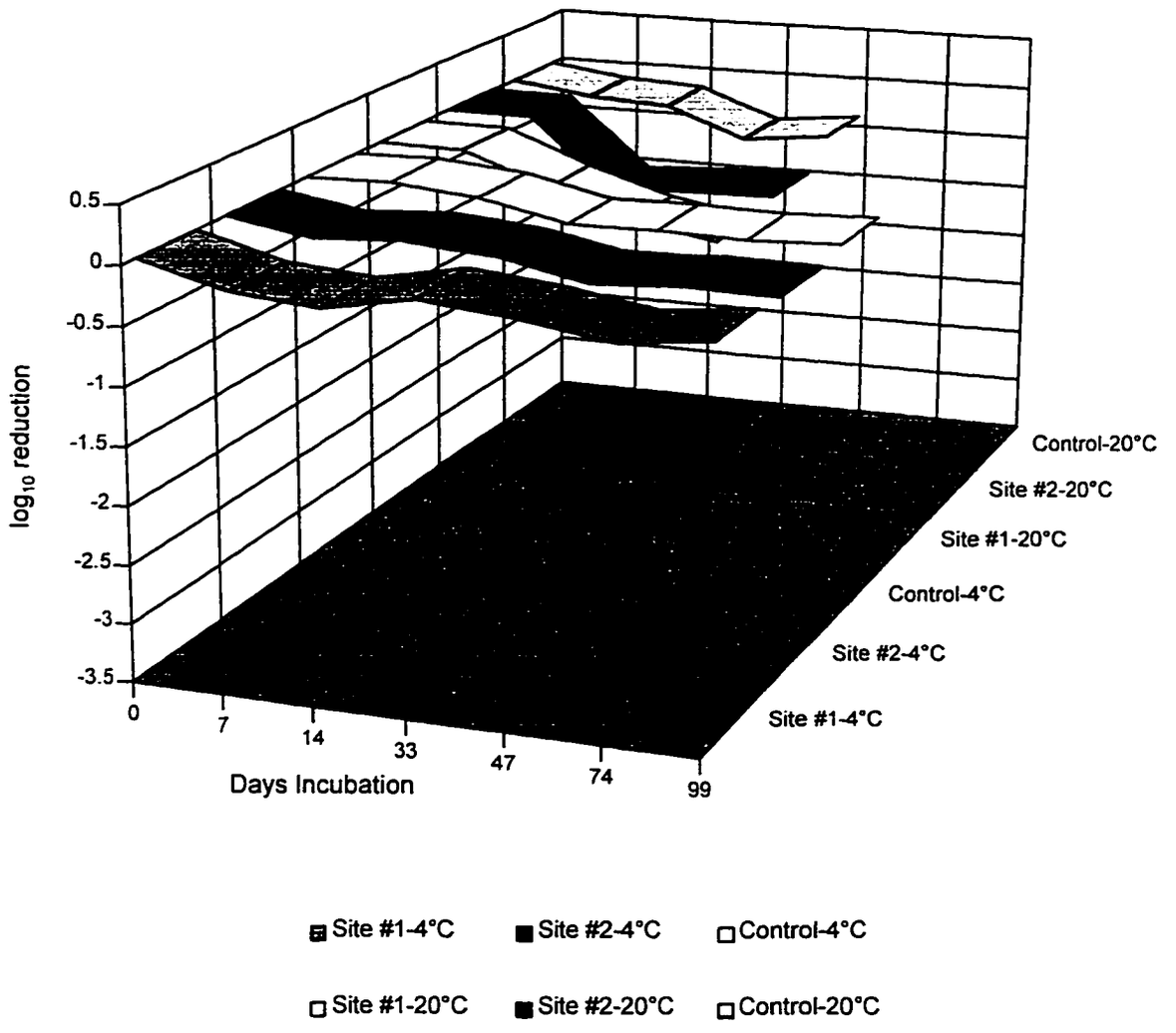


Figure 10: St. Lawrence River Log_{10} Reductions in Oocyst Viability, Winter, 1995.

Table 21: Spring, 1996-Grand River Total Oocyst Population Counts Over Testing Period

Treat-ment	Days Incubation							
	0	6	11-12	24-25	31-32	47-48	88	103
Site #1: 4°C	586842	68421	342105	239474	152632	168421	126316	86842
Site #2: 4°C	468421	697368	450000	405263	476316	328947	334211	242105
Control: 4°C	439474	589474	471053	434211	394737	457895	573684	331605
Site #1: 20°C	563158	378947	139474	26316	10526	10526	-	-
Site #2: 20°C	578947	447368	178947	221053	126316	34211	-	-
Control: 20°C	534211	502632	326316	510526	547368	468421	-	-

Table 22: Spring, 1996-Grand River Oocyst Populations Over Time as a Percentage of Original Population

Treatment	Days Incubation							
	0	6	11-12	24-25	31-32	47-48	88	103
Site #1 4°C	100	117	58	41	26	29	22	15
Site #2 4°C	100	149	96	87	102	70	71	52
4°C Control	100	134	107	99	90	104	131	75
Site #1 20°C	100	67	25	5	2	2	-	-
Site #2 20°C	100	77	31	38	22	6	-	-
20°C Control	100	94	61	96	102	89	-	-

Table 23: Spring, 1996-Grand River *In vitro* Excystation Over Testing Period

Treatment	Days Incubation							
	0	6	11-12	24-25	31-32	47-48	88	103
Site #1 4°C	89.0	85.5	81.0	87.0	84.3	77.0	89.5	89.0
Site #2 4°	89.0	85.5	77.0	87.5	89.7	79.6	86.5	88.0
4°C Control	89.0	78.0	77.5	83.5	76.0	82.0	89.0	83.0
Site #1 20°C	89.0	90.5	81.5	82.0	80.0	81.0	-	-
Site #2 20°	89.0	83.0	88.0	82.5	81.5	73.5	-	-
20°C Control	89.0	81.5	79.5	85.0	76.5	83.0	-	-

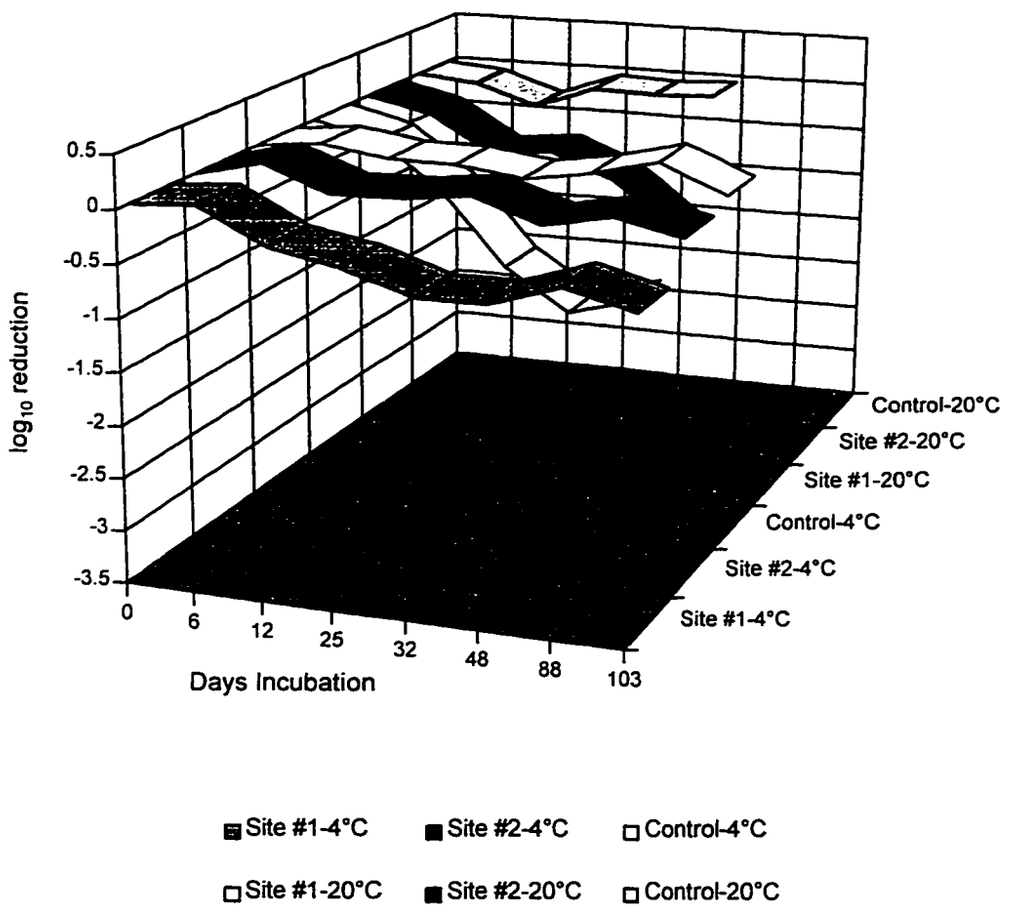


Figure 11: Grand River Log_{10} Reductions in Oocyst Viability, Spring, 1996.

Survival at 20°C, Survival in Summer, 1995, and Survival in Winter, 1995

Figure 12 and Figure 13 illustrate $\log_{10}$ reductions in oocyst viability over St. Lawrence and Grand Rivers at 20°C. It appears that the summer sampling season shows a more dramatic decrease in $\log_{10}$ reduction compared to all of the other seasons tested.

Figure 14 and Figure 15 illustrate the $\log_{10}$ reduction data for all rivers at both temperatures tested for the summer season. The results show that there appears to be differences among the rivers sampled.

Figure 16 and Figure 17 plot $\log_{10}$ reduction data from the Grand and St. Lawrence Rivers (Carp River was not tested in the winter) at both incubation temperatures. Results show that the St. Lawrence River may provide a more hospitable environment for oocyst survival than the Grand River for the winter season tested.

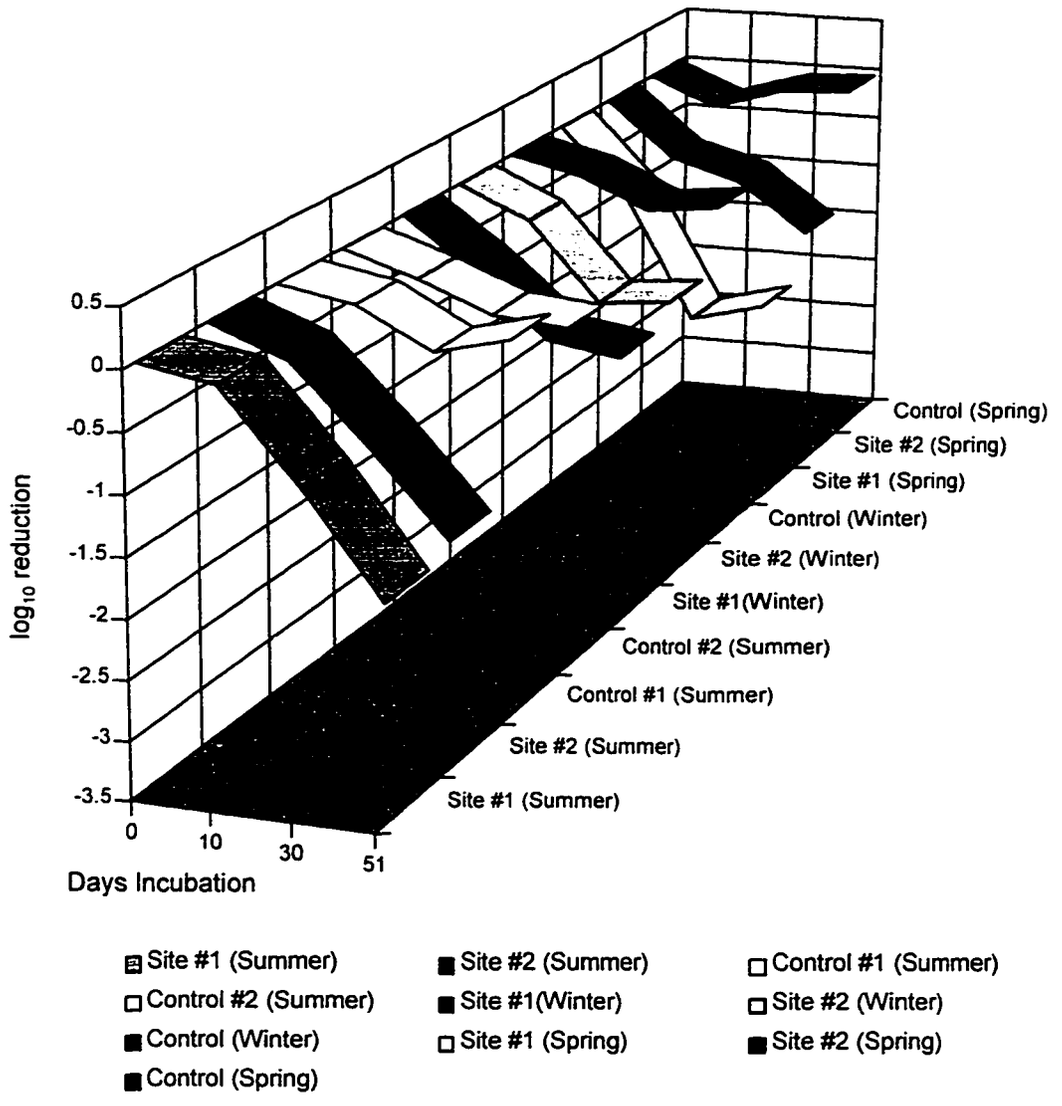


Figure 12: Grand River: *In vitro* Oocyst Survival at 20°C Over all Seasons Tested.

