MEASUREMENT OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS IN: SUPERNATANT, SLUDGE AND FIELD INACTIVATION TRIALS

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CHAPTER 1

INTRODUCTION

Statement of the Problem

Before the development of modern water and wastewater treatment facilities, waterborne diseases claimed thousands of lives, especially in urban areas. At the turn of the 19th century, the occurrence of waterborne diseases was reduced through the treatment of drinking water supplies.[28] Since then, the goal of public water utilities has been to provide a safe and plentiful supply of potable water. Despite these disinfection efforts, water utilities have been unable to inactivate *Cryptosporidium parvum* oocysts with the current prescribed doses of disinfectants used in water treatment facilities.[25] Cryptosporidiosis, an illness caused by oocysts, is creating concern in the water industry in several developed countries.[3, 26] Two decades ago, this parasite was virtually unknown; but currently it ranks as the leading cause of diarrhea.[14] This is due to a low infectious dose of oocysts, around 10 to 100 oocysts.[23]

Oocysts have been detected in highly variable numbers in surface waters.[16, 26] Oocysts have been detected in 90 percent of wastewater samples, 85 percent of surface waters samples, and 28 percent of drinking waters samples nationwide.[23] The presence of this organism in drinking water is of great concern to water utilities. The adverse effects of this parasite are well documented by health officials because of several outbreaks and the inability to control the outbreaks due to the lack of effective treatment for cryptosporidiosis.[11] Lessons learned from outbreaks in Milwaukee, Wisconsin; Talent, Oregon; and Carrolton, Georgia showed that oocysts can be present in finished drinking water.[8, 10] Due to the increase in outbreaks of the disease attributed to this parasite, public health officials and the water treatment industry have initiated numerous regulatory measures and research projects.[1] The US Environmental Protection Agency (EPA) has developed regulations on

oocysts to limit the reoccurrence of outbreaks related to the presence of oocysts in surface and drinking water by instituting a 2-Log oocyst removal requirement for systems that filter and a maximum contaminant level goal (MCLG) of zero for oocysts in the finished water.[30, 31] The US drinking water industry has given a number one priority to research related to the control of oocysts.[9] By the end of 1996, the American Water Works Research Foundation had spent 18.6 million dollars on oocyst related research.[1]

Documented outbreaks caused by drinking water from treatment facilities that were operating within established guidelines for producing safe water indicate the disinfection and filtration barriers installed at the treatment plants were breached. Previous investigators have reported finding oocysts in half of the two dozen filter effluent samples from a newly constructed plant using a slow sand filtration system.[20] This inability of treatment plants to capture oocysts, and the presence of this parasite in treated water, will be a continuous and serious threat to the public, especially if operating procedures of plants are not changed. The continued presence of oocysts in public drinking water supplies is mainly due to the inability of the physical and chemical water treatment processes to capture, remove, and inactivate oocysts. The filtration process alone cannot completely remove the parasites because they have a diameter of 4 to 6 μm[17] allowing the parasites to pass through filter.[23] In a pilot study, conducted in the Midwest, after the first four cycles, the cyst-sized particles removed using a slow sand filter ranged from 7 to 12 μm.[30]

Previous Studies and Findings

Johnson et al.[13] utilized polymerase chain reaction (PCR) method to detect oocysts in wastewater, surface water, and drinking water. In some water types, the presence of compounds inhibitory to PCR complicated detection of organisms. Several methods (flow cytometry, dot blot hybridization, and magnetic antibody capture) were tested to determine whether PCR sensitivity in the presence of inhibitors could be improved. Detection of purified oocysts of *Cryptosporidium* isolated from calves indicated a 10- to 100-fold increase in sensitivity using a DNA dot-blot procedure over ethidium-bromide stained agarose gels, depending on the age of the sample. The sensitivity of the PCR assay was found to decrease 100- to 1000-fold for oocyst-seeded

environmental samples compared with samples containing purified oocysts. However, when oocysts were separated from other particulates by flow cytometry prior to DNA extraction, detection was greatly improved.

Johnson et al.[13] noted that using magnetic antibody capture (MAC) would allow concentration of 250,000 oocysts into a 100-mL sample. In contrast, the concentrating capability of a conventional protocol was limited to only 25,000-oocysts/100 mL in a sample from similar starting volumes. MAC represented a 10-fold improvement over the conventional protocol. PCR detection of the sample that had not undergone MAC was not possible until the sample was diluted 100-fold, owing to interference from PCR inhibitors present in the environmental water. The MAC-treated sample containing oocysts, however, was detectable by PCR without further dilution due to the concentrating effect achieved by MAC.

Mayer and Palmer[18] compared PCR, nested PCR and fluorescent antibodies for detection of *Cryptosporidium* species in wastewater. The sensitivity achieved with nested PCR was 10^2 oocysts/L of primary wastewater influent. PCR products were confirmed by Southern blot, a technique used to detect specific DNA fragments so that a particular gene could be isolated from a complex DNA mixture. Correlation between PCR and immunofluorescent antibody (IFA) results ranged between 63 and 72 percent. IFA positive PCR negative results may have been due to the tendency of the IFA method to cross-react with nontarget organisms such as algae or to inhibitory substances present in the water that interfere with PCR enzymes such as humic acids. PCR positive, IFA negative results may have been caused by oocysts obscured by debris or by a greater sensitivity of the PCR method.

As many outbreaks of cryptosporidiosis are suspected to be the result of waterborne transmission of oocysts, the detection of *Cryptosporidium parvum* in drinking water has been an area of great interest to researchers. The use of PCR in the detection of oocysts in a water sample has proven to be a useful tool in achieving greater sensitivity over conventional microscopic methods, especially when coupled with nucleic acid hybridization methodologies. However, still greater sensitivity must be attained to reduce oocysts to levels that have been established for the minimum infective dose in humans.[33] The main challenge to increasing sensitivity is finding

methods to concentrate oocysts present in low numbers, while, at the same time, excluding both soluble and insoluble components that may interfere with detection.

Many investigators have measured the oocyst removal efficiency of various physical and chemical processes. Nieminski and Ongerth [20] achieved log removals of 1.6-4.0, using slow sand filtration and a 6 log removal using diatomaceous earth filtration. Alum was the primary coagulant used in the study. The authors used a microscope to detect and estimate the number of oocysts. Jacangelo et al., [12] reported using micro-filtration (MF) and ultrafiltration (UF) to examine oocysts removal efficiency in environmental samples. The results of their study, showed that absolute removal of oocysts is possible if the membrane filters are intact. The authors achieved greater than 6.0 log removal based on counts using an epifluorescent microscope. LeChevallier et al.[15] conducted pilot and full-scale studies using conventional treatment and achieved a log removal of 5.3 and 3.0, respectively. The authors used immunofluorescence antibodies (IFA) and a microscope for estimation of the oocysts. Studies conducted at the Metropolitan Water Quality District of Southern California by Yates et al. [27] showed that ferric chloride used in conjunction with filtration, removes a greater number of oocysts than alum with filtration at ambient pH values. Microscopic enumeration was used for oocyst estimation.

Ongerth and Hutton[21] performed a bench scale study to determine the overall applicability of diatomaceous earth (DE) filtration for the control of oocysts in surface water. Purified oocysts were spiked into the samples, which were filtered using DE filtration method. Quantitative measurement was conducted using IFA and a hemacytometer and established a 6-log removal.

Edzwald and Kelley,[6] conducted a pilot plant and a full-scale contact filtration study investigating the removal of oocysts with dual filtration operating at 7.3 m/hr. The authors achieved log removals of 1 – 2.5. Falk et al.[7] performed bench scale experiments to evaluate the membrane filtration method for recovery efficiency of oocysts. The authors used IFA and a hemacytometer to identify and count oocysts in the water sample. The results of their study showed a 42.1 percent oocysts recovery with 1.2 μm filter pore size.

Table 1-1 summarizes these articles, including their respective treatment methods, log removals, and analytical quantitative methods, for oocysts.

TABLE 1-1. SUMMARY OF THE *CRYPTOSPORIDIUM* REMOVAL EFFICIENCIES ESTIMATEDFOR VARIOUS PHYSICAL CHEMICAL PROCESS

Treatment process description	Bench Scale	Pilot Scale	Full Scale
• Coagulation + Gravity Settling	<1.0[A]	1.4-1.8[B]	0.4-1.7[G]
• Coagulation + Gravity Settling + Filtration		4.2-5.2[B]	1.6 – 4.0[E]
		>5.3[F]	<0.5-3.0[F]
• Coagulation + Dual filtration		2.1- 2.8[I]	1.0-2.5[G]
• Coagulation + Dissolved Air Flotation (DAF)	2.0-2.6[A]		
• Coagulation + DAF + Filtration		>4.7[A]	
• Slow Sand Filtration		>3.7[C]	
• Diatomaceous Earth (DE) Filtration		>6[C]	
• Coagulation + Microfiltration		>6.0[D]	
• Ultrafiltration + Microfiltration			>6.0[D]

Sources: References are as follows: A = Plummer et al., 1995; B = Nieminski, al., 1995; C = Ongerth and Hutton, 1997; D = Jacangelo et al., 1995; E = Nieminski and Ongerth, 1995; F = LeChevallier et al., 1991; G = Edzwald and Kelley, 1998; H = Falk et al., 1998; and I = Nieminski, 1995; Edzwald et al. 1996.

Expanding the Work of the Cited Researchers

Plummer et al.[22] conducted bench-scale studies to investigate the effectiveness of dissolved-air flotation (DAF) for the removal of oocysts from a drinking water supply. Oocysts were spiked into low turbidity water at a concentration of 3-4 x 10^5 oocysts/L. The efficiency of oocyst removal's relationship to ferric chloride dosage, pH, flocculation time, and recycle ratio was tested. Two experiments were performed to determine the recovery of oocysts using a jar testers. About 3 to 4 x 10^5 oocysts/L were added to each jar containing 2 to 3 mg/L of ferric chloride. The study indicates that oocyst levels were reduced to 2 log using a coagulant dose of 3-mg/L ferric chloride. The study also showed that application of 5 mg/L of ferric chloride resulted in 3.7 log removal. Oocyst removal was highest at pH 5. The authors stated that an increase in log removal of oocysts depended strongly on pH, an 8 percent recycle ratio, and flocculation time. The authors used a microscopic enumeration method for the quantification of C. oocysts. Their research did not evaluate the combination of clarification and filtration.

Nieminski and Ongerth[20] performed a pilot plant and full-scale study to investigate the removal of cysts and oocysts using conventional treatment and direct filtration methods. The study used

sand and anthracite as dual media filters. Alum was the coagulant used for conventional treatment and direct filtration. The authors stated that 12 mg/L of alum and 1.5 mg/L cationic polymer were used for conventional treatment, and 6 mg/L of alum and 3 mg/L of cationic polymer were used for direct filtration. Prior to cyst seeding, two tracer studies were conducted. Rhodamine was used in one tracer study to assess the hydraulic characteristics of the plant for cyst seeding, whereas table salt was used in another tracer study to define the optimum sampling times for the cysts. Separate pilot and full-scale runs were conducted with about 5 x 10^6 cysts and oocysts used for the pilot plant study. For the full-scale study, about 10^7 cysts and 10^7 oocysts were spiked into the influent water and treated. Ten experimental trials were performed in each study. In the pilot plant, the average log removal of cysts for conventional treatment was reported to be 3.40 with a percent removal of 99.9, whereas 2.98 log removal with a 99.4 percent was reported for oocysts. As for direct filtration, the average log removal for cysts was stated to be 3.30 with a 99.9 percent removal, and 2.97 log removal with a 98.0 percent removal for oocysts. In the full-scale runs, the authors reported an average log removal of 3.26 for cysts using conventional treatment; in direct filtration, 3.87 average log removal was obtained. The log removal for oocysts in both conventional treatment and direct filtration was reported to be 2.25 and 2.79, respectively.

Jacangelo et al.[12] used microfiltration (MF) and ultrafiltration (UF) to examine the removal of oocysts in environmental samples. The results of the study suggested that absolute removal of oocysts is possible if the membranes are intact. A greater than 6.0 log removal was achieved.

Edzwald et al.[34] conducted pilot studies to remove oocysts by in-line filtration, by dissolved air flotation (DAF) clarification alone, and by DAF followed by filtration. Water samples were taken from two reservoirs and characterized as low in turbidity, low in alkalinity, and low in TOC. Three runs were performed and 6-mg/L ferric chloride and 2.4 mg/L cationic polymer at pH 6 were used in the treatment process. Dual media filtration operating at the rate of 3 gpm/ ft^2 was used in the runs. Oocysts were spiked into the raw water prior to coagulation. Tracer tests were performed to determine the sampling times. The result of the first run shows a 4.7-accumulation log removal, as DAF achieved a log removal of only 0.6. In the second run, the concentration of ferric chloride was changed to 17.5 mg/L at pH 6, and the hydraulic loading rate remained at 3 gpm/ ft^2. The results indicate that the DAF achieved a log removal of 3.1. The dual filters achieved 1.9 log removal and

the combined log removal for the treatment was 5. The third and final run was performed with 20-mg/L alum at pH 6.5 using the same hydraulic loading rate. The result of the third run gave a total log removal of 4.9. A Hiaco-Royco instrument was used for enumeration of oocysts.

Ongerth and Hutton[21] performed a bench-scale study to determine the overall applicability of diatomaceous earth (DE) filtration for controlling oocysts in surface water. Oocysts were purified by isolation from fresh feces of calves. The purified oocysts were spiked into the samples at a concentration greater than 10^7/L to allow measurement of concentration reductions anticipated to be as much as 6 log. Two DE filter runs, at 1gpm/ft^2 and 2 gpm/ft^2, for the removal of oocysts were conducted. The results showed that runs conducted at a filtration rate of 2 gpm/ft^2 had a higher log removal than did runs conducted at a filtration rate of 1 gpm/ft^2, with average log removal of 6.095 and 5.38, respectively. The analysis for concentration of oocysts was performed using membrane filtration completed by IFA and microscopy. A hemocytometer was used for oocyst quantification.

Yates et al.[27] conducted pilot scale studies to optimize the removal of oocysts by coagulation and filtration processes. The essential treatment parameters examined by this study included combinations of coagulant and organic polymer, doses, chlorine, coagulation pH depression, and comparisons of dual and tri-media filtration. Alum and ferric chloride were evaluated in combination with cationic, anionic, and nonionic polymers to obtain optimal coagulation conditions for turbidity and particle removal. The authors reported that 108 oocysts were seeded directly into the influent of a single filter by a peristaltic pump, through Teflon tubing at approximately 0.83 mL/min for 60 minutes. The oocyst spike location was selected to minimize significant loss of oocysts in upstream unit processes and to better characterize removal during filtration. The seeded oocysts were not subject to the coagulation process, and results of oocyst removal reflect the filtration process only. The study showed equal to or less than 3-log removal for ferric chloride and 2-log removal for alum, showing that coagulation with ferric chloride provided greater oocyst removal than coagulation with alum. In addition, pilot study showed that the tri-media filters outperformed dual-media filters with respect to turbidity, particle, and oocyst removal.

LeChevallier et al.[15] assessed the impact of storage of potable water in open reservoirs by examining inlet and effluent water samples from six open finished water reservoirs used by four New Jersey utilities. About 120 samples were collected to determine the density and variation in

parasite concentrations. The parasite assay included positive control slides that were examined and confirmed before sample slides were examined. The authors prepared a negative membrane filter by utilizing phosphate buffered saline (pH 7.0) as the sample. In preparing the positive membrane filter, the authors used oocysts and cysts in the phosphate-buffer saline solution (PBS). An average of 1180 cysts and 1020 oocysts were spiked into tap water samples. A SAS statistical package was used to compare inlet and effluent values. In performing the quality control process, the authors evaluated 64 control filters to determine their recovery efficiencies; 32 of the filters were seeded with a known number of cysts and oocysts and evaluated to validate the recovery efficiencies. The results showed 39 percent recovery of cysts and oocysts. Furthermore, 32 negative control filters of polypropylene were evaluated and no cysts or oocysts were detected. Identification of cysts and oocysts in the seeded water sample was based on correspondence of morphological characteristics with positive controls. The IFA method that requires microscopic examination was used to count the parasites. Overall, the geometric mean for the detection limit of inlet samples (2.4oocysts/100L) was significantly different from that of the outlet samples (6.2oocysts/100L). LeChevallier et al. (1991) criticized the analytical method as being inefficient, variable, cumbersome, labor-intensive, time-consuming, expensive, and analyst- dependent.

Edzwald and Kelley[6] conducted a pilot plant contact filtration study to investigate the removal rate of oocysts with dual filtration at 7.3 m/hr. Oocysts were spiked into raw waters and the water was therefore treated. The results showed differences in the removal rates. These differences, as reported by the researchers, were due to coagulation, filter type, and filter rates.

Despite the interesting findings of the research reviewed above, a number of factors have not yet been investigated:

- *A* molecular-based method of detection and quantitation of *Cryptosporidium*, that allows the use of quantitative polymerase chain reaction (QPCR), an emerging technique capable of extreme sensitivity and accuracy,
- Measurement of oocysts in both the supernatant and sludge of a water treatment process,
- Determination of the number of oocysts lost in the overall experiments via a mass balance, and
- Investigation of the fate of oocysts in the sludge samples disposed of on agricultural land.

The current EPA-approved oocyst detection method (IFA) used by many of the authors reviewed, lacks the sensitivity (level of detection) and specificity (accurate identification) required for accurate detection of oocysts in water samples, and particularly in sludge samples.[13,18] In addition, the technique is cumbersome, and time consuming, could present false positive and negative detection, and exhibit low recovery.[18] The method does not recover small numbers of oocysts in small volumes of water samples.[13] Another shortcoming of the reviewed methods is the time interval between taking a sample and getting test results back from the laboratory using the EPA method, which may be as long as two weeks.[9] For faster tests, as well as more effective detection and quantitation of oocysts, a more sensitive method that detects deoxyribonucleic acid (DNA) of specific oocysts would be advantageous.

The purpose of this research is to address some of the gaps uncovered in the review of the literature. The research consists of three stages:

- Developing an improved molecular-based method of detection and quantitation of *Cryptosporidium parvum* oocysts, using QPCR
- Using this QPCR method to estimate the number of oocysts in the liquid as well as the sludge solids phase after conventional treatment processes, and
- Examining the fate of oocysts in the solid phase (sludge) exposed to agricultural land.

In the first stage of the research, a more sensitive and specific method of detection, QPCR will be applied to detecting oocysts in sludge and water samples.[27] The QPCR method of detection eliminates the false positive and negative detection of oocysts commonly found in the USEPA-approved methods.[13, 14] This first stage will entail optimization of QPCR methodology and use of this method to accurately detect and quantify the number of oocysts in sludge samples.

Because of the microscopic nature of oocysts, which are approximately 4 to 6 μm in diameter,[17] the inability of disinfectants to inactivate this organism,[9,13] frequent outbreaks, and lack of medicine to cure the disease of cryptosporidiosis,[9, 27] knowledge of the fate of oocysts in the liquid and the sludge solid phase is essential to determine the proper treatment method. The sensitivity and specificity of the QPCR method, and the ability of QPCR to detect and estimate oocysts in small water samples, justifies the use of this method to estimate the numbers of oocysts

in the liquid and solid phase of treated water samples in the second stage of the research. This second stage will focus on the effect of process variables on oocyst removal and the estimation of oocysts in supernatant and sludge samples.

The sludge produced in the second stage of study will be examined for the presence of oocysts. Conventional processes in water treatment facilities (WTF) also produce sludge. This sludge is occasionally used as soil amendments in agricultural and land reclamation.[27] Previous studies have suggested that a source of oocysts to humans and in drinking water, could be the application of sludge contaminated with oocysts to agricultural land.[17] This application of sludge to land poses a potential threat to public health due to the possibility of viable oocysts within the sludge surviving environmental pressures and returning to water treatment facilities through agricultural run-off.[27]

Little or no effort have been made to evaluate the presence of oocysts in settled sludge samples. This lack of effort ignores the potential of parasites present in sludge that may be transported back to treatment facilities through agricultural runoff if the sludge is applied to land.[27]

Also unknown at this time is the reduction in viability of oocysts applied to agricultural land (inactivation rate) and, more specifically, the impact this application may have on the potential infectivity of active oocysts. To determine the inactivation rate of oocysts, a method for assessing oocyst viability that differentiates live and dead oocysts will be applied (third stage). Live and dead oocysts will be differentiated on the basis of dye exclusion. Oocysts will be stained with dye trypan blue. The membranes of viable oocysts prevent dye uptake, but non-viable oocysts will be readily stained and identified by their blue color under a microscope. Such a test is vital to establishing the true potential health hazard posed by the presence of *Cryptosporidium* in the sludge samples.

Federal Regulations as Pertaining to *Cryptosporidium parvum*

To address the increasing problem of outbreaks of waterborne cryptosporidiosis, USEPA, through its commitment to the drinking water industry and to the public, implemented regulatory controls that protect public health against *Cryptosporidium* in drinking water supplies.[31] The Interim Enhanced Surface Water Treatment Rule (IESWTR) was the first step in that direction. The IESWTR applies to systems using surface water or groundwater under the direct influence of

surface water that serve 10,000 or more persons. The rule also includes provisions to conduct sanitary surveys for surface water systems regardless of system size. The rule builds upon the treatment technique requirements of the Surface Water Treatment Rule with the following additions and modifications.[31]

- Maximum contaminant level goal of zero for *Cryptosporidium,*
- 2-log *Cryptosporidium* removal requirements for water systems that use a filtration process,
- Inclusion of *Cryptosporidium* in the watershed control requirement for unfiltered public water,
- System using groundwater (under the influence of surface water) or surface water serving 10,000 or more persons must monitor for *Cryptosporidium,*
- Application of the new rule dealing with *Cryptosporidium* to system using groundwater under the direct influence of surface water,
- Individual filter turbidity monitoring provisions,
- Strengthened combined filter effluent turbidity performance standards,
- Requirements for covers on new finished water reservoirs,
- Disinfection profiling and benchmarking provisions, and
- Sanitary surveys, conducted by states, for all surface systems regardless of size.

The Interim Enhance Surface Water Treatment Rule, with tightened turbidity performance criteria and required individual filter monitoring, was designed to optimize treatment reliability and to enhance physical removal efficiencies to minimize the *Cryptosporidium* levels in finished water. In addition, the rule includes disinfection benchmark provisions to assure continued levels of microbial protection while facilities take the necessary steps to comply with new Disinfection Byproducts Rule (Table 1-2).[31]

TABLE 1-2. SCHEDULE OF MICROBIAL DISINFECTANT AND DISINFECTION BYPRODUCTS (M-DBP) RULES

Final Rule Dates	Interim Enhanced Surface Water Treatment Rule –Affected Stages
November 1998 -- Final Rule	Interim Enhanced Surface Water Treatment Rule and Stage 1 Disinfection Byproduct Rule
August 2000 – Final Rule	Filter Backwash Recycling Rule
November 2000 – Final Rule	Long Term 1 Enhanced Surface Water Treatment Rule and Ground Water Rule
May 2002 – Final Rule	Stage 2 Disinfection Byproduct Rule and Long Term 1 Enhanced Surface Water Treatment Rule

Source: USEPA Office of Ground Water and Drinking Water EPA 815-F-98-0014.[31]

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Chapter 2

Quantitative Polymerase Chain Reaction (QPCR) using the MIMIC Approach to Estimate *Cryptosporidium parvum* oocysts, an Intestinal Pathogen, in Municipal Water Treatment Sludge Samples

ABSTRACT

An accurate estimation of the number of *Cryptosporidium parvum* oocysts in water treatment plant sludge was determined using the Quantitative Polymerase Chain Reaction (QPCR) method. Approximately 8 x 10^6 purified viable oocysts were spiked into raw water and treated by conventional water treatment methods. The settled sludge was collected and the DNA extracted. The QPCR Mimic produced two competing products that were 300 and 435 base pair in size. The log ratios of the products were used in the standard curve to determine a final estimation of oocysts in the sludge sample. The final number of oocysts in the sludge sample was estimated at 258 oocyst per two liters of treated water. This is the first time sludge from a water treatment process has been tested for presence of *C. parvum* oocysts, which is a known contaminant of drinking water. The QPCR method can be used to test other sludge samples and help estimate the sanitary risks associated with using sludge to fertilize agricultural lands.

INTRODUCTION

Cryptosporidium parvum is a coccidian protozoan, a zoonotic parasite that is responsible for the gastrointestinal illness cryptosporidiosis in humans.[1] This parasite has been recognized as an important microbial contaminant in water and is characterized by the presence of oocysts.[2,3,4] Drinking water supplies are contaminated with oocysts through animal and human feces by way of

agricultural run-off and sewage effluents.[2] Unfortunately, oocysts are resistant to conventional water disinfectants such as chlorine, chloramine and ozone and are responsible for documented outbreaks of cryptosporidiosis.[3,4] During the water treatment process, sludge is known to accumulate. The accumulated sludge is commonly disposed of by applying it to land as fertilizer or to serve to increase the soil buffering capacity. [5] Land application was considered an alternative to traditional disposal methods because of its relatively low costs and potential as a long-term disposal solution. [5] On average, land that is fertilized with sludge contains 0.5 to 2.5 percent sludge. [5] This application process is a potential threat to public health as viable oocysts may be present in the sludge and may survive environmental pressures and make their way back to the water treatment facilities by agricultural run-off into surface water. [6,7]

To ensure safe drinking water, the United States Environmental Protection Agency approved several methods of identification of oocysts in water samples, such as immunoflorescence antibody and immuno-magnetic separation. [3,8] In addition, extensive efforts went into quantifying the number of oocysts in river and treated waters. [9,10] However, little if any work has been published on the detection of oocysts in water plant sludge. To test whether oocysts exist in sludge, a quantitative polymerase chain reaction (QPCR) method was used to determine the presence of oocysts in sludge generated in a bench scale version of a water treatment plant facility.

MATERIALS AND METHODS

Raw Water and Organism: Raw water was collected from Kaw Reservoir (Northern Oklahoma), which is the source of drinking water for the City of Stillwater, Oklahoma. Approximately 8 x 10^6 purified viable oocysts stored in phosphate buffer saline (PBS) were obtained from Waterborne Incorporated, New Orleans, Louisiana.

Composite and Specific Primer Construction and Generation of MIMIC Template: Composite primers for the generation of the MIMIC template (internal standard) were designed by using combined sequences from the *C. parvum* 18SrRNA gene and pBluescript SK (-) plasmid DNA. The forward composite primer sequence used was 5'AAGCTCGTAGTTGGATTTC TGTTCGAGCTTGGCGTAATCAT3') and the reverse primer sequence used was 5'TAAGGTGCT

GAAGGAGTA AGGTGAGCGAGGAAGCGGAAGAG 3'.The underlined sequence corresponds to the pBluescript SK (-) plasmid DNA and the non-underlined sequence correspond to the *C. parvum* 18SrRNA gene. In addition, specific *C. parvum* 18SrRNA forward and reverse primers were used which consisted of 5'AAGCTCGTAGTTGGATTTCTG3' which corresponds to nucleotides 601 – 621, and 5'TAAG GTGCTG A AGGAGTAAGG-3' which corresponds to nucleotides 1015 – 1035. [3,11] All the oligonucleotide primers were synthesized by the Recombinant DNA/Protein Resource Facility (Oklahoma State University, Stillwater, OK USA). The PCR reaction used to generate the MIMIC template consisted of 1μL of each of the composite primers (20 μM), 5μL of 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl; Perkin Elmer Cetus, Norwalk, Conn. USA), 3μL of 25 mM $MgCl_2$, 1μL of 10 mM dNTPs, 1μL of pBluescript SK (-) plasmid DNA (20 ng/μL) fragment, 0.2μL of Tag gold DNA polymerase (5 U/μL) and 37.8μL of sterile water for a total volume of 50μL. The sample was amplified in a DNA Thermocycler model 2400 (Perkin Elmer Cetus, Norwalk, Conn. USA), for 30 cycles using the following programmable profile: hot start (95 ° C for 60 seconds); denature (94 ° C for 15 seconds); anneal (58 ° C for 30 seconds); polymerize (72 ° C for 30 seconds); and a final polymerization (7 minutes). A 5-μL portion of the reaction was resolved on a 1.8- percent (w/v) ethidium bromide agarose gel. The intensity of the DNA product was analyzed using the Image Analysis System with Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The concentration of MIMIC was determined by comparing the intensity of the MIMIC template band with a known amount of DNA from a 100 base pair molecular weight marker sample.

The optimal amount of MIMIC template to be used in the competitive PCR reaction was determined using the specific primers. A 1μL amount of the primary reaction (above) was diluted to 100 μL of sterile water. A 2 μL amount of this dilution was added to a PCR reaction tube containing : 10 μL of 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl; Perkin Elmer Cetus, Norwalk, Conn. USA), 6 μL of 25 mM $MgCl_2$, 2 μL of 10 mM dNTPs, 2μL of each C. *parvum* 18SrRNA gene specific primer (20 μM), 0.2μL of Tag gold DNA polymerase (5 U/μL) and 65.8μL of sterile water for a total volume of 100μL. The sample was amplified for 30 cycles. A 10 μL portion of the PCR products were run on a 2 percent (m/v) agarose gel with ethidium bromide. The optimal amount of Mimic DNA template to use was 1000pg.

Construction of Standard Curve: Construction of the standard curve was based on the competitive PCR methodology. [12,13] DNA was extracted from 1 x 10^6 C. parvum oocysts using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Technologies Corporation, Madison, Wisconsin) and the amount of DNA extracted was used to estimate the amount of oocysts. Different amounts of C. parvum DNA (10-pg, 50-pg, 100-pg, 250-pg, 500-pg, 1000-pg, 2000-pg, 4000-pg, and 8000-pg) were added to the PCR reaction. As a competing template, 1μL of MIMIC DNA (1000-pg/μL) was added equally to all samples. The PCR conditions remained the same as above. The amplified sample was analyzed on conventional 2-percent (w/v) agarose gel stained with ethidium bromide. The competitive PCR product (300 and 435 base pair) concentrations were estimated by comparing the band intensity to known amounts of molecular weight size standards using the Image Analysis System. The standard curve was constructed by using the values from the log of the ratio of C. parvum DNA to MIMIC PCR, the log of the known concentration of C. parvum DNA and its corresponding number of oocyst values.

Oocysts Recovery and DNA Extraction from Sludge: A six-liter raw water sample was divided into three 2-liter containers. Each 2-liter container was spiked with approximately 333,333 oocysts (1 x 10^6 oocysts sample was divided equally into three parts). The spiked water sample was then treated by conventional methods (Udeh,P., maunuscript in preparation). After treatment, the settled sludge from each container was collected separately and dissolved in 250 ml deionized water and 2 drops of sulfuric acid. [14] The sample was incubated for 3 minutes at room temperature prior to filtration.

The dissolved sludge was decanted and filtered using a cellulose acetate membrane filter with a pore size of 1.2-μm (Advantec MFS, Inc. Pleasanton, CA). The entrapped oocysts in the filter were transferred to a 250-mL conical centrifuge tube and 200-mL of acetone was added. [15] The sample was agitated for 5 minutes and the dissolved matrix were spun at 650 x g for 10 minutes. The pellet was then washed with 95 percent acetone and 70 percent ethanol. [15] The pellet was then re-suspended in 750-μL lysis buffer: [120 mm NaCl, 10 mM EDTA, 25 mM Tris pH 7.5 and 1 percent (w/v) sarcosyl] and 0.5 mg/mL proteinase K, and incubated for 1 hour at 37 ° C. Oocysts walls were disrupted and sporozoite membranes lysed by the freeze-thaw (10 cycles) method. [16] The sample was frozen by liquid nitrogen (-195 °C) and incubation in a water-bath at 65 ° C for 10

min; repeated 10 times . The lysate was treated with additional 0.1 mg/mL of proteinase K and incubated at 37 °C for 2 hours. The lysate was pelleted by centrifugation at 16,000-x g for 15 minutes to remove the debris and the DNA in the supernatant was collected. The sample was further purified using the MasterPure Complete DNA and RNA Purification Kit (EPICENTRE TECHNOLOGIES, Madison, WI) according to the manufacturer's protocol. The amount of DNA was measured by the Image Analysis System.

Optimization of DNA Extraction and Purification: The method of DNA extraction by Chrisp and LeGendre[23] was first used, but it did not work. This method was modified to: first resuspended oocysts in 750 µL lysis buffer as described above with 1 % (w/v) sarcosyl and 0.5 mg/mL proteinase K) and incubated for 1hour at 37 ^{0} C. Ten freeze –thaw cycles was performed at –195 ^{0} C (liquid nitrogen) for 10 minute, and incubated at 65 ^{0} C for 10 minute. The lysate was treated with additional 0.1 mg/mL proteinase K and incubated at 37 ^{0} C for 2 hours. In addition, the DNA purification method described by Chrisp and LeGendre [23] was cumbersome, time consuming and resulted in a high lost of DNA. Therefore, the method was replaced with the MasterPure Complete DNA and RNA Purification Kit from the Epicentre Technologies Corporation, Madison, Wisconsin.

Following the DNA extraction, the sample was further purified by adding 300 µL of cell lysis solution containing 1µL of 50 µg/µL Proteinase K into the extracted DNA sample and spinning for 10 minutes at 16, 000 x g (Centrifuge, Sorvall RC – 5B refrigerated speed) in a microcentrifuge tube (MasterPure, Epicentre Technologies, Madison, WI). The DNA sample was then incubated at 65^{0}C for 15 minutes and votex (Model Genie 2, Fisher Scientific, Pittsburgh, PA) mixed every 5 minutes. After 15 minutes incubation, the sample was cooled to 37^{0}C and 1µL of 5 µg/µL RNase was added to the sample and mixed thoroughly to degrade and remove RNA that may contaminate the sample.

Following incubation at 37^{0}C for 30 minutes, the sample was placed on ice for 5 minutes. After 30 minutes incubation time, 150 µl of MPC Protein Precipitation Reagent was added to 300 µl of supernatant and vortexed for 10 seconds. The debris was pelleted by centrifugation for 10 minutes at 16,000 x g in a microcentrifuge . The supernatant was transferred into a clean microcentrifuge tube and the pellet discarded. A 500 µL aliquot of isopropanol was added to the recovered supernatant and the DNA pelleted by centrifugation at 16,000 x g at 4°C for 10 minutes in a

microcentrifuge. The isopropanol was poured off without dislodging the DNA pellet. The pellets were rinsed twice with 75 percent ethanol and vacuum dried with speed vac plus (Savant Instruments Inc., Holbrook, NY) to remove all residual ethanol. The chromosome DNA was resuspended in 50 μL of TE Buffer

In addition, an RNase substances was added to lysate solution to degrade and to remove ribonucleic acid (RNA) present in oocysts in order to prevent RNA from the interfering with PCR products and to improve PCR amplification.

