ABSTRACT

SUSAN LYNN BARBEE. Application of *In Vitro* Cell Culture Assay to *Cryptosporidium parvum* oocyst Inactivation by Thermal Processes (Under the direction of Mark D. Sobsey)

Cryptosporidium is an important waterborne pathogen and its oocysts are very persistent in the environment and very resistant to chemical disinfection. Inactivation of *Cryptosporidium parvum* oocysts by heat is a potential disinfection process for water, wastewater and biosolids. We studied the kinetics and extent of thermal inactivation of *C. parvum* oocysts in buffered water at 35°, 49°, 50°, 55° and 60°C, and by thermophilic anaerobic digestion of cow manure at 55°C using mammalian cell culture to quantify oocysts infectivity. Comparisons of infectious oocyst concentrations between untreated controls and thermally treated samples indicated significant losses of oocyst infectivity by higher temperature thermal treatment in both milieu. While there were only minor reductions of oocyst infectivity in buffered water after 24 hours at 23° and 35°C, temperatures of 55° and 60°C reduced oocyst infectivity by 99.9% within 60 minutes. Exposure at 55°C in anaerobically digested manure for 60 minutes reduced oocyst infectivity by 99.9%, but in the untreated manure oocyst infectivity was stable for many hours at 23°C. These data demonstrate that the infectivity of *C. parvum* oocysts is rapidly and extensively reduced in water and bovine manure at thermophilic temperatures as low as 55°C.

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CHAPTER ONE

INTRODUCTION

Cryptosporidium parvum represents a highly infectious, emerging disease, especially among immunocompromised individuals. The robust nature of the oocysts, a low infectious dose combined with resistance to commonly used disinfectants make these organisms difficult to control and a serious threat to public health. Oocysts follow a fecal oral route, and can be transmitted through multiple sources. Symptomatic and asymptomatic humans plus feral and domestic animals can shed oocysts, which can be carried into water courses and lead to outbreaks of enteric disease.

The US Environmental Protection Agency (USEPA), by the direction of the Safe Water Drinking Act. placed *Cryptosporidium* on its Drinking Water Priority List in 1986. The Enhanced Surface Water Treatment Rule currently requires monitoring of this organism by large utilities using surface and ground waters as directed by the regulatory negotiated Information Collection Rule (ICR). Information gained through this study will be used to develop regulations and evaluate the effectiveness of treatment measures and the possible cost of implementing control measures. Since detection of oocysts is plagued with problems, it is expected that regulation will require treatment processes to remove 99.9% of oocysts. Control of this organism is best made through a multiple barrier approach. A first barrier in protecting drinking water sources is wastewater treatment. Studies have shown that oocysts are present at high levels in raw sewage and that treatment processes alone cannot be realistically depended upon to prevent the release of oocysts into the environment. Wastewater treatment processes concentrate oocysts into the sewage sludges, therefore processes that treat sludge are important in prevention of release of oocysts into the environment. Studies to date have shown that oocysts are effectively inactivated by anaerobic and aerobic digestion both in the thermophilic and mesophilic range, as evidenced by commonly used viability methods. The objective of this research was to evaluate the inactivation

of oocysts by thermophilic anaerobic digestion using a cell culture infectivity assay. Anaerobic thermophilic digestion is an emerging treatment process for municipal sludges and one that is being used to treat waste at some large dairy facilities.

To assess the effectiveness of physical and chemical treatment process on the viability or infectivity of *C. parvum* three basic methods have been employed: 1) in vitro excystation of oocysts, 2) vital dye staining (DAPI/PI), and 3) in vivo infection of a suitable animal model. While animal models are accepted as the "gold standard" for assessing viability and infectivity, they are costly and do not always yield precise quantitative data. Viability has been addressed by application of in vitro excystation and staining with vital dyes. Questions remain as to the ability of these methods to predict infectivity reliably because results are often not correlated with predictions from animal models (Finch et al, 1993 & Korich et al, 1990). Recent advances in in vitro cell culture assay methods (Arrowood et al, 1996, Slifco et al,1996) make this an attractive alternative because they provide information on the viability of treated oocysts as determined by the ability to initiate infection and proliferate. The ability to detect quantitatively the infectivity of *C. parvum* oocysts in cell culture provides a convenient tool for better determination of water, wastewater and sludge treatment effectiveness and assessing risks to the public health.