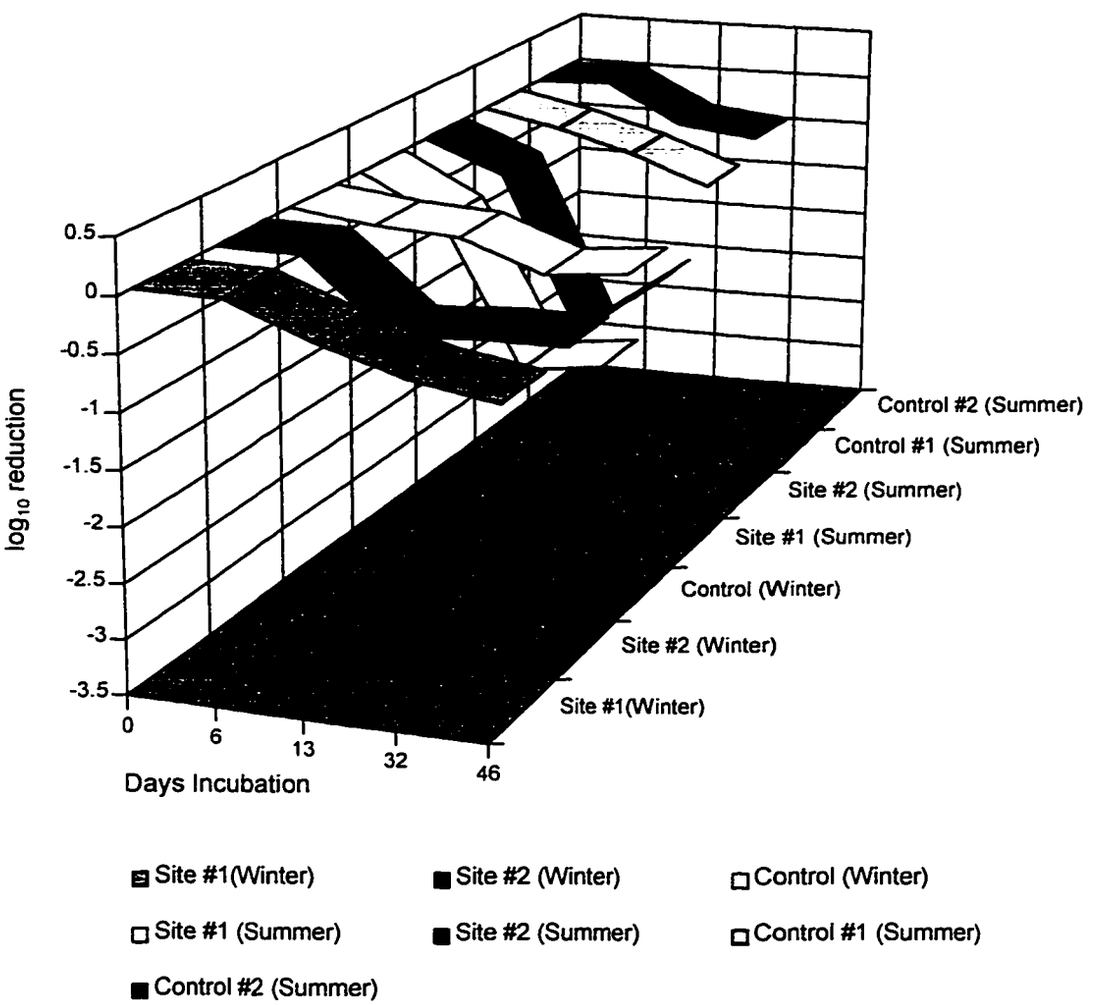


Figure 13: St. Lawrence River: *In vitro* Oocyst Survival at 20°C Over all Seasons Tested.

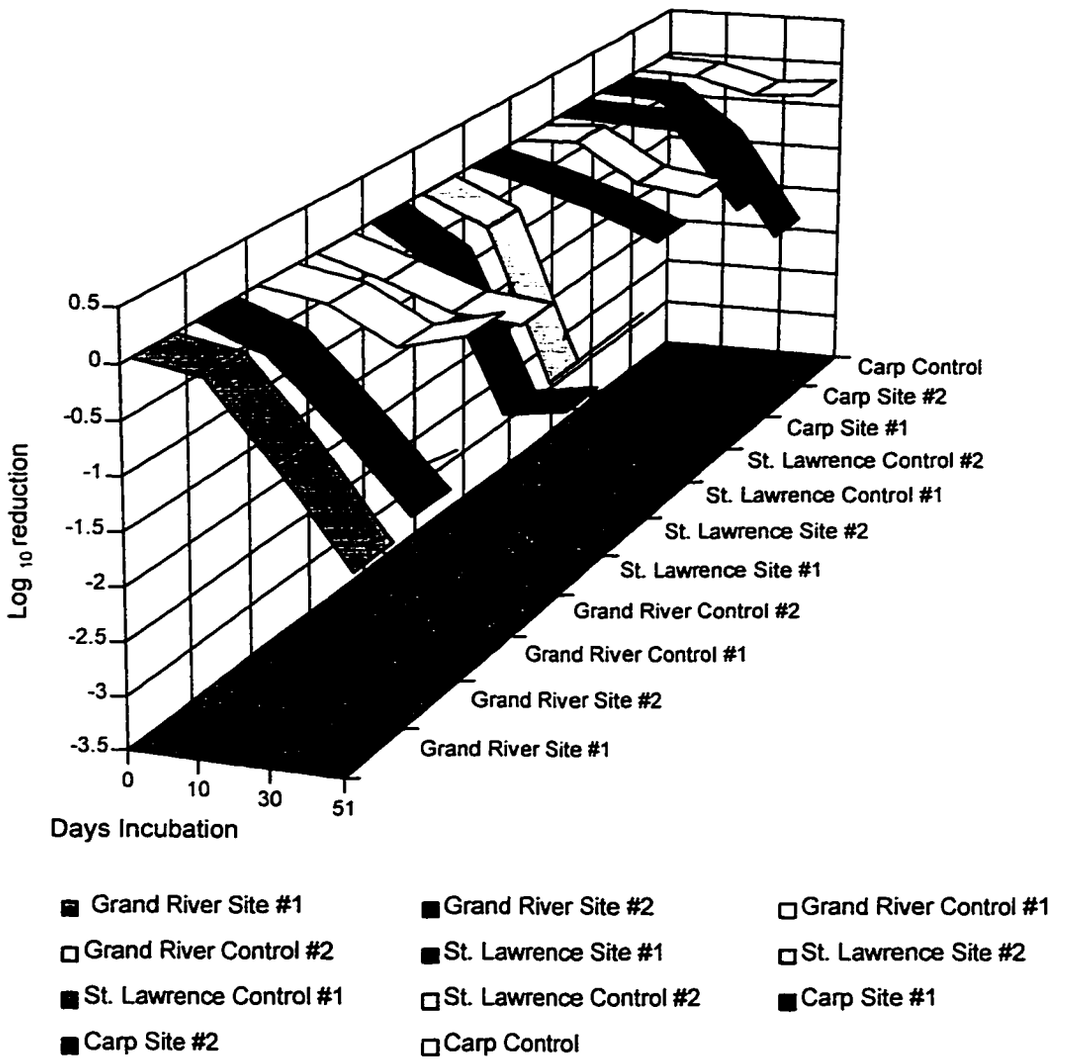


Figure 14: *In vitro* Oocyst Survival in the Grand, St. Lawrence and Carp Rivers: Summer, 1995 20°C. Arrows indicate $\log_{10}$ reductions which are less than or equal to values stated.

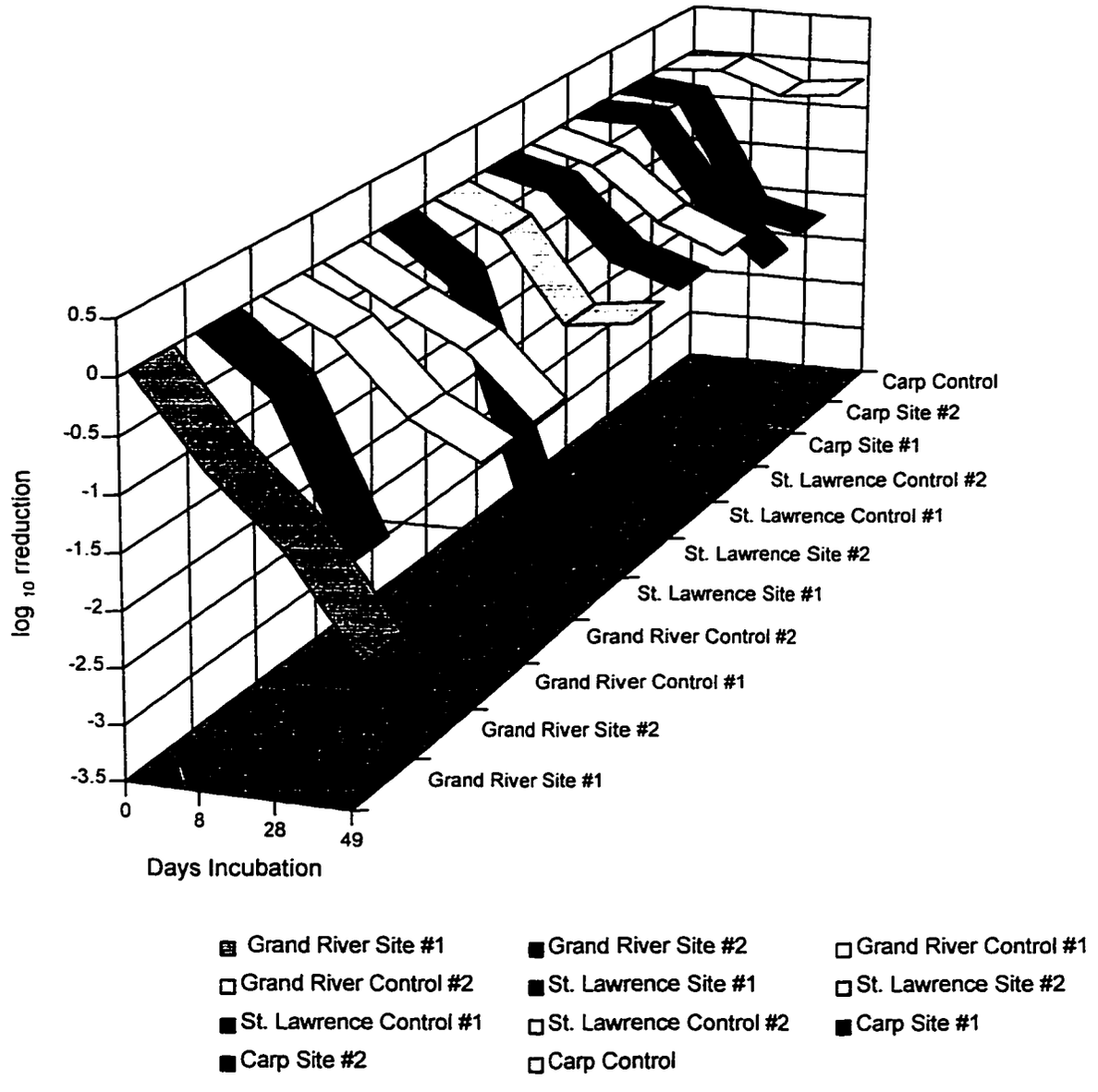


Figure 15: *In vitro* Oocyst Survival in the Grand, St. Lawrence and Carp Rivers: Summer, 1995 30°C. Arrows indicate $\log_{10}$ reductions which are less than or equal to values stated.

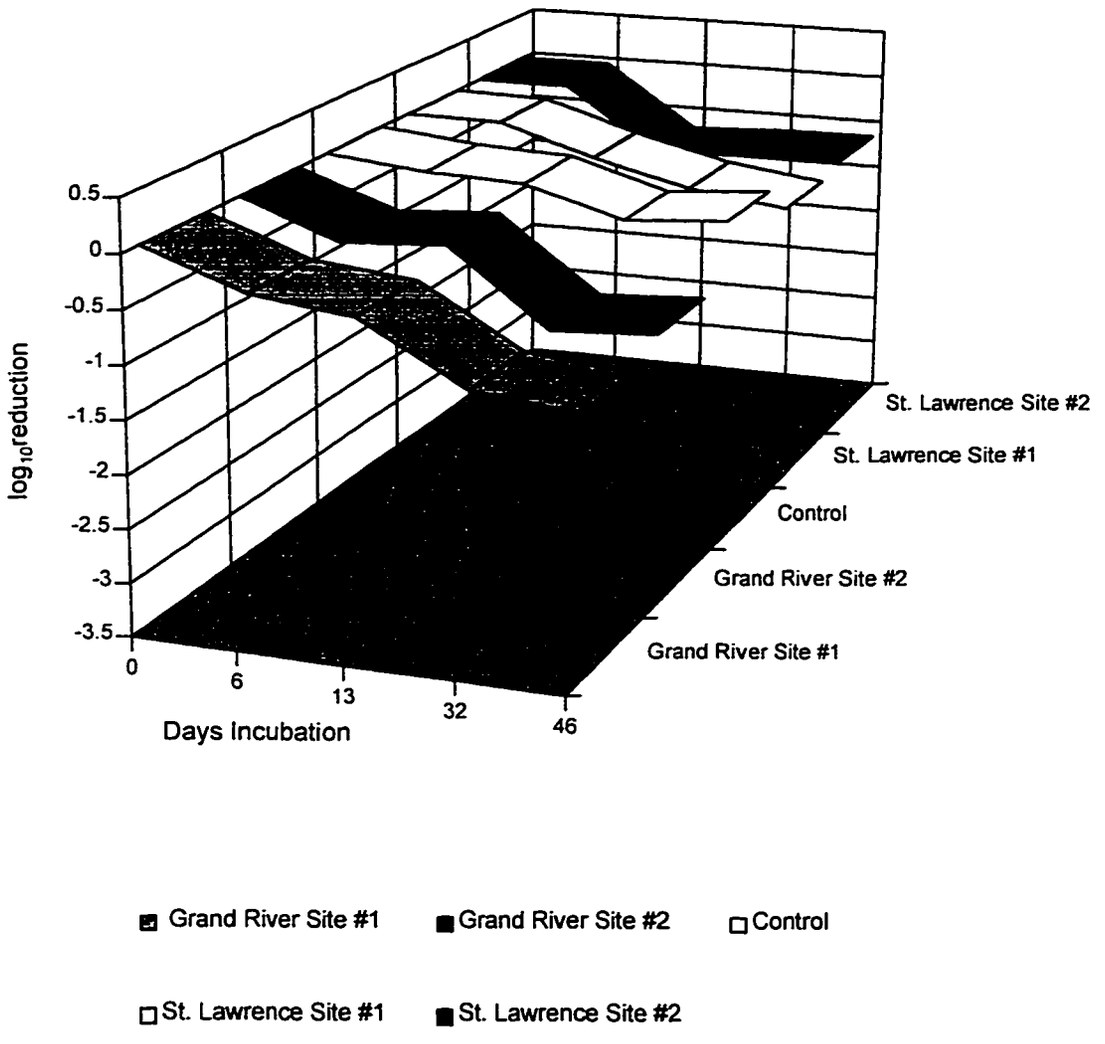


Figure 16: *In vitro* Oocyst Survival in the Grand and St. Lawrence Rivers: Winter, 1995 20°C.