Optimization of PCR amplification: To overcome the interfering substances in the supernatant and sludge and to improve the PCR amplifications, 100 μg/mL bovine serum albumin (BSA) was added.[16] Specificity and sensitivity of the PCR for oocysts were tested by the construction of a standard curve (Figure 2-B). The concentration of the PCR components such $MgCl_2$, and Tag gold DNA polymerase were varied to ensure optimal conditions for amplification of oocysts DNA. We compared AmpliTag (Fisher Scientific) and AmpliTag Gold (Perking Elma), AmpliTag Gold gave higher yields of PCR products. Annealing temperature was varied between 55^0 and 58^0C, and the optimal temperature for annealing was found to be 58^0C.

Image Densitometry: PCR product concentration was estimated using imaging densitometry to analyze product bands on conventional 2 percent (w/v) agarose gels stained with ethidium bromide. The Gel Doc 1000 Image Analysis System with Molecular Analyst Software (Bio-Rad Laboratories, Hercules, California) has such capabilities for band volume analysis. The software was used to generate volume integration reports of molecular weight size standard band intensities from which standard curves were constructed and quantitation of genomic oocysts DNA and MIMIC PCR products was achieved.

Quantitative PCR: DNA isolated from the sludge sample was added to the competitive PCR reaction. To prevent inhibition of the PCR reaction, 100μg of bovine serum albumin (BSA) was added to the competitive PCR reaction. [16] The ratio of sludge DNA to MIMIC DNA was measured to determine the initial estimated amount of oocysts, using the standard curve. The estimated number of oocysts was multiplied by the amount of C. *parvum* DNA extracted from the unknown,

by the amount of C. *parvum* DNA used for dilution factor, and by the dilution factor per amount of C. *parvum* DNA used for PCR amplification.

(Formula: Estimated number of oocysts = initial oocysts from standard curve X amount of *C. parvum* DNA extracted X amount of *C. parvum* DNA used for dilution X dilution factor per number of nμl of DNA used for PCR amplification)

RESULTS

Construction of Standard curve: Figure 2-1 demonstrates the steps used to generate the MIMIC template. In the 1° (1st) PCR reaction, a pBluescript SK (-) plasmid served as the template, as a 300 bp DNA fragment was amplified. To ensure the specific primers were working properly, a 2º (2nd) PCR reaction was done. For this, specific primers were added to the 1º PCR product and another 300-bp product was again generated (data not shown).

To determine the optimal amount of MIMIC DNA to be used in the competitive PCR reaction, a series of dilutions were tested and it was determined that 1000pg of Mimic was ideal for the PCR reaction (data not shown). Using a constant amount of MIMIC (1000-pg), a series of dilutions with known amounts of C. *parvum* DNA were added to the competitive PCR reaction. After amplification, a 300 bp and 435 bp DNA fragments were generated The different band intensities for both fragment sizes are shown in Figure 2-2A. The log of the ratio of the 435/300 bp band intensity, and the log of known concentration of *C. parvum* DNA and estimated amount of oocysts were used to construct the linear line for the standard curve (Figure 2-2B).

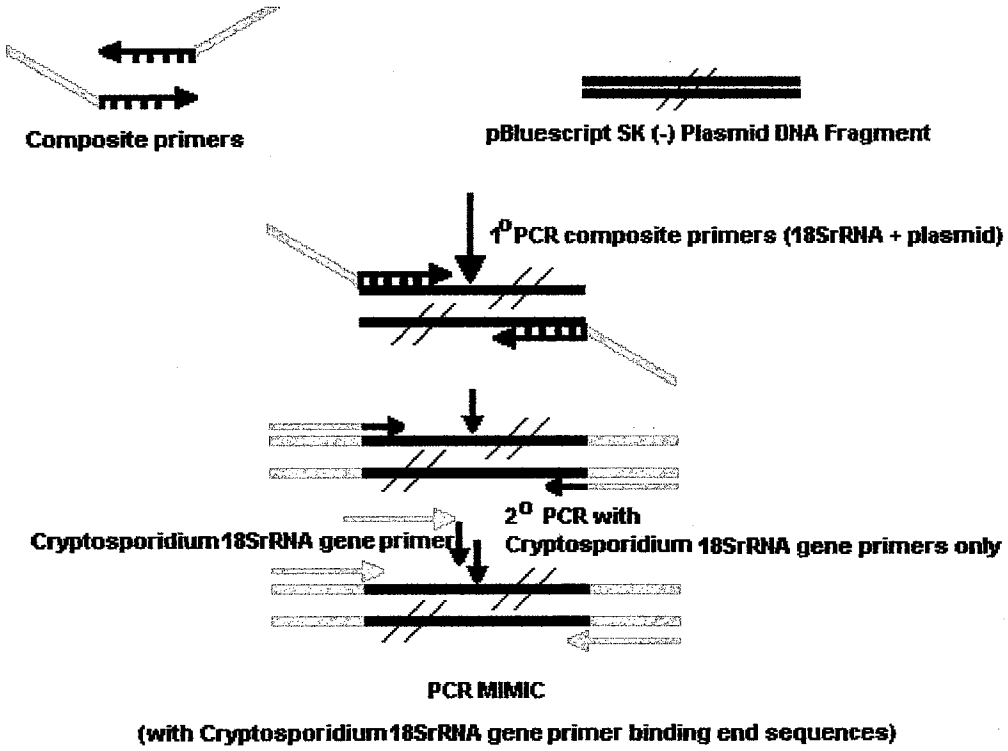
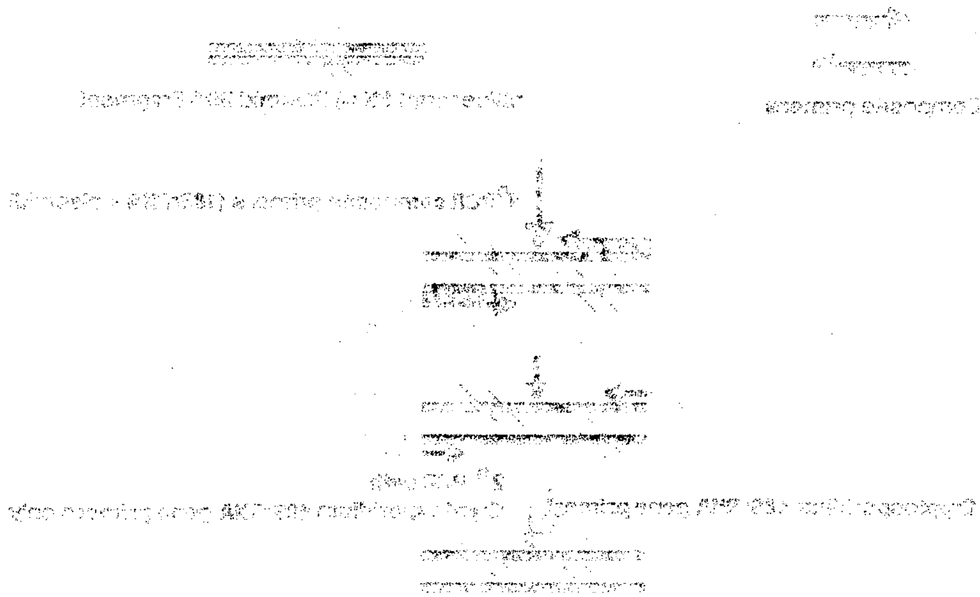


Figure 2-1. Flow chart illustrating the generation of competitive PCR MIMIC. The black portion of composite primer is specific for the pBluescript SK(-) plasmid DNA, and the light gray portion is specific for the C. *parvum* 18SrRNA gene. The gray only primer is specific for the C. *parvum* 18SrRNA gene.



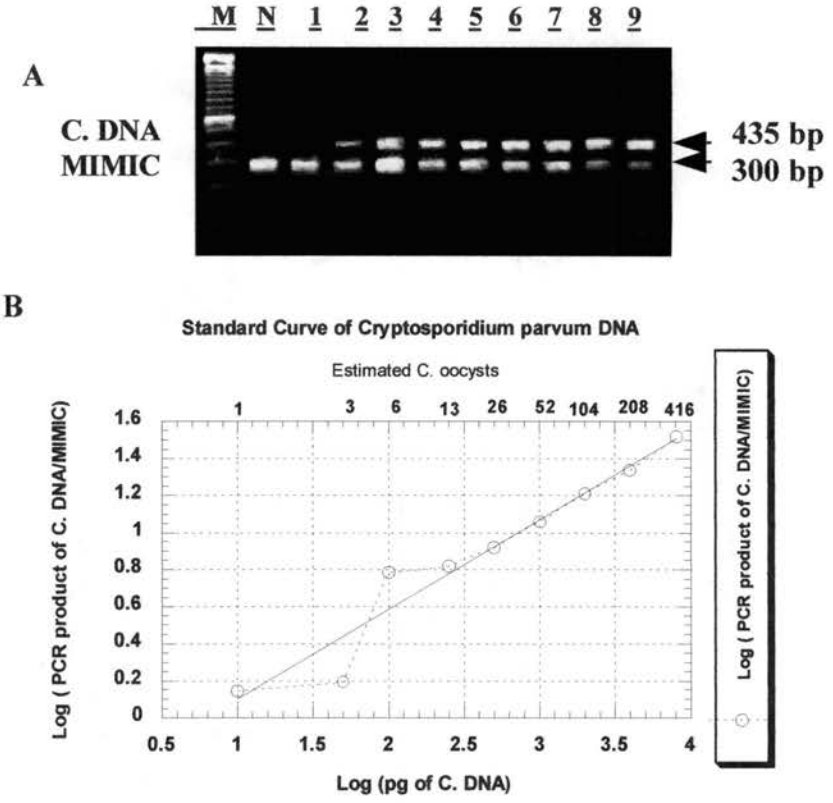


Figure 2-2. Agarose gel electrophoresis of PCR products from competitive amplification of different concentrations of *C. parvum* DNA. Two competitive products were amplified, a 435 bp that is specific for C. *parvum*, and a 300 bp MIMIC fragment. A) Lane M, 100 bp molecular weight marker (Life Technologies Inc., Gaithersburg, MD, USA); Lane N = negative control; Lanes 1-9: *C. parvum* DNA dilution in picogram added in an increasing fashion: 10, 50, 100, 250, 500, 1000, 2000, 4000, 8000-pg, respectively. B) Quantitative analysis of the competitive PCR experiment shown in (A). The ratio of *C. parvum* DNA and MIMIC was plotted against log (pg. C. DNA) to obtain the standard curve.

Recovery, Detection, and QPCR Assay of Sludge Samples Containing C. *parvum* DNA:

The amount of DNA extracted from sludge was 10.5μg. To obtain equivalent recovery efficiencies for the standard (oocysts in PBS) and the unknown (oocysts in sludge), deionized water and sulfuric acid was added to dissolve the sludge to achieve optimal filtration. In addition, the 1.2 -μm cellulose acetate membrane filter was dissolved in acetone. Because of low sedimentation of oocysts[10] in the treated water, low recovery efficiencies of oocysts in the sludge were expected[20]. To over come the interfering substances in the sludge that may affect the efficiency of the competitive PCR reaction, the addition of bovine serum albumin (BSA) was added.

The log of the ratio of the 435/300 bp band intensity was used to determined the concentration of the *C. parvum* DNA, based on the standard curve. The ratios of the three sludge samples (log *C. parvum* DNA/MIMIC) were 0.202, 0.197, and 0.192, respectively. These values were also used to determine the estimated oocysts, based on the standard curve. The initial estimated oocyst and the concentration of *C. parvum* DNA was then used to determine the estimated number of oocysts in the sludge sample. The average estimated number of oocysts was determined to be 258/2L (Figure 2-3).

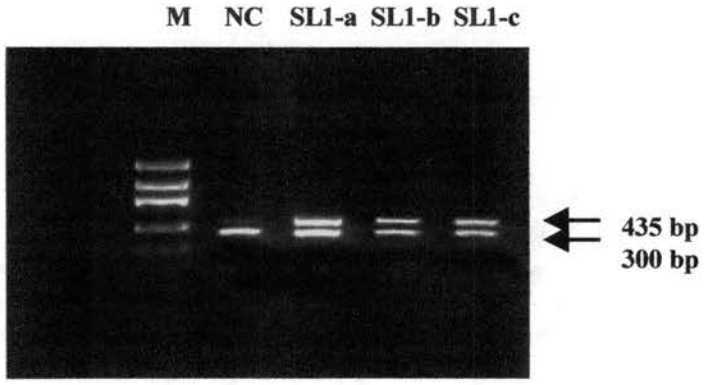


Figure 2-3. Agarose gel electrophoresis of competitive PCR products after amplification of DNA isolated from sludge samples. Lane M = 100 bp molecular size marker; lane NC = negative control. Lane SL1-a = 275 oocysts/2 liter container, SL1-b = 250 oocysts/2 liter container, SL1-c = 250 oocysts/2 liter container, total volume equaled 6 liters. For all three containers, an average number of 258 oocysts was estimated in the sludge samples from 1 x 10^6 oocysts spiked in the 6 liter of raw water.

DISCUSSION

The presence of oocysts in sludge has never been tested. However, since sludge is used as fertilizer for crops, the potential presence of oocysts in sludge poses a risk to public health.[20,2] The possibility of viable oocysts in sludge surviving environmental pressures and returning to the food chain via crops or livestock, on sludge fertilized land, as well as, the possibility that viable oocysts may be transported back to water treatment plants through agriculture run-off (during ran fall) into surface water, are both areas of concern. [17,1819,5,7,8,22] QPCR provides a method to accurately estimate the presence of oocysts in sludge.

QPCR is a rapid and sensitive method, but several factors can compromise the outcome. First, the accuracy of the standard curve is based on the extraction procedure used to obtain DNA. The amount of *C. parvum* DNA extracted from 1 x 10^6 oocysts was 19.25μg. To demonstrate consistency, two independent DNA extractions were performed using 1 x 10^6 oocysts. The amount of DNA extracted was between 18 and 19 μg of DNA from 1 x 10^6 oocysts. Secondly, the debris associated with sludge may reduce the oocyst recovery efficiency. To maximize oocyst recovery, the sludge was dissolved using a filter dissolution method. Thirdly, substances in the sludge that may interfere with the efficiency of the competitive PCR reaction was neutralized by adding BSA to the PCR reaction. [16] Fourthly, to allow for data analysis of PCR products in both the exponential phase or plateau phase, a competitive PCR method (MIMIC approach) was used as opposed to the co-amplification approach where only the exponential phase can be analyzed. [16] The use of MIMIC in the QPCR improves the accuracy of determining the number of oocysts in the sludge samples by minimizes the variability of PCR amplification from tube to tube among replications.[16] Fifthly, the validity of the ratio values used is based on the yield of the two products, which is defined by the following equation: $\log (Nn_1/N_2) = \log (No_1/No_2) + [n \times \log (eff_1/eff_2)]$. [12] where Nn_1 and Nn_2 are the amplification product concentrations, No_1 and No_2 are the initial template concentrations, n is the PCR cycles number, and eff_1 and eff_2 are the efficiencies of the two template amplification. The efficiencies of amplification of the two templates are the same ($eff_1 = eff_2$), if the ratios of the products (Nn_1/N_2) following any cycle (n) of PCR amplification depend

directly on the ratio of the concentrations of the initial templates (No_1/No_2) present. [13] Lastly, each time an unknown was tested, a standard curve was constructed. Based on the three samples (SL1-a, SL1-b, and SL1-c) tested , the slope of the linear line for each standard curve remain relatively constant. Therefore, reliability of the standard curve to accurately interpret the sample ratios was achieved.

Overall, the study showed that QPCR was an accurate method in the detection and estimation of small quantities of oocysts in sludge. The QPCR method applied in this study can be used to test various types of sludge and can help estimate the sanitary risks associated with using sludge to fertilize agriculture land.

CONCLUSION

Generation of MIMIC Template: As a result of discontinuing the distribution of MIMIC template by CLONTECH Laboratories Inc., Palo Alto, California in 1997, the need to generate MIMIC template was enormous. The composite primers for the generation of the MIMIC template were designed by using combined sequences from the *C. parvum* 18SrRNA gene and pBluescript SK (-) plasmid DNA. The use of MIMIC template as an internal standard, minimizes tube to tube variation as result of pipette error and improves the recovery of oocysts in the sludge samples

Optimization of DNA Extraction and Purification: The method of DNA isolation prescribed by Chrisp and LeGendre[23] was modified by resuspended oocysts in 750 μL lysis buffer with 1 % (w/v) sarcosyl and 0.5 mg/mL proteinase K) and incubated for 1hour at 37 ^{0} C. Ten freeze –thaw cycles was performed at –195 ^{0} C (liquid nitrogen) for 10 minute, and incubated at 65 ^{0} C for 10 minute. The lysate was treated with additional 0.1 mg/mL proteinase K and incubated at 37 ^{0} C for 2 hours. Because the method by Chrisp and LeGendre was cumbersome and time consuming, in addition to losing *Cryptosporidium* DNA, modification of extraction process was necessary.

DNA purification method described by Chrisp and LeGendre [23] was not used in DNA purification process. Instead MasterPure Complete DNA and RNA Purification Kit from the Epicentre Technologies Corporation, Madison, Wisconsin were used. In addition, an RNase

substances was added to lysate solution to degrade and to remove ribonucleic acid (RNA) present in oocysts in order to prevent RNA from the interfering with PCR products and to improve PCR amplification. The modifications were necessary to improve PCR sensitivity.

Optimization of PCR amplification: Addition of 100 μg/mL bovine serum albumin (BSA) in the PCR reactions, overcome the interfering substances in sludge samples that may affect the efficiency of the competitive PCR reaction. The concentration of the PCR components was varied to ensure optimal conditions for amplification of oocysts DNA. On comparisons of the efficiency of AmpliTag (Fisher Scientific) and AmpliTag Gold (Perking Elma), the AmpliTag Gold gave higher yields of PCR products. Annealing temperature was varied between 55^0 and 58^0C, and the optimal temperature for annealing was found to be 58^0C (pre-experimental trial).

Overall, PCR was method was very effective in the detection and quantitation of oocysts in sludge samples.

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CHAPTER 3

EFFECTS OF PROCESS VARIABLES OF WATER TREATMENT ON OOCYST RECOVERY FROM THE PREFILTERED SUPERNATANT AND SLUDGE SAMPLES

Abstract

Bench scale studies of a water treatment process were conducted to ascertain the effect of selected variables, such as aluminum sulfate (alum), polyaluminum chloride (PACl), cationic polymer, liquid pH levels, and temperatures on the detection of oocysts in prefiltered supernatant and sludge samples. The mass balances approach was used to determine the number of oocysts lost, and quantitative polymerase chain reaction (QPCR) was used to detect and estimate oocysts in the supernatant and sludge samples. Based on treating raw water spiked with 1.67×10^5 oocysts/L, the following general observations were made: (1) at colder temperatures, PACl was more effective than alum in turbidity removal and oocyst precipitation, (2) cationic polymer used in conjunction with either alum or PACl enhanced precipitation of oocysts in the sludge samples and improved turbidity and settling velocity, (3) a greater number of oocysts were recovered from the sludge samples when water was treated at a pH level of 6 as opposed to pH 8, (4) addition of chlorine during the treatment increased turbidity removal and oocyst recovery from the sludge samples, (5) on average, only 9.8 percent of oocysts were recovered from the sludge samples in 48 experiments, and (6) based on the mass balances, an average of 0.03 percent of oocysts were lost in the experiments.

INTRODUCTION

Cryptosporidium parvum is a coccidian protozoan that is parasitic in the intestinal tract and is characterized by the presence of oocysts.[3] The parasite has been recognized as an important

microbial contaminant of water that is introduced into the environment through animal feces, including human feces.[2, 6] *Cryptosporidium* contaminates surface water through agricultural run-off and sewage effluents.[2] The organism has been shown to be resistant to conventional disinfectants such as chlorine and chloramine, which are used in water treatment facilities. The oocyst resistance to disinfectants and the inability of some the filters to capture the parasite, are responsible for documented outbreaks of cryptosporidiosis.[2,5,21] These outbreaks have raised questions about the source of contamination of these protozoa in our finished water.[5,9] To address these concerns, the Environmental Protection Agency (EPA) has amended the surface water treatment rule with a maximum contaminant level goal of zero for oocysts and a requirement of a 2-Log oocyst removal for systems that filter.[11,14,15] To ensure compliance with this rule, EPA suggests the use of the immunoflorescence antibody (IFA) method to test water samples for the presence of oocysts.[6,8,14] Other methods are also used to test for this organism, including the flow-cytometer and hemocytometer.[4,6,13,19] The current problems with these methods include (1) the methods lack sensitivity and specificity to accurately estimate oocysts in the supernatant samples,[6] (2) the methods do not recover small numbers of oocysts in small volumes of water samples,[6] (3) EPA technique (the IFA) is cumbersome, time consuming, could present false positive and false negative detection and have a low recovery rates of oocysts,[11] and (4) oocysts in the sludge could not be measured with all these methods.[6,9]

Numerous studies have been conducted on detecting the presence of oocysts in surface and finished waters.[4,8,13,25] Little or no effort has been made to evaluate settled sludge samples for the presence of oocysts or the effects of various treatments. For example, investigators[13,20,24] have used microscopic enumeration to analyzed the removal of oocysts by conventional treatment processes (coagulation, flocculation, sedimentation, and filtration). However, two areas of these studies have not been analyzed: first, accurate enumeration of oocysts in sludge, along with its relationship to the number of oocysts lost during the analytical recovery process, and the use of QPCR to detect and quantify oocysts in the sludge samples, and second, mass balances have not been used to determine the number of oocysts lost during analysis of the supernatant and sludge samples.

This study will accurately (sensitivity and specificity) determining the presence of oocysts in supernatant and sludge and quantify the number of oocysts lost using a mass balance. The objectives of this study are as follows: (1) to use quantitative PCR to estimate and compare the number of oocysts in the settled sludge and supernatant, (2) to evaluate the relationship between the settled turbidity levels and oocysts recovered from the sludge and supernatant samples, (3) to evaluate the effects of alum, PACl, cationic polymer, pH levels (6 and 8), temperatures (14.5^0 and 22.1^0 C) on percent removal efficiency of turbidity and oocysts, and (4) to use mass balances to determine the number of oocysts lost through the analytical recovery process.

MATERIALS AND METHODS

Raw Water-Origin and Quality: The raw water utilized in this study originated in Kaw Reservoir (Ponca City, Oklahoma) and was sampled according to the sampling protocol established by the Information Collection Rules for protozoa and enteric virus.[12] The samples were collected from a tap connected to the main raw water supply line to the Stillwater Treatment Facility. Prior to collection, the raw water was purged for 3 minutes in order to remove residual debris from the main supply line or until the turbidity of the water became uniform. The raw water was then analyzed for 17 water quality parameters (Table 3-1). The analyses were conducted in triplicate prior to the study.

Organism: Approximately 1.6×10^7 purified viable oocysts stored in phosphate buffer saline (PBS) were obtained from Waterborne Inc. (New Orleans, Louisiana). An 8 mL aliquot of the purified oocyst stock (6.25×10^5 oocysts/mL) was recounted in triplicate using a hemacytometer to confirm the number of oocysts purchased from Waterborne Inc.

Examination of Raw Water for the Presence of Oocysts: The raw water samples were filtered through 1.2 µm pore-size cellulose acetate membrane filters (Advantec MFS Inc., Pleasanton, CA). To ensure capture of any oocyst in the raw water, the filtrate was refiltered through a 0.45 µm cellulose acetate membrane filter. After filtration, the filters, which were considered as part of the analytical process, were analyzed for the presence of oocysts by

performing DNA extraction and PCR detection.[23] The PCR method was used because of its sensitivity and specificity.[23]

Testing the Presence of Oocysts in Filtered Water of Supernatant and Sludge: After filtrating the supernatant and dissolved sludge (part of the analytical method), the filtrate samples were stained with Trypan Blue dye and examined at 400 X magnification using a Micro-master bright field Microscope (Fisher Scientific, Pittsburgh, PA) to confirm either the presence or the absence of oocysts in the filtered samples.

Spiking Samples: Prior to conducting any spiking study, 0.53 mL of the purified viable oocyst stock (1.67 x 10^5/L), was stained with Trypan Blue (0.2 percent in 0.85 percent saline) and counted in triplicate using a hemacytometer (Bright line Phase, Fisher Scientific, Pittsburgh, PA) to determine the initial count. The reasons for choosing this method of quantitation were that (1) the oocysts are purified and contained in a PBS stock solution, (2) the oocysts can be easily observed in the absence of foreign matter using a microscope, and (3) the dye permeability assay is easy and faster than the QPCR method. Based on the results of the count of 1.67 x 10^5 oocysts/L (0.53-mL oocysts stock) were spiked into 2 liters of raw water and treated.

Oocyst Recovery and Precision Test Using QPCR: Experiments were conducted to identify the precision of the PCR detection method,[23] and to demonstrate the recovery and quantitation of oocysts in treated water samples. These experiments provided data, which was used to determine the precision and accuracy of estimated oocysts in the supernatant (top) water layer, and sludge samples, based on EPA established IFA protocols. A 2L Gator Jar containing raw water was spiked with 3.33 x 10^5 oocysts. The raw water was then filtered with a 1.2 μm pore size cellulose acetate membrane filter and refiltered through a 0.45μm. After filtration, the filters were analyzed for the presence of oocysts by performing DNA extraction and PCR detection.[23] The QPCR precision tests in the measurement of oocysts recovered from the supernatant and sludge samples were conducted using the USEPA method 1662, which was approved for the IFA method. Accordingly, USEPA method 1662 precision was established by calculating the average percent recovery (P) and the standard deviation of percent recovery (Sr). The formula is expressed as P – 2 Sr to P + 2 Sr.[14]

TABLE 3-1. EQUIPMENT USED TO ANALYZE RAW AND SETTLED WATER WATER QUALITY PARAMETERS

Parameter Analyzed	Average Value	Method	Equipment Manufacturer/ Distributor
Color CU	103	Alpha Platinum Cobalt Standard method – 8025*	3000-DR, Spectrophotometer Hach Company, Loveland, CO
pH range	7.3 – 7.5	EC 30 pH-Meter	Hach Company, Loveland, CO
Temperature ^{0}C	14.5 and 22.1	EC 30 pH-Meter	Hach Company, Loveland, CO
Turbidity NTU	12.5 SD = 0.07	Nephelometric Method 8195*	2100 N Turbidimeter Hach Company, Loveland, CO
Total Dissolved Solids Mg/L	506 SD = 1.41	Digital Conductivity Meter Conductivity & TDS model	Fisher Scientific, Pittsburgh, PA
Conductivity µmho/cm	757 SD = 2.83	Digital Conductivity Meter Conductivity & TDS model	Fisher Scientific, Pittsburgh, PA
Total Hardness as $CaCO_3$ mg/L	260 SD = 7.1	EDTA- titrimetric method Standard Method -Section 314B	-
Total Alkalinity as $CaCO_3$ mg/L	188.5 SD = 2.1	H_2SO_4 -titrimetric method Standard Method - Section 403 B	-
Chloride as Cl^- Mg/L	20.2 SD = 1.48	Mercuric Thiocynate Method 8113*	3000-DR, Spectrophotometer Hach Company, Loveland, CO
Fluoride as F^- Mg/L	0.90 SD = 0.18	SPANDNS Method 8029*	"
Ferrous iron Mg/L	0.05 SD = 0.004	FerroVer Method 8008*	"
Nitrate as NO_3-N Mg/L	2.8 SD = 0.57	Cadium Reduction (Powder Pillow) Method 8039*	"
Manganese as Mn mg/L	0.27 SD = 0.014	PAN Method 8149*	"
Sulfate as S Mg/L	136.85 SD = 2.8	SulfaVer 4 Method 8051*	"
Hydrogen Sulfide as S Mg/L	0.31 SD = 0.02	Methylene Blue Method 8131*	"
Dissolved Oxygen Mg/L	10.6 SD = 0.14	HRDO Method 8166*	"
Silica as $CaCO_3$ Mg/L	1.92 SD = 0.028	Heteropoly Blue Method 8186*	"

SD = Standard deviation. * HacH Water Analysis Hand Book 3rd edition.[25]

Experimental Design and Operating Conditions: The bench scale study was conducted to determine the effect of selected process variables (alum, PACl, cationic polymer, chlorine, liquid pH, and temperature) on a group of dependent variables. Table 3-2 describes the experimental design and operating conditions. The turbidity and oocyst concentrations were selected as the dependent variables. The responses in terms of oocysts recovery were then measured. The process variables were changed systematically, by varying one, while holding the

others constant. Sixteen experiments were designed and conducted in triplicate and are listed in Table 2.

Chemicals: The chemicals used in this study are Polyaluminum chloride (PACl), which has a high basicity with a sulfate-to-aluminum molar ratio of 0.15 (Geo Speciality Chemical, Bastrop, LA), and aluminum sulfate with specific range of 8.3 and 0.01 total and free alumina, respectively (AL_2O_3-Ranger Chemical Company, Choctaw, OK),Polydimethyldiallylammonium (polyDADMAC) or cationic polymer (chloride 20 %,, HCL, USA, Distribution Company, Sand Springs, OK) and slaked lime (94.5 % $Ca(OH)_2$, Globlle Stone St. Clair, Marble City, Oklahoma).

Working Solution: The concentrations of liquid alum and PACl stock solutions that were used in the experiments were 2.1 M and 1.54 M, respectively. To make a working solution, 1- mL aliquot of each stock solution was dissolved in 1 liter of distilled water and stirred. The concentration of the diluted stock solution used in the treatment study was 9.0 mg/L for PACl and alum. To make a working solution of cationic polymer (polyDADMAC), 1-mL of stock solution of cationic polymer was dissolved in 1 liter of distilled water and stirred. The dose of cationic polymer used as a working solution was 4 mg/L. The dosage was determined based on 1mL of 0.1 percent stock solution added to 1liter of raw water. The working solution for slaked lime was obtained by dissolving 1mL aliquot of the stock solution of slaked lime in 1 liter of distilled water and stirring. The pH of the working solution was 10.89.

Chlorine stock solutions were prepared daily as needed. A 50-mL volume of sodium hypochlorite (Fisher Scientific, Pittsburgh, PA) with 6 percent available free chlorine was added to 1000-mL of deionized water to produce the chlorine solution. Dosages of 4 mg/L of chlorine solutions were used in the treatment study. The measurement of free and total chorine was performed according to Standard Methods (section 408 E).[22]

Preliminary Test to Establish Chemical Dosages: Series of alum or PACl, cationic polymer, Slaked lime, and chlorine doses were selected to determine the best dosage needed for treating raw water turbidity levels of 12.5 NTU. The selected dosages were 2, 4, 6, 8, 9, and 12 mg/L for alum and PACl; 0.25, 1.2, 3, 4, and 6 mg/L for cationic polymer; 1, 2, 3, 4, 5, and 6 mg/L for chlorine; and 0.25, 0.5, 1, 1.5, 2, and 3 mg/L for slaked lime. The chemicals were added in the sequential order of lime, chlorine, alum or PACl, and cationic polymer. Six jars were used in the

preliminary test, with the first jar representing under treatment and the last jar representing over treatment. The test was conducted twice to determine the consistency of results. The chemical dosages listed in Table 3-2 were selected based on the observed medium/large floc formed.

TABLE 3-2. EXPERIMENTAL DESIGN AND OPERATING CONDITIONS

Rapid mixing:	200 rpm for 1 minute 15 sec (G x t = 250 S^{-1} x 75 s = 18750). G = velocity gradient.							
Flocculation:	25 rpm for 25 minutes (G x t = 18 S^{-1} x 1500 s = 2.7 x 10^4). S = second							
Settling time:	0 rpm (no agitation) for 30 min.							
Filter:	1.2 and 0.45 μ m cellulose acetate membrane filters.							
Organism:	Approximately, 3.33 x 10^5 oocysts were spiked into 2 L of raw water.							
Parameter	Experiment Number							
	1-a*	1-b⊗	2-a	2-b	3-a	3-b	4-a	4-b
Coagulant and dose	Alum (9 mg/L)	PACl (9mg/L)	Alum (9 mg/L)	PACl (9mg/L)	Alum (9 mg/L)	PACl (9 mg/L)	Alum (9 mg/L)	PACl (9 mg/L)
Coagulant aids	Cat. Polymer (4 mg/L)	Cat. Polymer (4 mg/L)	-	-	-	-	Cat. Polymer (4 mg/L)	Cat. Polymer (4 mg/L)
Disinfectant	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)		
pH of Liquid	6	6	8	8	6	6	6	6
Alkalinity	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)	Slaked lime (2 mg/L)
Liquid Temperature	14.5 °C	14.5 °C	14.5 °C	14.5 °C	14.5 °C	14.5 °C	14.5 °C	14.5 °C
Experiment Number								
Parameter	5-a*	5-b	6-a	6-b	7-a	7-b	8-a	8-b
Coagulant and dose	Alum (9 mg/L)	PACl (9mg/L)	Alum (9 mg/L)	PACl (9mg/L)	Alum (9 mg/L)	PACl (9mg/L)	Alum (9 mg/L)	PACl (9mg/L)
Coagulant aids	Cat. Polymer (4 mg/L)	Cat. Polymer (4 mg/L)	-	-	-	-	Cat. Polymer (4 mg/L)	Cat. Polymer (4 mg/L)
Disinfectant	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)	Cl_2 (4 mg/L)		
pH of Liquid	6	6	8	8	6	6	6	6
Alkalinity	Slake Lime (2 mg/L)	Slake lime (2 mg/L)	Slake Lime (2 mg/L)	Slake lime (2 mg/L)	Slake lime (2 mg/L)	Slake lime (2 mg/L)	Slake lime (2 mg/L)	Slake lime (2 mg/L)
Liquid Temperature	22.1 °C	22.1 °C	22.1 °C	22.1 °C	22.1 °C	22.1 °C	22.1 °C	22.1 °C

*a = stands for experiments conducted with alum.⊗b = stands for experiments conducted with PACl. The temperature of 96 liters of raw water in the drum container was stored in an open area, where its temperature varied between 14° C and 16° C over the duration of these experiments. The raw water temperature was set by mixing the raw water at room temperature with cold water inside the drum, and then the pH was measured.