The purpose of this study was to evaluate the Madin Darby canine kidney (MDCK) cell culture assay method for use as a tool for quantitative assessment of treatment efficiencies; then apply this methodology to quantify the reductions of oocyst infectivity in thermal processes and anaerobic thermophilic digested sludge. This study presents data on the thermal inactivation of *C. parvum* oocysts in buffered water and in anaerobic thermophilically digested bovine manure.

CHAPTER TWO

REVIEW OF THE LITERATURE

Pathogen Characterization

History

Cryptosporidium parvum was first described as a new species in 1912 by the American parasitologist Ernest Edward Tyzzer (Tyzzer, 1929). It was distinguished from previously identified species of *Cryptosporidium* by its size (3-7 µM) and its restriction to the digestive tract of asymptomatic laboratory mice. Tyzzer's initial investigations of this genus of parasites elucidated the details of the complex life cycle of this organism which even today holds up as the definitive description of their reproduction development and morphology (Tyzzer, 1907).

Regarded as an opportunistic zoonosis with little if any medical interest or economic impact (Casemore and Jackson, 1984 & Schultz, 1983), *Cryptosporidium* received virtually no attention for more than sixty years after it was discovered. Even though numerous reports confirmed the presence of this parasite in many host species it remained relatively obscure. In 1971, *C. parvum* was associated with a case of bovine diarrhea and the interest of the veterinary community was stimulated as its role as a pathogen in animals was affirmed. The first description of a human case was reported five years later when in 1976 a three-year-old immunocompetent child from a rural farming community developed infection due to zoonotic transmission (Nime et al., 1976). Within a month, another resident of a farming community was diagnosed with cryptsporidiosis (Nime et al, 1976 & Meisel et al, 1976). Reports of human cryptosporidiosis remained rare until 1982 when physicians throughout the United States began reporting an ever increasing number of cases, especially among young males with acquired immunodeficiency disease, to the Centers for Disease Control. While apparently a self limited disease in healthy people, patients with suppressed immunities can develop chronic infections with high mortality rates. Since the early 80's, the number of outbreaks and cases of cryptosporidiosis have

continued to rise amoung immunocompetent and suppressed people.

Now accepted as a prominent agent of diarrhea in humans and domesticated animals throughout the world, *Cryptosporidium* is the leading cause of waterborne outbreaks for which an etiologic agent has been identified in the United States (Kramer, 1996). The CDC recently identified *C. parvum* as an emerging infectious disease due to evidence suggesting that it is newly spread and newly able to cause life threatening disease. It's role in the epidemic outbreak in Milwaukee in 1993 generated much public attention and concern for the potential for water borne transmission. It's ubiquitous nature, small size and a low infectious dose combined with its relative resistance to typical water treatment processes and disinfection makes it of particular concern to the public health.

Taxonomy

Cryptosporidium belongs to the genera of Coccidae protozoan parasites in the phylum Apicomplexa (the sporozoans). The members of this genus develop within the epithelial cells of the gastrointestinal and respiratory tracts of vertebrates. Species within this genus complete their life cycle within a single host and have the smallest oocysts among the coccidia. Although once considered strictly host specific, they are now known to be cross transmissible (Fayer and Ungar, 1986), between different hosts within the same vertebrate class.

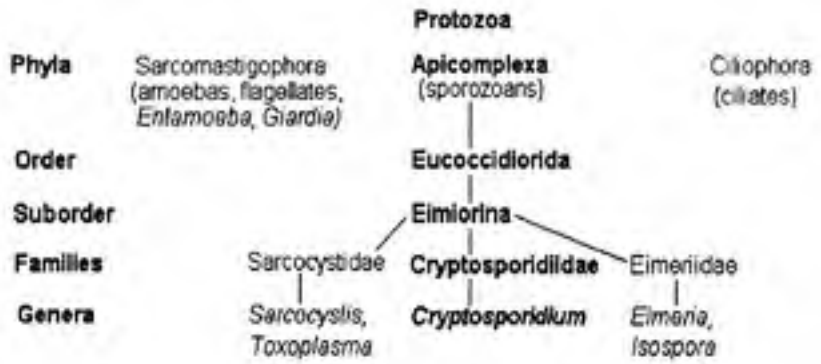
Initially, nomenclature was based on the specific host-parasite relationship (Fayer et al, 1990) and as many as twenty-one different species were identified. Taxonomic classification remains confusing due to the lack of host specificity and is therefore under revision. At present, six species of *Cryptosporidium* are accepted (Casemore, 1990); *C. parvum* infects mammals, including man; *C. muris* infect rodents; *C. bailey* and *C. meleagridis* infect birds; *C. crotali* infects reptiles; and *C. nasorum* infects fish. Variations within host specificity, sites of infection and pathogenicity may eventually lead to validation of additional species. Although cross transmission does occur, *C. parvum* is the species responsible for most human and animal infections and it is the only waterborne Coccid to infect humans (Curds, 1992).