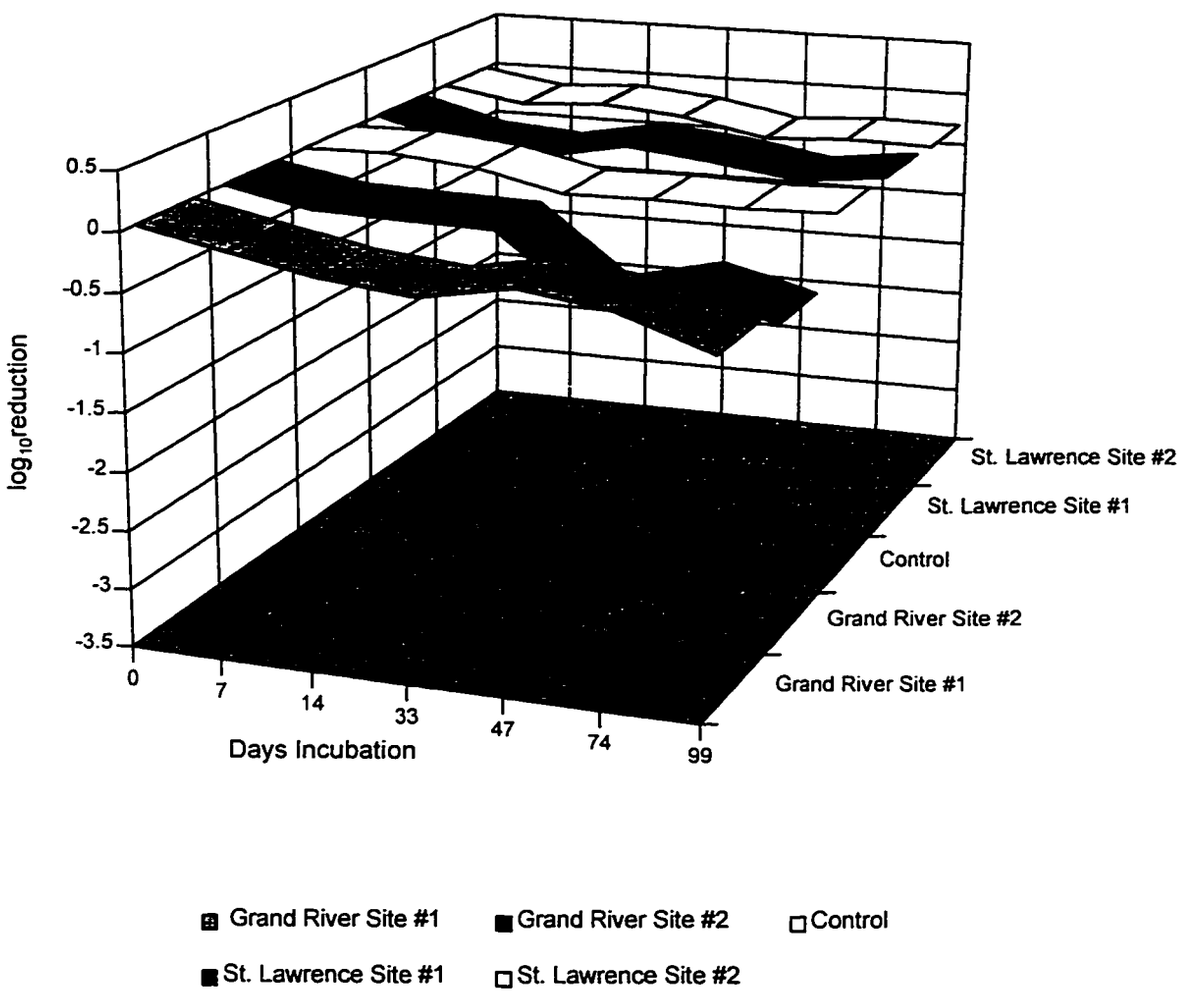


Figure 17: *In vitro* Oocyst Survival in the Grand and St. Lawrence Rivers: Winter, 1995 4°C.

Statistical Analysis

The following models were tested in order to assess the effect of the factors of river, site within a river, temperature and season of sampling, tested in the *in vitro* survival experiments presented.

1. Within river and sites, do seasons and temperature matter?

   example: in the St. Lawrence River site #1, do different seasons and temperatures affect oocyst survival?

2. Within rivers, sites and seasons, do temperatures matter?

   example: in the Grand River, site #1 in the spring, do different temperatures affect oocyst survival?

3. Within rivers, temperatures and seasons, do sites matter?

   example: in the Carp River, 20°C in the summer, does water collected from different sites affect oocyst survival?

4. Within temperatures, seasons and sites, do rivers matter?

   example: at 20°, in the summer at site #1, do different rivers affect oocyst survival differently?

5: Do rivers, sites seasons and temperature matter?

   example: do any or all factors tested affect oocyst survival?

Further statistical analysis was carried out using an F-test (128) to determine the significance of differences between all factors tested when the regression data was pooled.

Table 24: Model 1: The Effect of Season and Temperature on *In vitro* Oocyst Survival-Results of Statistical Analysis

Treatment	Important Factor	P-value	Specifics	P-value
GR site #1	-survival (4°C) > survival (20°C) > survival (30°C)	-p<0.05	-season not an important factor	-p>0.05
GR site #2	-survival (4°) > survival (20°C) > survival (30°C)	-p<0.05	no specifics	
	-survival (spring, winter) > survival (summer)	-p<0.05		
SL site #1	-survival (winter) > survival (summer)	-p<0.05	-temperature not an important factor	-p>0.05
SL site #2	- survival (winter) > survival (summer)	-p<0.05	-survival (4°) > survival (20°C)	-p<0.05
			-no difference in survival (20°C) and survival (30°C)	-p>0.05
CR site #1	- survival (20°C) > survival (30°C)	-p<0.05	no specifics	
CR site #2	-	-	-no difference in survival (20°C) and survival (30°C)	-p>0.05
CGR site #1	-survival (spring) > survival (summer)	-p<0.05	-no difference in survival (summer) and survival	-p>0.05
	-survival (20°C) > survival (30°C)	-p<0.05	(winter)	-p>0.05
			-no difference in survival (20°C) and survival (4°C)	
CGR site #2	-survival (4°C) > survival (20°C) > survival (30°C)	-p<0.05	-season not an important factor	-p>0.05
CSL site #1	-survival (20°C) > survival (30°C)	-p<0.05	-season not an important factor	-p>0.05
			-no difference in survival (4°C) and survival (20°C)	-p>0.05
CSL site #2	-survival (20°C) > survival (30°C)	-p<0.05	-season not an important factor	-p>0.05
CCR sites #1 and #2	-no difference in survival (20°C) and survival (30°C)	-p>0.05	season was not tested	-

GR: Grand River, CGR: Grand River control, SL: St. Lawrence River, CSL: St. Lawrence River control, CR: Carp River, CCR: Carp River control.
n=8-34

Table 25: Model 2: The Effect of Temperature on *In vitro* Oocyst Survival-Results of Statistical Analysis

Treatment	Important Factor	P-value	Specifics	P-value
Tests	-survival (4°C) > survival (20°)	-p<0.05	-no difference in survival (20°C) and survival (30°C)	-p>0.05
Controls	-survival (20°C) > survival (30°C)	-p<0.05	-survival (4°C, winter) > survival (20°C, winter) -no difference in survival (4°C, spring) and survival (20°C, spring)	-p<0.05 -p>0.05

n=7-14

Table 26: Model 3: The Effect of Site Within a River on *In vitro* Oocyst Survival-Results of Statistical Analysis

Treatment	Important Factor	P-value	Specifics	P-value
Grand River	-survival (site#2) > survival (site#1) in the spring	-p<0.05	-no difference in survival (site#1) and survival (site#2) at for all other seasons and temperatures	-p>0.05
St. Lawrence River	-survival (site#2) > survival (site#1) in the summer (30°C)	-p<0.05	-no difference in survival (site#1) and survival (site#2) at for all other seasons and temperatures	-p>0.05
Carp River	-no difference in survival (site#1) and survival (site#2) for all temperatures tested	-p>0.05	no specifics	-

n=7-16

Table 27: Model 4: The Effect of River on *In vitro* Oocyst Survival-Results of Statistical Analysis

Treatment	Important Factor	P-value	Specifics	P-value
Grand River	-survival (St. Lawrence) > survival (Grand) in the winter (4°C) -survival (Carp) > survival (Grand) in the summer (30°C)	-p<0.05 -p<0.05	-no difference in survival in any of the rivers for all other seasons and temperatures tested	-p>0.05

n=10-14

Table 28: Model 5: The Effect of All Factors Tested on *In vitro* Oocyst Survival-Results of Statistical Analysis

Treatment	Important Factor	F-value	P-value
Grand River	sites #1 and #2 are different from each other	[1, 192]	p<0.05
St. Lawrence River	sites #1 and #2 are not different from each other	[1, 192]	p>0.05
Carp River	sites #1 and #2 are not different from each other	[1, 192]	p>0.05
Grand, St. Lawrence and Carp Rivers	all of these rivers are different from each other	[5, 192]	p<0.05
Grand River and its Controls	the Grand River is different from its controls	[1, 192]	p<0.05
St. Lawrence River and its Controls	the St. Lawrence River is different from its controls	[2, 192]	p<0.05
Carp River and its Controls	the Carp River is different from its controls	[2, 192]	p<0.05
Spring and Winter Seasons	there is no difference between these two seasons	[1, 192]	p>0.05
4°C and 30°C Incubation Temperatures	there is a difference between the 4°C and 30°C incubation temperatures	[1, 192]	p<0.05

n=207

In vitro *Survival with a Spike of* Pseudomonas fluorescens

The results of the bacterial spiking trials can be found in Table 29, Table 30, Table 31, and Figure 18. It is apparent from observing these results that *P. fluorescens* had little or no effect on the *in vitro* survival of the oocysts. Raw water and 2.0 µm filtered water however, did show a dramatic decrease in total oocyst numbers, while excystation values did not decrease as significantly.

Table 29: *In vitro* Survival of *Cryptosporidium* in Waters Spiked with *P. fluorescens:* Total Oocyst Population Counts Over Testing Period

	Days Incubation						
Treatment	0	4	11	18	27	39	50
Test	457895	563158	365790	371053	363158	365790	344737
100 ppm water	647368	592105	597368	828947	436842	710526	636842
raw water	513158	352632	268421	115790	50000	**<36579**	**<10263**
0.22 µm water	818421	492105	513158	510526	426316	694737	463158
2.0 µm water	626316	265790	115790	81579	71053	13158	13158

Bolded values indicate those where one of the two counts used to calculate the mean was below the detection limit.

Table 30: *In vitro* Survival of *Cryptosporidium* in Waters Spiked with *P. fluorescens:* Oocyst Populations Over Time as a Percentage of Original Population

	Days Incubation						
Treatment	0	4	11	18	27	39	50
Test	100	123	80	81	79	80	75
100 ppm water	100	91	92	128	67	110	98
raw water	100	69	52	23	10	7	2
0.22 µm water	100	60	63	62	52	85	57
2.0 µm water	100	42	18	13	11	2	2

Table 31: *In vitro* Survival of *Cryptosporidium* in Waters Spiked with *P. fluorescens: In vitro* Excystation Over Testing Period

	Days Incubation						
Treatment	0	4	11	18	27	39	50
Test	83.0	80.0	87.0	80.3	85.0	87.0	87.5
100 ppm water	83.0	69.5	77.0	88.6	83.5	86.0	89.0
raw water	83.0	79.5	87.5	90.0	83.0	84.0	64.0
0.22 µm water	83.0	72.0	86.0	90.9	76.0	86.5	87.0
2.0 µm water	83.0	76.0	82.5	88.1	82.5	90.5	85.0

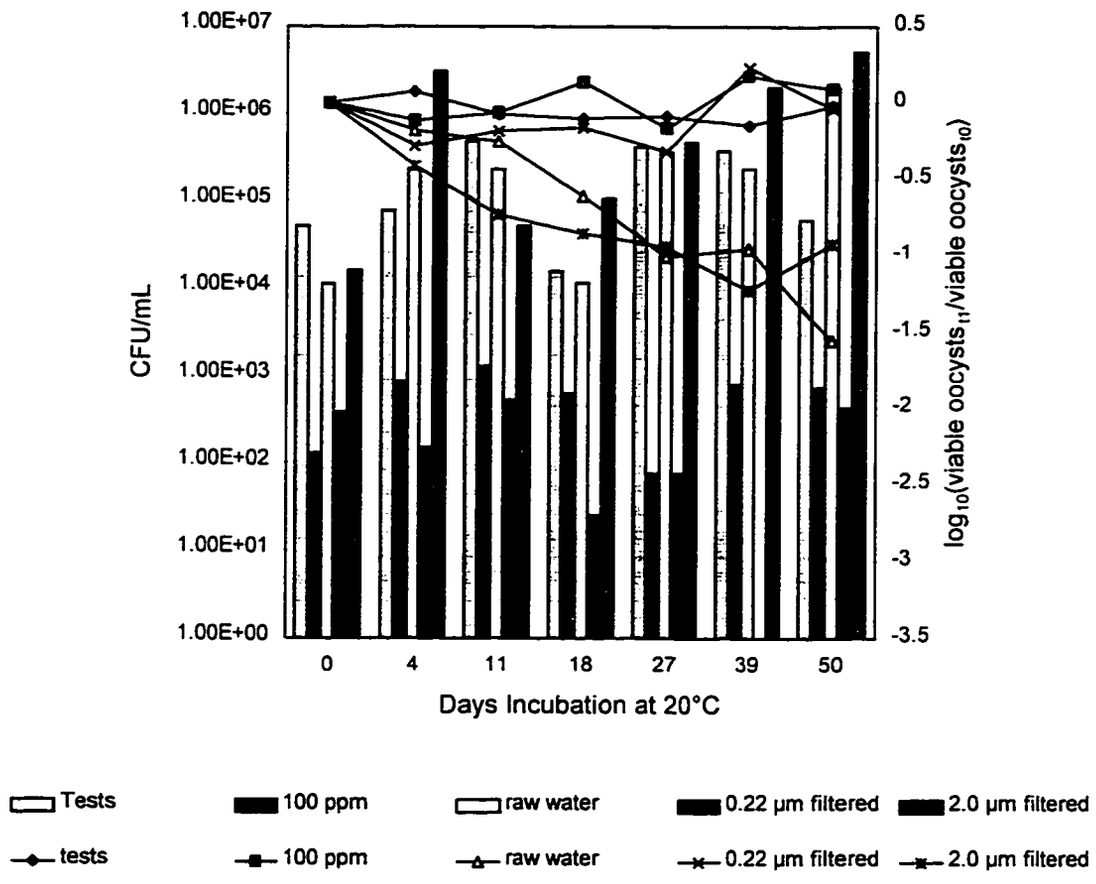


Figure 18: Bacterial Load Log_{10} Reductions in Carp River
Columns are HPCs in cfu/mL, while lines are log_{10} reductions.

Table 32: Statistical analysis of *in vitro* survival with a spike of *P. fluorescens* $\log_{10}$ reductions

Treatment	Is regression slope different from zero?	Upper 95% Confidence Interval	Lower 95% Confidence Interval	n
Test	no ($p>0.05$)	0.0022	-0.0061	7
100 ppm water	no ($p>0.05$)	0.0106	-0.0037	7
raw water	yes ($p<0.05$)	-0.02127	-0.03731	7
0.22 µm filtered water	no ($p>0.05$)	0.01502	-0.00667	7
2.0 µm filtered water	yes ($p<0.05$)	-0.00305	-0.03263	7

Because of the very limited data set, a more robust statistical analysis of the data was not possible. However, linear regression analysis of the data indicates that the oocyst $\log_{10}$ reduction regression curves obtained in raw water and 2.0 µm filtered water have slopes that are significantly different than zero ($p<0.05$, $n=7$), while test, 100 ppm synthetic and 0.22 µm filtered water all had slopes that were not significantly different than zero ($p>0.05$, $n=7$). This suggests that the bacterial load introduced into the test waters had no effect on oocyst survival compared to the 100 ppm synthetic and 0.22 µm filtered waters, while both raw and 2.0 µm filtered water had a detrimental effect compared to the other waters tested.