Coagulation Evaluation: Six liters of raw water sample were divided into 3 (2-liter) Gator Jars (containers). Each 2-liter container was spiked with approximately 3.33 x 10^5 oocysts (a 1 x 10^6 oocyst sample was divided equally into three parts). To treat the spiked raw water samples, predetermined pH control chemicals such as 2 drops of 5 N of sodium hydroxide (Fisher Scientific, Pittsburgh, PA) for raising the pH or 0.1N of hydrochloric acid concentration for lowering the pH were added first. Subsequently, the measured doses of chemicals (Table 3-2) were dispersed into each 2-Liter Gator Jar in sequential order: slaked lime, chlorine if used in the treatment, alum or PACl, and coagulant aid, and the mixture was treated using a conventional method of treatment.

The rapid mixing process conditions were described in Table 3-2. The G-value was determined from a "G-Curve Graph for Square-jars" (Phibbs and Birds, Richmond, VA). In addition, the rotational speed was confirmed using a torque meter and a revolution counter.

During flocculation (for process conditions, see Table 3-2), the destabilized particles were agglomerated into settleable flocs. During the process of sedimentation, samples were collected for measurement of settled water turbidity at an interval of 1,2, 5, 10, 20, and 30 minutes. Filtration was then conducted after 30 minutes of settling time as part of the analytical method. Therefore, the treatment during the treatment processes only evaluates coagulation, flocculation, and sedimentation.

Chlorine Residual: The concentration of chlorine was determined by the DPD-colorimetric procedure to produce a red color that was measured spectrophotometrically at 553 nm. In the absence of the iodide ion, only free available chlorine reacts with DPD. Potassium permanganate reacts with the DPD reagent to produce the same color as produced by chlorine. A standard solution of potassium permanganate was used instead of an unstable chlorine standard to generate the standard curve. The measurement of free and total chlorine was conducted using a Standard Method section 408 E.[22]

Aluminum Residual: After treatment, samples of settled water was collected and analyzed for alum residuals using Hach Aluminon Method 8012.[25] The collected samples were placed into a 50 mL graduated cylinders and mixed with AluVer 3 Aluminum Reagent Powder Pillow, and Ascorbic Acid Powder Pillow and hand shake for 30 seconds. The aliquot was divided into two and

Bleaching 3 Reagent Powder Pillow was added in one sample, which was used as blank. Both the prepared sample and the blank was given 15 minutes reaction time, prior to analysis.

Recovery of Oocysts from Supernatant and Sludge Samples: After the 30 minutes settling time, the supernatant water layer was filtered with a 90-mm diameter cellulose acetate membrane filter with a pore size of 1.2-μ m (Figure 3-1). Following the first filtration with a 1.2-μm cellulose acetate membrane filter, the supernatant water samples were re-filtered with a 0.45-μm cellulose acetate membrane filter (Figure 3-1). Although, both filters are smaller than the 4 – 6 μm diameter size of oocysts, the 0.45-μm cellulose acetate membrane filter was used to try to obtain absolute recovery of oocysts from the supernatant water. Both filters A and B (Figure 3-1) of the supernatant, containing the entrapped oocysts were transferred to a 250-mL conical centrifuge tube and 200-mL of acetone (Reagent Grade, Fisher Scientific, Pittsburgh, PA) was then added to dissolve the filters.[23] The aliquot was agitated for 5 minutes with a shaker (model R^2 - Fisher Scientific, Pittsburgh, PA) and the dissolved matrix was spun at 650-x g (Eppendorf model 5415C, Fisher Scientific, Pittsburgh, PA) for 10 minutes.

The sludge from each container was dissolved in 250 ml deionized water to which 2 drops of 6.0 N of sulfuric acid (Fisher Scientific, Pittsburgh, PA) had been added to obtain a pH level of 2. The reaction can be chemically expressed as: $2Al(OH)_3 + 3\ H_2SO_4 \Rightarrow Al_2\ (SO_4)_3$ (Alum) + $6\ H_2O$. Acid in excess of the chemical reaction was needed to establish a low pH value and for chemical decomposition of organic matter present in the sludge. The sample was allowed to sit for 3 minutes at room temperature to allow time for the sludge to dissolve, prior to filtration, which was part of the analytical process.[23] The dissolved sludge was decanted and filtered using a cellulose acetate membrane filter with a pore size of 1.2-μm (Figure 3-1) and then re-filtered through a 0.45-μm cellulose acetate membrane filter (Figure 3-1). In addition, both filters C and D of the sludge sample containing entrapped oocysts were dissolved in the same fashion as filters A and B of the supernatant.

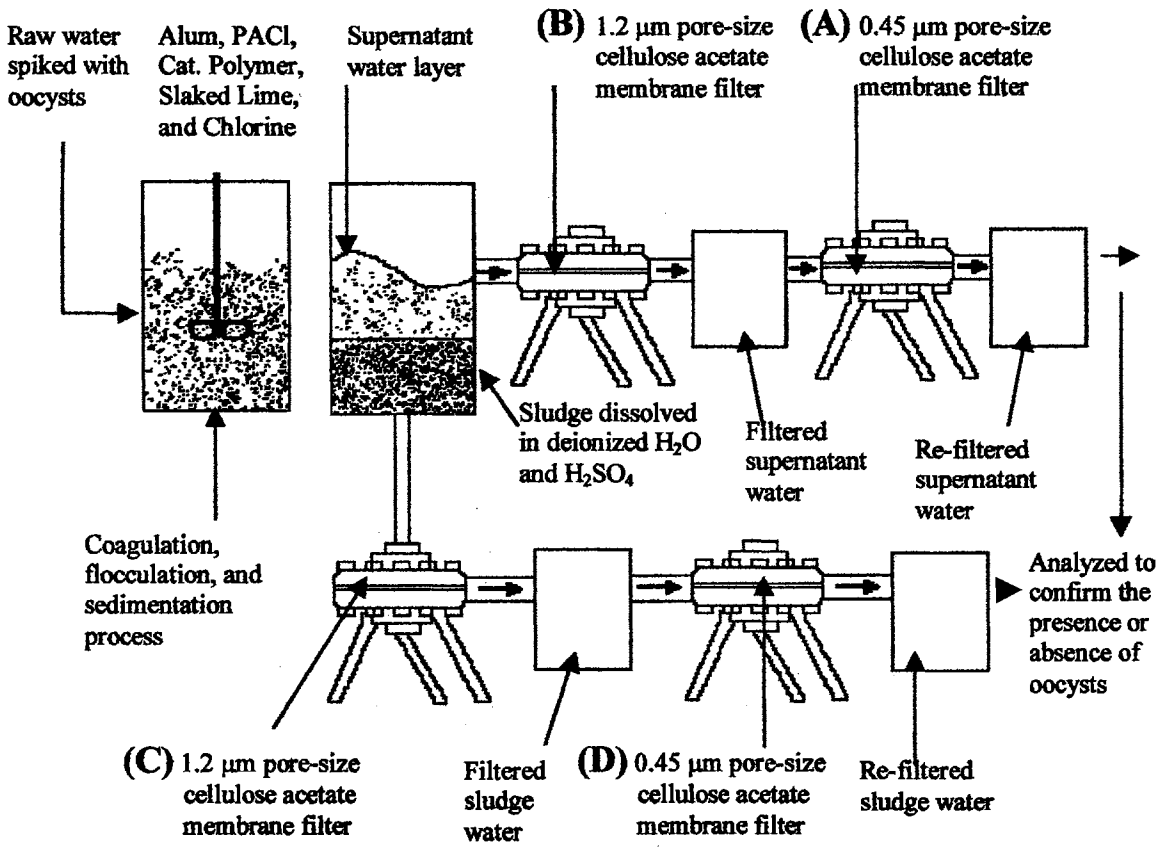

Figure 3-1. Recovery Process for *Cryptosporidium parvum* Oocysts
Filters were used to maximize oocyst recovery from the supernatant and sludge samples. Filters used were not considered part of the conventional water treatment process.

Quantitative PCR: QPCR was used to quantify the number of oocysts in the supernatant and sludge samples. Complete DNA extraction protocols, composite and specific primer construction methods, generation of MIMIC template, and details of the construction of the standard curve and overall QPCR were discussed in a previous publication.[23]

Mass Balance: The mass balance approach was used to determine the number of oocysts lost or unaccounted for in the treatment process. In this case, the mass balance can be defined as the initial number of oocysts spiked in the raw water prior to the treatment, minus the combined number of oocysts recovered from the supernatant, and sludge (Figure 3-1). The formula is expressed as: *Number of oocysts lost* = (# of oocysts spiked in the raw water) - [(# of oocysts in the supernatant) + (# of oocysts in the sludge) + (# of oocysts in the refiltered supernatant or sludge)].

Percent Recovery (PR): Percent recovery is the percent of the initial spiked dose recovered at the end of a treatment in a specific phase, sludge or liquid. The formula used in the calculation is:

$$\frac{\text{No of oocysts recovered}}{\text{Total No. of oocysts spiked in raw water}} \quad (3\text{-}1)$$

Statistical Analyses: JMP Start Statistics (SAS Institute Inc.-Student version, 1989 -1997) was used for statistical analyses of the data (Tables 3-6 and 3-7). One-way analysis of variance (ANOVA) was used to estimate the group means and differences. For all the statistical analyses, the significance level used was a 95 percent confidence interval (alpha = 0.05). The Student t-test, and mean comparison concepts of Least Significant Difference (LSD) were used to determine the significant difference between two means. The formula for LSD is defined as

$$t_{\alpha/2}\sqrt{\text{MSE}\,(1/r_i + 1/r_j)} \quad (3\text{-}2)$$

where $t_{\alpha/2}$ = tabulated value; MSE = mean square error from ANOVA; r_i = sample size for sample i; and r_j = sample size for sample j. For the 95 % confidence interval for regression coefficients formula used was

$$b \pm t_{\alpha/2}, n\text{-}2 \,.\, SE_b \quad (3\text{-}3)$$

$$SE_b = Se/\sqrt{TSS} \quad (3\text{-}4)$$

Where b = slope, SE_b = standard error of mean, Se = model standard deviation, and TSS = total sum of squares. Coefficient of determination, r^2 was calculated using the formula

$$r^2 = MSS/TSS \quad (3\text{-}5)$$

Where MSS = model sum of squares. The results of the statistical analyses for oocysts recovered from the sludge and supernatant samples are listed in Tables 3-6 and 3-7.

RESULTS AND DISCUSSION

Raw and Settled Water Quality Analyses: The results of raw water and settled water analyses are shown in Tables 3-1 and 3-3.

Organisms Recounted: The recount of 5 x 10^6 oocysts obtained from Waterborne Inc., counted in triplicate, showed an excess of (20 oocysts) 0.0005 percent. Because the difference in counts was negligible compared to the number of oocysts obtained from Waterborne Incorporated, the rest of the oocysts were not counted. Thus, the nominal number was used in the spiking study. The results of counts of 0.53 mL of the oocyst stock solution (1.67 x 10^5 oocysts/L), counted in triplicate using a hemacytometer, also showed an excess of (30 oocysts) 0.009 percent of oocysts in the stock. To verify the consistency of these counts (oocysts stock solution), one more independent count was performed and the result indicated an excess of (27oocysts) 0.008 percent.

Oocyst Recovery and Precision Test Using QPCR: To test for precision of recovery using QPCR, a 2 L Gator Jar containing raw water was spiked with 3.33 x 10^5 oocysts. The raw water was then filtered with a 1.2 μm pore size cellulose acetate membrane filter and refiltered through a 0.45μm. After filtration, which was part of the analytical process, the filters were analyzed for the presence of oocysts by performing DNA extraction and PCR detection.[23] The results of QPCR performance precision evaluation showed a range of 97.5 to 99.8 percent recovery of oocysts from the raw water, with an average recovery of 98.8 percent (n = 3, mean = 3.29 x 10^5, SD = 1.15, CV = 1.16).

TABLE 3-3. SETTLED WATER QUALITY ANALYSIS

Constituents	1-a	1-b	2-a	2-b	3-a	3-b	4-a	4-b
Color (CU)	4	4	6	6	8	8	6	9
Settling Turbidity (NTU)	2.4 SD =0.48	1.8 SD = .17	5.0 SD = 1.86	3.9 SD = 0.58	5.5 SD = 0.13	3.8 SD = 0.89	1.8 SD = 0.34	1.3 SD = 0.19
pH	6.3 – 6.4	6.3 – 6.5	8.2 – 8.4	8.1 – 8.3	6.3 – 6.5	6.3 – 6.4	6.4 –6.6	6.4 –6.5
Temp. (°C)	14.9	15.0	14.9	14.9	14.9	14.9	14.9	15.0
Total Dissolved Solid (mg/L)	498 SD =1.41	496 SD = 1.41	457 SD =1.41	460 SD =2.31	458 SD =2.0	461 SD = 1.0	459 SD = 1.0	463 SD = 2.5
Conductivity (µmho/cm)	750 SD = 1	741 SD = 1.5	682 SD = 1.0	687 SD = 2.0	684 SD = 3.0	688 SD = 1.0	685 SD = 3	690 SD = 0.58
Free Chlorine (mg/L)	0.15 SD = .006	0.17 SD = 0.01	0.19 SD =.0058	0.15 SD = 0.01	0.14 SD = 0.01	0.17 SD = 0.02	--	--
Total Chlorine (mg/L)	1.58 SD = .045	1.55 SD =0.071	1.63 SD = 0.05	1.49 SD =0.045	1.51 SD =0.029	1.68 SD = 0.05	--	--
Dissolved Oxygen(mg/L)	8.1 SD = 0.23	8.1 SD = 0.21	8.1 SD = 0.25	8.5 SD = 0.25	7.7 SD = 0.62	8.1 SD = 0.1	7.7 SD = 0.1	7.9 SD = 0.21
Alum Residual (mg/L)	0.08 SD = 0.03	0.04 SD =0.015	0.11 SD =0.015	0.13 SD =0.035	0.05 SD =0.029	0.07 SD =0.026	0.08 SD = 0.01	0.06 SD =0.006
Constituents	5-a	5-b	6-a	6-b	7-a	7-b	8-a	8-b
Color (CU)	4	4	7	7	5	5	4	5
Turbidity(NTU)	1.4 SD = 0.09	1.8 SD = 0.09	2.1 SD =0.113	2.8 SD = 0.5	2.3 SD = 0.14	2.5 SD = 0.64	1.5 SD = 0.08	2.0 SD =0.035
pH	6.3 – 6.5	6.3 – 6.5	8.1 – 8.3	8.0 – 8.3	6.3 – 6.4	6.3 – 6.4	6.3 –6.5	6.4 –6.5
Temp(° C)	22.5	22.5	22.6	22.5	22.6	22.5	22.6	22.5
Total Dissolved Solid (mg/L)	498 SD = 0.58	497 SD = 1.0	462 SD = 1.5	461 SD = 1.0	460 SD = 0.58	458 SD = 1.0	460 SD = 0.58	461 SD = 2.0
Conductivity (µmho/cm)	749 SD = 3.0	749 SD = 1.0	690 SD = 2.5	688 SD = 3.0	687 SD = 1.5	684 SD = 1.0	687 SD = 1.0	688 SD = 0.58
Free Chlorine (mg/L)	0.16 SD =.0058	0.17 SD =0.015	0.19 SD =0.006	0.18 SD = 0.01	0.20 SD =0.011	0.26 SD = 0.02	–	–
Total Chlorine (mg/L)	1.65 SD =0.025	1.60 SD =0.025	1.57 SD =0.035	1.54 SD =0.045	1.50 SD = 0.45	1.45 SD = 0.4	–	–
Dissolved Oxygen (mg/L)	8.3 SD = 0.26	8.2 SD = 0.49	8.1 SD = 0.06	8.1 SD = 0.44	8.0 SD = 0.15	8.1 SD = 0.41	8.2 SD = 0.1	8.2 SD = 0.1
Alum Residual (mg/L)	0.06 SD =0.006	0.03 SD =0.004	0.14 SD = 0.02	0.12 SD = 0.07	0.09 SD =0.025	0.08 SD =0.026	0.08 SD =0.006	0.05 SD =0.015

SD = Standard deviation. Experiment 2-a, 2-b, 6-a, and 6-b were conducted at pH 8. a = experiment conducted with alum, b = experiment conducted with PACl.

Coagulation Evaluation: Removal of oocysts and turbidity by coagulation depends on the nature and concentration of the colloidal contaminants, alum or PACl dosage, cationic polymer, and chemical characteristics of raw water, such as pH, temperature, and ionic character.[18] Coagulation was readily accomplished with dosage of 9.0 mg/L of alum or PACl. According to the domain diagram for alum, combination of sweep flocculation and adsorption was achieved in experiments conducted at pH 8.[18, 26] In addition, alum coagulation domain diagram showed that restabilization occurred in experiments conducted at pH 6. Because of the presence of silicate and a high concentration of sulfate (Table 3-1), charge reversal and restabilization was suppressed[18] Also, it is likely that the natural organic matter contributed to the control of 9.0 mg/L alum dosages required for coagulation and altered the zones of coagulation shown on the domain diagram. Kojima and Watanada[8] suggested that restabilization tend to occur when PACl is used to treat raw water at lower pH. However, due to a high level of sulfate, presence of silicate, and natural organic matter in the raw water, charge reversal and restabilization was suppressed[18] and sweep flocculation was achieved. Oocysts are therefore enmeshed in precipitated aluminum hydroxide due to addition of aluminum sulfate.

Taking the treatment conditions into consideration (Table 3-2), the level of raw water turbidity and the settling time determined the amount of sludge that settled in the jars. The number of oocysts obtained from the sludge in all the experiments varied due to different treatment conditions.

Percent Recovery of Oocysts from Prefiltered Supernatant and Sludge Samples: The average recovery of oocysts, irrespective of treatment conditions, for the supernatant ranged from 84.64 percent to 96.84 percent. For sludge samples, the average recovery of oocysts, ranged from 3.10 percent to 16.7 percent (Table 3-4). The QPCR precision tests in the measurement of oocysts recovered from the supernatant and sludge samples were conducted using the USEPA method 1662, which was approved for the IFA method. The IFA acceptance percent recovery under method 1662 ranged from 14 to 95 percent. This recovery precision range (14 to 95 percent) was based on average percent recovery (P) and standard deviation of percent recovery (Sr). Method 1662 was used to assess the recovery precision of the QPCR method. The results showed

a precision range of 82 percent to 98 percent and 2.1 percent to 18 percent for the supernatant and sludge samples, respectively (Table 3-4).

Figure 3-2 illustrates the average percent recovery of oocysts from supernatant and sludge samples. As shown in Figure 3- 2, experiments 2-a, 2-b, and 3-a have the highest percent recovery of oocysts from the supernatant samples, achieving 96.84, 96.63, and 96.32 percent, respectively. In addition, the lowest percent recoveries from the sludge samples were obtained from experiments 2-a, 2-b, and 3-a, achieving 3.10, 3.35, and 3.65 percent, respectively. Variations in the number of oocysts recovered, as shown in the graph (Figure 3-2), were attributed to effects of different chemicals used, pH's, and treatment conditions.

Relationship Between Settled Water Turbidity Levels and Oocysts Recovered from Sludge and Supernatant Samples: Multi-regression analyses were performed to determine the relationship between settled turbidity levels and the number of oocysts recovered from the sludge samples. The estimated numbers of oocysts as well as the settled turbidity levels were plotted using the power function as shown in Figure 3-3. Both power and linear functions were tried; however, the power function ($r = 0.80$) was selected because it has a better coefficient of determination than the linear function ($r = 0.75$). As shown in Figure 3-3, at the settled supernatant turbidity level of 2.0 NTU of the treated water, over 3×10^4 oocysts were recovered from the sludge samples. But as turbidity levels increased and passed 2.0 NTU, more oocysts remained in the supernatant, suggesting a relationship between settled turbidity levels and the number of oocysts in the sludge samples. The statistical analysis showed that the number of oocysts recovered from the sludge samples was significantly correlated with the value of settled water turbidity ($r = 0.801$; Figure 3-3). The relationship between settled water turbidity and number of oocysts recovered from the supernatant was also determined, as shown in Figure 3-4. Here, fewer oocysts (2.7×10^5 to 2.9×10^5 oocysts) were recovered when settled turbidity levels in the supernatant water were low (1 to 2.0 NTU). However, as settled turbidity levels in the supernatant exceeded 2.0 NTU, the number of oocysts (3×10^5 to 3.3×10^5 oocysts) recovered increases. This indicates that the lower the settled turbidity levels in the supernatant, the lower the number of oocysts recovered and the higher the settled turbidity levels, the greater the number of oocysts

recovered from the supernatant waters. A good relationship was observed between the number of oocysts and settled turbidity level ($r = 0.79$; Figure 3 - 4). Overall, the relationship between settled water turbidity and recovered oocysts showed that the level of settled turbidity in the supernatant samples could be used as an indicator of the concentration of oocysts in samples.

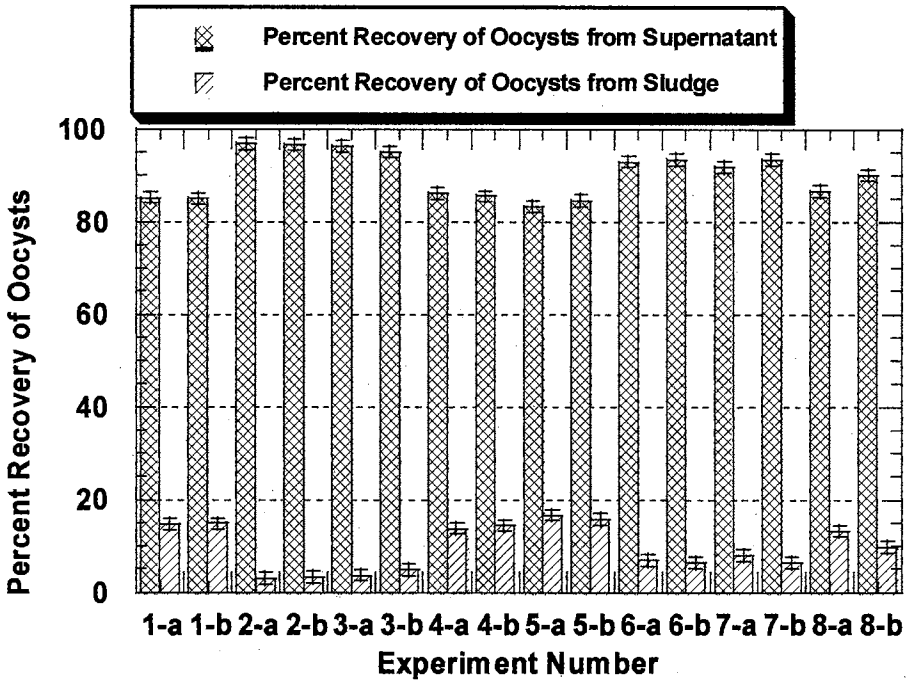

Figure 3-2 . Percent Recovery of Oocysts in Triplicate Supernatant and Sludge Samples.

TABLE 3-4. AVERAGE PERCENT RECOVERY OF OOCYSTS AND PRECISION

*Exp. No.	Average Percent Recovery (P)		Standard Deviation of Percent Recovery (Sr)		Precision Assessment (P – 2 Sr to P + 2 Sr)	Precision Assessment (P – 2 Sr to P + 2 Sr)
	Supernatant	Sludge	Super.	Sludge	Supernatant	Sludge
1-a	85.2	14.72	0.3	0.58	84.6 % to 85.8 %	13.6 % to 15.9 %
1-b	85.05	14.93	0.65	0.5	83.8 % to 86.34%	13.9 % to 15.9 %
2-a	96.84	3.10	0.59	0.48	95.7 % to 98%	2.1 % to 4 %
2-b	96.63	3.35	0.32	0.31	96 % to 97.3%	2.7 % to 4 %
3-a	96.32	3.65	0.14	0.17	96% to 96.6 %	3.3 % to 4 %
3-b	95.12	4.85	0.33	0.31	94.5 % to 95.8 %	4.2 % to 5.5 %
4-a	86.20	13.8	0.26	0.3	85.7% to 86.7%	13.2% to 14.4 %
4-b	85.57	14.4	0.32	0.3	84.9 % to 86.2%	13.8% to 15 %
5-a	83.30	16.70	0.64	0.64	82 % to 84.6%	15.4% to 18 %
5-b	84.64	15.83	0.46	0.38	83.7% to 85.6%	15% to 16.6 %
6-a	93.03	6.90	0.55	0.6	91.9 % to 94.1%	5.7% to 8.1 %
6-b	93.52	6.45	0.23	0.3	93 % to 94%	5.9% to 7 %
7-a	91.93	8.03	0.45	0.45	91% to 92.8%	7.1% to 9 %
7-b	93.50	6.47	0.56	0.61	92.4 % to 94.6%	5.3% to 7.7 %
8-a	86.73	13.28	0.25	0.23	86.2 % to 87.2%	12.8% to 13.7 %
8-b	90.15	9.83	0.41	0.47	89.3 % to 91%	8.9% to 10.8 %

*Experiments were conducted in triplicate.

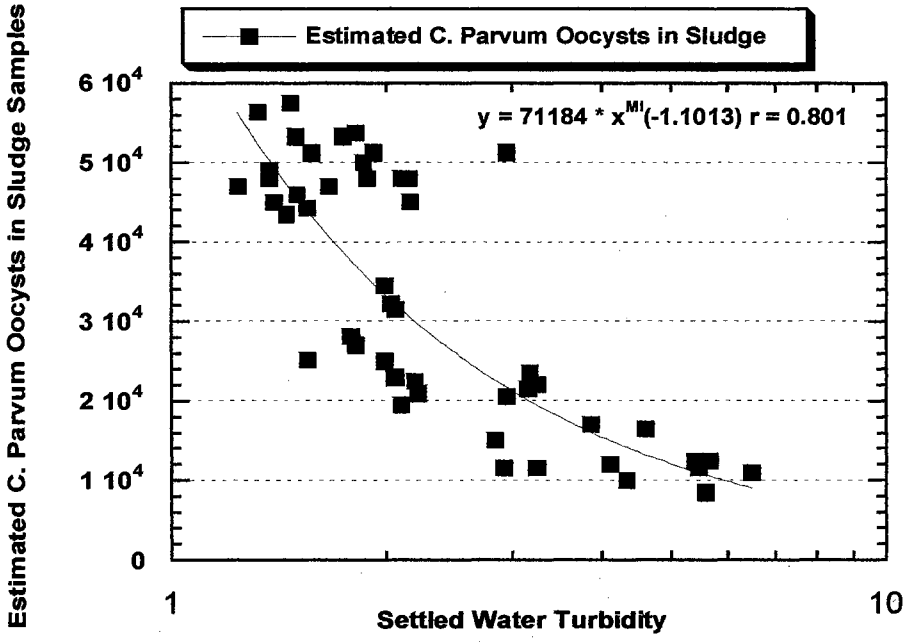

Figure 3-3. Relationship Between *Cryptosporidium* Recovered from the Sludge Samples and Settled Water Turbidity Levels after 30 Minutes Settled Time

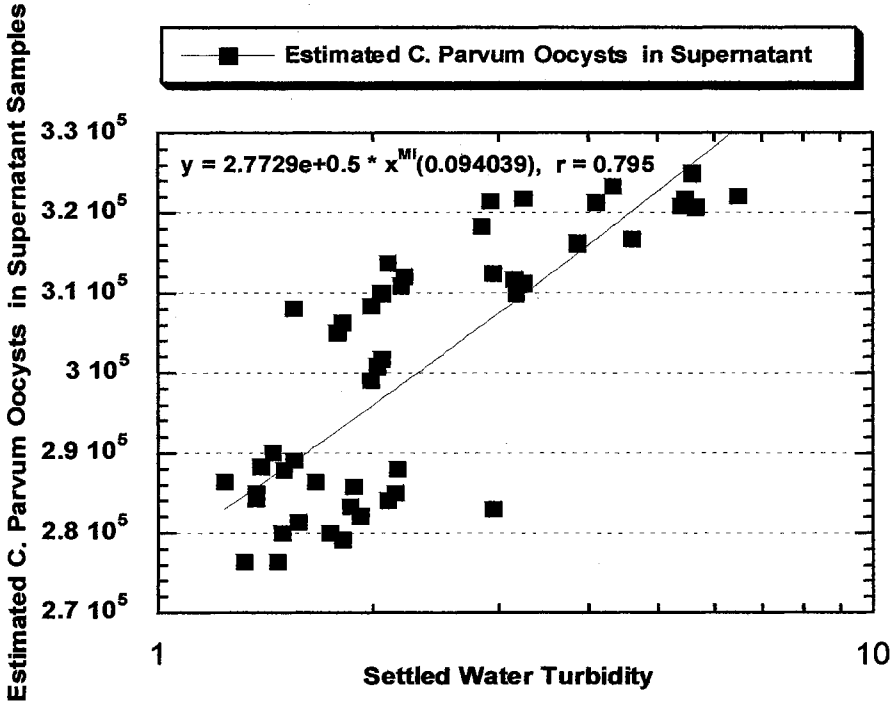

Figure 3-4. Relationship Between *Cryptosporidium* Recovered from the Supernatant Samples and Settled WaterTurbidity Levels after 30 Minutes Settling Time

Effects of Temperature on Oocysts Removal: Sixteen experiments were conducted in triplicate at liquid temperatures of 14.5^0 and 22.1^0 C. Each experiment was treated differently and the recovery of oocysts from each experiment treated at a different temperature was compared to determine the effects of temperature on removal of oocysts. The experiments compared were 1-a and 5-a, 1-b and 5 –b, 2-a and 6-a, 2-b and 6-b, 3-a and 7-a, 3-b and 7-b, 4-a and 8-a, and 4-b and 8-b (Table 3-2).

Each comparison in the Table 3-5 is given a unique designation (i.e. A, B, C) which can be used to identify the experiments compared. The results showed that oocyst removal by alum decreases in the sludge samples at low temperatures (Table 3-5, B, C, and N). The low recovery of oocysts in the sludge samples was likely due to the decrease in the efficiency of coagulation at the low temperature of 14.5^0 C. The decrease in efficiency of coagulation was likely due to an increase in viscosity and its effects on sedimentation. In addition to a decrease in the rate of (hydrolysis) of chemical reactions at colder temperatures, could be a contributing factor in the formation of smaller aggregates.[18]

The results showed that the PACl, with an average 10.7 percent recovery of oocysts from the sludge samples, appeared to be more effective than alum (an average percent 8.6) at 14.5 ^{0}C, under all treatment conditions (Table 3-5 Exp. A, B, C, D, N, Z-1). However, the differences (pair experimental comparisons) in average recovery of oocysts in sludge samples were relatively low.

Statistical analysis showed that the number of oocysts recovered from the supernatant of water treated at a temperature of 22.1^0 C (Table 3-6, 1-b and 5-b, 2-a and 6-a, 2-b and 6b, 3-a and 7-a, and 3-b and 7-b) was significantly different ($p < 0.0001$) from the number of oocysts when water was treated at 14.5^0 C ($n = 48$, $\alpha = 0.05$, $r^2 = 0.99$). The statistical analysis for the oocysts recovered from the sludge are shown in Table 3-7.The mean and standard deviation of oocysts recovered from the sludge and supernatant samples are shown in Table 3-8.

Effects of Cationic Polymer as Coagulant-aid on Oocyst Removal: The raw water was not treated with the cationic polymer alone; however, its effects on the percent recovery of oocysts was based on comparing those experiments conducted with alum or PACl with cationic polymer, to

those conducted without cationic polymer (Table 3-2). The experiments used to determine the effects of a coagulant-aid on oocyst removal include 1-a and 3-a, 1-b and 3-b, 5-a and 7-a, and 5-b and 7-b (Figure 3-5). The results showed that the use of all coagulants, plus cationic polymer appeared to improved settled water turbidity and removal efficiencies of oocysts (Figures 3-5 and 3-6). The reasons for turbidity removal, oocysts recovery, and settling velocity improvement, may be that cationic polymers tend to toughen the flocs when added with alum or PACl, and that cationic polymers bear positively charged groups (i.e., amino) which attract the negative charged particles such as the oocysts. When a polymer molecule comes in contact with a colloidal particle, some of these colloids adsorb at the positive site, leaving the remaining molecule extended out into the solution.[18] Also, when a second particle with available adsorption sites comes in contact with these extended segments, attachment can occur. A particle-polymer-particle or oocyst-polymer-oocyst complex is therefore formed in which the polymer serves as a bridge.[20] The enmeshed oocysts due to complex (aluminum) hydroxide flocs are then precipitated.

pH Effects: Four experiments were conducted at liquid pH levels of 8, while twelve experiments were conducted at pH levels of 6. All experiments were conducted in triplicate. The experiments that were compared for pH effects on removal of oocysts include 2-a and 3-a, 2-b and 3-b, 6-a and 7-a, and 6-b and 7-b (Table 3-2).

The statistical analysis showed that the number of oocysts recovered in sludge samples at a pH 6 and temperature of 14.5^0 C using alum as a primary coagulant, was not significantly different from the number of oocysts recovered at pH 8 at the same liquid temperature. For example, 3.7 percent of the oocysts were recovered in sludge samples in experiment 3-a conducted at pH 6, was not significantly different from the 3.1 percent oocysts from experiment 2-a conducted at pH 8 using alum (Table 3-7). The statistical analysis in Table 3-7, also showed that, there was a significant difference in the number of oocysts recovered in sludge samples when PACl was substituted as the primary coagulant (Exp. 2-b and 3-b, Table 3-7).

When the liquid temperature was changed to 22.1^0C, the number of oocysts recovered in the sludge at pH 6 was significantly different from the number oocyst recovered at pH 8 using alum

(Table 3-7, Exp. 6-a and 7-a). There was no significant difference in the number of oocysts recovered between pH 6 and 8 when PACl was substituted for alum (Table 3-7, Exp. 6-b and 7-b) at a liquid temperature of 22.1^0 C. Overall, the study showed that the pH effects on oocyst removal is dependent on several factors such as liquid temperature, type of coagulant used, and the effectiveness of the coagulant at that liquid temperature. However, less than 20 percent of oocysts were recovered in sludge samples.

Based on this work, water treated at pH 6 maintains a slightly ability to precipitate oocyst. This was because, when alum or PACl is added to water, soluble cationic aluminum species are formed that are complexed strongly by the negatively charged organic matter or oocysts. This complexation must be satisfied before aluminum hydroxide precipitation can occur[20] and cause enmeshment of the oocysts. At low pH, the dominant alum species is more highly positively charged and therefore has a greater capability for reducing the charge of the organic matter.

Effects of Chlorine on Removal of Oocysts: Eight experiments were compared to determine chlorine effects on oocyst removal. These experiments include 1-a and 4-a, 1-b and 4-b, 5-a and 8-a, and 5-b and 8-b (Table 3-2).