Subspecies and strains of *Cryptosporidium* are not currently recognized even though biological variation within species has been observed. In an attempt to differentiate host specific

strains, Mead et al (1990) compared protein patterns from two-dimensional gel electrophoresis of sporozoites from human, horse and calf sources. Four or five of these isolates showed sufficient differences in peptide properties to allow identification of the isolate. Yet, there is still insufficient evidence that isolates can be subdivided based on antigenic or other subtypes (Casemore, 1984). Other studies have identified differences between isolates at the genetic and phenotypic level, but aren't conclusive (Nichols et al, 1990, Awad-El-Kariem et al, 1995, Carraway et al, 1996).

In a recent dispatch in CDC's Emerging Infectious Disease journal, investigators described a genetic polymorphism within the TRAP-2 gene which may allow for the identification of the source of oocysts (Peng et al, 1997). The authors reported that bovine and human isolates can be distinguished based on these molecular techniques. This represents a significant breakthrough if they are able to verify this pattern with further research. Determination of sources of oocysts is important in the investigation of future outbreak investigations and control efforts.

Figure 2.1 Taxonomy of *Cryptosporidium*



Life Cycle

Cryptosporidium, a monecious parasite, completes its entire life cycle within a single host. Its life cycle is similar to other Coccidae in that it can be divided into six distinctive phases: excystation, merogony, gametogony, fertilization, oocysts wall formation, and sporogony (Current, 1987). Infection is initiated following ingestion of the thick walled, environmentally resistant oocyst which is shed in the feces or nasal secretions of the infected hosts. Oocysts can be present in contaminated food, water or the general environment and are extremely robust. Each oocyst

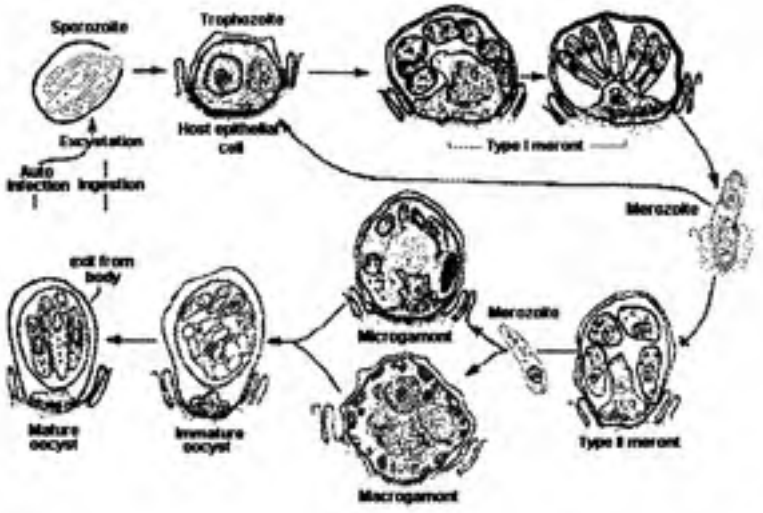
contains four motile, naked sporozoites, each capable of initiating infection. Sporozoites excyst from the oocysts in the gastrointestinal or respiratory tract and infect epithelial cells. All endogenous stages of the parasite are found at the luminal surface of the epithelium. While anatomical sites of infection usually involve the alimentary tract, other mucosal surfaces lined with epithelial cells are also permissive to infection (Casemore, 1988).

Excystation is thought to result from exposure to a combination of body temperature, CO_2, proteolytic enzymes and bile salts (Current, 1987). Upon attachment of the sporozoite to a cell, a feeder organelle develops between the sporozoites and cell cytoplasm. The parasite undergoes differentiation within the host's parasitophorous vacuole membrane to form an intracellular trophozoite. Asexual division, merogomy, results in the formation of type I meronts. These meronts contain six to eight merozoites and can infect a host cell to develop into a new Type I meront or Type II meronts. Type II meronts undergo asexual divisions to produce four merozoites that go on to develop into gamonts, the sexual stage. A majority of the meroites from Type II meronts go on to form macrogamets (80%), the remaining form microgamonts (20%), the female and male counterparts respectively (Current, 1987). Fertilization results in the production of a zygote that develops into either thin or thick walled oocysts which sporulates *in vivo*. Both types of oocyst contain four sporozoites. Most of the oocysts produced are thick walled and are shed in the feces. These are the environmentally resistant forms that can transmit the infection to other hosts. The thin walled oocyst is surrounded only by a unit membrane and excyst within the gastrointestinal tract of the host soon after release from a host cell and are capable of reinitiating the endogenous cycle. The potential for recycling sporozoites through the generation of thin walled oocysts is unique to this group of Coccidia's parasites (Current and Long, 1983). This feature is probably responsible for the low infective dose required to initiate infection and the persistent life threatening infection in absence of repeat oral exposure often seen in AIDS patients.