Unfortunately, if one observes the upper and lower 95% confidence intervals, it is apparent that a true, statistical difference between all of the curves, including raw water and 2.0 µm filtered water, cannot be ascertained, since both raw and 2.0 µm filtered waters' confidence intervals overlap with the test, 100 ppm synthetic and 0.22 µm filtered waters.

It can be noted in general though, that raw and 2.0 µm filtered water had the highest HPC levels and also had the greatest $\log_{10}$ reductions over the time frame tested.

Introducing a specific type of bacterium into the water system did not appear to have an effect on the *in vitro* survival of oocysts in natural waters.

Half-Life Analysis

Table 33: Half-life values (in days) for all rivers, seasons, temperatures and sites tested.

Season	River	Site	Temperature	r^2	1/2 Life
Summer, 1995	Grand	1	20°C	0.981	8.61
		2	20°C	0.999	9.42
	St. Lawrence	1	20°C	0.823	8.14
		2	20°C	0.612	10.04
	Carp	1	20°C	0.746	18.83
		2	20°C	0.930	10.39
	Grand Control	1	20°C	0.543	46.43
		2	20°C	0.885	30.13
	St. Lawrence Control	1	20°C	0.997	25.12
		2	20°C	0.812	30.13
	Carp Control	1 and 2	20°C	0.666	80.11
Summer, 1995	Grand	1	30°C	0.966	6.85
		2	30°C	0.999	4.24
	Grand Control	1	30°C	0.963	13.7
		2	30°C	0.886	9.42
	St. Lawrence	1	30°C	0.770	6.85
		2	30°C	0.798	13.1
	St. Lawrence Control	1	30°C	0.954	13.7
		2	30°C	0.974	14.35
	Carp	1	30°C	0.928	11.16
		2	30°C	0.914	10.39
	Carp Control	1 and 2	30°C	0.219	102.48
Winter, 1995	Grand	1	20°C	0.634	10.76
		2	20°C	0.867	14.35
	Grand Control	1 and 2	20°C	0.826	36.62
	St. Lawrence	1	20°C	0.994	19.69
		2	20°C	0.552	23.18
	St. Lawrence Control	1 and 2	20°C	0.826	36.62
Winter, 1995	Grand	1	4°C	0.709	69.03
		2	4°C	0.769	39.55
	Grand Control	1 and 2	4°C	0.640	124.46
	St. Lawrence	1	4°C	0.280	203.45
		2	4°C	0.695	122.83
	St. Lawrence Control	1 and 2	4°C	0.640	124.46

Season	River	Site	Temperature	r^2	1/2 Life
Spring, 1996	Grand	1	4°C	0.785	40.36
		2	4°C	0.710	76.26
	Control	1 and 2	4°C	0.102	34.49
		1	20°C	0.838	7.6
		2	20°C	0.912	13.3
		1 and 2	20°C	0.046	**-258.86**
Bacterial Load	Test	2	20°C	0.232	153.17
Spring, 1996	100 ppm water	2	20°C	0.232	**-88.14**
	Raw water	2	20°C	0.946	**-10.29**
	0.22 µm filtered water	2	20°C	0.164	**-72.2**
	2.0 µm filtered water	2	20°C	0.658	16.91

-1/2 Life determined by the following formula: 1/2 Life = $0.693/K_i$ where K_i is the slope of the log-linear plot (120). Bolded values indicate half-lives due to a positive slope of the regression curves.

Correlation Analysis

Spearman correlations were calculated between decay rates (given as half-life-Table 33), incubation temperature, HPC, TOC, pH and metals for Summer, Winter, 1995 and Spring, 1996, for all rivers tested except *in vitro* survival with a spike of *P. fluorescens*. Results of the analysis is presented in Table 34.

None of the metals or TOCs tested had any significant correlation with *in vitro* oocyst survival ($p>0.05$). However, temperature was significantly correlated ($p<0.05$) with decreased *in vitro* oocyst survival, and HPC, although not significant at the 5% level, does appear to be correlated with decreased oocyst survival.

Table 34: **Spearman Correlation Analysis**

Seasons Analyzed	Experimental component	Correlation coefficient	n
Summer, Winter 1995	Incubation Temperature	0.70 ($p<0.05$)	20
	HPC (day 0)	0.46 ($p<0.05$)	20
	pH	0.65 ($p<0.05$)	12
	TOC	0.07 ($p>0.05$)	20
Summer, 1995 Winter, 1995 Spring, 1996	Incubation Temperature	0.75 ($p<0.05$)	24
	HPC (day 0)	0.35 ($0.05<p<0.10$)	24
	pH	0.34 ($p>0.05$)	16
	TOC	0.12 ($p>0.05$)	24

Note: No significant correlations were obtained for any of the individual alkali-, alkaline earth- or heavy metals, or non-metals, tested.

DISCUSSION

The St. Lawrence River is part of the Great Lakes water basin which supplies potable water to a very large population in both Canada and the United States. The river flows through many communities that not only draw their water from it, but also discharge both domestic and commercial wastes back into it. The Grand River flows through a large agricultural area before passing through the urban region of Kitchener-Waterloo. It has been supplying the community with potable water since 1992. The Carp River is a relatively small river that also passes through an agricultural region before emptying into the Ottawa River, a river that serves the Ottawa region with drinking water. Since many communities obtain their drinking water from surface water sources, assessing the survival of *Cryptosporidium* oocysts in natural waters that have different chemical characteristics was considered desirable.

The results presented indicate that a very complex relationship exists between the *in vitro* survival of oocysts and the factors tested. However, it is apparent from examining

the results for the Grand River, presented in Table 6, Table 7 and Table 8, that the total population of oocysts within the samples shows a much larger decrease over time compared to the reduction in excystation rates over time. The same relationship can be shown to exist for the remaining rivers, seasons and temperatures tested. This suggests that when determining viability, as was done in these protocols, the total oocyst numbers present have the largest effect over determining the number of oocysts viable at the sampling point. It appears that oocysts decline in absolute number, in this *in vitro* system much faster and to a greater extent than excystation rates fall. This suggests that, although oocyst numbers may decline dramatically over time, the *in vitro* excystation rate will remain relatively high, and therefore remaining oocysts are possibly infectious. Therefore, total oocyst counts are important when examining the survival of oocysts in a water system.

The oocyst preparations were also an important factor for *in vitro* survival in natural and synthetic hard water. If one examines Table 24 and Table 25, it can be noted that there appears to be a difference in *in vitro* survival of oocysts in the control waters during different seasons. Model 2 suggests that there was a difference in survival between the 4°C and 20°C incubation temperatures in the winter ($p<0.05$), but this difference did not exist in the spring ($p>0.05$). This however, should not be the case since the controls have the *same* water, 100 ppm synthetic hard water, for all seasons tested. This control water does not change from season to season. The implication of this is that oocysts shed from different animals, collected and purified at different times (seasons), may have different survival capabilities in the water environment. If this was not so, there would be no difference in their survival over different seasons. The same disparity can be observed

with the results given for Model 1 Controls. Both the St. Lawrence and Grand River controls showed that season had an effect on oocyst survival; oocysts incubated in control waters with the summer season samples showed decreased survival compared to those incubated with the spring samples ($p<0.05$). Different batches and preparations of oocysts behaved differently in this study. This implies that oocysts shed from the same animal at different times or from different animals at the same time may have different survival capabilities in the natural environment.

Model 1 attempted to identify the combined effect of season and temperature *in vitro* oocyst survival. These two factors had to be combined in this particular model due to lack of adequate data to determine the effect of season alone. The results indicate that season and temperature had an effect on survival in this *in vitro* system; however, the effect was not at all consistent across rivers and sites. Since temperature and season were analyzed together, there was increased complexity introduced into this model, explaining the lack of ease of interpretation. However, from observing the data presented in Table 24, it is obvious that since different rivers did not behave the same and different sites did not necessarily behave the same, there must be a difference between rivers and possibly sites.

Model 2 approached the question of whether or not temperature had an effect on oocyst survival in this experimental system, given all other factors tested remained constant. The results presented in Table 25 indicate that temperature is an important factor effecting *in vitro* oocyst survival. The greatest reduction in oocyst viability occurs at the 30°C incubation temperature, followed by 20°C while the 4°C incubation temperature proved to be the most hospitable temperature tested for oocyst survival

($p<0.05$). The statistical significance of this is supported by the results presented in Table 28-results for Model 5. Therefore, the differences observed between the viability curves of different incubation temperatures detailed in Figure 6, Figure 7, Figure 8, Figure 9, Figure 10, Figure 11 are actually different ($p<0.05$).

Because the metals and TOC analyses indicated that there were slight differences in TOC and chemical composition, outside of acceptable experimental error, from different sites within the same river. Therefore, it was reasonable to test the model for their effect on *in vitro* oocyst survival. The model that addressed this question, Model 3-Table 26, indicate that the site from which the water was sampled is a factor that affects oocyst survival. An interesting result is that there appears to be increased survival at site #2 in the spring for the Grand River ($p<0.05$) for both temperatures tested, (Figure 11) and increased survival at site #2 in the summer at 30°C for the St. Lawrence River ($p<0.05$),(Figure 7). Both of these sites are at or downstream from the urban districts which the rivers flow through. There appears to be *something* occurring in the river as it flows through the urban districts that enhances oocyst survival compared to the site #1s of the two rivers during specific seasons and temperatures. When the results of the TOC and chemical analyses were correlated to $\log_{10}$ reductions, no significant correlation ($p>0.05$) between the chemicals examined and *in vitro* survival was observed (Table 34). However, a correlation did exist between temperature, and, not as strongly with, HPCs. This indicates that the microbial content of the host water may be a significant factor for influencing oocyst survival in them. The Carp River however, did not show any significant difference ($p>0.05$) in oocyst survival between sites tested (Figure 8. Figure 6,

Figure 9 and Figure 10). None of the differences between sites in the figures are significant ($p>0.05$).

The results of Model 1 combined with examining the TOC and metals analyses for the three rivers tested, indicated that a difference in survival of oocysts in different rivers may exist. The model developed to test this hypothesis, Model 4-Table 27, indicate that there is a difference between rivers, but only under certain restrictions. There is a significant increase in *in vitro* oocyst survival in the St. Lawrence River compared to the Grand River only in the winter at 4°C ($p<0.05$) (Figure 17). There was no significant difference between the Grand and St. Lawrence Rivers over all other seasons and temperatures tested ($p>0.05$). The Carp River also showed increased *in vitro* oocyst survival compared to the Grand River, but only during the summer season and at 30°C ($p<0.05$) (Figure 15). The remaining figures representing the river-to-river comparisons can be found in Figure 14, Figure 15 and Figure 16. Again, one would postulate that there are significant chemical differences between the rivers that affect oocyst survival, but attention must be again drawn to Table 34, since the results in this table clearly show that water chemistry is not correlated to oocyst survival ($p>0.05$). Not only the microbiology at the site of water collection may influence oocyst survival, but the microbiology of the entire river may also be a factor that affects the survival of oocysts in natural waters.

The final statistical model asked the question whether or not all factors tested, taken together, were important in the *in vitro* survival of *Cryptosporidium* oocysts. The results presented in Table 28 indicate that different sites within a river may affect survival differently, but it depends on the river. Different rivers however, are important such that they affect survival differently ($p<0.05$). Season is also an important factor, spring and

winter not affecting survival differently ($p>0.05$) but summer being different in its influence than spring and winter (Model 1) ($p<0.05$). The effect of the difference in incubation temperatures is also important, significantly so, for all of the temperatures tested ($p<0.05$). This final model, although it does not give the details that the previous models had, provides a statistical certainty such that it can now be said, for this experimental system, season, site of collection, river and temperature are all important factors that influence the *in vitro* survival of *Cryptosporidium* oocysts in natural waters.

However, the effect of HPCs on the *in vitro* survival of oocysts had until this point, only been touched upon. The hypothesis that microbiological antagonism decreases oocyst survival in water had recently been brought forth (31), and the statistical analysis of the previous *in vitro* studies had alluded to the possibility of microbial factors decreasing oocyst survival.

Pseudomonas fluorescens is a bacterium that is common in surface water systems. It can be isolated readily and easily cultured. Also, it was the only bacterium that could be subcultured from the river water sample. The aim of the bacterial spiking experiments was to determine if just high bacterial numbers were responsible for the decline in oocyst viability over time in a natural water *in vitro* system. Although Table 29, Table 30, and Table 31 show similar total oocyst population and excystation trends as was observed in previous survival experiments, it is obvious that oocysts inoculated into raw river water and 2.0 μm filtered river water show a much more dramatic reduction in oocyst viability than the other waters tested (filter sterilized raw water with *P. fluorescens*, 100 ppm synthetic hard water and 0.22 μm filter sterilized raw water). Although adequate statistical testing of $\log_{10}$ reduction data was not possible, it is apparent from Figure 18

that the presence of *P. fluorescens* in the filter sterilized river water had no effect on the survival of oocysts even though for the majority of the time the experiment was being conducted, it had one of the highest HPC values. Therefore, any decline observed was probably due to a temperature effect, as was discussed previously. The 2.0 μm filtered water showed a much steeper initial decline in oocyst viability than the raw water samples, but by the end of the experimental period, both the 2.0 μm filtered water and the raw water samples were similar. Chemical analysis of the different filtered waters again showed slight differences in the chemistries of the water, outside of experimental error, possibly due to the filter matrix. However previous correlation analysis (Table 34) clearly indicated that water chemistry was not a factor influencing oocyst survival in waters. Therefore, there appears to be a factor(s) in the water smaller than 2.0 μm but larger than 0.22 μm, that antagonizes *Cryptosporidium* oocysts. These factor(s) are yet unknown, but could be postulated to be bacteria, such as *Serratia marcescens*, and/or small protozoan predators.

It is apparent that although it has not been determined what microbiological agent(s) within a water system has such a serious impact on oocyst survival, it has been resolved that the physical components such as river, sites within a river, microbiology temperature and seasons of the year all have and effect on oocyst survival. The relationship between these factors and many other unknown elements is complex, thus further research is required to determine what the most important factors affecting oocyst survival are in the natural setting.