The use of a pre-oxidant such as chlorine changes the nature of colloidal-sized particles having a high surface charge, allowing the surface particles to agglomerate and be more readily removed by filtration.[24] Previous work has demonstrated that chlorine can improve turbidity and particle removal, and oocyst removal as well.[24] The effects of chlorine on the precipitation and recovery of oocysts in the sludge were tested. Overall, results showed differences in the numbers of oocysts recovered in sludge samples of water treated with chlorine and water not treated with it (Table 3-5 Exp. H, L, U, and X). Statistical analysis showed that pair experiments (Table 3-6, 4-a and 1-a, 8-b and 5-b, and 8-a and 5-a) compared were significantly different from each other. However, experiment 4-b was not significantly different from experiment 1-b (Table 3-6) Even though the statistical analysis showed 3 out of 4 paired experiments compared to be significantly different, the difference in the number of oocysts recovered in the sludge samples in each paired experiments were relatively small.

Determination of Oocysts Lost Using Mass Balance: A mass balance was used to determine the number of oocysts lost in the experiments. Based on the mass balance, the average

percentage of oocysts lost was 0.03 (ranged from 0.02 to 0.08 percent), in all sixteen experiments conducted in triplicate, regardless of the treatment conditions. Because of an absence of oocysts in all the filtered samples, it is likely that the loss of oocysts in the experiments may be the result of oocysts adhering to jars, sampling points, and tubing. This was the first time this type of information has been presented. Overall, the mass balance was effective in determining the number of oocysts lost.

TABLE 3-5. RESULTS OF MEASUREMENT OF EFFECTS OF PROCESS VARIABLES ON TURBIDITY REMOVAL AND OOCYST RECOVERY

Exp. 1-a & 1-b		A	Exp. 2-a & 2-b		B	Exp. 3-a & 3-b		C	Exp. 4-a & 4-b		D
	1-a	1-b		2-a	2-b		3-a	3-b		4-a	4-b
% Turbidity Removed:	81	86	% Turbidity Removed:	60	69	% Turbidity Removed:	56	70	% Turbidity Removed:	86	90
% Oocysts Recovered (Sup):	85.2	85	% Oocysts Recovered (Sup):	96.8	96.6	% Oocysts Recovered (Sup):	96.3	95.1	% Oocysts Recovered (Sup):	86.2	85.6
% Oocysts Recovered (SL):	14.7	14.9	% Oocysts Recovered (SL):	3.1	3.3	% Oocysts Recovered (SL):	3.7	4.9	% Oocysts Recovered (SL):	13.8	14.4
Exp. 1-a & 5-a		**E**	**Exp. 2-a & 6-a**		**F**	**Exp. 3-a & 7-a**		**G**	**Exp. 4-a & 1-a**		**H**
	1-a	5-a		2-a	6-a		3-a	7-a		4-a	1-a
% Turbidity Removed:	81	89	% Turbidity Removed:	60	83	% Turbidity Removed:	56	82	% Turbidity Removed:	86	81
% Oocysts Recovered (Sup):	85.2	83.3	% Oocysts Recovered (Sup):	96.8	93	% Oocysts Recovered (Sup):	96.3	92	% Oocysts Recovered (Sup):	86.2	85.2
% Oocysts Recovered (SL):	14.7	16.9	% Oocysts Recovered (SL):	3.1	6.9	% Oocysts Recovered (SL):	3.7	8	% Oocysts Recovered (SL):	13.8	14.7
Exp. 1-b & 5-b		**I**	**Exp. 2-b & 6-b**		**J**	**Exp. 3-b & 7-b**		**K**	**Exp. 4-b & 1-b**		**L**
	1-b	5-b		2-b	6-b		3-b	7-b		4-b	1-b
% Turbidity Removed:	86	86	% Turbidity Removed:	69	78	% Turbidity Removed:	70	80	% Turbidity Removed:	90	86
% Oocysts Recovered (Sup):	85	84.1	% Oocysts Recovered (Sup):	96.6	93.5	% Oocysts Recovered (Sup):	95.1	93.5	% Oocysts Recovered (Sup):	85.6	85
% Oocysts Recovered (SL):	14.9	15.8	% Oocysts Recovered (SL):	3.3	6.4	% Oocysts Recovered (SL):	4.9	6.5	% Oocysts Recovered (SL):	14.4	14.9
Exp. 1-a & 3-a		**M**	**Exp. 2-a & 3-a**		**N**	**Exp. 3-b & 1-b**		**O**	**Exp. 4-a & 8-a**		**P**
	1-a	3-a		2-a	3-a		3-b	1-b		4-a	8-a
% Turbidity Removed:	81	56	% Turbidity Removed:	60	56	% Turbidity Removed:	70	86	% Turbidity Removed:	86	88
% Oocysts Recovered (Sup):	85.2	96.3	% Oocysts Recovered (Sup):	96.8	96.3	% Oocysts Recovered (Sup):	95.1	85	% Oocysts Recovered (Sup):	86.2	86.7
% Oocysts Recovered (SL):	14.7	3.7	% Oocysts Recovered (SL):	3.1	3.7	% Oocysts Recovered (SL):	4.9	14.9	% Oocysts Recovered (SL):	13.8	13.3
Exp. 5-a & 5-b		**Q**	**Exp. 6-a & 6-b**		**R**	**Exp. 7-a &7-b**		**S**	**Exp. 8-a & 8-b**		**T**
	5-a	5-b		6-a	6-b		7-a	7-b		8-a	8-b
% Turbidity Removed:	89	86	% Turbidity Removed:	83	78	% Turbidity Removed:	82	80	% Turbidity Removed:	88	84
% Oocysts Recovered (Sup):	83.3	84.1	% Oocysts Recovered (Sup):	93	93.5	% Oocysts Recovered (Sup):	92	93.5	% Oocysts Recovered (Sup):	86.7	90.1
% Oocysts Recovered (SL):	16.9	15.8	% Oocysts Recovered (SL):	6.9	6.4	% Oocysts Recovered (SL):	8	6.47	% Oocysts Recovered (SL):	13.3	9.8
Exp. 5-a & 8-a		**U**	**Exp. 6-a & 7-a**		**V**	**Exp. 7-a & 5-a**		**W**	**Exp. 8-b & 5-b**		**X**
	5-a	8-a		6-a	7-a		7-a	5-a		8-b	5-b
% Turbidity Removed:	89	88	% Turbidity Removed:	83	82	% Turbidity Removed:	82	89	% Turbidity Removed:	84	86
% Oocysts Recovered (Sup):	83.3	86.7	% Oocysts Recovered (Sup):	93	92	% Oocysts Recovered (Sup):	92	83.3	% Oocysts Recovered (Sup):	90.1	84.1
% Oocysts Recovered (SL):	16.9	13.4	% Oocysts Recovered (SL):	6.9	8	% Oocysts Recovered (SL):	8	16.9	% Oocysts Recovered (SL):	9.8	15.8
			Exp. 6-b & 7-b		**Y**	**Exp. 7-b & 5-b**		**Z**	**Exp. 2-b & 3-b**		**Z-1**
				6-b	7-b		7-b	5-b		2-b	3-b
			% Turbidity Removed:	78	80	% Turbidity Removed:	80	86	% Turbidity Removed:	69	70
			% Oocysts Recovered (Sup):	93.5	93.5	% Oocysts Recovered (Sup):	93.5	84.1	% Oocysts Recovered (Sup):	96.6	95.1
			% Oocysts Recovered (SL):	6.4	6.5	% Oocysts Recovered (SL):	6.5	15.8	% Oocysts Recovered (SL):	3.3	4.9

SUP = Supernatant Samples. SL = Sludge Samples. a = Alum. b = PACl. Experiments 1 – 4 were conducted at temperature of 14.5^0 C. Experiments 5 – 8 were conducted at temperature of 22.1^0 C. Experiments 2-a, 2-b, 6-a, and 6-b were conducted at pH 8. The rest of the experiments were conducted at pH level of 6. Filtration was conducted after 30 minutes settling time as part of analytical method.

TABLE 3-6. STATISTICAL ANALYSIS FOR OOCYSTS RECOVERED FROM SUPERNATANT

Experiment Number Comparison	Means Comparisons (Mean 1 –Mean 2)	Means Comparisons Using Student t Abs (Diff) – LSD	Significant Different ($\alpha = 0.05$)
1-a & 1-b	510	-1876	NO
1-a & 3-a	37070	34683	YES
1-a & 4-a	3430	1044	YES
1-a & 5-a	6380	3994	YES
1-b & 3-b	33593	31207	YES
1-b & 4-b	1750	-636	NO
1-b & 5-b	3053	667	YES
2-a & 2-b	727	-1660	NO
2-a & 3-a	1740	-646	NO
2-a & 6-a	12700	10314	YES
2-b & 3-b	5000	2614	YES
2-b & 6-b	10360	7974	YES
3-a & 3-b	3987	1600	YES
3-a & 7-a	14653	12267	YES
3-b & 7-b	5417	3030	YES
4-a & 4-b	2190	-196	NO
4-a & 8-a	1680	-706	NO
4-b & 8-b	15260	12874	YES
5-a & 5-b	2817	430	YES
5-a & 7-a	28797	26410	YES
5-b & 7-b	31230	28844	YES
5-a & 8-a	11490	9104	YES
5-b & 8-b	20063	17677	YES
6-a & 6-b	1613	-773	NO
6-a & 7-a	3693	1307	YES
6-b & 7-b	57	-2330	NO
7-a & 7-b	5250	2864	YES
8-a & 8-b	11390	9004	YES

$P < 0.0001$. $r^2 = 0.995$.

TABLE 3-7. STATISTICAL ANALYSIS FOR OOCYSTS RECOVERED FROM SLUDGE SAMPLES

Experiment Number Comparison	Means Comparisons (Mean 1– Mean 2)	Means Comparisons Using Student t Abs(Diff) –LSD	Significant Different (α = 0.05)
1-a & 1-b	666.7	-1759	No
1-a & 3-a	36917	34491	Yes
1-a & 4-a	3083	657.6	Yes
1-a & 5-a	6611	4185	Yes
1-b & 3-b	33583	31158	Yes
1-b & 4-b	1750	-676	No
1-b & 5-b	3028	602	Yes
2-a & 2-b	833	-1592	No
2-a & 3-a	1833	-592	No
2-a & 6-a	12667	10241	Yes
2-b & 3-b	5000	2574	Yes
2-b & 6-b	10333	7908	Yes
3-a & 3-b	4000	1574	Yes
3-a & 7-a	14583	12158	Yes
3-b & 7-b	5500	3074	Yes
4-a & 4-b	2000	-426	No
4-a & 8-a	1750	-676	No
4-b & 8-b	15250	-2426	No
5-a & 5-b	2917	491	Yes
5-a & 7-a	28944	26519	Yes
5-b & 7-b	31111	28685	Yes
5-a & 8-a	11444	9019	Yes
5-b & 8-b	20028	17602	Yes
6-a & 6-b	1500	-926	No
6-a & 7-a	3750	1324	Yes
6-b & 7-b	1667	-2259	No
7-a & 7-b	5083	2658	Yes
8-a & 8-b	11500	9074	Yes

$P < 0.0001$. $r^2 = 0.994$.

TABLE 3-8. MEAN AND STANDARD DEVIATION FOR SUPERNATANT AND SLUDGE SAMPLES

Mean and Standard Deviations for Supernatant Samples		Mean and Standard Deviations for Sludge Samples	
Mean	Std Deviations	Mean	Std Deviations
1-a = 284000	1000	1-a = 49083	1876
1-b = 283490	2144	1-b = 49750	1639
2-a =322810	1918	2-a = 10333	1607
2-b = 322083	1100	2-b = 11168	1041
3-a = 321070	523	3-a =121668	577
3-b = 317083	1100	3-b =161668	1041
4-a = 287430	868	4-a = 46000	1000
4-b = 285240	1090	4-b = 48000	1000
5-a = 277620	2061	5-a = 55694	2138
5-b = 280437	1535	5-b = 52778	1339
6-a = 310111	1836	6-a = 23000	2000
6-b = 311722	752	6-b = 21500	1000
7-a = 306417	1507	7-a = 26750	1516
7-b = 311667	1909	7-b = 21667	2021
8-a = 289111	839	8-a = 44250	750
8-b = 300500	1364	8-b = 32750	1561

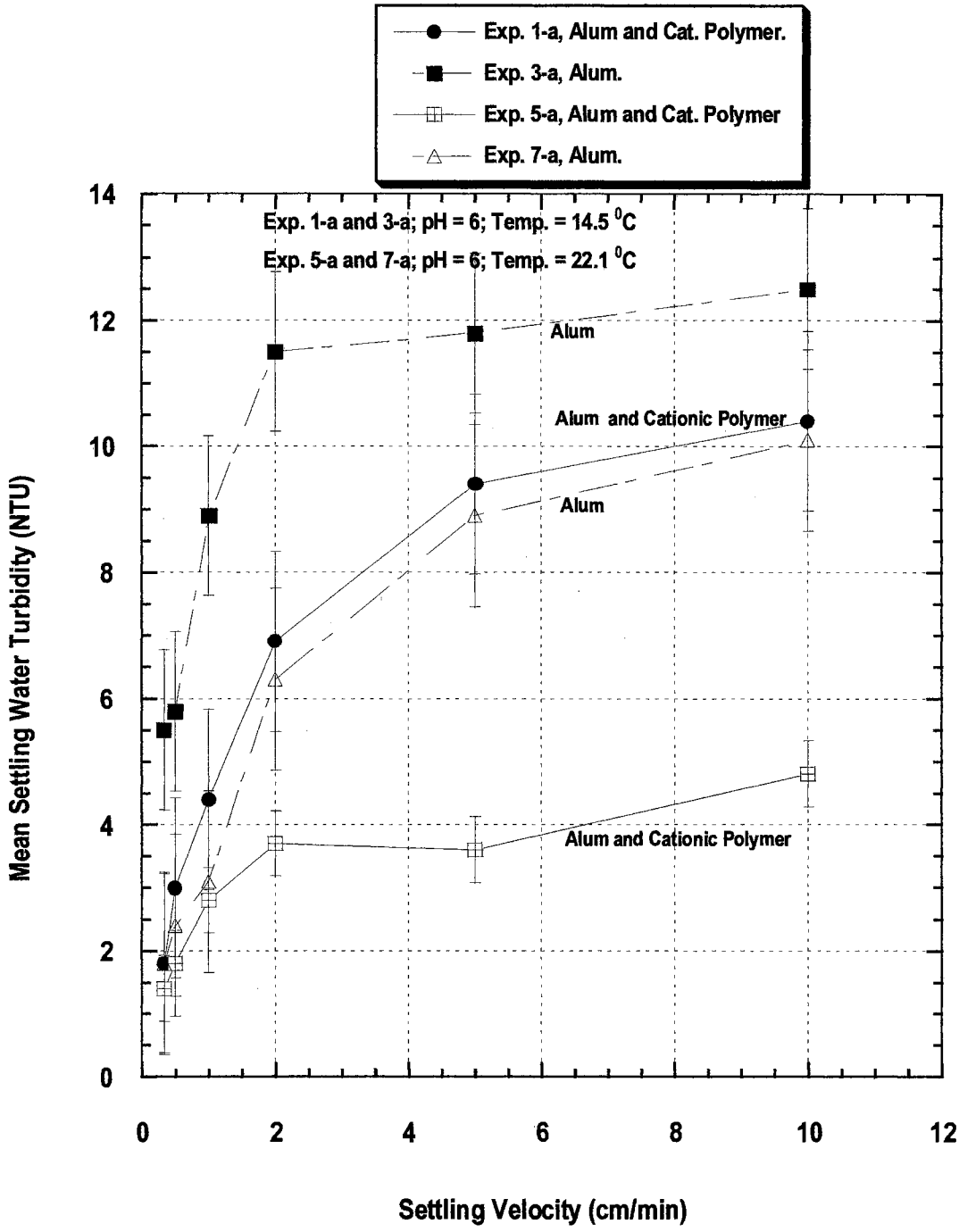

Figure 3-5. Effects of Alum with Cationic Polymer Versus Alum without Cationic Polymer.

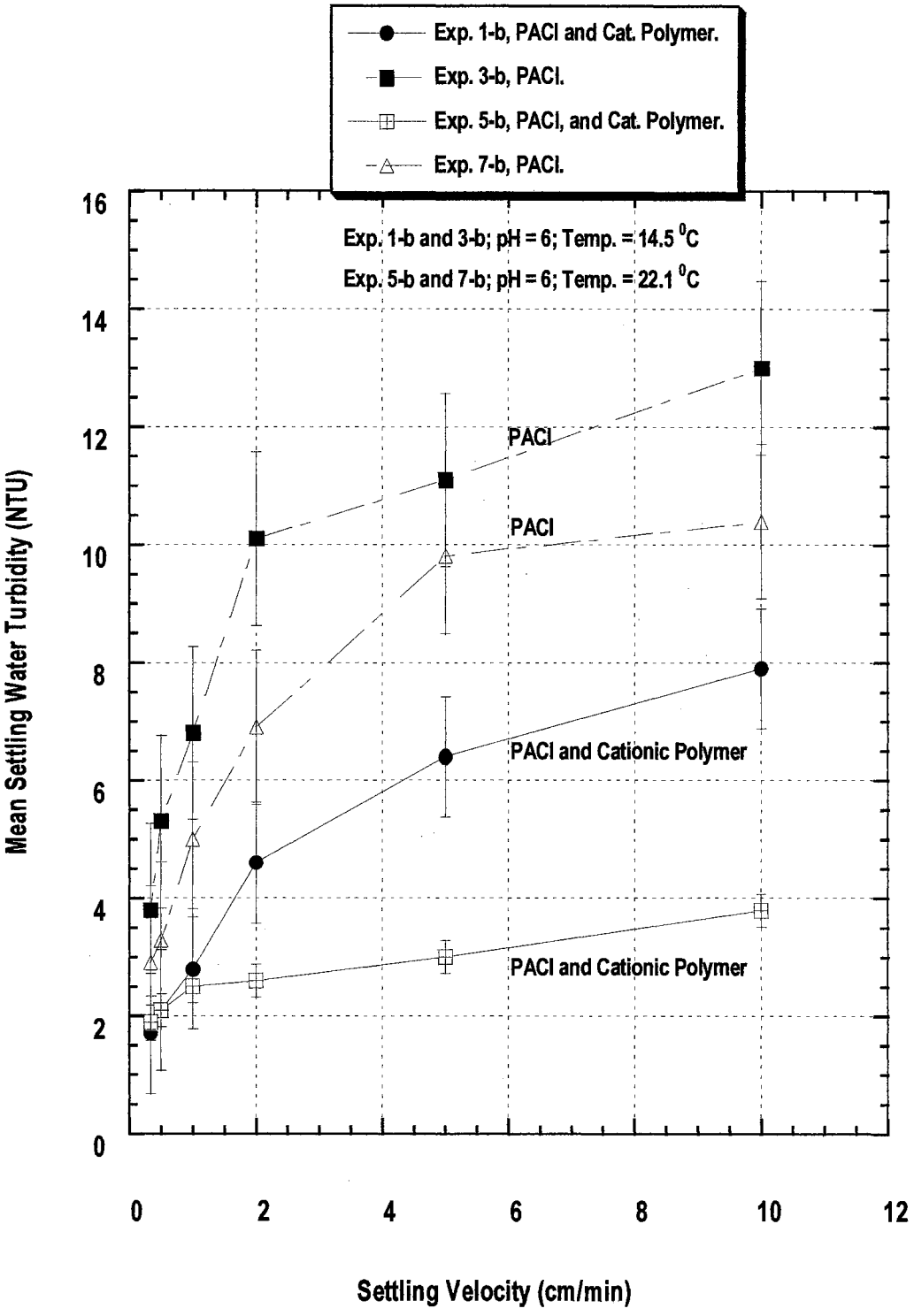

Figure 3-6. Effects of PACl with Cationic Polymer Versus PACl without Cationic Polymer.

Relationship Between Alum Residual Levels and Settled Water Turbidity: A multi regression model was used to examine the correlation between settled water turbidity and alum residual. Table 3-3 shows the average settled water quality analyses of 10 water quality parameters. The results showed that the water treated at pH 8 had a higher alum residual than water treated at pH 6. The levels of alum residuals for experiments 2-a, 2-b, 6-a, and 6-b, conducted at pH 8 are 0.11, 0.13, 0.14, and 0.12 mg/L, respectively. The highest level of alum residual at the pH level of 6 was 0.09 mg/L (Table 3-3). Figure 3-7 depicts the relationship between settled water turbidity and alum residual. Using settled water turbidity as a response in the model, results show a small correlation ($n = 48$, $r = 0.30$, $p > 0.04$) between settled water turbidity and alum residual. The paired t-test showed that the means are significantly different ($p < 0.0001$). Overall, at a pH of 6, lower levels of alum residual were obtained, which was consistent with the work of Amirtharajah and O' Melia[1], which suggested that adjusting the pH of water to 6 will reduce the level of alum residual in treated water.

Practical Application: Bench scale study using jar test is universally recognized as the most valuable and commonly used tool for coagulation control.[18] Settleability of oocysts spiked in the raw water using coagulation, flocculation, and sedimentation treatment processes, was the main focus of this study.

Pretreatment that is, prior to filtration, will only set a maximum of 20 percent of oocysts in sludge, leaving the filters to (recover 80 percent of oocysts in supernatant) do the bulk of the work. We suspected that the filters did all the work, but we have the data to back it up. In the actual plant operation processes, the percentage of oocysts recovered in sludge samples may be lower, since the bench scale study using jar testers were conducted in a laboratory-controlled environment. Oocysts have a low sedimentation rate. Based on a high percentage of the organism in the supernatant, the efficiency of the recovery of oocysts in the supernatant depended exclusively on the effect of filtration performance. Evidence of the importance of filtration was observed in 1993 Milwaukee, Wisconsin *Cryptosporidium* oocysts outbreaks incident, which was the result of the inefficiency of filtration performance.[6] To control a high percentage of oocysts in the supernatant, required high performance filters, and its efficiency should never be compromised.

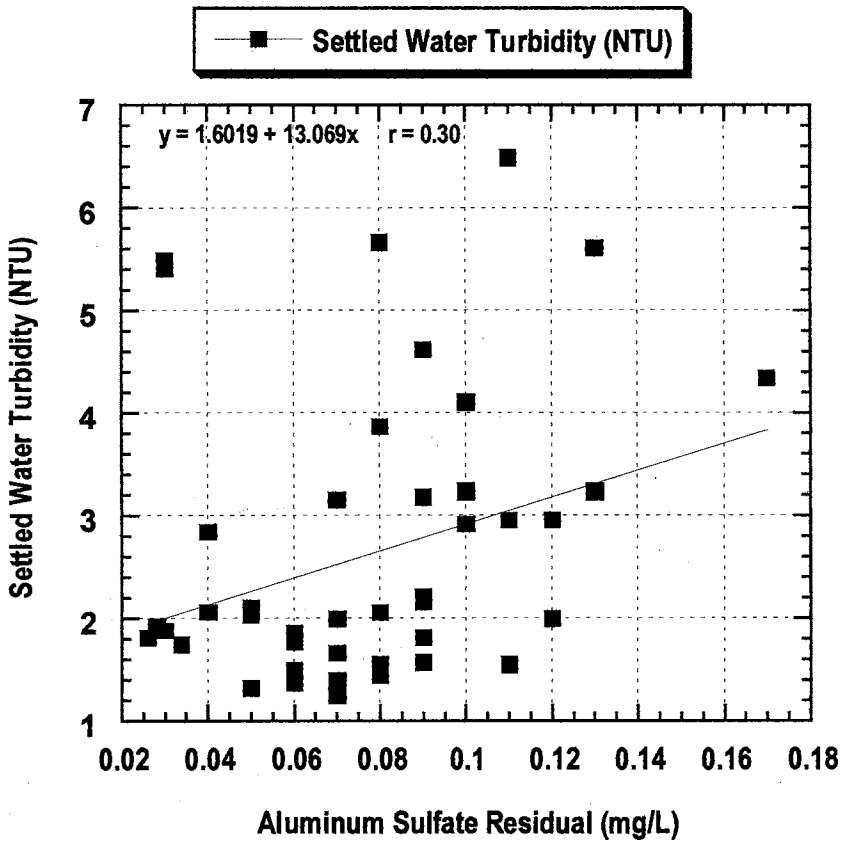

Figure 3-7. Relationship Between Alum Residual and Settled Water Turbidity

CONCLUSIONS

Treatment

- The average percent recovery of oocysts from the supernatant and sludge samples in all 16 experiments, conducted in triplicate, regardless of the treatment conditions or process variable used, was 90.2 and 9.8, respectively. Overall recovery of oocysts could be credited to the use of the PCR method of detection and quantitation, as well as the filter pore sizes of 1.2 and 0.45 μm, used in the filtration process, and the dissolution method used for oocyst recovery. In addition, high concentrations of oocysts in the supernatant (83.3 % – 96.8 %) indicates that: (1) oocysts have a low sedimentation rate (2) at best about 17 percent was recovered from the sludge samples and (3) Oocysts were present in the sludge samples (potential problem). The presence of oocysts in the sludge is an indicative of the sanitary risks associated with using sludge to fertilize agriculture land or for land reclamation.
- The use of PACl proved to be more effective in the recovery of oocysts from the sludge samples, regardless of the chemicals used to treat the water at 14.5^0 C. Alum was more effective when the liquid temperature was raised to 22.1^0C.
- Overall, the study showed that cationic polymer used in addition to alum or PACl was effective in enhancing turbidity removal, recovery of oocysts in sludge samples, and improving settling velocity. Although the studies were performed on one water type (Kaw reservoir), they do indicate a need to test coagulants on oocysts themselves. It is likely that the coagulants will react differently with different source waters.
- The addition of chlorine during treatment slightly improved the recovery of oocyst in the sludge samples.
- For physical and chemical removal of oocysts, indicator may include settled water turbidity. QPCR evaluation and performance tests using raw water showed an average recovery of 98.8

percent. The quality assurance (QA) test that was performed in each experiment, using the EPA method of precision assessment, provided a range of 82 to 98 percent recovery for supernatant and 2.1 to 16.6 percent for sludge samples in 16 experiments conducted in triplicate.

Monitoring

- The relationship between the settled water turbidity levels and recovered oocysts indicated that the level of settled water turbidity could be used as a surrogate indicator or predictor of the concentration of oocysts in the supernatant and sludge samples. While monitoring for oocysts directly may assist in building reference occurrence information, reliance on those measurements to indicate the safety of the treated water is not advised.

Measurement of Oocysts

- The mass balance approach, which was developed with intent to reconcile the number of oocysts recovered to the number of oocysts spiked into the raw water, was an effective approach in measuring the number of oocysts lost in the experiment. Overall oocyst recovery, regardless of how the water was treated or other variables used, was 99.97%. The loss of 0.03% of oocysts may have been due to oocysts adhering to jars, sampling points, and tubing.

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CHAPTER 4

FIELD INACTIVATION OF OOCYSTS EXPOSED TO AGRICULTURAL LAND

Abstract

Approximately 2.5 x 10^5oocysts were spiked into sentinel chambers containing a 1.0 g mixture of sludge and soil and exposed, at a depth of 10-cm, to the soil surface environment for 60 days in order to evaluate the field die-off rates of oocysts. Typical loading rates of sludge to land ranged from 0.5 to 2.5 percent (dry weight). This is the first inactivation study was conducted using a loading rate of 2 % sludge and 98 % soil, typical of that used in land application. The study was conducted from February to April 2000. The average daily soil temperature ranged from 7 ^{0} C to 19.8^0 C. The mass balances approach was used to determine the number of oocysts lost in the experiments. The results of the study showed that (1) oocysts could survive extreme environmental stress in soil, (2) the die-off rates of oocysts in the sentinel chambers from 0 to 17 and 45 to 60 days was – 0.0044 and - 0.012 day^{-1}, respectively, (3) die-off rates of oocysts in control units from 0 to 17 and 45 to 60 days was – 0.0021 and – 0.0025 day^{-1}, respectively, and (4) based on the mass balances, an average 4.8 percent of oocysts were lost in the experiments.

INTRODUCTION

Cryptosporidium is a coccidian protozoan, and a zoonotic parasite that is responsible for several documented outbreaks of the disease "cryptosporidiosis" associated with contaminated drinking water.[19] *Cryptosporidium* is ubiquitous in surface waters in the United States[10,19] and the organisms are detected in about 85 percent of surface water samples.[14] While *Cryptosporidium* does not multiply in the environment, [4] the oocyst form of the organism is very resistant to many

extremes of environmental conditions.[4, 20] The persistence of the organism contributes to its threat to drinking water sources.[20]

One source of oocysts in surface water is land application of sludge contaminated with oocysts which is used to improve soil fertility and land reclamation.[6] Land application of water treatment sludge is considered an alternative to traditional disposal methods because of its relatively low cost and potential is a long term disposal solution.[11,19] However, this application poses a potential threat to public health due to the possibility of viable oocysts within the sludge surviving environmental pressures and returning to the human food chain and drinking water via crops and livestock exposed to sludge-fertilized land. [4, 19]

Based on previous studies,[12, 20] it's known that oocysts can be transported back to water treatment facilities through surface water following the application of sludge to land. Unknown at this time is the survival rate of viable oocysts in a mixture of sludge and soil. In this study, a method prescribed by Jenkins et al. [7] which required the use of sentinel chambers was used to determine the survival rates of oocysts buried in the soil surface environment. The goals of this research include the following: (1) to use sentinel chambers to expose small quantities of sludge containing viable oocysts to ambient stress in soil, (2) to determine over a 60 day interval the die off rate of viable oocysts in a mixture of soil and sludge exposed under 10 cm of soil, and (3) to model the effects of soil temperature, soil pH, sludge salinity, alum and lime, and desiccation on viable oocysts.

MATERIALS AND METHODS

Field Spreading Site and Soil Sample Characterization: The field research plot (Figure 4-1), which belongs to the City of Stillwater, was used as the burial site for assessing environmental stress on the sentinel chambers containing oocysts. This site was previously used for crop cultivation and is located adjacent to the Stillwater Water Treatment Facility. All soil analyses were conducted at the Oklahoma State University Soil Testing Laboratory (Table 4-1).

TABLE 4-1. CHARACTERISTIC OF SOIL TYPE USED TO INVESTIGATE THE DIE-OFF RATE OF CRYPTOSPORIDIUM PARVUM OOCYSTS

⊗Soil Characteristic	Soil Type	Method of Analysis
	Silty loam	*ASTM 152H- Type Hydrometer Method
ξpH	6.72	
Sand %	62	
Silt %	20	
Clay %	17.5	

ξpH analysis was conducted using ASTM Calcium Chloride Method[1] c⊗Soil analysis was conducted at Oklahoma State University soil testing laboratory. * Western States Laboratory Proficiency Testing Program Soil and Plant Analytical Methods.[2]

Sludge Sample and Characterization: Characteristics of water treatment sludge differ from location to location due to differences in raw water characteristics and the type and amount of chemicals used in the treatment process. The sludge used in this study was collected in liquid form from the solids contact unit at the Stillwater Water Treatment Facility, Stillwater, OK. The Stillwater Water Treatment Facility employs conventional physical and chemical operations (coagulation, flocculation, sedimentation, and multi-media filtration) to treat surface water. The liquid sludge was placed in an 8-liter strainer consisting of Schleicher and Schnell 18.5-cm diameter size S&S filter paper (Hach Company, Loveland, CO). The sludge was allowed to drain, solidify, and air-dried for 8 days at an atmospheric temperature.

Determination of Soil and Sludge Moisture Content: The moisture content of soil and sludge was determined by the gravimetric method, Standard Method (Section 209 A)[2] by placing the samples in a drying oven at 105^{0}C for 48 hours until constant weight of the sample was attained.

Mixture of Soil and Sludge: A practical water treatment sludge application to land lies between 0.5–2.5 percent and 1.5 – 2.5 percent (mass of dry sludge per mass of dry soil).[6,11,18] The sludge loading of 2 percent (dry weight) was selected for this study because it is acceptable under most circumstances and is cost effective.[11] To obtain this 2 percent (dry weight) loading rates, moisture determination was conducted. Based on the moisture content determination, 2.35 g of wet sludge (2 g of dry weight) was added to 99.53 g of soil (98.0 g dry weight) and agitated in a shaker (Bio Dancer, New Brunswick Scientific, Edison, NJ) for 5 minutes to mix.

Description and use of Sentinel Chamber: The sentinel chambers used in this study were previously described by Jenkins et al. [7] The commercially produced microfiltration system (2.5 cm long, with an internal diameter of 0.7 cm; Osmonics, Livermore, CA) with a nylon 0.45 μm pore size filter encased in one end (Figure 4-2). The top of this chamber is a perforated cap used to secure the 60 μm nylon mesh filter (Spectra/Mesh, Markson, Hillsboro, OR), which allows maximum exposure and equilibration between the chamber containing mixture of soil and sludge and the field environment. At the bottom of the chamber is a 0.45 μm pore size filter that prevents the release of oocysts into the environment, but allows an exchange of the soil and sludge mixture inside the chamber with the surrounding field environment. The chambers were obtained from Dr. Bowman, Dept. of Veterinary Medicine at Cornell University, Ithaca, NY.

Oocysts are known to be nonmotile and do not replicate outside of a living host.[7] Therefore, if this organism is spiked into a mixture of soil and sludge, it is assumed that in the surface soil, there will not be a suffice flux of water into the chamber that would allow oocysts to be transported out of the 60 μm nylon mesh filter.[7] These chambers were designed to be installed vertically and to prevent environmental contamination by the oocysts (Figure 4-2).

Eight grams of the soil and sludge mixture (one gram in each chamber) were divided into eight sentinel chambers. The chambers were secured in microcentrifuge holders and placed in a 250 mL Pyrex glass vessel containing distilled water. The intent was to allow water to wick up and

equilibrate with the mixture of soil and sludge inside the chamber for 24 hours at room temperature, in order to achieve approximate field capacity.[7] This was done prior to the spiking of oocysts into the chamber.

Organism and Experimental Design: Approximately 2.5 x 10^5 purified viable oocysts stored in phosphate buffer saline solution (PBS) were obtained from Waterborne Inc., New Orleans, Louisiana, and used in all spiking studies. Prior to spiking, 8 mL of purified oocyst stock (6.25 x 10^5oocysts/mL) were recounted in triplicate using a hemacytometer (Bright Line Phase, Fisher Scientific, Pittsburgh, PA). The purpose was to confirm the number of oocysts obtained from Waterborne. Table 4-2, depicts the experimental design for sentinel and control oocysts buried in a 10-cm surface soil environment.