The time required to complete the entire life cycle of this parasite varies within hosts, but is reported to be between 18 - 24 hours within humans. The period between infection and oocyst shedding in humans varies from five to 21days (Anderson and Hall, 1981, Blagburn and Current, 1983 and Reese et al, 1982). Duration of oocyst shedding also varies among hosts and is dependent on host physiological state, but may be >30 days (Moon et al, 1988). During the acute

diarrhea stage oocyst may exceed 10^6 per gram feces (Casemore, 1990). The number of oocysts shed in feces appears to be associated with severity of symptoms, but asymptomatic carriers are also capable of shedding oocysts.

Figure 2.2 Life Cycle of *Cryptosporidium*



Cryptosporidiosis

The effect of ingesting *C. parvum* oocyst can range from asymptomatic infection, a few days of mild to severe diarrhea, cholera-like diarrhea requiring medical attention, hospitalization and potentially death. The most prominent and frequently identified manifestation of cryptospordiosis is profuse, watery diarrhea, associated with vomiting, abdominal pain, anorexia, weight loss, dehydration and low grade fever (Current and Garcia, 1991). Generally a self- limited infection of short duration in immunocompetent victims, the severity and duration of the illness appears to be connected to the immune system (Ungar, 1990). Symptoms usually onset one to two weeks after exposure, generally lasting between eight and twenty days, but can become chronic and lead to mortality in certain patients. The difference in clinical outcomes between the immunocompetent vs. immunosuppressed patients is probably due to an immune response which

is of sufficient magnitude to clear the parasite (Current and Garcia, 1991). This is supported by a report of a patient undergoing chemotherapy who was infected with *C. parvum*, but was able to recover and clear the infection following return of immune functions (Miller et al, 1983). Severe and chronic cryptosporidiosis has been reported to correlate well with CD4 counts. Of patients with the disease, those with CD4 counts >180 cells/mm^3 were able to clear infections, where only 13% of those with CD4 counts < 180 cells/mm3 had infections that cleared (Flanigan et al, 1992).

A recent study of *C. parvum* in human volunteers determined the infectious dose or ID_{50} at 132 oocysts. This estimate is based on a single isolate study in human volunteers who showed no evidence of previous exposure (Dupont et al, 1995). Other studies have reported infectivity as low as one oocyst in goats (Haas and Rose, 1995). The infectious dose varies between animal hosts and is dependent on the infectivity of an oocyst isolate. Oocyst infectivity is also dependent upon a variety of factors: age of oocyst and host, physiological state of host and oocyst, and immunity of the host. While little is known about the differences in virulence between oocyst isolates, this information could serve as a source of variation in infective doses as could the source of oocysts.

Cryptosporidiosis cannot be distinguished clinically from the numerous other illnesses that cause diarrhea. Definitive diagnosis requires detection of oocysts in feces and is usually made by acid fast or immunofluorescence staining. Of procedures available for the detection of oocysts within feces all suffer from low sensitivity, which might result in the under-diagnosis and under-reporting of this disease (Arrowood and Sterling, 1987). Development of newer techniques such as polymerase chain reaction (PCR) and flow cytometery provides a more sensitive, specific tool for use in clinical diagnosis (Laxer et al, 1992).

Despite widespread evaluation of hundreds of prophylactic and therapeutic compounds, none have been found with reliable cysticidal activity (O'Donoghue, 1995 & Wood et al, 1996). Paromomycin, azithromycin and spiramycin were found to have a modest benefit in controlled studies, but none were able to eradicate infection (White et al, 1994). A study by Forney (1996) reported significant reductions in *C. parvum* infection in cultured cells in the presence of serine protease inhibitors. Another study reported nitrazoxanide was effective against *C. parvum* infections in patients with AIDS (Doumbo et al, 1997), in decreasing parasites as well as diarrhea. This drug was also shown to have broad spectrum efficacy against other known intestinal

parasites. These reports represent significant advance in the treatment of infections in patients with AIDS. Treatment for patients of normal immunity is generally not necessary, but maintenance of fluids and symptomatic treatment of diarrhea can be applied.