IN VITRO EXCYSTATION VS. FLOW CYTOMETRY

Flow cytometry has recently been added to the list of a growing number of techniques available for *Cryptosporidium* research. As was described previously, flow cytometry is a relatively simple, and a very rapid method of analyzing cells in solution. Fluorochromes can be added as a marker of viability, or many other physiological conditions of the cell, or it can be conjugated to a monoclonal antibody specific for a particular component of the cell. Once the fluorochrome is added, the sample can be analyzed according to the non-fluorescing (non-stained) and fluorescing (stained) portions of the sample. These characteristics can then be used to give vital information with regards to the components of interest within the sample (121). For example, if one wanted to quantify the number of dead organisms within a culture, one may want to incubate the culture with a viability marker, such as propidium iodide (PI), and analyze it by flow cytometry. Usually, the entire process of labeling a sample and analyzing by flow cytometry is very rapid; hundreds of samples may be processed in the course of a single day. Yet, it is quite surprising that flow cytometry has not been adapted for use in oocyst viability studies.

Presently, the "gold standard" in *Cryptosporidium* viability and survival studies is animal (mainly mouse) infectivity. Normal neonatal (5,45,126) or immunodeficient mice can be used for this purpose. This method is time consuming, labour intensive and expensive. Although such a system can provide information on the infectivity of oocysts in a murine system, little is known about the applicability of the results to humans.

Because of the obvious limitations of using animal infectivity as the method of oocyst viability determination, alternative methods have been developed in hopes of

providing viability results easily, quickly and inexpensively. Many novel techniques, such as charge couple device (CCD) (19) have been developed, but because of either cost, lack of ease to use or perhaps inapplicability, these methods of viability assessment have not become commonplace in *Cryptosporidium* research.

In vitro excystation is another method commonly used in *Cryptosporidium* viability research, mainly due to its low cost. As was outlined previously, this technique, which simulates gut conditions, is a somewhat lengthy procedure that again, requires most of a day of a skilled microscopist. The end result, the percent excystation, is considered synonymous with percent viability and is thought to be closely related to infectivity. However, because *in vitro* excystation results may not necessarily agree with results obtained through animal infectivity studies (45), combined with the finding that different isolates of *Cryptosporidium* oocysts have different *in vitro* excystation efficiencies (21,110), viability and infectivity numbers derived from *in vitro* excystation experiments must be interpreted with caution.

Another common method used to determine *Cryptosporidium* oocyst viability is the use of vital dyes, such as the fluorescent stains DAPI and PI. PI readily penetrates dead cells (60) including oocysts (21) by only being able to pass through the cell membrane when it is disrupted (dead) (60) and is thus a good indicator of (non) viability when oocysts/sporozoites take up this stain. Oocysts that will take up DAPI but exclude PI will readily excyst under a suitable excystation protocol showing a very strong correlation between the DAPI/PI method and *in vitro* excystation (21). Experiments carried out with *Giardia* cysts revealed that all cysts positive for PI uptake never produced *Giardia* infection in test animals and thus cells positive for PI are dead and no

longer capable of infection (119). Recently, the kit described previously, Live/Dead BacLight® (Molecular Probes) and the single stain, SYTO9® (Molecular Probes), have been tested and show promise as vital stains (11). However, because the stains within the kit and the SYTO9® (Molecular Probes) require DNA to bind to and accumulate, they would not be appropriate to use in an automated system, such as flow cytometry, since empty oocyst shells would not be labeled as non-viable (which they are), and therefore results obtained from an automated system using either of these stains would need to be confirmed by microscopy, thus not improving existing viability methods greatly.

A membrane potential sensitive fluorochrome, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) is yet another fluorochrome that has entered the realm of vital stains. This molecule is very sensitive to voltage. It will enter depolarized cells (dead cells), and accumulate in lipid rich regions. Accumulation of the dye intensifies the fluorescence (62). $DiBAC_4(3)$ fluoresces green when excited by 488 nm light. There is no requirement of DNA to bind to and accumulate, thus having the potential to label empty oocyst shells as non-viable. This would then allow for total automation of a viability assay using this stain. Its use as a vital stain as analyzed by flow cytometry in a bacterial system has recently been demonstrated; it was found that viability as determined by $DiBAC_4(3)$ and flow cytometry was correlated to the standard CFU (colony forming units) count of the same bacterial system (62). Its usefulness in *Cryptosporidium* studies has yet to be determined. Thus it is apparent that there is much research devoted to developing new vital stains that give oocyst viability results similar to those obtained

from animal infectivity studies, since most vital staining procedures are ultimately the easiest and most cost effective of the available viability methods.

Flow cytometry using bacterial vital stains has also been increasing in popularity as a viability assessment tool in environmental bacteriology (105). Yet, its application in environmental microbiology, specifically *Cryptosporidium* analysis, has been limited to detection methodologies (136,138,139). The viability of *Cryptosporidium* oocysts could quickly and easily be determined using a double staining technique and flow cytometry. Monoclonal antibodies specific to *Cryptosporidium* oocysts conjugated to a fluorochrome could be used as a discriminating criterion within a sample to identify the oocysts from the contaminating debris. A vital stain of a different emission wavelength than the monoclonal antibody could then be used to discriminate between the oocysts as identified by the monoclonal antibody into live and dead fractions. Despite its ease of use and capability to process numerous samples in a day, there have been no reports to date that have used flow cytometry and a vital stain such as $DiBAC_4(3)$, to assess *Cryptosporidium* oocyst viability.

MATERIALS AND METHODS

All PBS used in flow cytometric and magnetic bead analyses contained 10% (v/v) normal goat serum (CELLect, ICN Biomedicals, Costa Mesa, CA).

When analyzing samples by flow cytometry, dimethylsulfoxide (DMSO) was run through the flow cytometer for 30 seconds to clear residual fluorochromes and debris. This was done after each tube.

Sorting of *Cryptosporidium* Oocysts Using Magnetic Beads

Primary antibody labeling of oocysts: Twenty μL of *Cryptosporidium* oocyst stock (C061296) was added to a 1.5 mL microcentrifuge tube (Sarstedt). To this, 18 μL of PBS was added and the contents vortexed. Two microlitres of Cy3-MAb (Cy3 labeled anti-*Cryptosporidium* oocyst mouse IgM monoclonal (Crypt-A-Glo™, Waterborne Inc., New Orleans, LA) was added, the contents vortexed, and incubated in the dark at room temperature for 30 minutes. After incubation, the contents were spun down at 13,500 x g for 10 minutes, the supernatant aspirated and discarded. The pellet was washed twice with PBS.

Sorting of oocysts using washed magnetic beads: Fifty μL of the beads (Dynabeads ® M-450, Product no. 110.15, Dynal A.S., N-0212 Oslo, Norway) was removed and washed according to the manufacturer's instructions. The washed beads were added to the labeled oocysts and the contents vortexed. The mixture was placed on a shaker (Vortex Genie2™, VWR Scientific, Mississauga, ON) for 60 minutes at 4°C (Dynal's instructions) to allow the beads and the labeled oocysts to bind. After incubation, the oocysts bound to the beads were removed using a sorting magnet (MPC ®-E-1, Dynal A.S.) according to the manufacturer's instructions. The separated beads were examined under bright field and epifluorescence microscopy (Leitz Laborlux).

Preparation of $DiBAC_4(3)$ Dilutions

To prepare a 1:1000 dilution, 10 µL of $DiBAC_4(3)$ (Molecular Probes, Eugene, OR) stock (1 mg/mL in ethanol) was added to 10 mL ethanol and mixed. Dilutions of 1:700, 1:500, 1:300, 1:200, 1:100 and 1:50 were all prepared in a similar manner.

To each of 20 five mL conical polystyrene centrifuge tubes (Sarstedt), 5 µL of *Cryptosporidium* oocyst stock was added. To each tube, 45 µL of PBS was added and the contents vortexed. Of the 20 tubes, 10 were placed in boiling water (a rolling boil) for 30 seconds, removed and immediately placed on ice. The remaining 10 tubes were left on ice. To all tubes, 349 µL PBS was added, and again, the contents vortexed. The Cy3-MAb was the first fluorochrome added. Sixteen sample tubes received 1 µL each of the Cy3-MAb (Waterborne, Inc.) and all were vortexed immediately. All sample tubes (20) were then placed in the dark, at room temperature, and incubated for 30 minutes, after which 599 µL of PBS was added to each tube and the second fluorochrome was added. One microlitre of $DiBAC_4(3)$ (Molecular Probes) was added to 14 of the Cy3-MAb labeled tubes at varying dilutions starting at a 1:1000 dilution of the stock solution and each of a 1:700, 1:500, 1:300. 1:200, 1:100 and 1:50 stock solution dilution. One unheated and one heated tube, both Cy3-MAb labeled, each received the same dilution in pairs. Two of the 4 unstained samples (one unheated, one heated) became the $DiBAC_4(3)$ only controls. These tubes received the **1:700** dilution of $DiBAC_4(3)$ only. Upon addition of the $DiBAC_4(3)$, all tubes were immediately vortexed. All 20 tubes were then incubated at room temperature, in the dark, for at least 10 minutes before analysis by flow cytometry.

The result of this procedure yielded 20 tubes labeled as follows:

Heated (Boiled)	Unheated
1 unstained control	1 unstained control
1 Cy3-MAb only control	1 Cy3-MAb only control
1 $DiBAC_4(3)$ only control	1 $DiBAC_4(3)$ only control
7 Tests (Cy3-MAb and $DiBAC_4(3)$-varying dilutions)	7 Tests (Cy3-MAb and $DiBAC_4(3)$-varying dilutions)

Analysis by Flow Cytometry: After incubation, the control tubes were used to set the appropriate voltages for the FL1-channel ($DiBAC_4(3)$) and FL2-channel (Cy3) and adjust the colour compensation for these two channels on the EPICS XL-MCL flow cytometer (Coulter Corporation, Miami, FL).

The optimization strategy for the $DiBAC_4(3)$ was as follows. The first tube to be analyzed was the 1:1000 unheated test. After DMSO cleaning, the 1:1000 heated test was analyzed and compared to the unheated test. This procedure was repeated, with DMSO cleaning between every sample, for every dilution down to 1:50. From these results, the optimal $DiBAC_4(3)$ working dilution was obtained.

Determination of *Cryptosporidium* Oocyst Background Staining with $DiBAC_4(3)$: Oocysts were purified from calf feces according to the oocyst purification procedure previously described. Five μL of fresh oocyst stock (C191296) was added to each of 8 1.5 mL microcentrifuge tubes (Sarstedt). Each tube received 394 μL PBS and the contents was vortexed. To 6 of the 8 tubes, 1 μL of Cy3-MAb (Waterborne Inc.) was added and the contents vortexed. After 30 minutes of incubation in the dark, at room temperature, 599 μL of PBS was added to each of the 8 tubes. One μL of $DiBAC_4(3)$ working dilution was added to 5 of the tubes that received the Cy3-MAb and to one of the tubes that did

not; one tube remained totally free of stains. All tubes were vortexed and incubated at room temperature, in the dark, for at least ten minutes.

The tests and controls were then analyzed by flow cytometry, each run stopping after approximately 5000 Cy3-positive events (*Cryptosporidium* oocysts).

In vitro Excystation vs. Flow Cytometry: *Cryptosporidium* Cultures Aged Over Time

This procedure was run in parallel for two different *Cryptosporidium* oocyst stock cultures (C240796 and C170796).

Preparation of *Cryptosporidium* Oocysts for One Stock Culture: Flow Cytometry: To each of five 5 mL conical polystyrene centrifuge tubes (Sarstedt), 5 μL of *Cryptosporidium* oocyst stock was added. To each tube, 394 μL of PBS was added and the contents vortexed. Next, 1 μL of Cy3-MAb (Waterborne, Inc.) was added to 3 tubes and the contents vortexed. All tubes were then incubated at room temperature, in the dark. After 30 minutes of incubation, all tubes received 599 μL of PBS and the contents vortexed. To 2 of the 3 tubes that received the Cy3-MAb and 1 of the 2 tubes that did not, 1 μL of the $DiBAC_4(3)$ (Molecular Probes) working dilution was added and the contents immediately vortexed. One tube remained as the unstained control. All tubes were then incubated at room temperature, in the dark, for at least 10 minutes. The result of this procedure yielded 5 tubes labeled as follows:

1 unstained control
1 Cy3-MAb only control
1 $DiBAC_4(3)$ only control
2 Tests (Cy3-MAb and $DiBAC_4(3)$)

The tests and controls were then analyzed by flow, each run stopping after approximately 5000 Cy3 positive events (*Cryptosporidium* oocysts).

Preparation of *Cryptosporidium* Oocysts for One Stock Culture: *In vitro* Excystation: To 4 labeled 1.5 mL microcentrifuge tubes (Sarstedt), 10 µL of *Cryptosporidium* oocyst stock was added. *In vitro* excystation was carried out as previously described.

***In vitro* Excystation vs. Flow Cytometry: *Cryptosporidium* Oocyst Cultures Heated at 55°C**

This procedure was run in parallel for two different *Cryptosporidium* oocyst stock cultures (C240796 and C100896).

Preparation of *Cryptosporidium* oocysts for one stock culture: Flow Cytometry. To each of 30 five mL conical polystyrene centrifuge tubes (Sarstedt), 5 µL of *Cryptosporidium* oocyst stock was added. To each of those, 45 µL of PBS was added and the contents vortexed. Five tubes were placed on ice, while the remaining 25 tubes were placed into a 55°C water bath (Fisher Scientific, model no. 15-458-10, Niles, IL) and allowed to warm up to bath temperature for one minute after which time 5 tubes at a time were removed for each of 30 seconds, 60 seconds, 90 seconds, 120 seconds and 180 seconds time intervals. All samples were immediately placed on ice after removal from the water bath.

Following treatment, to each tube 349 µL of PBS was added and the contents vortexed. Next, 1 µL of Cy3-MAb was added to each tube labeled "Cy3-MAb only control" and to each of the two tests at each time interval and the contents were vortexed.

All tubes were then incubated at room temperature, in the dark. After 30 minutes of incubation, all tubes received 599 µL of PBS and the contents vortexed. At each time interval, to 2 of the 3 tubes that received the Cy3-MAb and 1 of the 2 tubes that did not, 1 µL of the $DiBAC_4(3)$ working dilution was added and the contents immediately vortexed. One tube of each time interval remained completely unstained as the unstained control. All tubes were then incubated at room temperature, in the dark, for at least 10 minutes. The results of this procedure yielded 30 tubes labeled as follows:

Sample	Control: unstained	Control: Cy3-MAb	Control: $DiBAC_4(3)$	Tests: Cy3-MAb and $DiBAC_4(3)$
unheated control	1	1	1	2
30 second test	1	1	1	2
60 second test	1	1	1	2
90 second test	1	1	1	2
120 second test	1	1	1	2
180 second test	1	1	1	2

The tests and controls were then analyzed by flow cytometry, each run stopping after approximately 5000 Cy3-positive events (*Cryptosporidium* oocysts).