Controls: Eight 1.5-mL microcentrifuge tubes containing distilled water were spiked with oocysts (2.5 x 10^5 oocysts/1.5-mL microcentrifuge tube) and used as controls. Eight control microcentrifuge tubes were necessary since eight sentinel chambers were used to conduct the experiment.

Installation of Sentinel Chamber with Spiked Oocysts: Before installing sentinel chambers in the field site, a 0.4-mL aliquot of purified oocysts (2.5 x 10^5) was injected into one gram of the wetted mixture of soil and sludge with a syringe. After spiking, 16 holes about 10 cm deep and 2.5 mm diameter were dug to install the experimental equipment (microcentrifuge tubes and sentinel chambers). The experiment began on February 11 and ended on April 12, 2000.

Measurements of Soil pH and Temperature: Two standard test methods for soil pH (deionized water and calcium chloride), prescribed by the American Society of Testing Materials (ASTM D 4972 – 95a) were used to measure the pH of the soil.[1] The pH values obtained using calcium chloride solution (1.0M) were slightly lower than those measured in deionized water due to the release of more aluminum ions which then hydrolyses.

Two thermometers (Ertco Mercury thermometers, Fisher Scientific, Pittsburgh, PA) placed in a thermometer holder were buried in 10 cm surface soil and were used to monitor soil temperature on a daily basis.

Dye Permeability Assay: The dye Trypan Blue was used to determined the viability of oocysts on the basis of dye exclusion. Intact membranes of viable oocysts prevent dye uptake, but

nonviable oocysts are readily stained and identified by their blue color using a hemacytometer. [8] To prepare Trypan Blue, 0.85 g of sodium chloride were mixed with 100 mL of distilled water to produced 0.85 percent saline solution. One mL of this solution was placed in a 1.5 mL microcentrifuge tube and mixed with 0.00256-g of Trypan Blue to produce 0.2% Trypan Blue in 0.85% saline solution.[8] After the extraction of oocysts from the mixed soil and sludge, 200 μL of phosphate buffer saline solution (80 g NaCl, 11.5 g Na_2HPO_4, 2 g KCl, 2 g KH_2PO_4, [pH 7.4] in 1 Liter of distilled water) were added to the pellet, which was stained with 200 μL of Trypan Blue (0.2% in 0.85% saline solution). Observations were performed at 400X magnification using a hemacytometer (Bright Line Phase, Fisher Scientific, Pittsburgh, PA).

Figure 4-1. Agricultural land used to bury sentinel chambers containing a mixture of soil and sludge spiked with viable oocysts (arrow). The survival rate of this organism was monitored for 60 days. This study location was protected from field mowing with an iron fence.

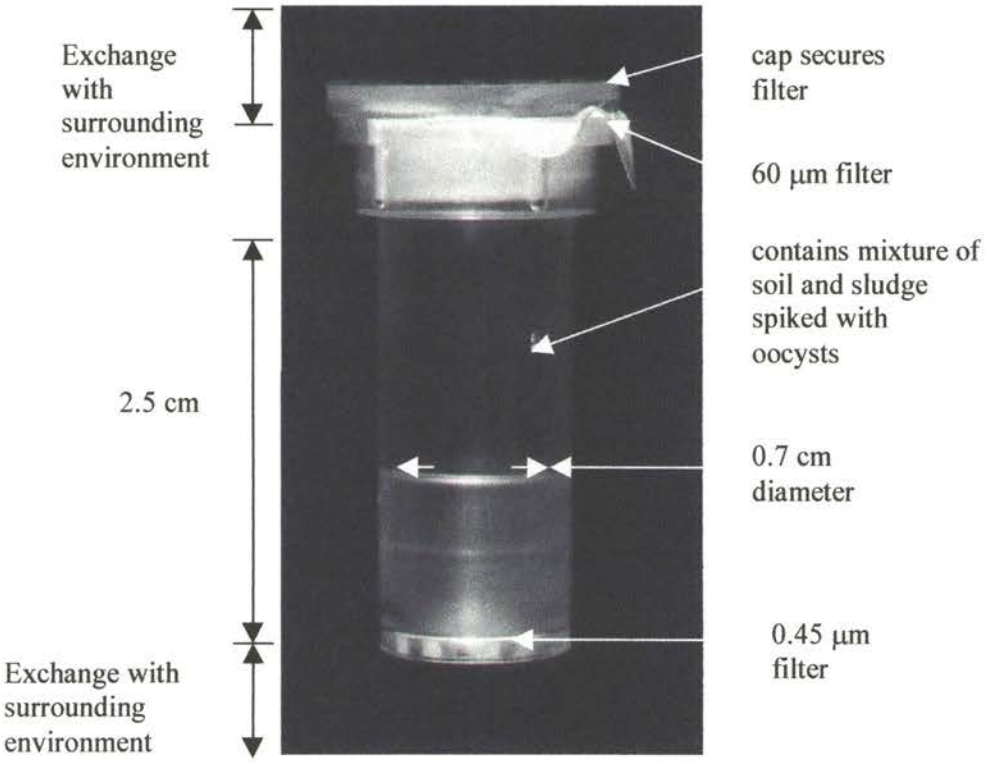

Figure 4-2. Sentinel chamber used in the field experiment

TABLE 4-2. EXPERIMENTAL DESIGN FOR SENTINEL AND CONTROL OOCYSTS BURIED IN 10-CM SURFACE SOIL ENVIRONMENT.

Oocysts Inactivation Study					
Sentinel Chamber Containing A Mixture of Soil and Sludge and Spiked Oocysts		Microcentrifuge tubes Containing Distilled Water and Spiked Oocysts		Sample Period (Day)	Extraction Dates
Number of Sentinel Chambers Per Experiment	Number of Oocyst Spiked Per Gram of Mixture of Soil and Sludge	Number of Controls Per Experiment	Number of Oocyst Spiked in 1.5 mL Microcentrifuge Tubes Containing Distilled Water		
2	2.5×10^5	2	2.5×10^5	17	February 28
2	2.5×10^5	2	2.5×10^5	30	March 14
2	2.5×10^5	2	2.5×10^5	45	March 29
2	2.5×10^5	2	2.5×10^5	60	April 11

Total number of sentinel chambers used in the spiking studies = 8; Total number of microcentrifuge tubes used for controls = 8; Total number of oocysts used in the study = 4×10^6 oocysts. Note: Based on preliminary assessment, the extraction efficiency of 250,000 oocysts from the spiked mixture of soil and sludge ranged from 89.6 to 92.3 %.

Extraction Solution: The solution used for the extraction of oocysts from a mixture of soil and sludge was prepared by adding 6.057 grams of 50 mM TRIS into 995 mL distilled water containing 5 mL of Tween 80.[20] The aliquot was autoclaved for 20 minutes at temperature of 121^{0}C. A cold sucrose solution (specific gravity 1.18) was prepared by adding 20 grams of sucrose (EM Industries, Gibbstown, NJ) into 28.58 mL of distilled water and stirring for 20 minutes. The sucrose solution was refrigerated at 4^{0}C prior to use.

Extraction of Oocysts from Soil-Sludge: A protocol described by Walker et al.[20] was used to extract oocysts from the sludge. A one-gram aliquot of mixed soil and sludge was washed by placing the it soil into a 50-mL centrifuge tube containing 20 mL of 50 mM Tris and 0.5% (vol/vol) of Tween 80.The centrifuge tube was then vortexed (Gene 2, Fisher Scientific, Pittsburgh, PA) for 2 minutes and spun at 1600-x g (Sorvall RC-5B refrigerated speed) for 10 minutes. The supernatant layer was discarded following this first wash. The wash was repeated. After the second wash with Tris and Tween 80, the pellet was re-suspended in 10-mL of the same extraction solution and agitated for 10 minutes using a shaker (Bio Dancer, New Brunswick Scientific, Edison, NJ).

The resulting suspension was underlaid with a 10-mL cold sucrose solution (specific gravity 1.18) and centrifuged (1600-x g) for 15 minutes. The interface (10-mL) was removed to a clean 50-

mL centrifuge tube and washed three times in distilled water. After the final wash with distilled water, the supernatant layer was removed down to a final volume of 1 mL and the aliquot was stained with a 200 μL solution of 0.2 percent of Trypan Blue (Sigma, St. Louis, MO).

Extraction Efficiency and Recovery Test for Spiked Oocysts in the Mixture of Soil and Sludge: To evaluate the efficiency of recovery of oocysts, 2.5 x 10^5 oocysts were spiked in 1.0 g of the mixture of soil and sludge, and the extraction solution and the procedure described above (Extraction of Oocysts from Soil-Sludge) was used in the recovery process. The recovery efficiency test was conducted in triplicate. This was done prior to setting out the sentinel chambers. Oocysts were stained with Trypan blue dye and counted using a hemacytometer.

Temperature Experiments: To assess the effect of temperatures that may be generated in the soil, 0.4-ml suspensions of viable oocysts (6.25 x 10^5 oocysts mL^{-1}) in the distilled water (pH 6.32) were incubated in 1.5 mL microcentrifuge tubes at temperatures of 7° and 14°C, using a thermocycler (model 2400 Perkin Elmer, Cetus, Norwalk, CONN)) for a 17-day period. The experiment was conducted in triplicate. The temperatures were selected based on temperatures of soil measure the field. The samples were removed and analyzed after a 17-day incubation period.

Sludge and Soil Salinity Experiment: To assess the effects of the sludge and soil salinity to which the oocysts were exposed, a 0.4-mL suspension of viable oocysts (2.5 x 10^5 oocysts) was placed in a 1.5-mL microcentrifuge tube containing 0.6 mL of salt solution (Table 4-3). The total soluble salts was 845 mg/L (salinity). This value was based on the 1280 μmhos/cm conductivity salt measurement, which was converted to total soluble salt using an empirical factor of 0.66.[2] In addition, the total soluble salt of 845 mg/L that was used in the spiking study was only 5 mg/L less than the combined total soluble salts from the soil and sludge samples (850 mg/L Table 4-3) as analyzed by the OSU Agricultural Testing Laboratory. Table 4-3 shows the measured sludge and soil salinity parameters as well as the salts used to prepare the milliequivalent weight per liter of salinity solution. The duplicate aliquots were incubated in 1.5-mL micro-centrifuge tubes at 7^0 and 14^0C for a period of 17 days.

Alum Experiment: The concentration of aluminum sulfate (Ranger Chemical Company, Choctaw, OK) used in this study was 2.1 M. To assess the effect of alum in the sludge containing viable oocysts, a 0.4 ml suspension of the organism (2.5 x 10^5 oocysts/1.5 mL microcentrifuge

tube) was exposed to an aluminum sulfate solution with a working concentration of 9 mg/L (1 mL of alum, in 1 liter of deionized water, pH 6, Table 4-4), and incubated in a thermocycler (Model 2400, Perkin Elmer, Cetus Norwalk, CONN) at 7^0 and 14^oC for a 17-day period.

Slaked Lime Experiment: To assess the effects of slaked lime (94.5 % $Ca(OH)_2$, Globe Stone St. Clair, Marble City, OK) in the sludge containing viable oocysts, 2.5x 10^5 oocysts were exposed to a solution of slaked lime (1 mL of slake lime, 1L of deionized water, working conc. = 2 mg/L, pH 10.89 Table 4), and incubated for 17days at various temperatures (7^o C, and 14^o C) by using a thermocycler.

Cationic Polymer Experiment: The stock cationic polymer (Polydimethyldiallylammonium – 20 % chloride) was obtained from HCL Distribution Company, Sand Springs, OK. The working solution was prepared by dissolving 1-mL of the stock solution in 1 liter of deionized water with pH 5.8. The concentration (dosage) in mg/L was determined by adding 1 mL of 0.1 percent of cationic polymer to l liter of distilled water. The dosage used in the spiking study was 4 mg/L (Table 4-4).

TABLE 4-3. MEASURED SLUDGE AND SOIL SALINITY PARAMETERS AND SALTS USED TO PREPARE MILLIEQUIVALENT WEIGHT PER LITER OF SALINITY SOLUTION

Sludge Salinity Parameter (as the ion)	Concentration Measured From Soil Sample (Mg/L)	Concentration Measured From Sludge Sample (Mg/L)	Salts Used For Salinity Solution	Cation (Meq/L)	Anion (Meq/L)
*Calcium	55	61	$CaCl_2$	2.9	-
*Magnesium	2	21	$MgCl_2$	0.95	-
⊗Chloride	128	10		-	3.89
*Bicarbonate	90	242	$Ca(HCO_3)_2$	-	5.44
*Sodium	88	5	NaCl	4.04	-
*Sulfate	105	23	Na_2SO_4	-	2.67
*Potassium	10	8	KCl	0.46	-
⊗Nitrate	2	-	$NaNO_3$	-	0.14
Total soluble salt (mg/L)	480[ξ]	370[ξ]	-	-	-
Total concentration of cations and anions in 1 liter of distilled water				8.35	12.14

[ξ]850 mg/L = Concentration of total soluble salts of soil and sludge. Analysis was conducted by Saturated Paste Extract (SPE). *Analyzed with Inductable Coupled Plasma (ICP Method).[23] ⊗Analysis was conducted with Flow Injection Analyzer(FIA).[23]

Desiccation Experiment: Desiccation has been suggested to be catastrophic for oocysts under experimental conditions.[16] To assess the possible effects of desiccation of a mixture of soil and sludge on the inactivation of oocysts, a method described previously was used. [16] A 50 μL stock suspension (approximately 31,250 oocysts per mL of distilled water) was placed on glass slides and air dried at a room temperature of 20^{0}C for 24 hours. The experiment was conducted in duplicate. After the incubation period, the slide was stained with Trypan Blue dye and the oocysts observed using a hemacytometer. In a separate experiment, 50 μL stock suspension of oocysts were injected into glass vials containing 1.0 g of a mixture of sludge and soil and exposed at room temperatures from 20^0 to 22 ^{0} C for 17 days. After the 17 day incubation time, the oocysts were extracted and counted using a hemacytometer.

TABLE 4-4. SLUDGE PARAMETERS TESTED FOR OOCYST INACTIVATION

Parameter	Applied Dose	Measured Residual Level	pH of Chemical	Number of Oocysts Spiked
Alum	9 mg/L	0.22 mg/L	6	2.5 x 10^5 per 1.5 mL of Microcentrifuge Tubes
Cationic Polymer	4 mg/L	N/A	5.8	
Salinity	*TSS = 845 mg/L	N/A	7.57	
Slaked lime	2 mg/L	N/A	10.89	

*TSS = Total soluble salts.

Mass Balance: The mass balance approach was used to determine the number of oocysts lost or unaccounted for in the experiment. In this case, the mass balance can be defined as the initial number of oocysts spiked in the sentinel chambers containing a mixture of soil and sludge prior to incubation, minus the combined number of estimated viable and non viable oocysts after each incubation. The formula is expressed as: Number of oocysts lost = (Initial # of oocysts spiked in a mixed soil and sludge) - [(Estimated # of viable oocysts after incubation) + (Estimated # of non viable oocysts after incubation)].

Calculation: After the extraction of oocysts from the mixture of soil and sludge, cells were stained by withdrawing a 10 μL aliquot of diluted oocyst suspension and were injecting it into the hemacytometer. The entire plate of the hemacytometer was scanned and all the oocysts were counted. Since the entire hemacytometer was used as a counting chamber, the number of oocysts per mL was calculated using the formula

$$\frac{\text{Number of oocysts counted} \times 1{,}000\ \mu\text{L}}{10\ \mu\text{L} \times \text{mL}} \qquad (4\text{-}1)$$

With the use of Trypan Blue (dye) and the hemacytometer, viable oocysts were distinguished from nonviable oocysts. The viable oocysts, those are not stained from the Trypan blue dye, were observed, differentiated from the nonviable, and counted. Following the estimation of viable

oocysts, percent or log inactivation efficiency, as well as die-off rates were calculated using the following equations.

Inactivation rate: First and second order reactions were plotted to determine the best linear fit. The formula for first order reaction is as follows[3]:

$$\ln\frac{N_t}{N_0} = -kt \qquad (4\text{-}2)$$

where N_t is the number of oocysts at the time t; N_0 is the number of oocysts at time 0; N_t/N_0 is the surviving fraction of oocysts; k is the rate constant of inactivation; and t is the inactivation period.

The formula for second order reaction is expressed as

$$\frac{1}{N_t} - \frac{1}{N_0} = k\,t \qquad (4\text{-}3)$$

RESULTS

Soil Temperature and pH Measurement: Soil (10 cm deep) temperature was measured daily. The average daily soil temperatures ranged from 7 to 19.8^{0}C. The results of the measurements showed a pH range of 6.70 to 6.75 using a calcium chloride ($CaCl_2$) solution, and a pH range of 6.98 to 7.05, using distilled water. The pH levels of 9.4 and 7.89 were measured from the sludge and a sample of the mixture of sludge and the soil, respectively.

Organism: Five million of the 25 million viable oocysts, which were purchased from Waterborne Incorporated, were recounted in triplicate using a hemacytometer to verify the accuracy of the number as well as the viability of the organisms. The viability of oocysts was determined by vital staining using Trypan Blue dye. The results of the counting and viability tests showed an excess of 20 viable oocysts over what was expected and 4 nonviable oocysts. Since the difference in count was negligible compared to the number of oocysts obtained from Waterborne Incorporated, the rest of oocysts were not recounted; hence the nominal number was used in the spiking study.

Extraction Efficiency and Recovery Test for Spiked Oocysts in a Mixture of Soil and Sludge: The results of the extraction efficiency test of oocysts spiked in a mixture of soil and

sludge ranged from 89.6 to 92.3%, with a mean of 91.2%, a standard deviation of 1.4% and a coefficient of variation of 1.5.

Inactivation of Sentinel Oocysts: After 17, 30, 45, and 60 day designated periods of inactivation of oocysts exposed to soil environmental stresses, viable oocysts were differentiated from nonviable oocysts with Trypan Blue dye and visualized with the aid of a microscope at 400X. Figure 4-3-A is a photograph of *Cryptosporidium parvum* showing four viable oocysts without visible sporozoites. Figure 4-3-B is a photograph of *Cryptosporidium parvum* embossed to expose sporozoites as shown by the arrow. Figure 3-C is a negative view of *Cryptosporidium parvum* showing one nonviable and two viable oocysts.

Table 4-5 illustrates the average results of the estimated viable oocysts in the sentinel chambers containing a mixture of soil and sludge exposed in agricultural land for a period of 60 days. The observed percent viable and nonviable oocysts ranged from 92.6 to 49.3 for viable, and 3.2 to 45.2 for nonviable (Table 4-5). Figure 4-4-A illustrates the percent viable and nonviable and control oocysts that were exposed to surface soil experiments as determined by a dye permeability assay. Each data point in the figure represents the average percentage estimates and standard error of two replicates. As shown in Figure 4-4-A, at 45 days, inactivation kinetics of sentinel oocysts significantly diverged from the control oocysts, showing a 25 percent inactivation. Figure 4-4-B represents the average daily temperatures of the surface soil (10-cm) where the sentinel chambers and controls were buried. As shown in Figure 4-4-B, the temperatures fluctuated with a general trend toward warmer temperatures. The highest temperature observed in this study was 19.8^{0}C, while the lowest was measured at 7^{0}C.

Table 4-6 illustrates the die-off rates of oocysts in the sentinel and control units buried in a 10-cm surface soil environment. First order kinetics were used to calculate the die-off rates of oocysts in the sentinel because the data for the control units have the best fit (r^2 = 0.88) compared to the second order data of the control units (r^2 = 0.59). Based on the first order kinetics, an initial slow die-off of -0.0044 day^{-1} was observed in the first 17 days at temperatures ranging from 7^0 to 15^0 C, and was followed by an increase die-off rate of -0.0032 day^{-1} in the subsequent weeks, that is, after 30 days (Table 4-6). In addition, a slow die-off rate of -0.0043 day^{-1} was observed after 45 days at temperatures ranging from 11^0 to 17^0 C for oocysts in the sentinel chambers. A rapid die-off rate of

-0.012 day^{-1} was observed after 60 days inactivation period, with temperatures ranging from 10.5^0 to 19.8^0 C.

It is not clear whether the differences in the die-off rates between the control oocysts and oocysts in the sentinel chambers were caused by chemical or biological phenomenon in the soil matrix or by other factors.

Figure 4-5A, illustrates the effects of rainfall in the inactivation of oocysts. Figure 4-5A is the rainfall data for City of Stillwater plotted against the 60 days inactivation period. The rainfall data obtained from MESONET CLIMATOLOGICAL DATABASE was for the period of February 12 to April 11, 2000. Figure 4-5B, illustrates the estimated viable oocysts per period of inactivation plotted against the time (day). Table 4-7 shows the rainfall in inches per day for 60 days. As shown in Table 4-7 and Figure 5-A, the total inches of rainfall for the City of Stillwater for period of 0 – 17, 18 to 30, 31 to 45, and 46 to 60 days was 1.23, 2.21, 2.03, and 1.31 inches, respectively. Based on the rainfall data, it is likely that desiccation could not have been a factor in the die-off rate of oocysts in the sentinel and control units. Therefore, the die-off rates of oocysts were the result of a natural death due to time.

TABLE 4-5. AVERAGE ESTIMATED VIABLE OOCYSTS IN SENTINEL CHAMBERS CONTAINING MIXTURE OF SOIL AND SLUDGE EXPOSED IN AGRICULTURAL LAND

Inactivation Period (Day)	Mean Estimated Viable Oocysts per Sentinel	Mean Percent Viable Sentinel Oocysts	Mean Estimated Non-Viable Oocysts per Sentinel	Mean Percent Non-Viable Sentinel
0 – 17	2.32×10^5	92.6	7.9×10^3	3.2
18 – 30	2.27×10^5	90.6	1.1×10^4	4.3
31 – 45	2.06×10^5	82.4	3.1×10^4	12.5
46 – 60	1.24×10^5	49.3	1.13×5	45.2

Number of oocysts spiked per sentinel and per control was 2.5×10^5.

TABLE 4-6. SURVIVAL OF CRYPTOSPORIDIUM PARVUM OOCYSTS BURIED IN 10-CM SURFACE SOIL ENVIRONMENT

Time (Day)	Die- off Rate of Sentinel Oocysts Day^{-1} (1st Order)	Die- off Rate of Sentinel 1/Oocysts/Day (2nd Order)	Die-off Rate of Control Oocysts Day^{-1} (1st Order)	Die-off Rate of Control 1/Oocysts/Day (2nd Order)
0 – 17	-0.0044	1.8 E – 8	-0.0021	9 E – 9
18 – 30	-0.0032	1.4 E – 8	-0.0019	8 E – 9
31 – 45	-0.0043	1.9 E – 8	-0.0017	1.8 E – 9
46 – 60	-0.012	6.8 E – 8	-0.0025	1.1 E – 8
0 – 60	-0.012	-	-0.0025	-
	$r^2 = 0.61$	$r^2 = 0.71$	$r^2 = 0.88$	$r^2 = 0.59$

TABLE 4-7. TOTAL RAINFALL (INCHES) VERSUS TIME (DAY)

Time (Day)	*Total Rainfall (Inches)
0 – 17	1.23
18 – 30	2.21
31 – 45	2.03
46 – 60	1.31

* Rainfall data for the City of Stillwater was obtained from MESONET CLIMATOLOGICAL DATA BASE

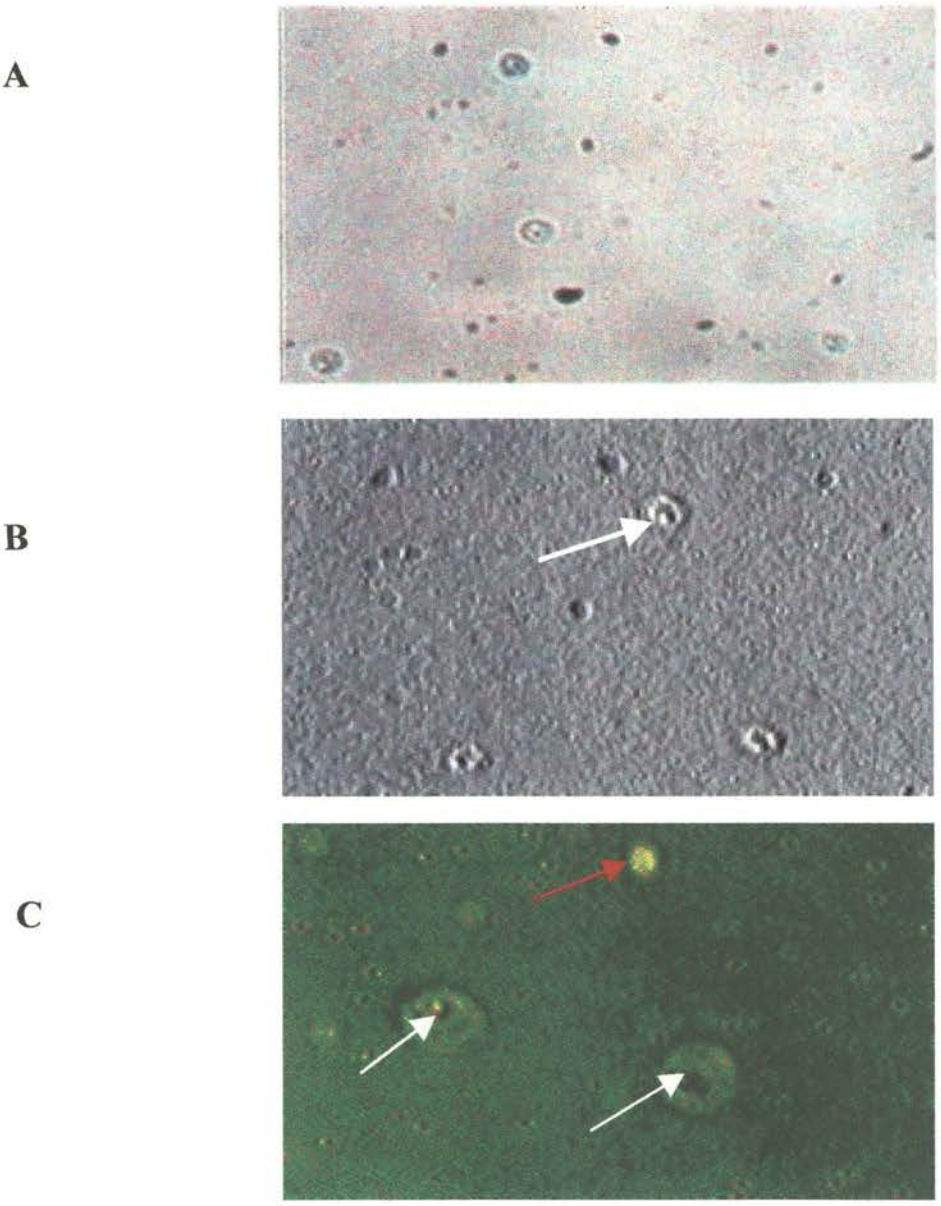

Figure 4-3. *Cryptosporidium Parvum* Oocysts: Photo (A) Illustrates 4 Viable Oocysts without Visible Sporozoites (the Cause of Disease Cryptosporidiosis in Humans). Photo (B) Illustrates 3 Viable Oocysts Elevated to Show Sporozoites (within the Oocyst). The Arrow with White Color Points to the Location of the 4 Sporozoites. Photo (C) Shows A Negative View of Two Viable Oocysts and One Non-Viable Oocyst. The Red Arrow Points to the Dead Stained Oocyst, While the Two White Arrows Point to the Two Viable Oocysts. The Photo Was Taken with A Sony SSC-S20 Color Video Camera Attached to A Fisher Micro-Master Bright Field Microscope.

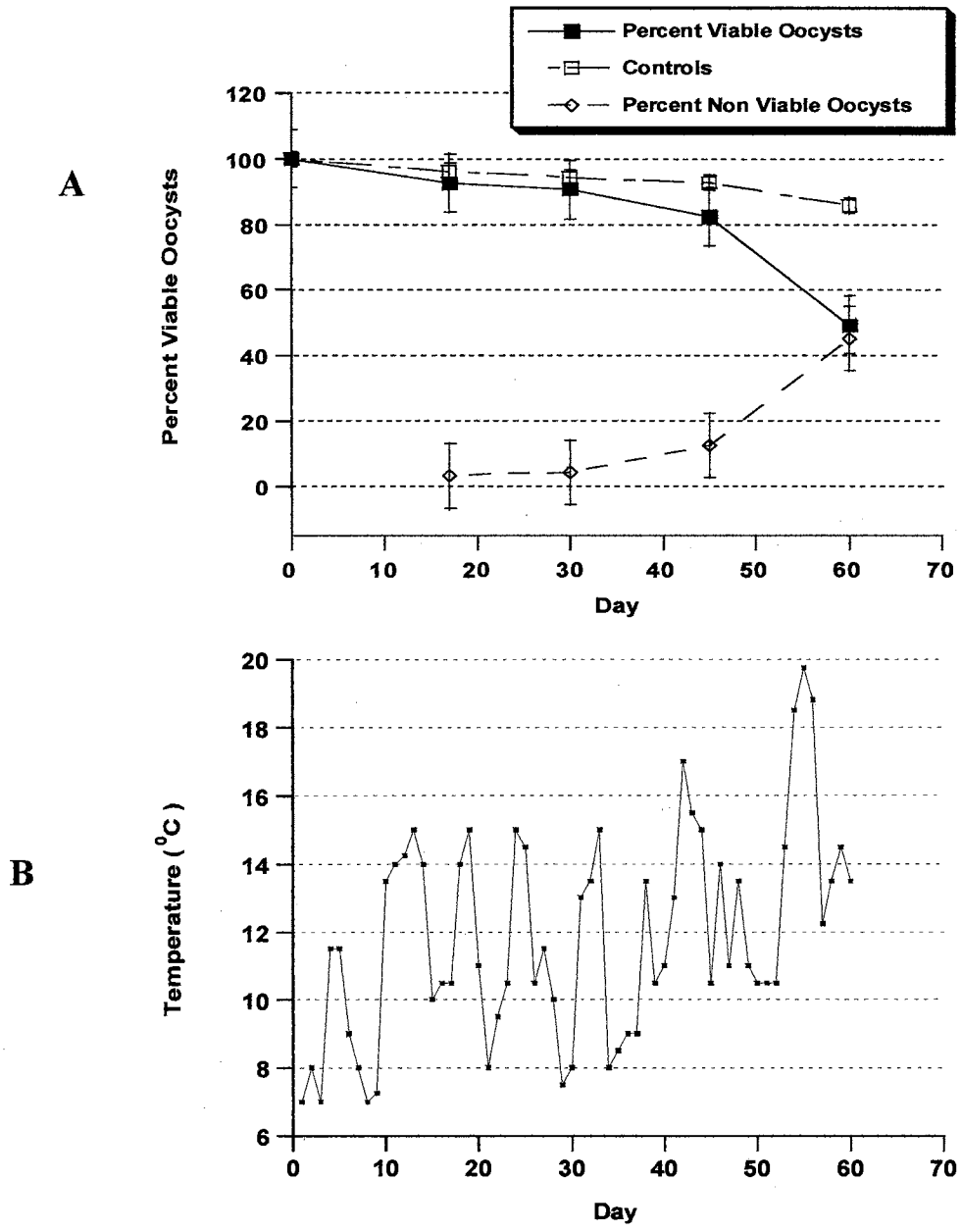

Figure 4-4. Oocyst survival and temperature observed in a 10-cm surface soil experiment (February to April). (A) Percent viable and nonviable sentinel and control oocysts that were exposed to a surface soil environment, as determined by the dye permeability assay. (B) Average daily temperature of the surface soil (10-cm) where sentinel chambers and controls were buried.

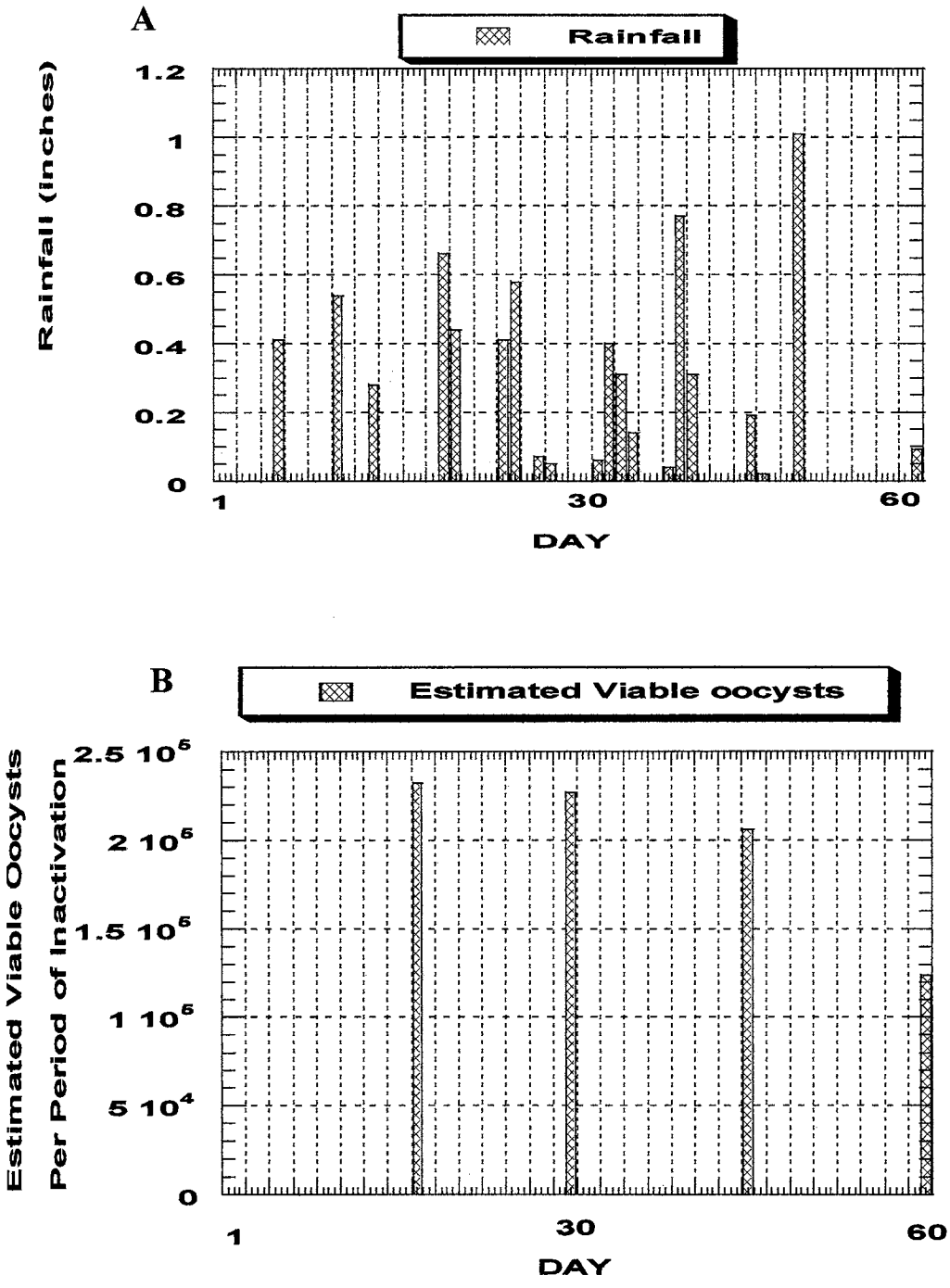

Figure 4-5. Effect of Rainfall on the Inactivation of Oocysts. Plot A = Rainfall versus Day. Plot B = Estimated Viable Oocysts Per Period of Inactivation versus Day.