Transmission

Cryptosporidium is transmitted by the fecal oral route through ingestion of oocysts that have been excreted in the feces of infected humans and animals. Infection may occur as a result of direct contact with fecal material, through person-person or animal-person contact. Indirect transmission may occur by contact with fecally contaminated food and water and environmental surfaces. In terms of importance to human disease it is unknown how these different routes rank.

Person-person transmission is a major route in day care and nursing facilities (Alpert et al, 1986), among health care providers (Baxby et al, 1983), sexual partners and family members (Heijbel et al, 1987). Zoonotic transmission occurs mainly among those associated with farm and research animals (Anderson et al, 1982). While no documented cases are available, transmission from domestic animals is possible. In one case, a domestic animal was infected by a human (personal communication, M. J. Arrowood).

While few cases of food-borne transmission have actually been reported, the potential of contamination of foodstuff posses a serious risk for development of infection. Epidemiologic features of this parasite support the assumption that the incidences of cryptsporidiosis due to contaminated foodstuffs are probably under-reported (Laberge and Griffiths, 1996). Proof of food-borne transmission is hampered by the limitations in recovery methodology, low number of oocysts present in contaminated items as well as the insensitivity of epidemiology survey methods. In October 1994, the first well documented food-borne outbreak associated with *C. parvum* was reported in Maine. The outbreak resulted from the consumption of fecally contaminated, hand-pressed apple cider (Millard et al, 1994). Other cases of cryptsporidiosis among humans have implicated raw milk and other raw sources, but conclusive demonstration of transmission has not been established. Consumption of livestock and their products such as raw milk and meat products pose a high risk due to the high prevalence of *C. parvum* among dairy herds. Contaminated water also poses a risk to the food industry, since it is used for cleaning raw

produce or can be included in the products themselves (LaBerge and Griffiths, 1996)

Waterborne transmission is well documented and may be the most significant route of transmission. Outbreaks associated with contaminated drinking water and recreational waters have been reported and are well documented in the literature. At least eleven outbreaks of cryptsporidiosis attributed to drinking water have been recognized in the USA (Solo-Gabriele, 1996). Other outbreaks have been caused by the inadvertent consumption of contaminated water while swimming (Anon., 1993), at water slides and amusement parks (Levins et al, 1990, Sorvillo et al, 1992, and Bell et al, 1993). Contamination of drinking water sources has been linked to sewage and septic contamination and agricultural practices within the watershed.

Waterborne Disease Outbreaks

A large number of outbreaks and epidemics of cryptospordiosis have been traced to the consumption of contaminated drinking water. The leading causes of drinking and recreational water outbreaks in the United States between 1991 and 1994 were *Giardia* and *Cryptosporidium*, but *Cryptosporidium* accounted for substantially more cases than *Giardia* (Guerrat, 1997). During this period 30 waterborne disease outbreaks were reported; of the 25 with known etiology, 40% were caused by either Giardia or Cryptosporidia, and of the 405,360 persons ill 403,000 had cryptosporidiosis (Anon., 1993). To date there have been eleven documented outbreaks of cryptosporidiosis in drinking water, including the largest known outbreak ever documented in Milwaukee (Solo-Gabriele, 1996).

A disease outbreak in 1984 in Braun Station, Texas was the first documented case of cryptospordiosis caused by contaminated ground water that had been disinfected, but not filtered (D'Antonio, 1985). Subsequently, waterborne outbreaks have been reported in numerous states throughout the United States. Outbreaks have occurred in both small, rural communities with small water supplies (i.e., Washington State) and large urban areas (i.e., Milwaukee and Las Vegas) with more sophisticated water supply systems. Raw water sources have included both surface (rivers and lakes) and groundwater (springs and wells) supplies. Roughly half of the reported outbreaks were associated with contaminated groundwater, yet none have been reported for filtered groundwater. The majority of the cases were served by surface water supplies (Solo-Gabriele,

1996).

While the actual source of oocysts contamination has not been confirmed in most waterborne outbreaks, suspected sources include both agricultural and wastewater contamination. All the waterborne outbreaks in the US between 1984 and 1993 occurred in communities where utilities met local and federal standards for acceptable drinking water quality. Treatment deficiencies and suboptimal operational practices were noted during some, while optimal treatment was achieved by others. These outbreaks indicate that even with compliance with USEPA water treatment standards adequate protection against waterborne *Cryptosporidium* may not be provided and illustrates the importance of increasing our knowledge of treatment, source and transmission of these oocysts.