Preparation of *Cryptosporidium* oocysts for one stock culture: *In vitro* Excystation. To each of 12 conical polystyrene centrifuge tubes (Sarstedt), 10 µL of *Cryptosporidium* oocyst stock was added. Forty µL of PBS was added to each tube and the contents vortexed. Of the 12 tubes, 10 of them were placed in the 55°C water bath with the flow cytometry tubes, giving two tubes for each of the sampling points mentioned previously. The two remaining tubes were left on ice as untreated controls. After heating for the specified times following a one minute warm-up period, the tubes were removed and immediately placed on ice. Before the *in vitro* excystation procedure was started, the

contents of all 12 tubes were transferred by micropipettor to 12 appropriately labeled 1.5 mL microcentrifuge tubes (Sarstedt). Each tube was then rinsed with 500 µL sterile PBS, with the rinses being added to the appropriate tubes. All tubes were then spun down for 10 minutes at 13,500 x g, the supernatant aspirated and discarded leaving only the pellet. *In vitro* excystation was then carried out as previously described *(General Materials and Methods: In vitro Excystation)*.

In vitro Excystation vs. Flow Cytometry: _Cryptosporidium_ Oocysts-γ Irradiation

Preparation of *Cryptosporidium* oocysts: Flow Cytometry. To each of 26 microcentrifuge tubes (1.5 mL, Sarstedt), 10 µL of *Cryptosporidium* oocyst stock (C061296) was added. To each of those, 40 µL of PBS was added and the contents vortexed. All tubes were appropriately labeled according to the scheme outlined. Test tubes were irradiated for 5, 15, 30, 45, and 60 minutes (692, 2075, 4149, 6224, 8298 Gy, respectively; three repeats for each time point), and 75, 90, 105 and 120 minutes (10373, 12447, 14522, 16596 Gy respectively, one repeat for each time point) in the 10-position temperature-controlled stand (Nordion Gammacell 220 Serial Number 59) at a temperature of 25°C while the untreated controls were left at 25°C outside of the irradiation chamber for a total of 120 minutes. After each irradiation, samples were immediately removed and placed on ice and the irradiation chamber was refilled with new samples until all samples were irradiated.

Following treatment, the contents of the 120-minute irradiation tube was split into three separate samples, one as the treated test, one to be the treated unstained control, and one to be the treated Cy3-MAb only control. The volume of these split samples was

brought up to 399 µL with PBS and the contents vortexed. Next, 1 µL of Cy3-MAb was added to each irradiated tube and all tubes labeled "Cy3-MAb only control" and the contents were vortexed. All tubes were then incubated at room temperature, in the dark. After 30 minutes of incubation, all tubes received 599 µL of PBS and the contents vortexed. Again, all irradiated tubes received 1 µL of the $DiBAC_4(3)$ working dilution as well as the tubes labeled "untreated $DiBAC_4(3)$ only control" and the contents immediately vortexed. One tube of each time interval remained completely unstained as the unstained control. All tubes were then incubated at room temperature, in the dark, for at least 10 minutes. After incubation, the contents of each tube was transferred by micropipettor to appropriately labeled conical polystyrene centrifuge tubes (Sarstedt), and analyzed by flow cytometry as described previously. The set-up of this procedure yielded 26 tubes labeled as follows:

No. of tubes	**Exposure Time**	**Staining**
3 of each	5, 15, 30, 45, 60 minutes	Cy3-MAb and $DiBAC_4(3)$
1 of each	75, 90, 105, 120 minutes	Cy3-MAb and $DiBAC_4(3)$
2	0 minutes	Cy3-MAb and $DiBAC_4(3)$
1 of each	0 and 120 minutes	unstained
1	0 minutes	Cy3-MAb only control
1	0 minutes	$DiBAC_4(3)$ only control
1	120 minutes	Cy3-MAb only control

Preparation of *Cryptosporidium* oocysts: *In vitro* Excystation. To each of 21 microcentrifuge tubes (1.5 mL, Sarstedt), 10 µL of *Cryptosporidium* stock (C061296) was added. Forty µL of PBS was added to each tube and the contents vortexed. The tubes were labeled and irradiated in the Nordion Gammacell according to the following scheme:

No. of tubes	Exposure Time
3	5 minutes
3	15 minutes
3	30 minutes
3	45 minutes
3	60 minutes
1	75 minutes
1	90 minutes
1	105 minutes
1	120 minutes
2	0 minutes-control

All tests were irradiated at 25°C and immediately placed on ice upon end of time interval. Unirradiated controls were held at 25°C for 120 minutes and immediately placed on ice.

Upon completion of the irradiations, all samples were spun down at 13,500 x g for 10 minutes and the supernatant aspirated and discarded. *In vitro* excystation was carried out as previously described.

RESULTS

$DiBAC_4(3)$ and FITC both fluoresce green when excited by 488 nm light. Therefore, FITC-positive events could not be distinguished from $DiBAC_4(3)$-positive events. In view of this, a different dye, Cy3, was used to label the MAbs for use with the oocysts. Cy3 fluoresces red when excited by 488 nm light and is easily discernible by both flow cytometry and epifluorescence microscopy, from the green emission of $DiBAC_4(3)$. Oocysts labeled with MAbs conjugated to Cy3 can be observed in Figure 19.

Since the Coulter EPICS XL-MCL flow cytometer was not equipped with a sorting device, the Cy3-positive population observed by flow cytometry (Figure 20)

could not be sorted on to a microscope slide and verified as a pure sample of *Cryptosporidium* oocysts. Instead, this verification was made by sorting the Cy3-MAb-labeled population from the unlabeled fraction by magnetic beads (13) and verifying by microscopy. The results of magnetic bead sorting can be observed in Figure 21. The purity of the sort indicated that there was very little non-specific binding occurring with the MAb. Therefore, the Cy3-positive population observed in Figure 20 was a highly pure population of oocysts.

Because $DiBAC_4(3)$ had previously never been used in oocyst studies, the proper concentration of this fluorochrome for use in oocyst investigations was required. The results of *Determination of $DiBAC_4(3)$ Working Dilution* can be observed in Figure 22. It is to be noted that the *entire* Cy3-positive population, i.e. all oocysts, move into the $DiBAC_4(3)$-positive zone when the sample was heated. This occurred at the 1:100 dilution of the stock solution. Therefore, the working dilution of $DiBAC_4(3)$ was 1:100. Figure 23 shows oocysts stained with the working dilution of $DiBAC_4(3)$.

Once the MAb and the working concentration of the vital stain were sorted out, the oocyst viability obtained by the vital stain and analyzed by flow cytometry had to be compared to an accepted method of oocyst viability assessment. *In vitro* excystation was used for this purpose, since this comparison of viability had to correspond over a number of different oocyst stresses. Flow cytometry and *in vitro* excystation were tested separately after natural aging, heat stress and gamma irradiation.

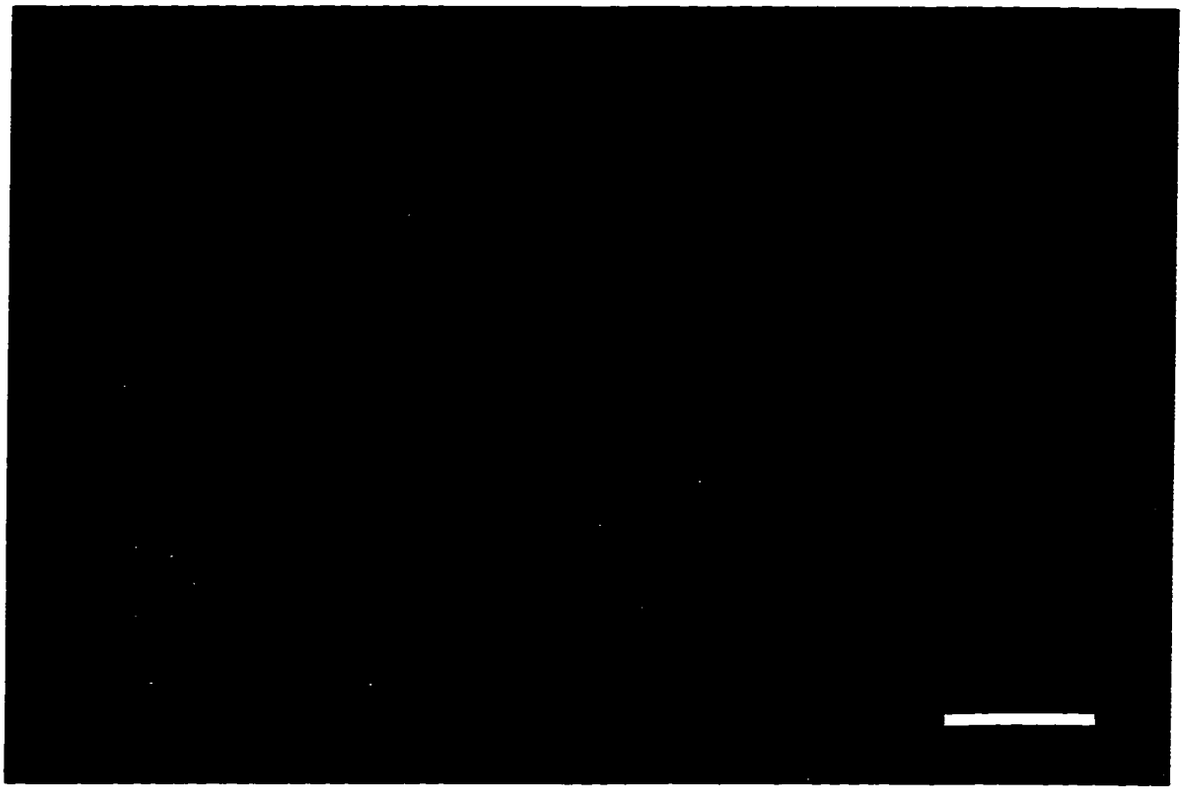


Figure 19: *Cryptosporidium* oocysts labeled with Cy3-conjugated monoclonal antibodies. Bar=10 μm.

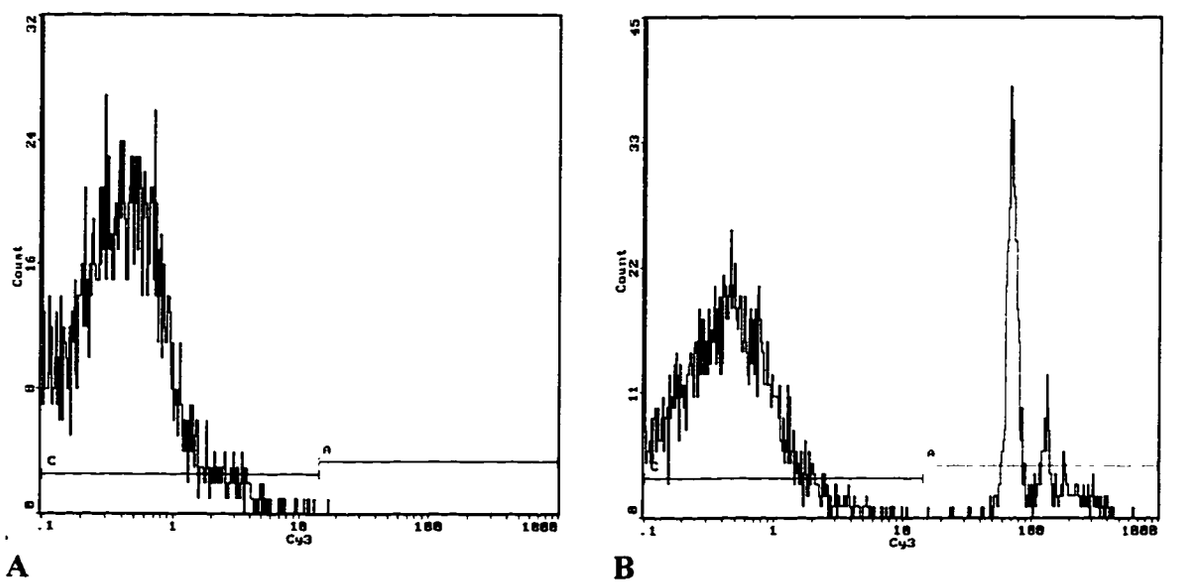


Figure 20: Cy3-labeled oocysts: Analysis by flow cytometry. A. Output from the EPICS XL-MCL flow cytometer while analyzing an unstained sample of oocysts and discriminating on the Cy3 signal vs. number of events. B. Output from the flow cytometer while analyzing a sample of oocysts labeled with the Cy3-conjugated MAb.

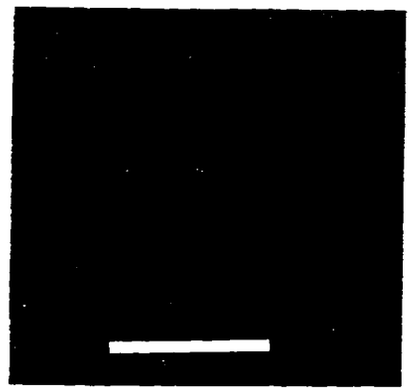


Figure 21: Cy3-MAb-labeled *Cryptosporidium* oocysts sorted with magnetic beads (Dynabeads ® M-450, Dynal A.S.). The field of view is debris free, indicating the high specificity of the MAb.