Determination of Oocysts Lost Using Mass Balance: A mass balance was used to determine the number of oocysts lost in the incubation experiments. Based on the mass balance, the average percentage of oocysts lost from 0 to 17, 18 to 30, 31 to 45, and 46 to 60 days was 4.0, 4.8, 5.2, and 5.2 percent, respectively. The average percent lost of oocysts in all four experiments conducted in duplicate, regardless of number of days the organism was incubated was 4.8 percent.

Inactivation of Control Oocysts: Table 4-8 shows the measurements of oocysts exposed in distilled water and exposed to agricultural land and used as a control. The observed percent of viable oocysts, was highest after a 17-day period of incubation, at 96.2%. The viability of oocysts decreased with an increase in the period of inactivation, down to 85.8% after 60 days. The die-off rates of oocysts in the control units ranged from -0.0021 to -0.0025 day^{-1} (Table 4-8).

TABLE 4-8. ESTIMATED VIABLE OOCYSTS EXPOSED IN DISTILLED WATER AND AGRICULTURAL LAND: THE CONTROLS

Inactivation Period (Day)	Mean Estimated Viable Oocysts per Microcentrifuge Tube	Mean Percent Viable	Die-off Rate of Control Oocysts Day^{-1}
0-17	2.41×10^5	96.2	-0.0021
18-30	2.36×10^5	94.3	-0.0019
31-45	2.32×10^5	92.7	-0.0017
46-60	2.15×10^5	85.8	-0.0025

Comparisons of Viability and Die-off Rates of Controls and Sentinel Oocysts: As discussed above, the kinetic of sentinel oocysts significantly diverges from that of the control oocysts (Figure 4-4). It was likely that this diverging of the plot was the result of the environmental stresses between the soil matrix and the sentinel oocysts, causing increased inactivation of the oocysts.

Statistical analysis was conducted to determine any significant difference between the observed viability as well as the die-off rates of oocysts in the control and the sentinel units (Table 4-9). The results of the statistical analysis showed that (1) there was a significant difference in the number of viable oocysts in the control and sentinel units after the 17-day period ($P>0.40$, $\alpha = 0.05$, $r^2= 0.36$) and also a significant difference in the die-off rates of oocysts in the control and sentinel units after 17 days ($P > 0.41$, $\alpha = 0.05$, $r^2 = 0.35$); (2) the number of viable oocysts in the sentinel units after 30 days, was significantly different from the number of viable oocysts in the control units ($P>0.19$, $\alpha = 0.05$, $r^2=0.65$) and a significant difference was observed between the die-off rates of oocysts in the sentinel and control units ($P> 0.27$, $\alpha = 0.05$, $r^2 = 0.54$); (3) after 45 days, the number of viable oocysts obtained from the sentinel units was significantly different from the number of oocysts recovered from the control units ($P>0.64$, $\alpha = 0.05$, $r^2=0.13$) as well as the die-off rate of oocysts in the sentinel units being significantly different from the die-off rate in the controls units ($P> 0.87$, $\alpha = 0.05$); and (4) there was a significant difference in the number of viable oocysts in the sentinel and control units ($P>0.85$, $\alpha = 0.05$,) with low coefficient of determination, ($r^2= 0.02$), and the die-off rate of oocysts in the sentinel units was significantly different ($P> 0.16$, $\alpha = 0.05$, $r^2 = 0.70$) from the die-off rate in the control unit.

Overall, the difference between the sentinel and control units in the die-off rates of oocysts, suggests that the presence of environmental factors other than the temperature affected the rates.

Effects of Soil and Sludge Parameters: Table 4-4 shows the initial doses and the level of the chemical residuals used to assess the survivability of oocysts incubated at temperatures of 7^0 and 14^0C for a period of 14 days. Figures 4-6A and B illustrates the results of the survival rate of oocysts spiked with the above mentioned chemicals and incubated at 7^0 and 14^0C using a thermocycler. As shown in Figures 4-6A and B, samples incubated at a temperature of 7^0C showed pattern of plotted lines of survival rates to be similar to samples incubated at a temperature of 14^0C. Also, as shown in Figure 4-6A, at an incubating temperature of 7^0C, the survival rates of oocysts in the liquid alum, saline solution, and cationic polymer, appeared to be similar because the error bar lines overlapped each other (Figure 4-6A). However, the survival rate of oocysts in water containing slaked lime was lower (Figure 4-6A). In Figure 4-6B, there was no significant difference in the survival rates of oocysts spiked in the liquid alum and the saline solution at 14^0C,

based on the error bars overlapped one another. The survival rates of oocysts in the liquid lime were lowest (Figures 4-6A and B). This is likely due to high pH effects in the slaked lime. This result was consistent with previous studies that reported pH as a factor in the inactivation of oocysts.[5,15] Finally, the result of the desiccation study showed no survival of oocysts in both experiments conducted. This study was consistent with the previous work that showed no survival of oocysts due to desiccation.[16] Overall, the study showed that the chemicals could individually kill oocysts if the contact time with the oocyst is longer than 17 days. The study also revealed that the slaked lime was more effective in oocyst inactivation, than the other of the chemicals tested. This was likely due to high pH level of 10.89. Previous authors have suggested that increases or decreases in pH could affect the survival rate of oocysts.[17]

The die-off rates of oocysts incubated in liquid alum, slaked lime, salinity, and cationic polymer at 7^0 C was -0.0031, -0.0042, -0.0034, and -0.0033 day^{-1}, respectively. In addition, the die-off rates of oocysts incubated at 14^0 C in liquid alum, slaked lime, salinity, and cationic polymer was -0.0039, -0.0045, -0.0046, and -0.0034 day^{-1} respectively.

TABLE 4-9. COMPARISONS OF VIABLE AND INACTIVATION RATE OF CONTROLS AND SENTINEL OOCYSTS OBSERVED FROM 17 TO 60 DAYS SOIL EXPOSURE

Sample # Comparisons	Means Comparisons of Viable Oocysts Using Paired t – test	Significant Difference for Viable Oocysts in the Control and Sentinel	Significant Difference (Die-off Rate)	Coefficient Determination for Viable Oocysts r^2	Coefficient of Determination Oocysts for Die-off Rate r^2
17d Control & 17d Sentinel	9100	YES P >0.40	YES P > 0.41	0.36	0.35
30d Control & 30d Sentinel	9388	YES P >0.19	YES P > 0.27	0.65	0.54
45d Control & 45d Sentinel	25650	YES P >0.64	YES P > 0.87	0.13	0.02
60d Control & 60d Sentinel	93021	YES P > 0.85	YES P > 0.16	0.02	0.70

Statistical analysis was conducted to compare the survival rates of oocysts in the control samples to the rates of survival in alum, lime, salinity, and cationic polymer. The results showed that the survival rates of oocysts in the control samples (oocysts spiked in distilled water pH 6.32),

incubated at 7 ^{0} C was significantly different from the survival rates of oocysts in all the tested parameters (Table 4-10).

TABLE 4-10. STATISTICAL ANALYSIS FOR COMPARISON OF SURVIVAL RATES OF VIABLE OOCYSTS AND CONTOLS INCUBATED AT VARIOUS TEMPERATURES USING A THERMOCYCLER

Samples	Mean comparisons of survival rate of oocysts Log (N_t/N_0)	Significant difference using Paired t-Test	Correlation coefficient	Coefficient of determination (R^2)	Standard Deviation
7 ^{0} C- Control & Salinity	-0.0157	Yes	0.94 P > 0.06	0.88	0.003
7 ^{0} C- Control & Alum	-0.0158	Yes	-0.83 P > 0.17	0.69	0.003
7 ^{0} C- Control & Slaked lime	-0.009	Yes	0.091 P > 0.9	0.008	0.002
7 ^{0} C- Control & Cat. Polymer	-0.016	Yes	0.77 P > 0.23	0.59	0.006
14 ^{0} C- Control & Salinity	-0.142	Yes	0.16 P > 0.84	0.025	0.0016
14 ^{0} C- Control & Alum	-0.014	Yes	0.77 P > 0.22	0.60	0.0012
14 ^{0} C- Control & Slaked lime	-0.008	Yes	0.64 P > 0.36	0.41	0.0051
14 ^{0} C- Control & Cat Polymer	-0.018	Yes	0.94 P > 0.06	0.88	0.0012

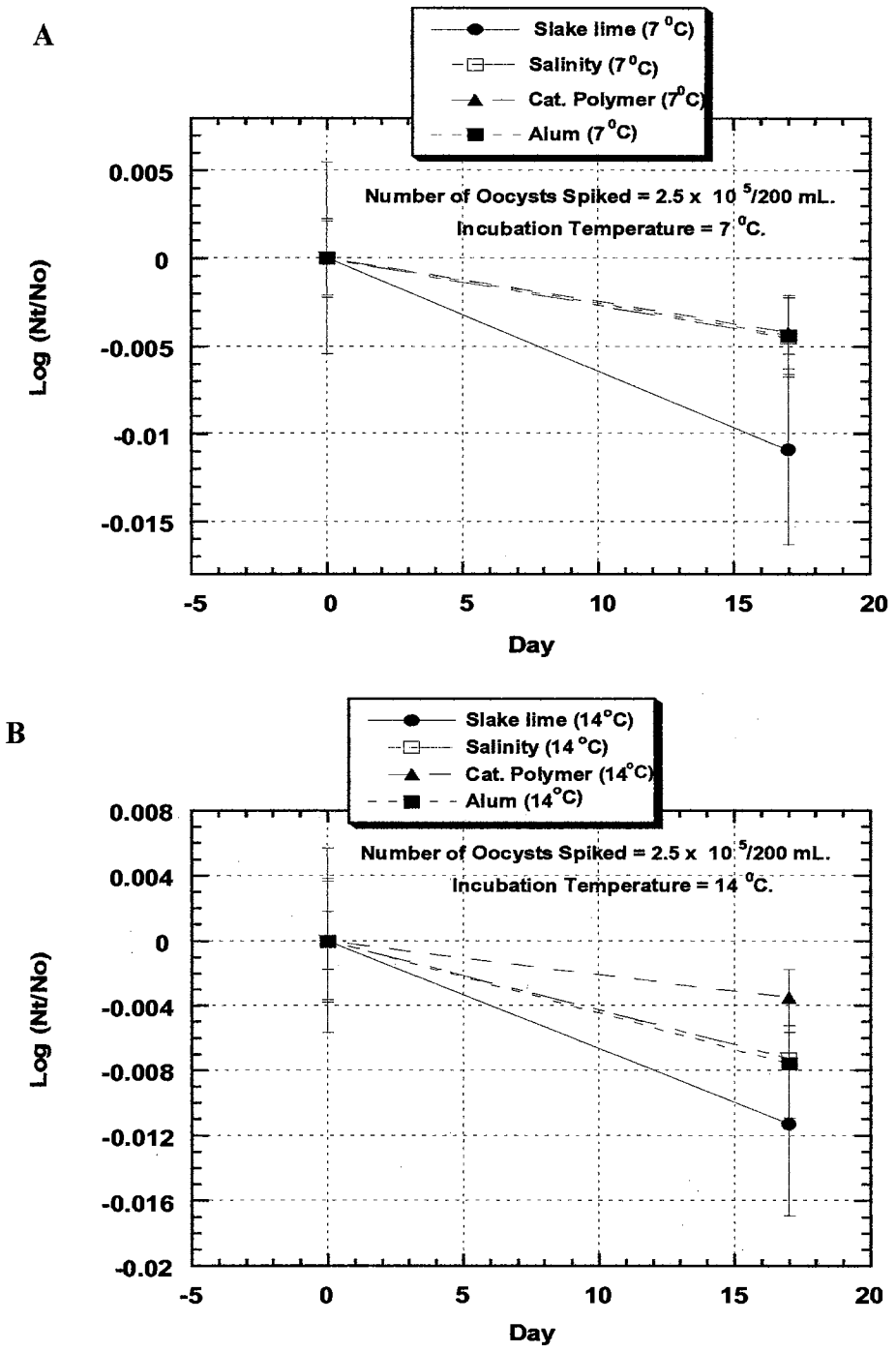

Figure 4-6. Survival rates of oocysts incubated in liquid alum, salinity, slaked lime, and cationic polymer at temperature of 7^0 C and 14^0 C. (A) Samples incubated at 7^0 C, and (B) Samples incubated at 14^0 C.

DISCUSSION

The sentinel chamber used in this study was an effective method for equilibrating the external environment of test units containing oocysts and the surrounding soil environment. The chamber prevented the release of oocysts into the environment and effectively exposed the oocysts to stresses due to the soil environment.[7] The average percent lost of oocysts, based on the mass balance, in all four experiments conducted in duplicate, regardless of number of days the organism was incubated was 4.8 percent. This loss of 4.8 percent of oocysts (dead and alive) in the experiments may be due to the extraction of oocysts from the mixed soil and sludge. In addition, the loss of 4.8 oocysts shows that no oocysts escaped the sentinel chambers during rainfall when the soil contents in the chambers were saturated with water. This study did not refute the statement of the previous authors who stated that because oocysts are known to be nonmotile and do not replicate outside the living host, therefore, there will not be a flux of water that would allow the organism to be transported out of the 60 μm of nylon mesh filter.[7] This was the first time this type of information has been presented. Overall, the mass balance was effective in determining the number of oocysts lost.

Because the volume of the sentinel chambers was very small, the use of many small-sized replicates, which served as independent samples at each sample interval, was necessary. The differences in the die-off rates of oocysts in the sentinel and in the control units indicated that the presence of environmental factors other than temperature affects the survival of oocysts. It was not clear from these data (die-off rates) if this difference was the reflection of a biological or biochemical phenomenon in the soil matrix.

However, based on rainfall data, desiccation was unlikely a major factors in the die-off rates of sentinel oocysts. Therefore, it is clear that the die-off rates of oocysts in the sentinel chambers were natural death due to time.

Laboratory models of the effects of alum, salinity, cationic polymer, and slaked lime on the viability of oocysts, suggested that these chemicals could individually kill oocysts. However, rapid inactivation of oocysts with individual chemicals will probably depend on the dosage applied, pH and the contact time.

The results of laboratory models showed high die-off rates of oocysts incubated in these chemicals. For examples, the survival rate of oocysts incubated in liquid alum of (9 mg /L) ranged from -0.0031 day^{-1} at 7^0C, to -0.0039 day^{-1} at 14^0C. In addition, the die-off rates of oocysts in the liquid cationic polymer (Polydimethyldiallylammonium) at a concentration of 4 mg/L were -0.0033 day^{-1} at both 7^0 and 14^0C, respectively. Also, the die-off rates of oocysts for 2 mg/L of slaked lime, was -0.0046 day^{-1} at 7^0 C and -0.042 day^{-1} at 14^0C, respectively. The reasons for low die-off rates of oocysts in these chemicals was that the concentrations of chemicals used in this study were similar to those normally used at the treatment facilities.[16] The dosages of these chemicals do not have a significant impact on the viability of oocysts.

The results of this study, reflect the months and the season the study was conducted. However, different outcome is possible if conducted in different months and season. Overall, the study showed that oocysts could survive extreme soil environmental stress for more than 2 months. Prescott et al.[15] suggested that oocysts could remain viable in a moist environment for up to 6 months. Because of low die-off rates of oocysts and the ability of this organism to survive extreme soil environmental conditions for long periods of time, it is possible that oocysts in water treatment plant sludge that is used for agricultural fertilizer or pH buffer, could be transported back to treatment facilities through surface water and agricultural run-off. Also, because the chemicals used in the treatment facilities did not completely inactivate the oocysts, the presence of oocysts in the sludge samples, and the sanitary risks associated with using sludge to fertilize agricultural land, satisfied the significance of this study.

CONCLUSIONS

- Between the 0 to 17 and 18 to 60 day observations of surface soil exposure, die- off rates of oocysts in the sentinel units ranged from -0.0044 to -0.012 day^{-1}. The die-off of oocysts was slow for the first 45 days and increased rapidly after the 45-day period, achieving 25% oocyst inactivation.
- An average total of 4.8 percent of oocysts were lost in all the experiments, regardless of the incubation period.
- The sentinel chambers used in this study was effective at equilibrating the mixture of soil and sludge contained in sentinel units and the surface soil environment. Since the volume of the sentinel chambers was very small, the use of many replicates to serve as independent samples at each sample interval was necessary. The use of this technique was appropriate, due to the study results that showed differences between survival rates of oocysts in the sentinel chamber and in the of controls.
- Overall, the study showed that (1) desiccation was most likely not a factor in the die-off rates of oocysts exposed to surface soil because of the availability of rainfall all through the 60 days, (2) temperature may not likely be a factor because of low die-off rates of oocysts in the control units, and (3) the difference in the die-off rates of oocysts between the sentinel and control units showed that the die-off rates of oocysts was due to soil matrix or natural die-off due to time.
- Laboratory models of the effects of alum, cationic polymer, salinity, and lime showed that each chemical could kill oocysts; however, the magnitude of the inactivation of oocysts inside the sentinel chamber containing a mixture of soil and sludge and exposed to surface soil could not be determined. The model iterated common knowledge, that the chemicals used in the treating water contaminated with oocysts, can not completely inactivate the organism based on used chemical dosages, and contact time.

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CHAPTER 5

RECOMMENDATIONS and RESEARCH NEEDS

General Recommendations

The association of *Cryptosporidium parvum* oocysts with recent waterborne disease outbreaks has caused concern for many water utilities using surface water as their source water. The extensive distribution of oocysts in the surface water and field environment, the broad range of other organisms harboring this parasite, and the ability of parasites to resist conventional disinfectants are characteristics that increase the risk of disease transmission via water. It would therefore be advantageous for water utility laboratories to not only positively identify this parasite but to also evaluate oocyst viability and relate this to the potential for infectivity.

Conventional water treatment plants using coagulation, flocculation, sedimentation, filtration, and disinfection can provide effective treatment to protect drinking water from oocysts. Treatment facilities receiving their source water from surface water should be watchful during periods when intake water has high turbidity levels resulting from storm water runoff, snow melt runoff, and lake overturns.

Detection

- The PCR method of detection was very effective in the detection of oocysts, with performance ranging between 82 % to 98 % for the supernatant. The percent recovery of oocysts in sludge ranged from 2.1 to 18 %. Therefore, it is recommended that treatment facilities consider using the PCR detection method for oocyst detection. The PCR method of

detection requires trained personnel, but it is worth the investment for sensitivity and efficiency of detection.

Occurrence

- Treatment facilities should stop the practice of re-treating recycled lagoon water already containing backwash water. This is because the lagoon water is exposed to animals and may contain animal feces, which are the source of oocysts. Utilities should first ozonate lagoon water prior to mixing with raw water in the storage tank. However, the residual of 0.2 mg/L of ozone, as observed in Stillwater Water Treatment Facility, may not inactivate oocysts because of the contact time and fast dissipation of ozone residual.
- Treatment facilities should consider using PACl, in addition to a coagulant aid during the winter season. PACl was observed to be more effective than alum in the precipitation of oocysts when water was treated at temperature of 14.5 ^{0}C. PACl is more expensive than alum, but it is worth using for the purpose of removal efficiency of oocysts at cold temperatures.

Monitoring

- Treatment facilities using solid contact clarification should consider monitoring turbidity levels on a daily basis and maintain less than 2 NTU. Based on the present study, the lower the settled turbidity in the supernatant, the fewer numbers of oocysts were recovered from the supernatant. The study also showed that when the final settled water turbidity is at 2 NTU or less, greater numbers of oocysts were recovered from sludge samples. However, when the settled water turbidity levels were greater than 2 NTU, a greater number of oocysts were recovered from the supernatant.
- Treatment facilities should test for the presence of oocysts in the source and effluent water at least once a month to monitor the influx of this organism in the plant. In addition, utilities should take it upon themselves to test at least twice a month for the presence and viability of oocysts in sludge samples. This is important because larger number of organisms in

sludge samples may indicate a massive influx from source water. Because viable oocysts can withstand extreme environmental stresses, sludge samples containing viable oocysts, if applied to agricultural land, could be transported back to treatment facilities due to agricultural runoff.

Research needs

- Further research is needed to determine how treatment facilities can improve treatment of oocysts in water, especially the impact of oocyst removal from solid contact clarification.
- Further research is needed to monitor the die-off rate of oocysts in the spiked raw water, by treating the water using a conventional treatment method. The study should examine the effect of each chemical added, the effects of rapid mixing and flocculation at different velocity gradients (G-value), and settling times.
- Further research is required for process control for consistent effluent water quality. A process control approach should be derived using a suite of online water quality parameters that will provide advance warning of water quality movements that may permit oocysts to pass through the treatment plant.
- Further research is needed to determine the appropriate disinfection practices for oocysts. These studies should be conducted under field conditions similar to drinking water treatment.
- Research is needed to determine the impact of cold water on survival and treatment of oocysts.
- Studies are needed for pretreatment processes for removal of oocysts such as evaluating the potential for the application of pretreatment processes; that is, riverbank filtration and soil passage to remove oocysts from the surface water.
- Studies should be conducted to determine the concentration of viable oocysts in source water, specifically Kaw Reservoir.

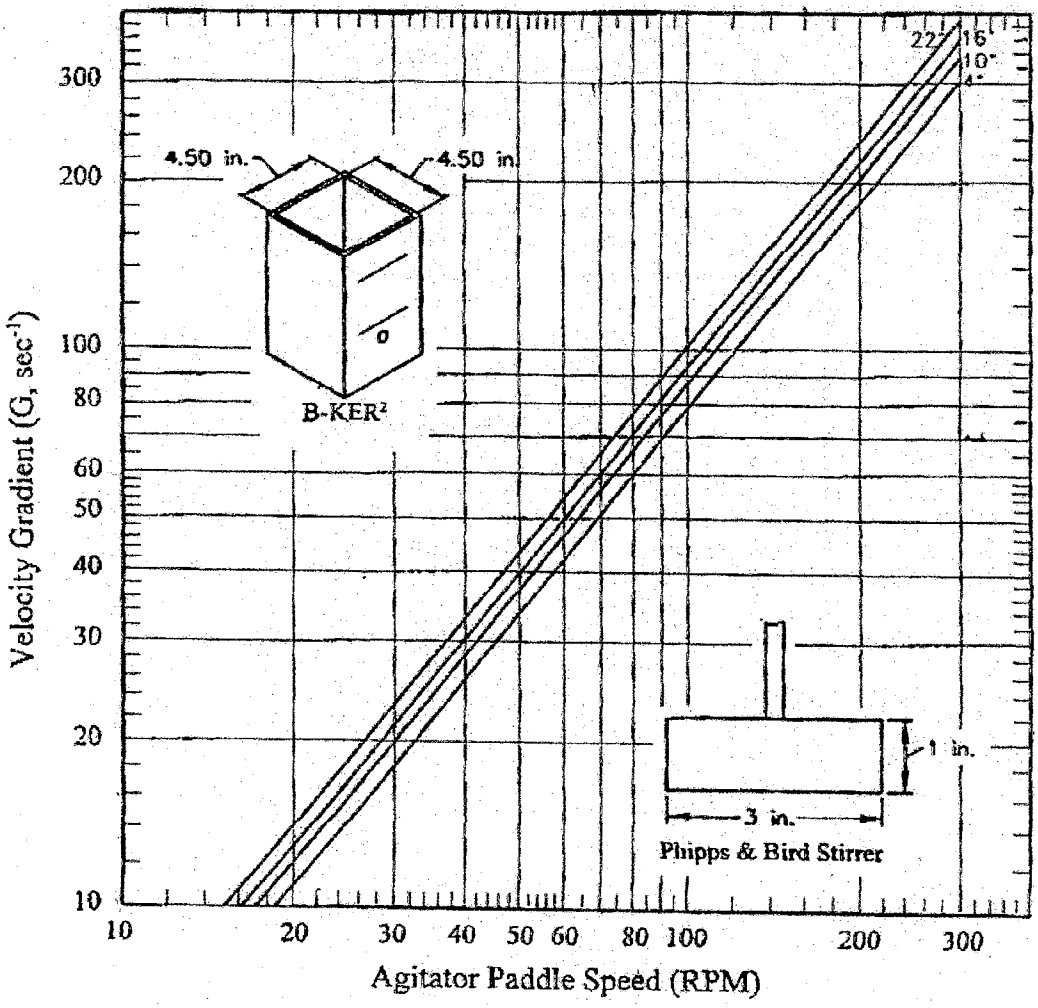
- More research is needed to determine the effects of alum sludge on oocysts. The study should utilize newly produced alum sludge as well as old alum sludge from the treatment facility.

APPENDICES

APPENDIX A

Velocity Gradient (G – Value)

The G-value concept is a rough approximation of mixing intensity. It is based upon the input power, basin volume, and viscosity. In this study, during rapid mixing, 200 revolution per minute (rpm) was used and the corresponding velocity gradient (G-value) was 250 S^{-1} based on the water temperature of 22.1. The G – value was determined from the G – Curve. This G – Curve is for the Gator Jar (Square), with a 1 x 3-inch Phipps and Bird stirrer paddle.



Source: Phipps & Bird

Velocity Gradient vs. Agitation Speed for a 2 – liter Square Beaker Using a Phipps & Bird Stirrer.

CONCENTRATION VERSUS % TRANSMITTANCE FOR CHLORINE AND TOTAL CHLORINE

% T*	0	1	2	3	4	5	6	7	8	9
10	-	4.70	4.58	4.46	4.34	4.22	4.10	3.98	3.88	3.76
20	3.66	3.56	3.40	3.26	3.18	3.08	3.03	2.90	2.81	2.72
30	2.63	2.55	2.47	2.39	2.31	2.24	2.17	2.11	2.05	1.99
40	1.94	1.88	1.83	1.78	1.73	1.68	1.63	1.58	1.54	1.49
50	1.45	1.41	1.37	1.33	1.29	1.25	1.21	1.18	1.14	1.10
60	1.07	1.03	1.0	0.97	0.93	0.90	0.87	0.84	0.81	0.78
70	0.75	0.72	0.69	0.66	0.63	0.60	0.57	0.55	0.52	0.49
80	0.47	0.44	0.41	0.39	0.36	0.34	0.32	0.29	0.27	0.24
90	0.22	0.2	0.17	0.15	0.13	0.11	0.09	0.06	0.04	0.02

%* = Percent transmittance, which was used to determine the concentration of chlorine. The concentration is determined by first obtaining the reading of the transmittance from the spectrophotometer. For example, say the transmittance reading is 55 and the value of chlorine concentration should be where the 50 % T, and number 5 at the top of the table meet. In this case the chlorine concentration is 1.25 mg/L.

RECOUNTING OF OOCYSTS FROM WATERBORNE INC.

Number of oocysts From waterborne	Actual number of viable oocysts counted	Percent Counted (sub-count)	Standard deviation	Coefficient of variation	Significant difference
5×10^6	6.10006×10^6	122.012	1,100,030	22	Yes
	5×10^6	100.0			
	3.9×10^6	78.0			
Average	5.00002×10^6	100.004			P < 0.0001

RECOUNTING OF OOCYSTS IN 0.53 ML OOCYSTS STOCK SOLUTION

Predicted # of oocysts in 0.53 mL Stock solution	Actual # of oocysts in 0.53 mL stock solution	Percent Counted (sub-count)	Standard deviation	Coefficient of variation	Significant difference
333,333	333,630	100.089	325.9	0.1	Yes
	333,460	100.04			
	333,000	99.9			
Average	333,363	100.01			P > 0.165

APPENDIX B

Computation of Electrophoretic Mobility and Zeta Potential

Zeta potential and electrophoretic mobility was determined by using the prescribed protocols by Zeta Meter Incorporated. The Helmholtz – Smoluchowski equation is the most elementary expression of Zeta Potential, and in some cases it only approximates the values obtained from more sophisticated calculations, yet it is sufficient for most technical work. The formula for Zeta Potential shows a direct relation between ZP and electrophoretic mobility and can be expressed as:

$ZP = 4\pi V_t/D_t \times EM$

Where EM = electrophoretic mobility at actual temperature

V_t = viscosity of the of water at temperature "t"

D_t = Dielectric Constant of water at temperature "t"

$4\pi = 12.57$

ZP = voltage in electrostatic units

However, it is preferable to calculate the ZP in "practical" millivolts instead of in electrostatic units. The formula then becomes:

$ZP = 113{,}000\ V_t/D_t \times EM$ ZP = millivolts.

However, at any given temperature the term $113{,}000\ V_t/D_t$ becomes a constant, thus the equation can be expressed as:

$ZP = C_t \times EM$ where: C_t = correction temperature for ZP.

The equation for EM = 160 microns/t x 10 cm/V

Where 160 micron = distance traversed for one full micrometer division of cell and ocular micrometer; 10 cm = length of the cell tube; t = time to traverse one full voltage.

EM = 1600/t x V; V = applied voltage. The units for EM are microns per second/ volts per centimeter.

APPENDIX C

QPCR Standard curves and estimated C. parvum oocysts

The log of the ratio of the 435/300-bp band intensity and the log of known concentration of oocysts and estimated number of oocysts were used to construct the linear line for the standard curve. The final number of oocysts were estimated based on the formula: initial oocysts from the standard curve per pg. X the amount of C. DNA extracted in microgram X the amount of C. DNA used for dilution X the dilution factor per amount of micro- liter used in PCR amplification.

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND LOG (C. DNA)
AND ESTIMATED OOCYSTS FOR RECOVER PRECISION TEST

EXP. #	PCR Product of C. DNA	MIMIC Template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
1	181.74	28.15	6.46	0.81	2.26
2	191.89	30.06	6.38	0.81	2.28
3	180.38	27.11	6.65	0.82	2.26
Exp. #	Initial Oocysts from Standard curve per pg.	Amount of C. Parvum DNA Extracted (μg/2L)	Amount of C. Parvum DNA Used for Dilution	Dilution Factor per amount of μL used for PCR amplification	Estimated Number of Oocysts per 2 Liter water sample
1	13.0/pg	50 μg	1 μL	10^{-3} per 2 μL	325,000
2	13.2/pg	50 μg	1 μL	10^{-3} per 2 μL	330,000
3	13.3/pg	50 μg	1 μL	10^{-3} per 2 μL	332,500
				Average	329,167

* pg. = pico gram = 1 x 10^{-12} g. $^{\xi}$μg = microgram = 1 x 10^{-6} g.