Analysis of Cryptosporidium

Methods of detecting and quantifying *Cyptospordium* oocysts in environmental samples represent adaptations from methods originally developed for use with *Giardia* cysts and other Coccidium oocysts. Recovery and detection of *Cryptosporidium* oocysts in environmental samples can be divided into several basic steps: recovery and concentration, isolation, purification, identification and enumeration. Methods for the detection of *C. parvum* in environmental samples can be divided into three types: detection of oocyst antigens, detection of nucleic acids and detection of oocyst infectivity or viability.

Concentration and Recovery

Water Samples

Recovery and concentration of oocysts from water samples rely most commonly on methods involving microporous filtration, a process that moves water through barriers with porosities that allow the flow of water, but trap the pathogen within its structure. Concentration is necessary due to the relatively low number of oocysts found in water sources. Three methods which rely on filtration have typically been used to recover waterborne oocysts: American Society for Testing and Materials (ASTM-19, 1993), the Standard Methods for the Examination of Water and Wastewater (Anon., 1995), and the Alternative Method (AM) (Graczyk et al, 1997). Following

the numerous waterborne outbreaks of *C. parvum* in the US, EPA chose the ASTM as a reference method for the detection of *C. parvum* oocyst in water. The ASTM analytical procedure was selected and is currently mandated by the EPA for ICR monitoring. However, the method is frought with inconsistent, low recoveries and poor reproducibility. Much work is going forward toward the development of improved methodologies.

The ASTM method involves the use of a yarn-wound polypropylene filter cartridge of nominal-porosity (one µm). This filter cartridge is widely used and accepted due to its application to a broad range of water sources. In addition, they are relatively inexpensive and can be used to process large volumes of water (100L filtered - 1,000 L raw). In experiments with seeded samples the retention rates of these filters are reported to be as high as 84% -86% (Musial et al, 1987, LeChevallier et al, 1995), with a pass through of 5 - 30% (Versey and Slade, 1991). A disadvantage of cartridge filters is the difficulty in subsequent processing steps, which may lead to variable recovery rates.

Elution of retained particles from filters is accomplished by washes in a detergent-based solution (nonionic and ionic surfactants) followed by reconcentrating by centrifugation. Most oocyst losses can occur during the centrifugation step (LeChevallier et al, 1995). Centrifugation speeds recommended in the EPA-ASTM method (1,500 x g) leave oocysts in the supernatant. LeChevallier (1995) evaluated the ASTM procedure and showed that improvements in recoveries could be realized by increasing the number of filter washes and centrifugation speed (5,000 x g).

Membrane filtrations offer an alternative to cartridge filtration. Several products are available for use including: polycarbonate filtration (Ongerth and Stibbs, 1987), micro/ultra porous filtration (Juliano, 1997), vortex-flow filtration (Jonas et al, 1995), and cellulose acetate membrane filters (Aldom, 1995). All alternatives show promise for future applications with considerable improvements over cartridge filtration recovery efficiencies and ease of processing (Nieminski et al, 1995).

Recovery efficiency by filtration is dependent upon the turbidity of water, relative oocyst concentration and sample volume (Musial et al, 1987). A major flaw of the filtration recovery method is that other particulates (i.e., algae) compact with oocysts and there is a non-selective isolation of objects within the size range of oocysts. These objects tend to interfere with further

purification and enumeration of isolated oocysts.

Chemical flocculation (Versey and Slade, 1993) is being successfully used by researchers in the UK for oocysts recovery. Samples treated with calcium chloride or sodium carbonates, adjusted to pH 10, form particles that precipitate from solution. When filtration and chemical flocculation were compared, chemical flocculation resulted in the highest recoveries, with an average of 68% (Shepherd and Wyn-Jones, 1995). The method is limited by its application to samples of small volumes (<10 L).

Immunocapture (IMC) has recently been evaluated for recovering oocysts from environmental samples and shows promise for future application. When IMC is combined with messenger RNA-PCR (Stinear et al, 1996) it provides a very sensitive method for isolation and detection of oocysts from small volumes.

Organic Samples - Feces, Sludge and Sewage

Several techniques are available for isolating oocysts from feces and materials consisting of high concentrations particulates such as raw sewage and activated sludge. Efficiency of recovery varies from sample to sample, and is dependent on the consistence of the matrix and the presence of high numbers of oocyst within the starting material.