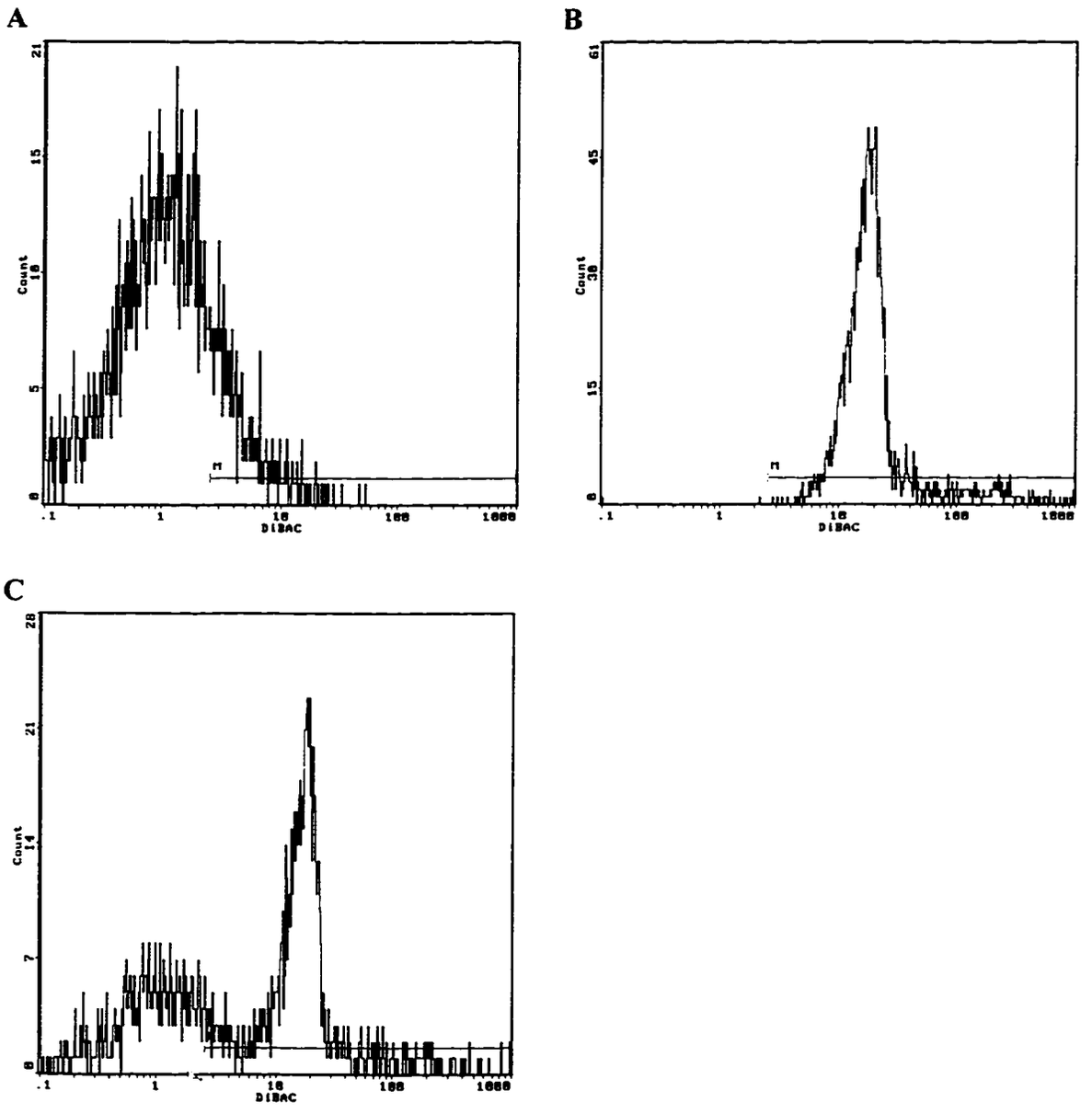
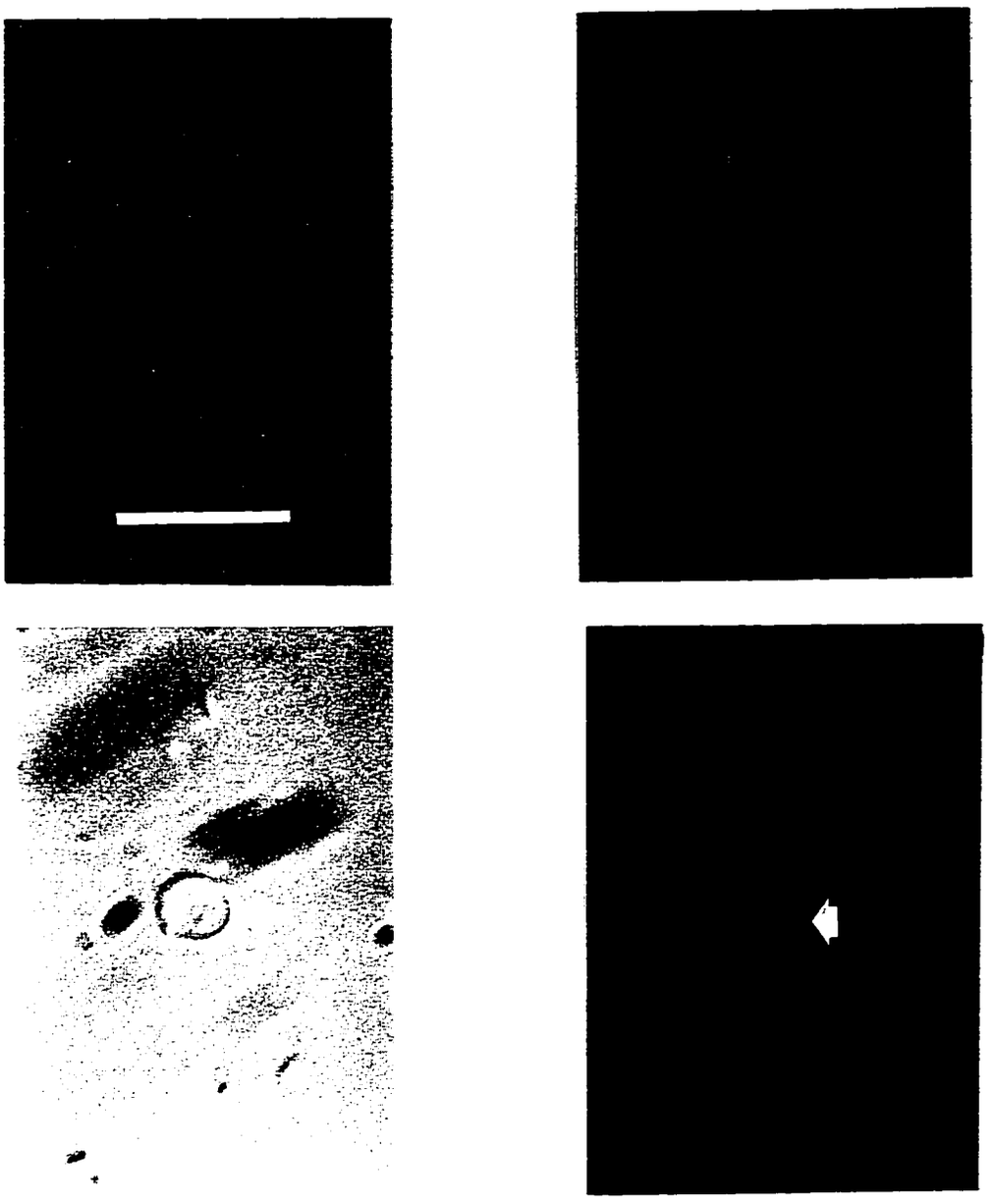


Figure 22: Determination of $DiBAC_4(3)$ Working Dilution. The population shown in these figures is Cy3-positive. These figures show the positive population being analyzed on the $DiBAC_4(3)$ emission characteristics of the sample. A. Unheated sample with $DiBAC_4(3)$ stain. B. Heated sample with $DiBAC_4(3)$ stain. C. Mix (1:1) of heated and unheated samples with $DiBAC_4(3)$ stain.



A B

Figure 23: *Cryptosporidium* oocysts stained with the working dilution of $DiBAC_4(3)$ and viewed under epifluorescence microscopy. A. Bright field images of column B. Arrows indicate empty oocyst shell. Bar = 10μm

Two cultures were aged over time and viability was assessed by both *in vitro* excystation and flow cytometry. Figure 24 shows that when comparing *in vitro* excystation with flow cytometry, both methods give similar viability results. There is only one point at week 1 where the results are not comparable.

Over the course of heat treatment at 55°C, however, it is apparent from Figure 25 that the two viability assays do not give similar results. Flow cytometry only compares to *in vitro* excystation at time 0 (i.e. no heat treatment). Therefore, viability assays in this instance are not comparable.

Gamma irradiation was not carried out with two different cultures. The results of viability analysis after gamma irradiation as determined by flow cytometry and *in vitro* excystation can be found in Figure 26. It is apparent that flow cytometry and *in vitro* excystation give similar viability results over the course of gamma irradiation treatment. A similarity comparison of the two methods cannot be made beyond the 8298 Gy mark since from 10373 to 16596 Gy, the points are single experimental sample points (i.e. n=1). The experiment was limited by the size of the irradiation chamber. The overlap of error bars between the two viability assays occurs during the middle of irradiation; the start point and end points are not similar between the two assays (±2 x s.e.m.).

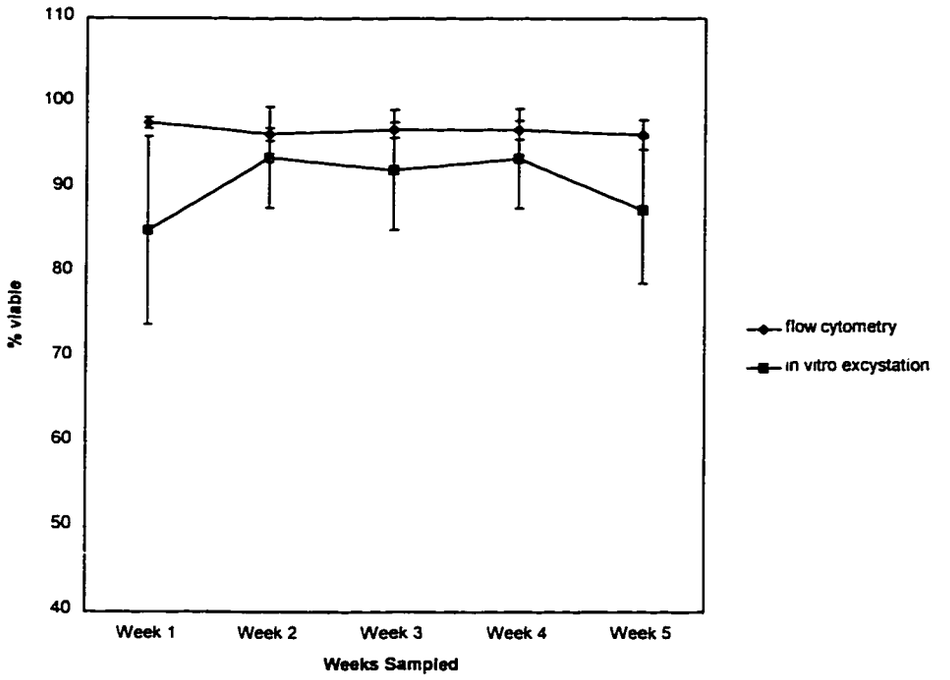


Figure 24: Flow Cytometry vs. *In vitro* Excystation: Two Cultures Aged Over Time. The vertical error bars represent 2 times the standard error from the mean of both cultures combined, therefore twelve observations for each x value.

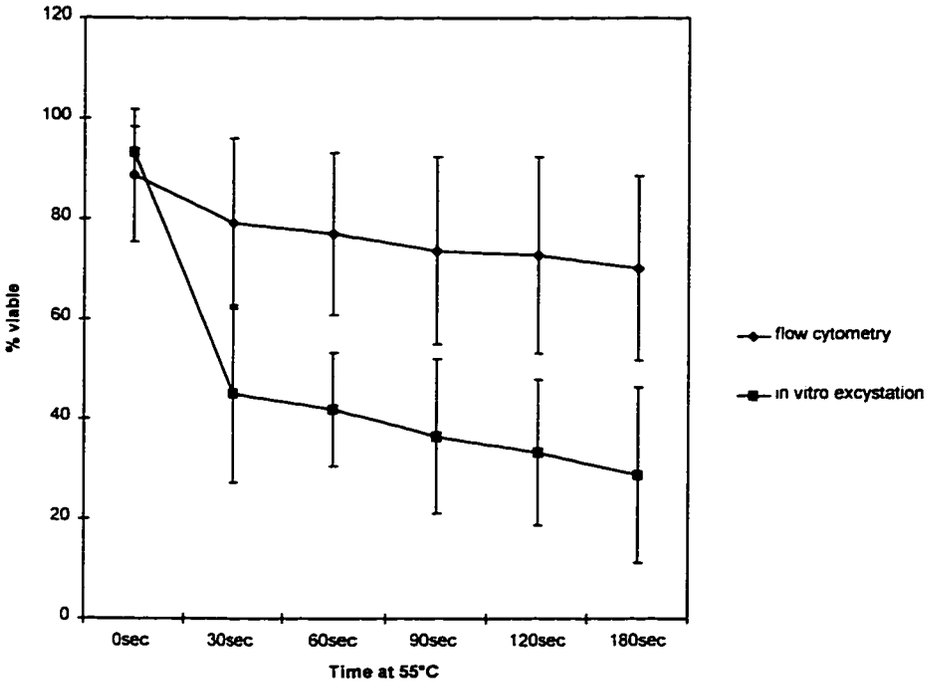


Figure 25: Flow Cytometry vs. *In vitro* Excystation: Heating at 55°C for 180 seconds. The vertical error bars represent 2 times the standard error from the mean of both cultures combined, therefore twelve observations for each x value.

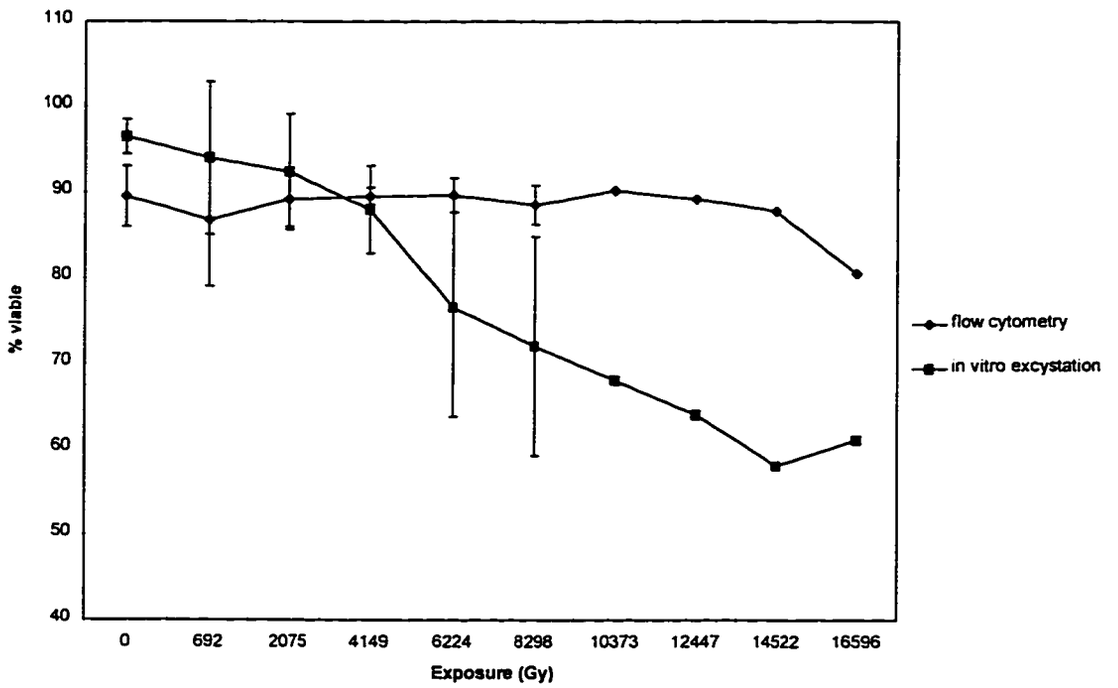


Figure 26: Flow Cytometry vs. *In vitro* Excystation: γ Irradiation. The vertical error bars represent 2 times the standard error from the mean of three observations for each x value. Points without error bars are single points without repeats, therefore no error could be calculated.