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND
LOG (C. DNA) FOR THE STANDARD CURVE

No of Samples	PCR product of C. DNA	Mimic template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
1					
2	10	16.145	0.6194	-0.2080	1.0
3	50	32.03	1.561	0.1934	1.699
4	100	16.368	6.1094	0.7860	2.0
5	250	37.992	6.5804	0.8183	2.398
6	500	60.386	8.280	0.9180	2.699
7	1000	87.093	11.482	1.06	3.0
8	2000	123.32	16.218	1.210	3.301
9	4000	184.527	21.677	1.336	3.602
10	8000	246.571	32.810	1.516	3.908

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND
LOG (C. DNA) FOR SUPERNATANT SAMPLES

EXP. #		PCR Product of C. DNA	MIMIC Template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
SUPERNATANT SAMPLES						
1a	1	207.45	31.77	6.53	0.82	2.32
	2	177.65	25.20	7.05	0.85	2.25
	3	234.66	29.15	8.05	0.91	2.37
1b	1	779.56	65.73	11.86	1.07	2.89
	2	1069.3	69.98	15.28	1.18	3.03
	3	687.04	73.43	9.35	0.97	2.84
2a	1	402.82	42.18	9.55	0.98	2.61
	2	440.40	51.75	8.51	0.93	2.64
	3	627.74	49.86	12.59	1.10	2.80
2b	1	185.64	27.79	6.68	0.83	2.27
	2	256.13	34.06	7.52	0.88	2.41
	3	204.05	30.73	6.64	0.82	2.31
3a	1	342.49	28.47	12.03	1.08	2.53
	2	293.66	32.20	9.12	0.96	2.47
	3	353.98	37.94	9.33	0.97	2.55
3b	1	214.15	33.15	6.46	0.81	2.33
	2	171.33	25.92	6.61	0.82	2.23
	3	217.57	30.73	7.08	0.85	2.34
4a	1	350.36	42.11	8.32	0.92	2.54
	2	526.64	50.30	10.47	1.02	2.72
	3	695.62	64.89	10.72	1.03	2.84
4b	1	538.50	53.85	10.00	1.00	2.73
	2	651.02	62.18	10.47	1.02	2.81
	3	787.51	76.98	10.23	1.01	2.90

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND
LOG (C. DNA) FOR SUPERNATANT SAMPLES

EXP. #		EXP. # PCR Product of C. DNA	MIMIC Template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
SUPERNATANT SAMPLES						
5a	1	143.96	17.92	8.04	0.90	2.16
	2	162.47	18.15	8.95	0.95	2.21
	3	155.63	19.14	8.13	0.91	2.19
5b	1	394.92	33.61	11.75	1.07	2.60
	2	370.44	38.79	9.55	0.98	2.57
	3	389.86	29.58	13.18	1.12	2.59
6a	1	315.99	40.79	7.76	0.89	2.50
	2	446.11	39.76	11.22	1.05	2.64
	3	274.98	37.98	7.24	0.86	2.44
6b	1	224.30	22.43	10.00	1.0	2.35
	2	289.29	29.61	9.77	0.99	2.46
	3	2.34.38	25.70	9.12	0.96	2.37
7a	1	282.11	33.15	8.51	0.93	2.45
	2	192.36	25.96	7.41	0.87	2.28
	3	256.59	30.84	8.32	0.92	2.41
7b	1	213.09	27.46	7.76	0.89	2.33
	2	198.71	26.18	7.59	0.88	2.30
	3	144.51	19.96	7.24	0.86	2.16
8a	1	160.96	18.48	8.71	0.94	2.21
	2	329.94	37.03	8.91	0.95	2.51
	3	327.04	34.25	9.55	0.98	2.51
8b	1	252.00	27.01	9.33	0.97	2.40
	2	453.19	44.30	10.23	1.01	2.66
	3	446.96	42.69	10.47	1.02	2.65

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND
LOG (C. DNA) FOR SLUDGE SAMPLES

EXP. #		PCR Product of C. DNA	MIMIC Template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
SLUDGE SAMPLES						
1a	1	214.86	76.19	2.82	0.45	2.33
	2	234.15	69.48	3.37	0.53	2.37
	3	241.56	75.96	3.18	0.50	2.38
1b	1	97.82	27.79	3.52	0.55	1.99
	2	87.46	34.03	2.57	0.41	1.94
	3	154.98	47.98	3.23	0.51	2.19
2a	1	144.84	79.58	1.82	0.26	2.16
	2	160.63	84.10	1.91	0.28	2.21
	3	165.52	82.76	2.0	0.30	2.22
2b	1	90.46	46.39	1.95	0.29	1.96
	2	125.55	47.20	2.66	0.43	2.10
	3	79.92	39.96	2.0	0.30	1.90
3a	1	173.45	64.48	2.69	0.43	2.24
	2	157.96	58.72	2.69	0.43	2.20
	3	139.38	69.69	2.00	0.30	2.14
3b	1	169.54	60.12	2.82	0.45	2.23
	2	197.62	71.86	2.75	0.44	2.30
	3	213.72	74.21	2.88	0.46	2.33
4a	1	226.31	69.85	3.24	0.51	2.35
	2	244.79	72.21	3.39	0.53	2.39
	3	252.55	76.30	3.31	0.52	2.40
4b	1	306.04	82.27	3.72	0.57	2.49
	2	272.96	76.89	3.55	0.55	2.44
	3	221.40	80.51	2.75	0.44	2.35

STANDARD CURVE OF LOG (C. DNA/MIMIC) AND
LOG (C. DNA) FOR SLUDGE SAMPLES

EXP. #		EXP. # PCR Product of C. DNA	MIMIC Template	$\frac{C.DNA}{MIMIC}$	$\text{Log}\left(\frac{C.DNA}{MIMIC}\right)$	Log (C. DNA)
SLUDGE SAMPLES						
5a	1	43.62	16.28	2.68	0.43	1.64
	2	58.95	18.13	3.25	0.51	1.77
	3	52.96	17.30	3.06	0.49	1.72
5b	1	76.53	27.14	2.82	0.45	1.88
	2	83.08	25.10	3.31	0.52	1.92
	3	53.75	19.98	2.69	0.43	1.73
6a	1	74.17	22.75	3.26	0.54	1.87
	2	117.32	30.16	3.89	0.59	2.07
	3	101.03	28.46	3.55	0.55	2.0
6b	1	86.03	31.98	2.69	0.43	1.93
	2	115.86	35.76	3.24	0.51	2.06
	3	108.57	32.80	3.31	0.52	2.04
7a	1	76.19	41.86	1.82	0.60	1.88
	2	140.08	39.46	3.55	0.55	2.15
	3	161.24	37.76	4.27	0.63	2.21
7b	1	161.95	51.25	3.16	0.50	2.21
	2	206.81	62.48	3.31	0.52	2.32
	3	248.72	60.11	4.07	0.61	2.40
8a	1	258.34	64.91	3.98	0.60	2.41
	2	264.99	68.12	3.89	0.59	2.42
	3	284.94	70.01	4.07	0.61	2.45
8b	1	208.24	58.66	3.55	0.55	2.32
	2	179.04	54.09	3.31	0.52	2.25
	3	193.52	55.77	3.47	0.54	2.29

ESTIMATED NUMBER OF OOCYSTS USING QPCR

Exp. #		Initial Oocysts from Standard curve per pg*.	Amount of C. Parvum DNA Extracted (µg/2 L)	Amount of C. Parvum DNA Used for Dilution	Dilution Factor per amount of µL used for PCR amplification.	Estimated Number of Oocysts. (Oocysts/ 2 L water sample)
		Supematant Samples				
1a	1	14.2/pg*	50 µg$^{\xi}$	2 µL	10^{-3} per 5 µL	284,000
	2	22.8/pg	50 µg	1 µL	10^{-3} per 4 µL	285,000
	3	28.3/pg	50 µg	1 µL	10^{-3} per 5 µL	283,000
					Average	284,000
1b	1	39.4/pg	50 µg	1 µL	10^{-3} per 7 µL	281,429
	2	40.0/pg	50 µg	1 µL	10^{-3} per 7 µL	285,714
	3	34.0/pg	50 µg	1 µL	10^{-3} per 6 µL	283,333
					Average	283,492
2a	1	32.5/pg	50 µg	1 µL	10^{-3} per 5 µL	325,000
	2	32.2/pg	50 µg	1 µL	10^{-3} per 5 µL	322,000
	3	45.0/pg	50 µg	1 µL	10^{-3} per 7 µL	321,429
					Average	322,810
2b	1	19.4/pg	50 µg	1 µL	10^{-3} per 3 µL	323,334
	2	25.7/pg	50 µg	1 µL	10^{-3} per 4 µL	321,250
	3	19.3/pg	50 µg	1 µL	10^{-3} per 3 µL	321,667
					Average	322,084
3a	1	44.9/pg	50 µg	1 µL	10^{-3} per 7 µL	320,714
	2	38.5/pg	50 µg	1 µL	10^{-3} per 6 µL	320,833
	3	38.6/pg	50 µg	1 µL	10^{-3} per 6 µL	321,667
					Average	321,071
3b	1	19.0/pg	50 µg	2 µL	10^{-3} per 6 µL	316,667
	2	19.1/pg	50 µg	2 µL	10^{-3} per 6 µL	318,333
	3	25.3/pg	50 µg	2 µL	10^{-3} per 8 µL	316,250
					Average	317,083
4a	1	28.8/pg	50 µg	1 µL	10^{-3} per 5 µL	288,000
	2	40.1/pg	50 µg	1 µL	10^{-3} per 7 µL	286,429
	3	40.3/pg	50 µg	1 µL	10^{-3} per 7 µL	287,857
					Average	287,429
4b	1	39.8/pg	50 µg	1 µL	10^{-3} per 7 µL	284,286
	2	40.1/pg	50 µg	1 µL	10^{-3} per 7 µL	286,429
	3	39.9/pg	50 µg	1 µL	10^{-3} per 7 µL	285,000
					Average	285,238

* pg. = pico gram = 1 x 10^{-12} g. $^{\xi}$µg = microgram = 1 x 10^{-6} g.

ESTIMATED NUMBER OF OOCYSTS USING QPCR

Exp. #		Initial Oocysts from Standard curve per pg*.	Amount of C. Parvum DNA Extracted (µg/ 2 L)	Amount of C. Parvum DNA Used for Dilution	Dilution Factor per amount of µL used for PCR amplification.	Estimated Number of Oocysts. (Oocysts/ 2 L water sample).
		Supernatant Samples				
5a	1	28/pg	50 µg	1µL	10^{-3} per 5 µL	280,000
	2	38.7/pg	50 µg	1µL	10^{-3} per 7 µL	276,429
	3	33.2/pg	50 µg	1µL	10^{-3} per 6µL	276,667
					Average	277,699
5b	1	39.2/pg	50 µg	1µL	10^{-3} per 7 µL	280,000
	2	33.5/pg	50 µg	1µL	10^{-3} per 6 µL	279,167
	3	39.5/pg	50 µg	1µL	10^{-3} per 7 µL	282,143
					Average	280,437
6a	1	31.5/pg	50 µg	1µL	10^{-3} per 5 µL	312,000
	2	37.0/pg	50 µg	1µL	10^{-3} per 6 µL	308,334
	3	24.8/pg	50 µg	1µL	10^{-3} per 4 µL	310,000
					Average	310,112
6b	1	37.5/pg	50 µg	1µL	10^{-3} per 6 µL	312,500
	2	37.4/pg	50 µg	1µL	10^{-3} per 6 µL	311,667
	3	31.1/pg	50 µg	1µL	10^{-3} per 5 µL	311,000
					Average	311,722
7a	1	30.8/pg	50 µg	1µL	10^{-3} per 5 µL	308,000
	2	24.5/pg	50 µg	1µL	10^{-3} per 4 µL	306,250
	3	30.5/pg	50 µg	1µL	10^{-3} per 5 µL	305,000
					Average	306,417
7b	1	25.1/pg	50 µg	1µL	10^{-3} per 4 µL	313,750
	2	24.9/pg	50 µg	1µL	10^{-3} per 4 µL	311,250
	3	24.8/pg	50 µg	1µL	10^{-3} per 4 µL	310,000
					Average	311,667
8a	1	28.9/pg	50 µg	1µL	10^{-3} per 5 µL	289,000
	2	29.0/pg	50 µg	1µL	10^{-3} per 5 µL	290,000
	3	34.6/pg	50 µg	1µL	10^{-3} per 6 µL	288,333
					Average	289,111
8b	1	29.9/pg	50 µg	1µL	10^{-3} per 5 µL	299,000
	2	36.1/pg	50 µg	1µL	10^{-3} per 6 µL	300,833
	3	36.2/pg	50 µg	1µL	10^{-3} per 6 µL	301,667
					Average	300,500

* pg. = pico gram = 1 x 10^{-12} g. ^{5}µg = microgram = 1 x 10^{-6} g.

ESTIMATED NUMBER OF OOCYSTS USING QPCR

Exp. #		Initial Oocysts from Standard curve per pg*.	Amount of C. Parvum DNA Extracted (µg/2 L)	Amount of C. Parvum DNA Used for Dilution	Dilution Factor per amount of µL used for PCR amplification.	Estimated Number of Oocysts (Oocysts/ 2 L water sample)
		Sludge Samples				
1a	1	3.2/pg	25 µg	3 µL	10^{-3} per 5 µL	48,000
	2	4.8/pg	25 µg	2 µL	10^{-3} per 5 µL	48,000
	3	4.1/pg	25 µg	2 µL	10^{-3} per 4 µL	51,250
					Average	49,083
1b	1	4.1/pg	25 µg	2 µL	10^{-3} per 4 µL	51,250
	2	3.2/pg	25 µg	3 µL	10^{-3} per 5 µL	48,000
	3	4.0/pg	25 µg	2 µL	10^{-3} per 4 µL	50,000
					Average	49,750
2a	1	1.7/pg	25 µg	2 µL	10^{-3} per 10 µL	85,00
	2	2.2/pg	25 µg	2 µL	10^{-3} per 10 µL	11,000
	3	2.3/pg	25 µg	2 µL	10^{-3} per 10 µL	11,500
					Average	10,334
2b	1	2.0/pg	25 µg	2 µL	10^{-3} per 10 µL	10,000
	2	2.4/pg	25 µg	2 µL	10^{-3} per 10 µL	12,000
	3	2.3/pg	25 µg	2 µL	10^{-3} per 10 µL	11,500
					Average	11,167
3a	1	2.5/pg	25 µg	2 µL	10^{-3} per 10 µL	12,500
	2	2.5/pg	25 µg	2 µL	10^{-3} per 10 µL	12,500
	3	2.3/pg	25 µg	2 µL	10^{-3} per 10 µL	11,500
					Average	12,167
3b	1	3.3/pg	25 µg	2 µL	10^{-3} per 10 µL	16,500
	2	3.0/pg	25 µg	2 µL	10^{-3} per 10 µL	15,000
	3	3.4/pg	25 µg	2 µL	10^{-3} per 10 µL	17,000
					Average	16,167
4a	1	4.5/pg	25 µg	4 µL	10^{-3} per 10 µL	45,000
	2	4.7/pg	25 µg	4 µL	10^{-3} per 10 µL	47,000
	3	4.6/pg	25 µg	4 µL	10^{-3} per 10 µL	46,000
					Average	46,000
4b	1	4.9/pg	25 µg	4 µL	10^{-3} per 10 µL	49,000
	2	4.7/pg	25 µg	4 µL	10^{-3} per 10 µL	47,000
	3	3.2/pg	25 µg	6 µL	10^{-3} per 10 µL	48,000
					Average	48,000

* pg. = pico gram = 1 x 10^{-12} g. $^{\xi}$µg = microgram = 1 x 10^{-6} g.

ESTIMATED NUMBER OF OOCYSTS USING QPCR

Exp. #		Initial Oocysts from Standard curve per pg*.	Amount of C. Parvum DNA Extracted (µg/ 2L)	Amount of C. Parvum DNA Used for Dilution	Dilution Factor per amount of µL used for PCR amplification.	Estimated Number of Oocysts (Oocysts/2 L water sample)
		Sludge Samples				
5a	1	3.2/pg	25 µg	2 µL	10^{-3} per 3 µL	53,333
	2	4.5/pg	25 µg	2 µL	10^{-3} per 4 µL	56,250
	3	4.6/pg	25 µg	2 µL	10^{-3} per 4 µL	57,500
					Average	55,694
5b	1	3.2/pg	25 µg	2 µL	10^{-3} per 3 µL	53,333
	2	4.3/pg	25 µg	2 µL	10^{-3} per 4 µL	53,750
	3	4.1/pg	25 µg	2 µL	10^{-3} per 4 µL	51,250
					Average	52,778
6a	1	4.2/pg	25 µg	2 µL	10^{-3} per 10 µL	21,000
	2	5.0/pg	25 µg	2 µL	10^{-3} per 10 µL	25,000
	3	4.6/pg	25 µg	2 µL	10^{-3} per 10 µL	23,000
					Average	23,000
6b	1	4.1/pg	25 µg	2 µL	10^{-3} per 10 µL	20,500
	2	4.3/pg	25 µg	2 µL	10^{-3} per 10 µL	21,500
	3	4.5/pg	25 µg	2 µL	10^{-3} per 10 µL	22,500
					Average	21,500
7a	1	6.7/pg	25 µg	3 µL	10^{-4} per 2 µL	25,125
	2	5.4/pg	25 µg	2 µL	10^{-3} per 10 µL	27,000
	3	7.5/pg	25 µg	3 µL	10^{-4} per 2 µL	28125
					Average	26,750
7b	1	3.9/pg	25 µg	2 µL	10^{-3} per 10 µL	19,500
	2	4.4/pg	25 µg	2 µL	10^{-3} per 10 µL	22,000
	3	6.2/pg	25 µg	3 µL	10^{-4} per 2 µL	23,250
					Average	21,583
8a	1	5.9/pg	25 µg	3 µL	10^{-3} per 10 µL	44,250
	2	5.8/pg	25 µg	3 µL	10^{-3} per 10 µL	43,500
	3	6.0/pg	25 µg	3 µL	10^{-3} per 10 µL	45,000
					Average	44,250
8b	1	4.6/pg	25 µg	3 µL	10^{-3} per 10µL	34,500
	2	4.3/pg	25 µg	3 µL	10^{-3} per 10 µL	32,250
	3	4.2/pg	25 µg	3 µL	10^{-3} per 10 µL	31,500
					Average	32,750

* pg. = pico gram = 1 x 10^{-12} g. $^{\xi}$µg = microgram = 1 x 10^{-6} g.

APPENDIX D

SLUDGE TEST RESULTS

Sludge reaction		NO_3-N (mg/L)		Test Index	
pH:	8.7	Surface:	2	P (mg/L):	16
Buffer Index				K (mg/L):	56
Secondary nutrients				Micro-nutrients	
Surface SO_4-S (mg/L):	176	Ca (mg/L):	31676.5	Fe (mg/L):	11
Subsoil SO_4-S (mg/L):		Mg (mg/L):	4729	Zn (mg/L):	0.26
				B (mg/L):	0.22

Sludge Salinity Test Results

Cations		Anions		Other	
Sodium (mg/L)	88	Nitrate-N (mg/L)	2	pH	7.1
Calcium (mg/L)	55	Chloride (mg/L)	128	EC (µmhos/cm)	726
Magnesium (mg/L)	2	Sulfate (mg/L)	105	Texture	fine
Potassium (mg/L)	10	Carbonate (mg/L)	0	Boron (mg/L)	0.07
		Bicarbonate (mg/L)	90		

Derived Values		Derived Values (cont'd)	
Total Soluble Salts (TSS in mg/L)	480	Exchangeable Sodium Percentage (ESP)	3.3
Sodium Adsorption Ratio (SAR)	3.2	Exchangeable Potassium Percentage (EPP)	5.5
Potassium Adsorption Ratio (SPR)	0.2		

SOIL TEST RESULTS

Soil reaction		NO_3-N (mg/L)		Test Index	
pH:	6.7	Surface:	3	P (mg/L):	8.5
Buffer Index				K (mg/L):	152.5

Secondary nutrients				Micro-nutrients	
Surface SO_4-S (mg/L):	30	Ca (mg/L):		Fe (mg/L):	23.3
Subsoil SO_4-S (mg/L):		Mg (mg/L):	635	Zn (mg/L):	14.10
				B (mg/L):	0.71

Textural Class	Sand (%)	Silt (%)	Clay (%)
Sandy Loam	62.5	20.0	17.5

Soil Salinity Test Results

Cations		Anions		Other	
Sodium (mg/L)	5	Nitrate-N (mg/L)	< 1	pH	8.0
Calcium (mg/L)	61	Chloride (mg/L)	10	EC (µmhos/cm)	434
Magnesium (mg/L)	21	Sulfate (mg/L)	23	Texture	Medium
Potassium (mg/L)	8	Carbonate (mg/L)	0	Boron (mg/L)	0.14
		Bicarbonate (mg/L)	242		

Derived Values		Derived Values (cont'd)	
Total Soluble Salts (TSS in mg/L)	370	Exchangeable Sodium Percentage (ESP)	< 0.1
Sodium Adsorption Ratio (SAR)	0.1	Exchangeable Potassium Percentage (EPP)	4.8
Potassium Adsorption Ratio (SPR)	0.1		

Interpretations for Comprehensive Salinity from saturated paste extract: Total soluble salts and the level of individual chemicals in this soil are within normal ranges for a productive soil and neither salinity should be factors limiting crop production. NO_3-N, P, and K are plant availability. ppm = mg/L = lbs/acre

ESTIMATION OF VIABLE AND NONVIABLE SENTINAL OOCYSTS

Sample #	# of Oocysts Spiked g^{-1}	Estimated Oocysts per gram of mixture of soil and sludge		Percent Viable (%)	Percent Non-Viable	Log Inactivation	Inactivation Rate	Inactivation Period
		Viable	Non viable					
Count 1	2.5×10^5	2.35×10^5	7×10^3	94	2.8	0.027	0.0036	
Count 2		2.30×10^5	9×10^3	92	3.6	0.036	0.0049	
Avg. #1		2.33×10^5	8×10^3	93	3.2	0.032	0.0043	
								17
Count 1	2.5×10^5	2.32×10^5	6.12×10^3	92.8	2.4	0.032	0.0044	
Count 2		2.29×10^5	9.5×10^3	91.6	3.8	0.038	0.0053	
Avg. #2		2.30×10^5	7.8×10^3	92.2	3.1	0.035	0.0048	
Combined Avg. 1 & 2	2.5×10^5	**2.32×10^5**	**7.9×10^3**	**92.6**	**3.2**	**0.034**	**0.0045**	
Count 1	2.5×10^5	2.30×10^5	9×10^3	92.0	3.6	0.036	0.0028	
Count 2		2.26×10^5	11.6×10^3	90.4	4.6	0.044	0.0034	
Avg. # 1		2.28×10^5	10.3×10^3	91.1	4.1	0.041	0.0031	
								30
Count 1	2.5×10^5	2.26×10^5	9.4×10^3	90.4	3.8	0.044	0.0034	
Count 2		2.25×10^5	13×10^3	90.0	5.2	0.046	0.0035	
Avg. #2		2.26×10^5	11.2×10^3	90.2	4.5	0.045	0.0035	
Combined Avg. 1 & 2	2.5×10^5	**2.27×10^5**	**10.8×10^3**	**90.6**	**4.3**	**0.043**	**0.0033**	
Count 1	2.5×10^5	2.07×10^5	30×10^3	82.8	12.0	0.082	0.0042	
Count 2		2.05×10^5	32×10^3	82.0	12.8	0.086	0.0044	
Avg. #1		2.06×10^5	31×10^3	82.4	12.4	0.084	0.0043	
								45
Count 1	2.5×10^5	2.07×10^5	31×10^3	82.8	12.4	0.082	0.0042	
Count 2		2.05×10^5	32×10^3	82.0	12.8	0.086	0.0044	
Avg. #2		2.06×10^5	31.5×10^3	82.4	12.6	0.084	0.0043	
Combined Avg. 1 & 2	2.5×10^5	2.06×10^5	**31.3×10^3**	**82.4**	**12.5**	**0.084**	**0.0043**	
Count 1	2.5×10^5	1.26×10^5	1.11×10^5	50.4	44.4	0.30	0.011	
Count 2		1.21×10^5	1.15×10^5	48.4	46.0	0.32	0.012	
Avg. # 1		1.24×10^5	1.13×10^5	49.4	45.2	0.307	0.012	60
Count 1	2.5×10^5	1.25×10^5	1.11×10^5	50.0	44.5	0.30	0.011	
Count 2		1.22×10^5	1.15×10^5	48.8	46.1	0.31	0.012	
Avg. #2		1.24×10^5	1.13×10^5	49.4	45.3	0.31	0.012	
Combined Avg. 1 & 2	2.5×10^5	1.24×10^5	1.13×10^5	**49.4**	**45.2**	**0.31**	**0.012**	

ESTIMATION OF VIABLE AND NONVIABLE CONTROL OOCYSTS

	# of Oocysts Spiked mL^{-1}	Estimated Oocysts mL^{-1}		Percent Viable (%)	Percent Non-Viable	Log Inactivation	Inactivation Rate	Inactivation Period (Day)
		Viable	Non viable					
Sample #		Controls						
Count 1	2.5×10^5	2.41×10^5	-	96.4	-	0.015	0.0022	17
Count 2		2.41×10^5	-	96.4	-	0.016	0.0022	
Count 3		2.4×10^5	-	96.0	-	0.018	0.0024	
Count 4		2.4×10^5	-	96.0	-	0.019	0.0024	
Average		**2.41×10^5**	**-**	**96.2**	**-**	**0.017**	**0.0023**	
Count 1	2.5×10^5	2.37×10^5	-	94.8	-	0.023	0.0018	30
Count 2		2.36×10^5	1.0×10^3	94.4	0.4	0.025	0.0019	
Count 3		2.35×10^5	1.2×10^3	94.0	0.48	0.027	0.0021	
Count 4		2.35×10^5	1.8×10^3	94.0	0.72	0.027	0.0021	
Average		**2.36×10^5**	1.0×10^3	**94.3**	**0.40**	**0.025**	**0.0019**	
Count 1	2.5×10^5	2.34×10^5	1×10^3	93.6	0.40	0.029	0.0015	45
Count 2		2.33×10^5	6×10^3	93.2	2.4	0.031	0.0016	
Count 3		2.31×10^5	7×10^3	92.4	2.8	0.034	0.0018	
Count 4		2.29×10^5	9×10^3	91.6	3.6	0.038	0.0019	
Average		**2.32×10^5**	**5.8×10^3**	**92.7**	**2.3**	**0.033**	**0.0017**	
Count 1	2.5×10^5	2.17×10^5	2.1×10^3	86.8	8.4	0.061	0.0023	60
Count 2		2.17×10^5	2.1×10^3	86.8	8.4	0.061	0.0023	
Count 3		2.15×10^5	2.2×10^3	86.0	8.8	0.066	0.0025	
Count 4		2.09×10^5	2.7×10^3	83.6	10.8	0.078	0.003	
Average		2.15×10^5	2.28×10^3	85.8	9.1	0.067	0.0025	
		SALINITY @ 7 °C						
Count 1	2.5×10^5	2.34×10^5	-	96.0	-	0.018	0.0024	17
Count 2		2.37×10^5	-	94.8	-	0.023	0.0031	
Count 3		2.35×10^5	1.8×10^3	94.0	0.72	0.027	0.0036	
Count 4		2.32×10^5	8×10^3	92.8	3.2	0.032	0.0044	
Average		**2.36×10^5**	**2.45×10^3**	**94.4**	**.98**	**0.025**	**0.0034**	
		SALINITY @ 14 °C						
Count 1	2.5×10^5	2.36×10^5	1.8×10^3	94.4	0.72	0.025	0.0034	17
Count 2		2.34×10^5	2×10^3	93.6	0.8	0.028	0.0039	
Count 3		2.34×10^5	5×10^3	93.6	2.0	0.028	0.0039	
Count 4		2.31×10^5	8×10^3	92.4	3.2	0.034	0.0046	
Average		2.34×10^5	**4.2×10^3**	**93.6**	**1.7**	**0.029**	**0.0046**	
		SLAKE LIME @ 7 °C						
Count 1	2.5×10^5	2.34×10^5	2×10^3	93.6	0.8	0.029	0.0039	17
Count 2		2.34×10^5	5×10^3	93.6	2.0	0.029	0.0039	
Count 3		2.33×10^5	6×10^3	93.2	2.4	0.031	0.0041	
Count 4		2.30×10^5	8×10^3	92.0	3.2	0.036	0.0049	
Average		2.33×10^5	5.25×10^3	**93.1**	**2.1**	**0.031**	**0.0042**	
		SLAKE LIME @ 14 °C						
Count 1	2.5×10^5	2.34×10^5	1×10^3	93.6	0.4	0.029	0.0039	17
Count 2		2.32×10^5	6×10^3	92.8	2.4	0.032	0.0044	
Count 3		2.31×10^5	7×10^3	92.4	2.8	0.034	0.0046	
Count 4		2.30×10^5	9×10^3	92.0	3.6	0.036	0.0049	
Average		**2.32×10^5**	**5.75×10^3**	**92.7**	**2.3**	**0.033**	**0.0045**	

ESTIMATION OF VIABLE AND NON VIABLE OOCYSTS

	# of Oocysts Spiked mL^{-1}	Estimated Oocysts per mL		Percent Viable (%)	Percent Non-Viable	Log Inactivation	Inactivation Rate	Inactivation Period
CATIONIC POLYMER @ 7 ° C								
Sample #		Viable	Non viable					
Count 1	2.5×10^5	2.4×10^5	-	96.0		0.018	0.0024	
Count 2		2.38×10^5	-	95.2		0.021	0.0029	
Count 3		2.37×10^5	1×10^3	94.8	0.4	0.023	0.0031	17
Count 4		2.30×10^5	2×10^3	92.0	0.8	0.036	0.0049	
Average		**2.36×10^5**	**7.2×10^2**	**94.5**	**0.72**	**0.025**	**0.0033**	
CATIONIC POLYMER @ 14° C								
Count 1	2.5×10^5	2.37×10^5	1.0×10^3	94.8	0.40	0.023	0.0031	
Count 2		**2.36×10^5**	**2.0×10^3**	94.4	0.80	0.025	0.0034	
Count 3		2.36×10^5	3.1×10^3	94.4	1.24	0.025	0.0034	17
Count 4		2.35×10^5	3.6×10^3	94	1.44	0.027	0.0036	
Average		**2.36×10^5**	**2.43×10^3**	**94.4**	**0.97**	**0.025**	**0.0034**	
	CHLORINE @ 7 ° C							
Count 1	2.5×10^5	2.37×10^5	3.0×10^3	94.8	1.2	0.023	0.0031	
Count 2		2.35×10^5	3.2×10^3	94.0	1.28	0.027	0.0036	
Count 3		2.34×10^5	4.5×10^3	93.6	1.80	0.029	0.0039	17
Count 4		2.32×10^5	9.2×10^3	92.8	3.68	0.032	0.0044	
Average		**2.35×10^5**	**5.0×10^3**	**93.8**	**2.0**	**0.028**	**0.0038**	
CHLORINE @ 14 ° C								
Count 1	2.5×10^5	2.35×10^5	1.8×10^3	94.0	0.72	0.027	0.0036	
Count 2		2.34×10^5	3.2×10^3	93.6	1.28	0.029	0.0039	
Count 3		2.34×10^5	6.1×10^3	93.6	2.44	0.029	0.0039	17
Count 4		2.33×10^5	8.0×10^3	93.1	3.2	0.031	0.0041	
Average		**2.34×10^5**	**4.78×10^3**	**93.6**	**1.91**	**0.29**	**0.0039**	
ALUM @ 7 ° C								
Count 1	2.5×10^5	2.40×10^5	-	96.0	-	0.018	0.0024	
Count 2		2.37×10^5	1×10^3	94.8	0.4	0.023	0.0031	17
Count 3		2.36×10^5	2×10^3	94.4	0.8	0.025	0.0034	
Count 4		2.36×10^5	2.5×10^3	94.4	1.0	0.025	0.0034	
Average		**2.37×10^5**	**1.38×10^3**	**94.9**	**0.55**	**0.023**	**0.0031**	
ALUM @ 14 ° C								
Count 1	2.5×10^5	2.35×10^5	2.0×10^5	94.0	0.8	0.027	0.0036	
Count 2		2.34×10^5	2.5×10^5	93.6	1.0	0.029	0.0039	
Count 3		2.33×10^5	4.9×10^5	93.2	1.96	0.031	0.0041	17
Count 4		2.33×10^5	6.0×10^5	93.2	2.4	0.031	0.0041	
Average		2.34×10^5	3.85×10^5	93.5	1.54	0.030	0.0039	
TEMPERATURE @ 7 ° C (Distilled Water)								
Count 1	2.5×10^5	2.40×10^5	-	96.0	-	0.018	0.0024	
Count 2		2.39×10^5	-	95.6	-	0.019	0.0026	
Count 3		2.38×10^5	-	95.2	-	0.021	0.0029	17
Count 4		2.37×10^5	-	94.8	-	0.023	0.0031	
Average		2.39×10^5	-	95.4	-	0.02	0.0028	
TEMPERATURE @ 14 ° C (Distilled Water)								
Count 1	2.5×10^5	2.40×10^5	-	96.0	-	0.018	0.0024	
Count 2		2.38×10^5	1×10^3	95.2	0.4	0.021	0.0029	
Count 3		2.37×10^5	1×10^3	94.8	0.4	0.023	0.0031	17
Count 4		2.36×10^5	2.2×10^3	94.4	0.88	0.025	0.0034	
Average		2.38×10^5	1.05×10^3	95.1	0.84	0.022	0.0029	

DAILY SOIL TEMPERATURE MEASUREMENT

Day	Soil pH	Temperature (^{0} C)		Average Measurement Temperature	Data/Month Measured
		Morning	Evening		
1		5	9	7	2 – 12 - 2000
2		8	8	8	2 – 13 – 2000
3		5	9	7	2 – 14 – 2000
4		11	12	11.5	2 – 15 – 2000
5		11	12	11.5	2 – 16 – 2000
6		9	9	9	2 – 17 – 2000
7		8	8	8	2 – 18 – 2000
8		5	9	7	2 – 19 - 2000
9		5	9.5	7.25	2 – 20 – 2000
10		13	14	13.5	2 – 21 – 2000
11		14	14	14	2 – 22 – 2000
12		14	14.5	14.25	2 – 23 – 2000
13		15	15	15	2 – 24 – 2000
14		13.5	14.5	14	2 – 25 – 2000
15	Measurement using $CaCl_2$ pH range 6.70 to 6.75	9	12	10.5	2 – 26- 2000
16		7	14	10.5	2 – 27 – 2000
17		8	13	10.5	2 – 28 – 2000
18		13	15	14	2 – 29 – 2000
19		14	16	15	3 – 01 – 2000
20		10	12	11	3 – 02 – 2000
21		8	8	8	3 – 03 – 2000
22		5	14	9.5	3 – 04 - 2000
23		8	13	10.5	3 – 05 – 2000
24		13	17	15	3 – 06 – 2000
25		12	17	14.5	3 – 07 – 2000
26		9	12	10.5	3 – 08 - 2000
27		10	13	11.5	3 – 09 – 2000
28		10	10	10	3 – 10 – 2000
29		5	10	7.5	3 – 11- 2000
30		5	11	8	3 – 12 – 2000
31		10	16	13	3 – 13 – 2000
32	Measurement using Deionized water pH range 6.98 to 7.05	12	15	13.5	3 – 14 – 2000
33		13	17	15	3 – 15 - 2000
34		8	8	8	3 – 16 – 2000
35		8	9	8.5	3 – 17 – 2000
36		9	9	9	3 – 18 - 2000
37		7	11	9	3 – 19 – 2000
38		13	14	13.5	3 – 20 – 2000
39		10	11	10.5	3 – 21 – 2000
40		10	12	11	3 – 22 - 2000
41		12	14	13	3 – 23 – 2000
42		16	18	17	3 – 24 – 2000
43		12.5	18	15.25	3 – 25- 2000
44		12	18	15	3 – 26– 2000
45		10	11	10.5	3 – 27 – 2000
46		12	16	14	3 – 28 – 2000
47		10	12	11	3 – 29 - 2000
48		12	15	13.5	3 – 30 – 2000
49		10	12	11	3 – 31 – 2000
50		9	12	10.5	4 – 01 - 2000
51		9.5	11	10.25	4 – 02 – 2000
52		10	11	10.5	4 – 03 – 2000
53		13	16	14.5	4 – 04 – 2000
54		16	21	18.5	4 – 05 - 2000
55		15.5	24	19.75	4 – 06 – 2000
56		18	19	18.5	4 – 07 – 2000
57		9.5	15	12.25	4 – 08 – 2000
58		9	18	13.5	4 – 09 – 2000
59		10	19	14.5	4 – 10 – 2000
60		9	18	13.5	4 – 11 - 2000

APPENDIX E, Statistical Analysis for Oocysts Estimated from the Supernatant Samples

Oneway Anova

Summary of Fit

RSquare	0.994621
RSquare Adj	0.9921
Root Mean Sq	1434.78
Mean of Resp	300674.4
Observations	48

Analysis of Variance

Source	DF	Sum of Squar	Mean Square	F Ratio
Model	15	1.22E+10	8.12E+08	394.4717
Error	32	65875000	2058594	Prob>F
C Total	47	1.22E+10	2.61E+08	<.0001

Means for Oneway Anova

Level	Number	Mean	Std Error
1-a	3	284000	828.37
1-b	3	283490	828.37
2-a	3	322810	828.37
2-b	3	322083	828.37
3-a	3	321070	828.37
3-b	3	317083	828.37
4-a	3	287430	828.37
4-b	3	285240	828.37
5-a	3	277620	828.37
5-b	3	280437	828.37
6-a	3	310110	828.37
6-b	3	311723	828.37
7-a	3	306417	828.37
7-b	3	311667	828.37
8-a	3	289110	828.37
8-b	3	300500	828.37