In the initial recovery step, the amount of organic debris and some of the fecal fat that may be present in the sample is minimized. This step is accomplished by straining the material through a series of wire screen sieves of decreasing pore sizes, then rinsing the retained debris to flush through any trapped oocysts. United standared concentration grade standard mesh sieves are most commonly used in pore sizes ranging from 20 through 100 µM mesh. Addition of non-ionic and ionic surfactants to samples is used to improve oocyst recoveries from organic samples (Whitmore and Robertson, 1995, Leng et al, 1995). Detergents facilitate the release of oocysts from any hydrophobic associations and disperse trapped particulate material into solution.

Sieving steps are followed by sequential washes in detergent-based solutions and centrifugation. Ethyl ether or chloroform extraction of the supernatant may be included to remove excess lipid material and fecal fat that can interfere with further process steps, but the use of organic compounds in oocysts isolation may alter the antigenicity of the oocyst (Current, 1990).

After centrifugation and wash steps, a low speed centrifugation or a settling step sediments the heavier fecal debris to clarify samples. Washed oocysts can be resuspended in either an antibiotic cocktail solution, formalin or 2.5% potassium dichromate. Studies have shown that oocysts stored in potassium dichromate solution retain their viability and infectivity over a longer period of time than oocysts stored in phosphate buffers (Arrowood and Sterling, 1987). Potassium dichromate is compatible with nucleic acid techniques and effectively kills most microbes that may be present in the sample.

While methods described above are effective with samples containing high numbers of oocysts, more sensitive, specific methods are needed for rapid recovery and for the detection of small numbers of oocysts in dilute environmental samples

Purification

Purification methods are necessary to isolate oocysts from contaminating microorganisms and debris that co-isolate with oocysts during the concentration steps. Purification of oocysts is of particular value for clinical use, *in vitro* and immunologic applications, where clean, relatively microbe and antigen-free preparations are needed. Typically, purification of oocysts from environmental and organic samples is achieved by density gradient centrifugation which isolates oocysts based upon buoyant density. Oocyst purification techniques have been based on a number of flotation methods including: sucrose(sg=1.2 through 1.0640 (Current and Haynes, 1984, Heine et al, 1987), percol-percol (sg = 1.05 & 1.09), percol-sucrose , cesium chloride (1.4 - 1.05 g/ml) (Arrowood and Donaldson, 1996, Kilani and Sekia, 1987), and zinc sulfate (sg=1.18 &1.2). Percol-percol floatation is the technique included in the ASTM-EPA method. This technique was found to be less osmotically stressful than flotation involving zinc sulfate, and the recovery of oocysts were higher than percol-sucrose (Nieminski, 1995), but less than differential sucrose (Suresh and Rehg, 1996). A major disadvantage to this method is the cost of the percol and it applicability to small samples.

A number of improvements in the purification process have been introduced over the years to increase the quality and quantity of oocysts with the flotation technique. Arrowood and Sterling (1987) introduced the use of two sequential discontinuous sucrose gradients (sg=1.103 &

1.064) followed by an isopycnic-percol gradient. They reported oocysts obtained by this method were virtually free of debris and bacteria and represented a recovery of 34% of the original oocysts in suspension. Suresh and Rehg (1996) evaluated the addition of either a percol, glass bead columns or dialysis step following differential sucrose purification of oocysts from rat feces. In this study all approaches yielded infective oocysts, but a final percol isolation step resulted in recovery of the most viable oocysts and proved to be the only one free of bacterial contamination.

Further improvements in purification have been achieved by adding a cesium chloride gradient following differential sucrose gradients (Arrowood and Donaldson, 1996). Cesium chloride gradient purification results in highly purified oocysts which can be easily separated from purification media unlike percol preps (Current, 1990). The ease of use, inexpensive components plus the quality of the oocysts make this an attractive method for the purification of samples for biological use. A disadvantage of the sucrose gradient method is the inclusion of phenol in Sheather's (sucrose) solution which may alter antigenicity of the oocysts (Heyman et al, 1986), and the need to use only fresh (<60 days) oocysts to maximize recovery from cesium chloride gradients (Kilani and Sekla, 1987).