DISCUSSION

It is apparent from the results that flow cytometry gave consistent results in all experiments conducted. It was comparable to an accepted viability assay, *in vitro* excystation, while following two oocyst cultures over time (Figure 24) and the majority of the gamma irradiation trials (Figure 26). Viability results obtained by flow cytometry during heat stress were significantly different than viability results obtained through *in vitro* excystation. The question remains as to why.

Differences in viability among the assays during heat treatment may be attributable to loss of epitopes on the oocyst wall of dying oocysts during heating. If epitopes were lost during heat inactivation, only healthy oocysts would be labeled with the Cy3-MAb and further discriminated to be analyzed for $DiBAC_4(3)$ staining intensity. The result of such analysis would give higher viability results than is actually the case. However, this scenario is not likely. Figure 22 clearly shows that even after boiling oocysts, the Cy3-MAb is still capable of binding to oocyst wall epitopes, indicating that surface epitopes are not destroyed during the heat treatments studied. Therefore, another explanation is required.

An alternative interpretation for the observed discrepancy between methods may be due to protein/enzyme degradation during heating. If proteins/enzymes necessary for excystation were heat labile and were destroyed during heat treatment, then the sporozoites would not be released from within the oocyst, and therefore the oocyst would be counted as non-viable. However, if the oocyst is still alive but unable to excyst, it is falsely counted as non-viable, according to *in vitro* excystation. This explanation is

consistent with the observed results, yet the implications of this scenario are important to describe. If, as in this case, oocysts are counted as viable, yet are unable to excyst, the oocysts would not pose any threat to human health. This method of viability assessment would not then correspond to animal infectivity results.

The results for the gamma irradiation trials were difficult to interpret. Correspondence to *in vitro* excystation was lacking at the start of the experiment and at the end, yet during the course of the experiment, both *in vitro* excystation and flow cytometry gave similar viability results. The discrepancy near the middle of the irradiation time course (6224-8298 Gy) may be due to the creation of highly reactive substances within the oocyst due to the ionizing radiation, which in turn caused the degradation and therefore decrease in fluorescence of the $DiBAC_4(3)$ molecule. This would give increased viability results as obtained by flow cytometry, which is exactly what was observed.

These gamma irradiation trials are important at another level. Gamma irradiation is increasingly being used in industry as a sterilization technology. It is used in the food industry to decrease the levels of microbial contamination of foods. However, the results presented here (Figure 26) suggest that, even after over 16 kGy of gamma irradiation, 50-60% of the seeded oocysts remained viable, as determined by *in vitro* excystation. Yet gamma irradiation levels used in industry to inactivate contaminating microorganisms rarely reach 10 kGy (103) to achieve a 90% inactivation of the microbial population (D_{10} value). *E. coli* on poultry has a D_{10} value of 0.39 kGy, *Clostridium botulinum* type E in beef stew has a D_{10} value of 1.37 kGy (103) while coxsackievirus on beef has a D_{10} value of 7.6 kGy (103). Clearly, the D_{10} value of *Cryptosporidium* oocysts under the outlined

experimental conditions is much higher than any of these organisms, and therefore they would not be inactivated using standard irradiation exposures.

Although $DiBAC_4(3)$ used as the vital dye in flow cytometry did not correspond to the results of an accepted viability assay, *in vitro* excystation, over all stresses tested, $DiBAC_4(3)$ does hold some promise as a vital dye. As can be seen in Figure 23, $DiBAC_4(3)$ stains empty oocyst shells and therefore labels shells as non-viable. Because $DiBAC_4(3)$ does not require DNA to bind to, it accumulates in the oocyst wall where there is lipid. In this way, oocyst shells (dead) will take up the stain and fluoresce. This is the first vital dye of this sort to be used in *Cryptosporidium* studies, and it is the first studied with the capability of being able to label oocyst shells as dead, hence non-infectious. However, the presented studies show that flow cytometry using $DiBAC_4(3)$ as the vital dye may not be an acceptable assay over all stress and inactivation protocols.

Flow cytometry on the other hand is very rapid, straightforward and easy. Cy3-positive events were very easy to discern and manipulate. It is a promising approach to pursue for future viability assays.

GENERAL DISCUSSION

Since *Cryptosporidium* was first implicated as a cause of human gastroenteritis in 1976 (99), many waterborne outbreaks have occurred throughout the world, with the outbreak of 1993 in Milwaukee, WI, (52,83) being the most notable. During this outbreak, an estimated 403,000 people were affected due to contaminated drinking water (52,83). Although there have been numerous reports regarding the distribution of *Cryptosporidium* oocysts within water systems (30,32,55,63,76,84,100,108,114,116,124), few have addressed the survival of oocysts within natural water sources (109). Therefore, this study was designed to address the gap in our knowledge.

Three rivers were chosen in the south and eastern regions of Ontario, Canada. The Grand, St. Lawrence and Carp Rivers were chosen to determine the effect of waters of different chemistry and from different geographical locations on the *in vitro* survival of *Cryptosporidium* oocysts. Water was sampled from these rivers at two different locations, and when possible, were sampled over different seasons of the year. Oocysts were purified from calf feces and inoculated into the test samples and incubated at 4°C, 20°C and 30°C. These temperatures were chosen because they were thought to best represent the water temperatures achieved during the seasons sampled. Viability was determined by a standardized *in vitro* excystation assay, an assay that was thought be an acceptable, inexpensive and rapid replacement for the "gold standard" of viability assessment, animal infectivity.

In vitro excystation however, was still a lengthy procedure, and only a limited number of samples could be processed daily. Because of this, the sample sizes used for

statistical analyses were relatively small, unless all of the data was pooled. Nevertheless, interesting trends were derived from the analyses.

The findings presented in this study show that *Cryptosporidium* oocysts are capable of surviving in natural water for extended periods. This is consistent with the results of limited studies published previously (109). However, the complexity of the factors that affect *in vitro* survival is large. This study has found that although incubation temperature, river sampled, site sampled and season of water sampling all have a significant effect on the *in vitro* survival of *Cryptosporidium* oocysts in natural waters; only incubation temperature had a significant effect across the board.

Although it was shown that HPCs within a river did affect *in vitro* survival ($0.05>p>0.10$), it was determined that water chemistry had no effect on *in vitro* survival of oocysts ($p>0.05$). This has been shown previously (31).

Another result from analyzing the data was that oocysts appeared to have different survival capabilities at different sample sites within a river. It was found that the Grand River, at the site downstream of the urban district (site #2) in the spring, showed increased oocyst survival over site #1 ($p<0.05$). Additionally, the St. Lawrence River, at the site downstream from the urban district (site #2) in the summer (at 30°C), also showed increased oocyst survival over site #1 ($p<0.05$). This finding suggests that the microbial ecology of the river at these locations may be different from the upstream sites, hence possibly providing a more hospitable environment for the oocysts. It was also shown that increased bacterial numbers alone, such as with increased numbers of *P. fluorescens*, did not contribute to a decline in oocyst viability over time. Therefore, there appears to be a significant microbial factor(s), less than 2.0 μm in size but larger than

0.22 μm, that is serving as an oocyst antagonist. What this factor is has yet to be determined. It must also be noted that bacterial numbers and incubation temperature are intrinsically related; warmer water temperatures may stimulate bacterial growth.

At this point, it must be stressed that this study was conducted using an *in vitro* assay, using small volumes of water with large numbers of artificially inoculated oocysts. This may not best represent the natural river water conditions where small numbers of oocysts exist in very large volumes of water. Also, viable HPC bacteria enumerated from *in vitro* samples may not necessarily be those which predominate under field conditions. Therefore, although it is hoped that the results presented here are applicable to the natural water environment, the results should be interpreted with caution.

Animal infectivity studies require extended periods of time with great monetary expense. Because of this, there is a definite need for a simple, rapid and inexpensive oocyst viability assessment assay that can be directly related to the "gold standard". Flow cytometry is very rapid and simple. However, this technology has only been applied to *Cryptosporidium* research as a detection tool (136,138,139).

In the present study, flow cytometry was successfully adapted for oocyst analysis with the use of a Cy3-conjugated monoclonal antibody. Oocysts could easily be discerned from contaminating debris by the outlined protocol. However, the fluorescent stain under investigation as a vital dye, $DiBAC_4(3)$, did not give consistent viability results with those obtained by *in vitro* excystation under the stresses tested. There was correspondence between the two methods while aging two cultures over time and gamma irradiation, but stressing the oocysts by heat gave dissimilar viability results for flow cytometry compared to *in vitro* excystation.

Unlike the vital stains previously under investigation, $DiBAC_4(3)$ does have the ability to stain empty shells and hence label them as non-viable. Unfortunately, the results presented here indicate that $DiBAC_4(3)$ may not be as general of a vital dye as was originally hoped; it may only be useful under specific conditions. However, it was determined that flow cytometry is a very rapid and simple tool to use. It shows great promise for future research involving *Cryptosporidium*, and further studies should continue to examine its role as a tool for rapid viability assessment of oocysts.

CONCLUDING REMARKS

The experiments carried out in this study were listed in the objectives previously outlined on page 43. The results presented show that all of the objectives have been met and that the following conclusions can be drawn from the results.

In vitro Cryptosporidium Oocyst Survival in Natural Waters

1. The *in vitro* survival of *Cryptosporidium* oocysts in three different watersheds was addressed and determined. It was found that overall, there was at most a 2.5 $\log_{10}$ reduction in oocysts over the course of the experiments.
2. Incubation temperature has a dramatic effect on *in vitro* oocyst survival in natural waters. Four degrees centigrade is the most hospitable temperature for oocysts, followed by 20°C, and 30°C showing the greatest negative impact on *in vitro* oocyst survival in natural waters ($p<0.05$).
3. Season also had an effect on the *in vitro* survival of *Cryptosporidium* oocysts; statistical analysis showed that the relationship between these two variables is complex. Season only had an effect on survival under specific conditions detailed previously.
4. Sites within a river also affected the *in vitro* survival of *Cryptosporidium* oocysts. Again, the relationship of site and survival was complex, such that location within a river only affected survival under specific conditions detailed previously.

5. Water chemistry did not have an effect on oocyst survival ($p>0.05$). The microbiology of the water however, did appear to be of significance to *in vitro* oocyst survival in natural waters. This is consistent with previous reports (31).

Development of a Rapid *Cryptosporidium* Oocyst Viability Assay Using Flow Cytometry

1. A flow cytometric assay was developed for the analysis of *Cryptosporidium* oocysts in suspension. This procedure was rapid and simple and defined a population of oocysts from a mixture containing contaminating debris that is relatively debris-free. Thus, a very clean population of oocysts could be analyzed further.
2. The rapid assessment of *Cryptosporidium* oocyst viability using flow cytometry was attempted with moderate success. The flow cytometric protocol established was rapid and simple; however, the fluorescent stain, $DiBAC_4(3)$, did not appear to be as general as was originally hoped. More work must be done to determine the usefulness of this fluorochrome as a vital dye.

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PRESENTATIONS

PRESENTATIONS AND POSTERS

1. November 15-16, 1994 — **Environment and Energy Conference of Ontario, Toronto, Ontario**
   Presented a poster entitled "Autoclaves for the Decontamination of Biomedical Waste" V.S. Springthorpe, **M. Heisz**, S.A. Sattar
2. October 17-18, 1995 — **Ottawa Life Sciences Conference and Exhibition, Ottawa, Ontario**
   Poster presentation entitled "Rapid Assessment of *Cryptosporidium* Viability by Flow Cytometry" **M. Heisz**, C. Chauret, S. Springthorpe, S.A. Sattar.
3. April 21-24, 1996 — **Ontario Water Works Association/Ontario Municipal Water Association Joint Annual Conference, London, Ontario**
   Presented a paper entitled "*In vitro* survival of *Cryptosporidium* Oocysts in Natural Waters and Its Impact on Watershed Management" **M. Heisz**, P. Chen, C. Chauret, S. Springthorpe, S.A. Sattar.
4. October 6-10, 1996 — **International Association on Water Quality (IAWQ) Conference on Health Related Water Microbiology, Mallorca, Spain**
   Poster presentation entitled "Survival of *Cryptosporidium parvum* Oocysts in Surface Waters" C. Chauret, **M. Heisz**, P. Chen, K. Nolan, S. Springthorpe, S. Sattar
5. March 1-5, 1997 — **International Symposium on Waterborne *Cryptosporidium*, Newport Beach, California.**
   Presented a poster entitled, "*In vitro Cryptosporidium* Survival in Natural Waters" **M. Heisz**, C. Chauret, P. Chen, S. Springthorpe, S. Sattar.

CONFERENCES ATTENDED

1.	February 6-7, 1996	**Thirty First Central Canadian Symposium on Water Pollution and Research, Canada Centre for Inland Waters, Burlington, Ontario**
2.	May 26-30, 1996	**American Water Works Association Annual Conference and Exposition, Toronto, Ontario**
3.	November 17-20, 1996	**American Water Works Association Water Quality Technology Conference, Boston, Massachusetts**

APPENDIX

Example of Statistical Output from Model 3 (n=7-16)
Within Rivers, Temperatures and Seasons, do Sites Matter?

Site	4°C Winter	4°C Spring	20°C Winter	20°C Spring	20°C Summer	30°C Summer	
Time*Grand River	-0.072	-0.101	-0.211	0.288	-0.323	-0.500	coefficient
Site #1	0.011	0.009	0.025	0.026	0.072	0.085	std. error
	-6.532	-11.75	-8.330	-11.043	-4.481	-5.912	t-statistic
Time*Grand River Site	-0.029	0.072	0.026	0.133	0.002	0.013	coefficient
#2	0.016	0.012	0.036	0.037	0.101	0.150	std. error
	-1.839	5.866	0.728	3.594	0.016	0.087	t-statistic
Time*St. Lawrence	-0.043	-	-0.117	-	-0.433	-0.531	coefficient
River Site #1	0.006		0.023		0.093	0.091	std. error
	-7.159		-4.991		-4.652	-5.804	t-statistic
Time*St. Lawrence	0.002	-	-0.017	-	0.061	0.260	coefficient
River Site #2	0.008		0.033		0.131	0.129	std. error
	0.186		-0.524		0.460	2.012	t-statistic
Time*Carp River Site	-	-	-	-	-0.096	-0.217	coefficient
#1					0.049	0.043	std. error
					-1.935	-5.109	t-statistic
Time*Carp River Site	-	-	-	-	-0.82	-0.022	coefficient
#2					0.070	0.062	std. error
					-1.176	-0.373	t-statistic

bolded sites: these are the reference groups. These sites' statistics are determining if the slope is significantly different from zero. The other site (same river, non-reference group) is relative to the refernce group for all statistics (i.e., coefficient and t-statistic).

coefficient: slope of regression curve.

t-statistic: a t-statistic with an absolute value of >2.0 is significant (i.e. $p<0.05$).