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean
1-a	3	284000	1000	577.4
1-b	3	283490	2144.48	1238.1
2-a	3	322810	1917.89	1107.3
2-b	3	322083	1099.88	635
3-a	3	321070	523.07	302
3-b	3	317083	1099.88	635
4-a	3	287430	868.85	501.6
4-b	3	285240	1090	629.3
5-a	3	277620	2061.14	1190
5-b	3	280437	1532.39	884.7
6-a	3	310110	1837.47	1060.9

6-b	3	311723	751.42	433.8
7-a	3	306417	1506.93	870
7-b	3	311667	1909.41	1102.4
8-a	3	289110	840.42	485.2
8-b	3	300500	1365.25	788.2

Means Comparisons

Dif=Mean[i]-M	2-a	2-b	3-a	3-b	6-b	7-b	6-a	7-a	8-b	8-a	4-a	4-b	1-a	1-b	5-b	5-a
2-a	0	726.7	1740	5726.7	11086.7	11143.3	12700	16393.3	22310	33700	35380	37570	38810	39320	42373.3	45190
2-b	-726.7	0	1013.3	5000	10360	10416.7	11973.3	15666.7	21583.3	32973.3	34653.3	36843.3	38083.3	38593.3	41646.7	44463.3
3-a	-1740	-1013.3	0	3986.7	9346.7	9403.3	10960	14653.3	20570	31960	33640	35830	37070	37580	40633.3	43450
3-b	-5726.7	-5000	-3986.7	0	5360	5416.7	6973.3	10666.7	16583.3	27973.3	29653.3	31843.3	33083.3	33593.3	36646.7	39463.3
6-b	-11086.7	-10360	-9346.7	-5360	0	56.7	1613.3	5306.7	11223.3	22613.3	24293.3	26483.3	27723.3	28233.3	31286.7	34103.3
7-b	-11143.3	-10416.7	-9403.3	-5416.7	-56.7	0	1556.7	5250	11166.7	22556.7	24236.7	26426.7	27666.7	28176.7	31230	34046.7
6-a	-12700	-11973.3	-10960	-6973.3	-1613.3	-1556.7	0	3693.3	9610	21000	22680	24870	26110	26620	29673.3	32490
7-a	-16393.3	-15666.7	-14653.3	-10666.7	-5306.7	-5250	-3693.3	0	5916.7	17306.7	18986.7	21176.7	22416.7	22926.7	25980	28796.7
8-b	-22310	-21583.3	-20570	-16583.3	-11223.3	-11166.7	-9610	-5916.7	0	11390	13070	15260	16500	17010	20063.3	22880
8-a	-33700	-32973.3	-31960	-27973.3	-22613.3	-22556.7	-21000	-17306.7	-11390	0	1680	3870	5110	5620	8673.3	11490
4-a	-35380	-34653.3	-33640	-29653.3	-24293.3	-24236.7	-22680	-18986.7	-13070	-1680	0	2190	3430	3940	6993.3	9810
4-b	-37570	-36843.3	-35830	-31843.3	-26483.3	-26426.7	-24870	-21176.7	-15260	-3870	-2190	0	1240	1750	4803.3	7620
1-a	-38810	-38083.3	-37070	-33083.3	-27723.3	-27666.7	-26110	-22416.7	-16500	-5110	-3430	-1240	0	510	3563.3	6380
1-b	-39320	-38593.3	-37580	-33593.3	-28233.3	-28176.7	-26620	-22926.7	-17010	-5620	-3940	-1750	-510	0	3053.3	5870
5-b	-42373.3	-41646.7	-40633.3	-36646.7	-31286.7	-31230	-29673.3	-25980	-20063.3	-8673.3	-6993.3	-4803.3	-3563.3	-3053.3	0	2816.7
5-a	-45190	-44463.3	-43450	-39463.3	-34103.3	-34046.7	-32490	-28796.7	-22880	-11490	-9810	-7620	-6380	-5870	-2816.7	0

Alpha= 0.05

Comparisons for each pair using Student's t

t 2.03692

Abs(Dif)-LSD	2-a	2-b	3-a	3-b	6-b	7-b	6-a	7-a	8-b	8-a	4-a	4-b	1-a	1-b	5-b	5-a
2-a	-2386.2	-1659.6	-646.2	3340.4	8700.4	8757.1	10313.8	14007.1	19923.8	31313.8	32993.8	35183.8	36423.8	36933.8	39987.1	42803.8
2-b	-1659.6	-2386.2	-1372.9	2613.8	7973.8	8030.4	9587.1	13280.4	19197.1	30587.1	32267.1	34457.1	35697.1	36207.1	39260.4	42077.1
3-a	-646.2	-1372.9	-2386.2	1600.4	6960.4	7017.1	8573.8	12267.1	18183.8	29573.8	31253.8	33443.8	34683.8	35193.8	38247.1	41063.8
3-b	3340.4	2613.8	1600.4	-2386.2	2973.8	3030.4	4587.1	8280.4	14197.1	25587.1	27267.1	29457.1	30697.1	31207.1	34260.4	37077.1
6-b	8700.4	7973.8	6960.4	2973.8	-2386.2	-2329.6	-772.9	2920.4	8837.1	20227.1	21907.1	24097.1	25337.1	25847.1	28900.4	31717.1
7-b	8757.1	8030.4	7017.1	3030.4	-2329.6	-2386.2	-829.6	2863.8	8780.4	20170.4	21850.4	24040.4	25280.4	25790.4	28843.8	31660.4
6-a	10313.8	9587.1	8573.8	4587.1	-772.9	-829.6	-2386.2	1307.1	7223.8	18613.8	20293.8	22483.8	23723.8	24233.8	27287.1	30103.8
7-a	14007.1	13280.4	12267.1	8280.4	2920.4	2863.8	1307.1	-2386.2	3530.4	14920.4	16600.4	18790.4	20030.4	20540.4	23593.8	26410.4
8-b	19923.8	19197.1	18183.8	14197.1	8837.1	8780.4	7223.8	3530.4	-2386.2	9003.8	10683.8	12873.8	14113.8	14623.8	17677.1	20493.8
8-a	31313.8	30587.1	29573.8	25587.1	20227.1	20170.4	18613.8	14920.4	9003.8	-2386.2	-706.2	1483.8	2723.8	3233.8	6287.1	9103.8
4-a	32993.8	32267.1	31253.8	27267.1	21907.1	21850.4	20293.8	16600.4	10683.8	-706.2	-2386.2	-196.2	1043.8	1553.8	4607.1	7423.8
4-b	35183.8	34457.1	33443.8	29457.1	24097.1	24040.4	22483.8	18790.4	12873.8	1483.8	-196.2	-2386.2	-1146.2	-636.2	2417.1	5233.8
1-a	36423.8	35697.1	34683.8	30697.1	25337.1	25280.4	23723.8	20030.4	14113.8	2723.8	1043.8	-1146.2	-2386.2	-1876.2	1177.1	3993.8
1-b	36933.8	36207.1	35193.8	31207.1	25847.1	25790.4	24233.8	20540.4	14623.8	3233.8	1553.8	-636.2	-1876.2	-2386.2	667.1	3483.8
5-b	39987.1	39260.4	38247.1	34260.4	28900.4	28843.8	27287.1	23593.8	17677.1	6287.1	4607.1	2417.1	1177.1	667.1	-2386.2	430.4
5-a	42803.8	42077.1	41063.8	37077.1	31717.1	31660.4	30103.8	26410.4	20493.8	9103.8	7423.8	5233.8	3993.8	3483.8	430.4	-2386.2

Positive values show pairs of means that are significantly different.

APPENDIX F, Statistical Analysis for Oocysts Estimated from the Sludge Samples

Oneway Anova

Summary of Fit

RSquare	0.994456
RSquare Adj	0.991858
Root Mean Sq	1458.557
Mean of Resp	32565.96
Observations	48

Analysis of Variance

Source	DF	Sum of Squar	Mean Square	F Ratio
Model	15	1.22E+10	8.14E+08	382.6902
Error	32	68076435.3	2127389	Prob>F
C Total	47	1.23E+10	2.61E+08	<.0001

Means for Oneway Anova

Level	Number	Mean	Std Error
1-a	3	49083.3	842.1
1-b	3	49750	842.1
2-a	3	10333.3	842.1
2-b	3	11166.7	842.1
3-a	3	12166.7	842.1
3-b	3	16166.7	842.1
4-a	3	46000	842.1
4-b	3	48000	842.1
5-a	3	55694.3	842.1
5-b	3	52777.7	842.1
6-a	3	23000	842.1
6-b	3	21500	842.1
7-a	3	26750	842.1
7-b	3	21666.7	842.1
8-a	3	44250	842.1
8-b	3	32750	842.1

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean
1-a	3	49083.3	1876.39	1083.3
1-b	3	49750	1639.36	946.5
2-a	3	10333.3	1607.28	928
2-b	3	11166.7	1040.83	600.9
3-a	3	12166.7	577.35	333.3
3-b	3	16166.7	1040.83	600.9
4-a	3	46000	1000	577.4
4-b	3	48000	1000	577.4
5-a	3	55694.3	2138.35	1234.6
5-b	3	52777.7	1339.33	773.3

6-a	3	23000	2000	1154.7
6-b	3	21500	1000	577.4
7-a	3	26750	1515.54	875
7-b	3	21666.7	2020.73	1166.7
8-a	3	44250	750	433
8-b	3	32750	1561.25	901.4

Means Comparisons

Dif=Mean[i]-M	5-a	5-b	1-b	1-a	4-b	4-a	8-a	8-b	7-a	6-a	7-b	6-b	3-b	3-a	2-b	2-a
5-a	0	2916.7	5944.3	6611	7694.3	9694.3	11444.3	22944.3	28944.3	32694.3	34027.7	34194.3	39527.7	43527.7	44527.7	45361
5-b	-2916.7	0	3027.7	3694.3	4777.7	6777.7	8527.7	20027.7	26027.7	29777.7	31111	31277.7	36611	40611	41611	42444.3
1-b	-5944.3	-3027.7	0	666.7	1750	3750	5500	17000	23000	26750	28083.3	28250	33583.3	37583.3	38583.3	39416.7
1-a	-6611	-3694.3	-666.7	0	1083.3	3083.3	4833.3	16333.3	22333.3	26083.3	27416.7	27583.3	32916.7	36916.7	37916.7	38750
4-b	-7694.3	-4777.7	-1750	-1083.3	0	2000	3750	15250	21250	25000	26333.3	26500	31833.3	35833.3	36833.3	37666.7
4-a	-9694.3	-6777.7	-3750	-3083.3	-2000	0	1750	13250	19250	23000	24333.3	24500	29833.3	33833.3	34833.3	35666.7
8-a	-11444.3	-8527.7	-5500	-4833.3	-3750	-1750	0	11500	17500	21250	22583.3	22750	28083.3	32083.3	33083.3	33916.7
8-b	-22944.3	-20027.7	-17000	-16333.3	-15250	-13250	-11500	0	6000	9750	11083.3	11250	16583.3	20583.3	21583.3	22416.7
7-a	-28944.3	-26027.7	-23000	-22333.3	-21250	-19250	-17500	-6000	0	3750	5083.3	5250	10583.3	14583.3	15583.3	16416.7
6-a	-32694.3	-29777.7	-26750	-26083.3	-25000	-23000	-21250	-9750	-3750	0	1333.3	1500	6833.3	10833.3	11833.3	12666.7
7-b	-34027.7	-31111	-28083.3	-27416.7	-26333.3	-24333.3	-22583.3	-11083.3	-5083.3	-1333.3	0	166.7	5500	9500	10500	11333.3
6-b	-34194.3	-31277.7	-28250	-27583.3	-26500	-24500	-22750	-11250	-5250	-1500	-166.7	0	5333.3	9333.3	10333.3	11166.7
3-b	-39527.7	-36611	-33583.3	-32916.7	-31833.3	-29833.3	-28083.3	-16583.3	-10583.3	-6833.3	-5500	-5333.3	0	4000	5000	5833.3
3-a	-43527.7	-40611	-37583.3	-36916.7	-35833.3	-33833.3	-32083.3	-20583.3	-14583.3	-10833.3	-9500	-9333.3	-4000	0	1000	1833.3
2-b	-44527.7	-41611	-38583.3	-37916.7	-36833.3	-34833.3	-33083.3	-21583.3	-15583.3	-11833.3	-10500	-10333.3	-5000	-1000	0	833.3
2-a	-45361	-42444.3	-39416.7	-38750	-37666.7	-35666.7	-33916.7	-22416.7	-16416.7	-12666.7	-11333.3	-11166.7	-5833.3	-1833.3	-833.3	0
Alpha=	0.05															

Comparisons for each pair using Student's t

t	2.03692															
Abs(Dif)-LSD	5-a	5-b	1-b	1-a	4-b	4-a	8-a	8-b	7-a	6-a	7-b	6-b	3-b	3-a	2-b	2-a
5-a	-2425.8	490.9	3518.6	4185.2	5268.6	7268.6	9018.6	20518.6	26518.6	30268.6	31601.9	31768.6	37101.9	41101.9	42101.9	42935.2
5-b	490.9	-2425.8	601.9	1268.6	2351.9	4351.9	6101.9	17601.9	23601.9	27351.9	28685.2	28851.9	34185.2	38185.2	39185.2	40018.6
1-b	3518.6	601.9	-2425.8	-1759.1	-675.8	1324.2	3074.2	14574.2	20574.2	24324.2	25657.6	25824.2	31157.6	35157.6	36157.6	36990.9
1-a	4185.2	1268.6	-1759.1	-2425.8	-1342.4	657.6	2407.6	13907.6	19907.6	23657.6	24990.9	25157.6	30490.9	34490.9	35490.9	36324.2
4-b	5268.6	2351.9	-675.8	-1342.4	-2425.8	-425.8	1324.2	12824.2	18824.2	22574.2	23907.6	24074.2	29407.6	33407.6	34407.6	35240.9
4-a	7268.6	4351.9	1324.2	657.6	-425.8	-2425.8	-675.8	10824.2	16824.2	20574.2	21907.6	22074.2	27407.6	31407.6	32407.6	33240.9
8-a	9018.6	6101.9	3074.2	2407.6	1324.2	-675.8	-2425.8	9074.2	15074.2	18824.2	20157.6	20324.2	25657.6	29657.6	30657.6	31490.9
8-b	20518.6	17601.9	14574.2	13907.6	12824.2	10824.2	9074.2	-2425.8	3574.2	7324.2	8657.6	8824.2	14157.6	18157.6	19157.6	19990.9
7-a	26518.6	23601.9	20574.2	19907.6	18824.2	16824.2	15074.2	3574.2	-2425.8	1324.2	2657.6	2824.2	8157.6	12157.6	13157.6	13990.9
6-a	30268.6	27351.9	24324.2	23657.6	22574.2	20574.2	18824.2	7324.2	1324.2	-2425.8	-1092.4	-925.8	4407.6	8407.6	9407.6	10240.9
7-b	31601.9	28685.2	25657.6	24990.9	23907.6	21907.6	20157.6	8657.6	2657.6	-1092.4	-2425.8	-2259.1	3074.2	7074.2	8074.2	8907.6
6-b	31768.6	28851.9	25824.2	25157.6	24074.2	22074.2	20324.2	8824.2	2824.2	-925.8	-2259.1	-2425.8	2907.6	6907.6	7907.6	8740.9
3-b	37101.9	34185.2	31157.6	30490.9	29407.6	27407.6	25657.6	14157.6	8157.6	4407.6	3074.2	2907.6	-2425.8	1574.2	2574.2	3407.6
3-a	41101.9	38185.2	35157.6	34490.9	33407.6	31407.6	29657.6	18157.6	12157.6	8407.6	7074.2	6907.6	1574.2	-2425.8	-1425.8	-592.4
2-b	42101.9	39185.2	36157.6	35490.9	34407.6	32407.6	30657.6	19157.6	13157.6	9407.6	8074.2	7907.6	2574.2	-1425.8	-2425.8	-1592.4
2-a	42935.2	40018.6	36990.9	36324.2	35240.9	33240.9	31490.9	19990.9	13990.9	10240.9	8907.6	8740.9	3407.6	-592.4	-1592.4	-2425.8

Positive values show pairs of means that are significantly different.

APPENDIX G

Statistical Analysis for Estimated Viable Oocysts in Sentinel Chamber

Oneway Anova

Summary of Fit

RSquare	0.997628
RSquare Adj	0.997035
Root Mean Square Error	2447.905
Mean of Response	196803.1
Observations (or Sum Wgt:	16

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	3	3.02E+10	1.01E+10	1682.623
Error	12	71906875	5992240	Prob>F
C Total	15	3.03E+10	2.02E+09	<.0001

Means for Oneway Anova

Level	Number	Mean	Std Error
17d	4	231375	1224
30d	4	226475	1224
45d	4	206050	1224
60d	4	123313	1224

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean
17d	4	231375	2809.95	1405
30d	4	226475	2129.75	1064.9
45d	4	206050	1405.94	703
60d	4	123313	3092.03	1546

Means Comparisons

Dif=Mean[i]-M	17d	30d	45d	60d
17d	0	4900	25325	108063
30d	-4900	0	20425	103163
45d	-25325	-20425	0	82738
60d	-108063	-103163	-82738	0

Alpha= 0.05

Comparisons for each pair using Student's t

t

2.17882

Abs(Dif)-LSD	17d	30d	45d	60d
17d	-3771	1129	21554	104291
30d	1129	-3771	16654	99391
45d	21554	16654	-3771	78966
60d	104291	99391	78966	-3771

Positive values show pairs of means that are significantly different.

APPENDIX H

Statistical Analysis for Viable Sentinel and Control Oocysts

17d (Sentinels) By 17d (Controls)

Mean Fit

Mean	231375
Std Dev [RMSE]	2809.953
Std Error	1404.976
SSE	23687500

Linear Fit

17d (Sentinels) = -241227 + 1.96529 17d (Controls)

Summary of Fit

RSquare	0.364019
RSquare Adj	0.046028
Root Mean Square Error	2744.523
Mean of Response	231375
Observations (or Sum Wgts)	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	8622690	8622690	1.1447
Error	2	15064810	7532405	Prob>F
C Total	3	23687500		0.3967

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-241227	441716	-0.55	0.6398
17d (Controls)	1.9652856	1.836839	1.07	0.3967

Paired t-Test

17d (Controls) - 17d (Sentinels)

Mean Difference	9100	Prob > |t|	0.0047
Std Error	1195.303	Prob > t	0.0024
t-Ratio	7.61313	Prob < t	0.9976
DF	3		

30d (Sentinels) By 30d (Controls)

Mean Fit

Mean	226475
Std Dev [RM	2129.75
Std Error	1064.875
SSE	13607500

APPENDIX H

Statistical Analysis for Viable Sentinel and Control Oocysts

Linear Fit

30d (Sentinels) = -205893 + 1.83314 30d (Controls)

Summary of Fit

RSquare	0.654884
RSquare Adj	0.482326
Root Mean Square Error	1532.345
Mean of Response	226475
Observations (or Sum Wgts)	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	8911337	8911337	3.7952
Error	2	4696163	2348082	Prob>F
C Total	3	13607500		0.1908

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%
Intercept	-205893.3	221943	-0.93	0.4515	-1.16E+06
30d (Controls)	1.8331369	0.940979	1.95	0.1908	-2.215615
					Upper 95%
Paired t-Test					749061.1
30d (Controls) - 30d (Sentinels)					5.8818885

Mean Differe	9387.5	Prob > |t|	0.001
Std Error	738.0648	Prob > t	0.0005
t-Ratio	12.71907	Prob < t	0.9995
DF	3		

45d (Sentinels) By 45d (Controls)

Mean Fit

Mean	206050
Std Dev [RM	1405.94
Std Error	702.9699
SSE	5930000

Linear Fit

45d (Sentinels) = 145824 + 0.25993 45d (Controls)

Summary of Fit

RSquare	0.131938
RSquare Adj	-0.30209
Root Mean S	1604.308
Mean of Res	206050
Observations	4

APPENDIX H

Statistical Analysis for Viable Sentinel and Control Oocysts

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	782392.1	782392	0.304
Error	2	5147607.9	2573804	Prob>F
C Total	3	5930000		0.6368

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%
Intercept	145824.01	109237.4	1.33	0.3136	-324191.6
45d (Controls)	0.2599309	0.471448	0.55	0.6368	-1.768567

	Upper 95%
Paired t-Test	615839.6
45d (Controls) - 45d (Sentinels)	2.2884286

Mean Differe	25650	Prob > |t|	0.0001
Std Error	978.5193	Prob > t	<.0001
t-Ratio	26.21308	Prob < t	0.9999
DF	3		

60d (Controls) By 60d (Sentinels)

Linear Fit

60d (Controls) = 222278 – 0.04821 60d (Sentinels)

Summary of Fit

RSquare	0.023874
RSquare Adj	-0.46419
Root Mean S	1167.266
Mean of Res	216333.3
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	66649	66649	0.0489
Error	2	2725017.8	1362509	Prob>F
C Total	3	2791666.8		0.8455

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	222277.54	26882.85	8.27	0.0143	106608.68	337946.4
60d (Sentinels)	-0.048205	0.217955	-0.22	0.8455	-0.985998	0.8895879

Paired t-Test

60d (Sentinels) - 60d (Controls)

Mean Differe	-93020.8	Prob > |t|	<.0001

APPENDIX H

Statistical Analysis for Viable Sentinel and Control Oocysts

Std Error	1689.151	Prob > t		1
t-Ratio	-55.0695	Prob < t	<.0001	
DF	3			

APPENDIX I

Statistical Analysis for Die-off Rate of Oocysts in the Sentinel and Control Units

17d (Sentinels) By 17d (Controls)
Linear Fit
17d (Sentinels) = 0.00188 + 2.88889 17d (Controls)
Summary of Fit

RSquare	0.349896
RSquare Adj	0.024845
Root Mean S	0.000723
Mean of Res	-0.00455
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000056	5.63E-07	1.0764
Error	2	0.00000105	5.23E-07	Prob>F
C Total	3	0.00000161		0.4085

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	0.0018778	0.006206	0.3	0.7908	-0.024824	0.02858
17d (Controls)	2.8888889	2.784436	1.04	0.4085	-9.091706	14.869484

Paired t-Test
17d (Controls) - 17d (Sentinels)

Mean Differe	0.002325	Prob > |t|	0.0058
Std Error	0.000328	Prob > t	0.0029
t-Ratio	7.098065	Prob < t	0.9971
DF	3		

30d (Sentinels) By 30d (Controls)
Linear Fit
30d (Sentinels) = -1e-5 + 1.7 30d (Controls)
Summary of Fit

RSquare	0.540187
RSquare Adj	0.31028
Root Mean S	0.000248
Mean of Res	-0.00333
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000014	1.45E-07	2.3496

APPENDIX I

Statistical Analysis for Die-off Rate of Oocysts in the Sentinel and Control Units

Error	2	0.00000012	6.15E-08	Prob>F
C Total	3	0.00000027		0.265

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	-0.00001	0.002166	0	0.9967	-0.009331	0.0093105
30d (Controls)	1.7	1.109054	1.53	0.265	-3.071925	6.4719251

Paired t-Test
30d (Controls) - 30d (Sentinels)

Mean Differe	0.001375	Prob > |t|	0.0011
Std Error	0.000111	Prob > t	0.0006
t-Ratio	12.40216	Prob < t	0.9994
DF	3		

45d (Sentinels) By 45d (Controls)
Linear Fit
45d (Sentinels) = -0.0041 + 0.1 45d (Controls)
Summary of Fit

RSquare	0.014815
RSquare Adj	-0.47778
Root Mean S	0.000182
Mean of Res	-0.00428
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	1.00E-09	1.00E-09	0.0301
Error	2	0.00000007	3.33E-08	Prob>F
C Total	3	0.00000007		0.8783

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	-0.004105	0.000984	-4.17	0.053	-0.008341	0.000131
45d (Controls)	0.1	0.576628	0.17	0.8783	-2.381058	2.5810579

Paired t-Test
45d (Controls) - 45d (Sentinels)

Mean Differe	0.002575	Prob > |t|	0.0002
Std Error	0.000111	Prob > t	<.0001
t-Ratio	23.22586	Prob < t	0.9999
DF	3		

APPENDIX I

Statistical Analysis for Die-off Rate of Oocysts in the Sentinel and Control Units

60d (Sentinels) By 60d (Controls)

Linear Fit

60d (Sentinels) = -0.0052 + 2.58621 60d (Controls)

Summary of Fit

RSquare	0.705329
RSquare Adj	0.557994
Root Mean S	0.000637
Mean of Res	-0.01175
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000194	0.000002	4.7872
Error	2	0.00000081	4.05E-07	Prob>F
C Total	3	0.00000275		0.1602

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	-0.005155	0.003031	-1.7	0.2311	-0.018196	0.0078858
60d (Controls)	2.5862069	1.182009	2.19	0.1602	-2.499625	7.6720388

Paired t-Test

60d (Controls) - 60d (Sentinels)

Mean Differe	0.0092	Prob > |t|	0.0001
Std Error	0.000358	Prob > t	<.0001
t-Ratio	25.68136	Prob < t	0.9999
DF	3		

APPENDIX J

Statistical Analysis for Survival Rates of in the Control and Chemicals @ 7oC

Salinity @ 7 oC By Controls @ 7 oC

Linear Fit

Salinity @ 7 oC = 0.01861 + 1.14133 Controls @ 7 oC

Summary of Fit

RSquare	0.882389
RSquare Adj	0.823584
Root Mean S	0.001276
Mean of Resp	-0.0045
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00002442	0.000024	15.0052
Error	2	0.00000326	0.000002	Prob>F
C Total	3	0.00002768		0.0606

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	0.018612	0.006	3.1	0.0901	-0.007206	0.0444302
Controls @ 7 oC	1.1413333	0.29464	3.87	0.0606	-0.126412	2.4090788

Paired t-Test

Controls @ 7 oC - Salinity @ 7 oC

Mean Differe	-0.01575	Prob > |t|	<.0001
Std Error	0.00055	Prob > t	1
t-Ratio	-28.6364	Prob < t	<.0001
DF	3		

Slake Lime @ 7 oC By Controls @ 7 oC

Linear Fit

Slake Lime @ 7 oC = -0.0095 + 0.068 Controls @ 7 oC

Summary of Fit

RSquare	0.008283
RSquare Adj	-0.48758
Root Mean S	0.002278
Mean of Resp	-0.01093
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000009	8.67E-08	0.0167
Error	2	0.00001038	0.000005	Prob>F
C Total	3	0.00001047		0.909

APPENDIX J

Statistical Analysis for Survival Rates of in the Control and Chemicals @ 7oC

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	-0.009548	0.010715	-0.89	0.4669	-0.055652	0.0365555
Controls @ 7 oC	0.068	0.526138	0.13	0.909	-2.195815	2.3318145

Paired t-Test

Controls @ 7 oC - Slake Lime @ 7 oC

Mean Differe	-0.00933	Prob > |t|	0.0082
Std Error	0.001491	Prob > t	0.9959
t-Ratio	-6.2553	Prob < t	0.0041
DF	3		

Cat. Polymer @ 7 oC By Controls @ 7 oC

Linear Fit

Cat. Polymer @ 7 oC = 0.03245 + 1.80987 Controls @ 7 oC

Summary of Fit

RSquare	0.591299
RSquare Adj	0.386949
Root Mean S	0.004607
Mean of Res	-0.0042
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00006142	0.000061	2.8936
Error	2	0.00004245	0.000021	Prob>F
C Total	3	0.00010387		0.231

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	0.0324548	0.021668	1.5	0.2729	-0.060777	0.1256868
Controls @ 7 oC	1.8098667	1.063973	1.7	0.231	-2.768092	6.3878249

Paired t-Test

Controls @ 7 oC - Cat. Polymer @ 7 oC

Mean Differe	-0.01606	Prob > |t|	0.0049
Std Error	0.002136	Prob > t	0.9976
t-Ratio	-7.51643	Prob < t	0.0024
DF	3		

APPENDIX J

Statistical Analysis for Survival Rates of in the Control and Chemicals @ 7oC

Alum @ 7 oC By Controls @ 7 oC

Linear Fit

Alum @ 7 oC = -0.0242 – 0.976 Controls @ 7 oC

Summary of Fit

RSquare	0.691743
RSquare Adj	0.537614
Root Mean S	0.001995
Mean of Res	-0.0044
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00001786	0.000018	4.4881
Error	2	0.00000796	0.000004	Prob>F
C Total	3	0.00002582		0.1683

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-0.024164	0.009382	-2.58	0.1235
Controls @ 7 oC	-0.976	0.460701	-2.12	0.1683

Paired t-Test

Controls @ 7 oC - Alum @ 7 oC

Mean Differe	-0.01585	Prob > |t|	0.0089
Std Error	0.002601	Prob > t	0.9956
t-Ratio	-6.09428	Prob < t	0.0044
DF	3		

Paired t-Test

Controls @ 7 oC - Alum @ 7 oC

Mean Differe	-0.01585	Prob > |t|	0.0089
Std Error	0.002601	Prob > t	0.9956
t-Ratio	-6.09428	Prob < t	0.0044
DF	3		

APPENDIX K

Statistical Analysis of Survival Rates of the Control and Chemicals @14oC

Salinity @ 14 oC By Controls @14 oC
Linear Fit
Salinity @ 14 oC = -0.0051 + 0.10263 Controls @14 oC
Summary of Fit

RSquare	0.024624
RSquare Adj	-0.46306
Root Mean S	0.001991
Mean of Resp	-0.00728
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.0000002	2.00E-07	0.0505
Error	2	0.00000793	0.000004	Prob>F
C Total	3	0.00000813		0.8431

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	-0.005068	0.00987	-0.51	0.6587	-0.047537	0.0374006
Controls @14 oC	0.1026316	0.456744	0.22	0.8431	-1.8626	2.0678634

Paired t-Test
Controls @14 oC - Salinity @ 14 oC

Mean Differe	-0.01423	Prob > |t|	0.002
Std Error	0.001391	Prob > t	0.999
t-Ratio	-10.2245	Prob < t	0.001
DF	3		

Slake Lime @ 14 oC By Controls @14 oC
Linear Fit
Slake Lime @ 14 oC = 0.01479 + 1.31579 Controls @14 oC
Summary of Fit

RSquare	0.41377
RSquare Adj	0.120655
Root Mean S	0.004827
Mean of Resp	-0.0135
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00003289	0.000033	1.4116
Error	2	0.00004661	0.000023	Prob>F
C Total	3	0.0000795		0.3568

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0147895	0.023932	0.62	0.5996
Controls @14 oC	1.3157895	1.107454	1.19	0.3568

Paired t-Test

APPENDIX K

Statistical Analysis of Survival Rates of the Control and Chemicals @14oC

Controls @14 oC - Slake Lime @ 14 oC

Mean Differe	-0.008	Prob > |t|	0.0284
Std Error	0.00201	Prob > t	0.9858
t-Ratio	-3.97933	Prob < t	0.0142
DF	3		

Cat. Polymer @ 14 oC By Controls @14 oC

Paired t-Test

Controls @14 oC - Cat. Polymer @ 14 oC

Mean Differe	-0.01805	Prob > |t|	0.0022
Std Error	0.001819	Prob > t	0.9989
t-Ratio	-9.92243	Prob < t	0.0011
DF	3		

Linear Fit

Cat. Polymer @ 14 oC = -0.0128 – 0.43684 Controls @14 oC

Summary of Fit

RSquare	0.882187
RSquare Adj	0.823281
Root Mean S	0.000492
Mean of Res	-0.00345
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000363	0.000004	14.9761
Error	2	0.00000048	2.42E-07	Prob>F
C Total	3	0.00000411		0.0608

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-0.012842	0.002439	-5.26	0.0342
Controls @14 oC	-0.436842	0.112882	-3.87	0.0608

Alum @ 14 oC By Controls @14 oC

Linear Fit

Alum @ 14 oC = -0.0153 – 0.35789 Controls @14 oC

Summary of Fit

RSquare	0.596491
RSquare Adj	0.394737
Root Mean S	0.000907
Mean of Res	-0.0076
Observations	4

Analysis of Variance

Source	DF	Sum of Squa	Mean Square	F Ratio
Model	1	0.00000243	0.000002	2.9565
Error	2	0.00000165	8.23E-07	Prob>F
C Total	3	0.00000408		0.2277

Parameter Estimates

APPENDIX K

Statistical Analysis of Survival Rates of the Control and Chemicals @14oC

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-0.015295	0.004498	-3.4	0.0767
Controls @14 oC	-0.357895	0.208144	-1.72	0.2277

Paired t-Test

Controls @14 oC - Alum @ 14 oC

Mean Differe	-0.0139	Prob > |t|	0.0042
Std Error	0.001748	Prob > t	0.9979
t-Ratio	-7.95043	Prob < t	0.0021
DF	3		

APPENDIX L

Statistical Analysis for Inactivation Rate (K) By Experiment Number

Oneway Anova

Summary of Fit

RSquare	0.477994
RSquare Adj	0.347492
Root Mean S	0.002785
Mean of Resp	0.005356
Observations	16

Analysis of Variance

Source	DF	Sum of Square	Mean Square	F Ratio
Model	3	0.00008521	0.000028	3.6627
Error	12	0.00009305	0.000008	Prob>F
C Total	15	0.00017826	0.000012	0.044

Means for Oneway Anova

Level	Number	Mean	Std Error
17d	4	0.00455	0.00139
30d	4	0.003325	0.00139
45d	4	0.004275	0.00139
60d	4	0.009275	0.00139

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean
17d	4	0.00455	0.000733	0.00037
30d	4	0.003325	0.000299	0.00015
45d	4	0.004275	0.00015	0.00008
60d	4	0.009275	0.005511	0.00276

Means Comparisons

Dif=Mean[i]-M	60d	17d	45d	30d
60d	0	0.004725	0.005	0.00595
17d	-0.00473	0	0.000275	0.001225
45d	-0.005	-0.00028	0	0.00095
30d	-0.00595	-0.00123	-0.00095	0
Alpha=	0.05			

Comparisons for each pair using Student's t

t

2.17882

Abs(Dif)-LSD	60d	17d	45d	30d
60d	-0.00429	0.000435	0.00071	0.00166
17d	0.000435	-0.00429	-0.00402	-0.00307
45d	0.00071	-0.00402	-0.00429	-0.00334
30d	0.00166	-0.00307	-0.00334	-0.00429

Positive values show pairs of means that are significantly different.

VITA

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