Detection Analysis

The identification of oocysts in the concentrates obtained from environmental samples is most often accomplished using antibody based immunofluorescent assays (IFA) combined with microscopic observation. This method is routinely used in labs and is the technique included in the EPA-ASTM method. In this method the concentrated sample is filtered through a cellulose acetate membrane and then stained with labeled antibody. Both monoclonal and polyclonal antibodies have been used in direct and indirect immunofluorescent procedures (Ongerth and Stibbs, 1986, Arrowood and Sterling, 1987, and Garcia et al,8197), but monoclonals have been used exclusively for commercial kits. Antibodies contained in the kits cross-react with species other than human strain *C. parvum*: i.e., *C. meleagridis* (turkeys), *C. serpentis* (snakes), *C. wrairi* (quinea pigs), *C. muris* (mice).

Identification of oocysts is based on fluorescence, size and shape of objects and internal morphological features (Jabubowski et al, 1996). This assay is generally used as a positive or

negative test with results expressed as presumptive or confirmed. A positive test indicates the organism is present, but provides no information on its concentration in the source. A negative result does not mean that the organism is not present, only that it was not detected in the sample. While the IFA method provides rapid results, is easy to use, and inexpensive, it requires a skilled analyst and a lot of time. Disadvantages include the inaccuracy of the method, the detection of oocysts which may not be of public health significance and the inability to distinguish viable from nonviable oocysts.

Alternative methods are currently available and others are under development for oocysts identification. Many of these alternative methods address the limitations inherent to the IFA method. These methods include flow cytometry (Versey et al, 1990, Arrowood et al, 1995), PCR (Johnson et al, 1993, Rochelle et al, 1995), ELISA (de la Cruz, 1994), confocal microscopy (Anquist, & Ghiorse, 1997, and electron rotation (USEPA, 1995), and UV spectrophotometer (Patten, 1994). Similar to IFA, all methods require purified material. Flow cytometry coupled with cell sorting procedures are published. When combined with vital staining (4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) they are capable of providing information on oocyst viability (Versey et al, 1993), this method is currently being used in a number of countries.

Indicators

Methods used to detect and identify oocysts in water are both complex and inefficient, therefore the development of an indirect method for monitoring for contamination of this pathogen in water is warranted (Chauret et al, 1995). Indicators are generally used in leu of pathogen detection due to cost and ease of methodology. Since protozoans are more resistant than total/fecal coliforms to environmental stresses and disinfectants, these microbes are not reliable indicators for the presence of *Cryptosporidium* oocysts (Chauret, 1995). Several studies have investigated the relationship of oocysts with standard surrogates. In 1988, Rose reported that neither Giardia nor *Cryptospordium* were significantly correlated with turbidity, total or fecal coliforms. LeChevallier (1991), in a study of 66 public operation treatment works, found that turbidity levels could not be correlated to oocysts numbers. He suggested that particle monitoring in the size range of oocysts may be a useful method for the detection of wastewater treatment

breakthroughs. Chauret (1995) evaluated *Clostridium perfringens* and fecal streptococcus as possible surrogates for oocysts. Although Chauret found correlations with fecal streptococcus, it was highly variable with water source. Payment and Franco (93) reported an apparent relationship of *C. perfringens* with oocysts in raw waters, yet in studies by Chauret they were unable to find any correlation, but did report of a correlation with *Giardia*.

Methods for Evaluating Oocysts Viability

Detecting oocysts in environmental samples is a cause for concern, but detection by traditional methods provides no information on the viability or infectivity of the oocysts. Methods that detect viable or infectious oocysts enable the estimation of health risk, the level of contamination, and the efficacy of disinfection or sterilization procedures. Determination of oocyst viability after exposure to disinfectants has significant public health importance since injured oocysts may retain their infectivity. Several methods are available which provide a surrogate estimation of oocysts viability and new ones are under development. The suitability of each of these methods may be dependent on the quality of the sample or the particular application (Korich et al, 1990).

Animal Infectivity

The neonatal mouse infectivity model has been put forth as the "gold standard", or method of choice for measuring the ability of oocysts to initiate infection. Animal studies provide information not only on the viability of the oocysts, but also establish oocysts infectivity *in vivo* (Rose, 1988, Sterling, 1990, Schaeffer, 1987). In side by side comparison with other viability assessment methods, animal models proved more sensitive than either vital dye staining (DAPI/PI) or *in vitro* excystation (Black et al, 1996, Finch et al, 1993). While Korich (1990) reported that *in vitro* excystation gave results similar to those of the animal model, Finch (1993) reported that excystation and vital dye methods consistently underestimate the effectiveness of treatment in disinfection experiments when compared to the animal model. The difference in their excystation

results may be due to the different excystation procedures used to score viability and in the method of oocysts enumeration.

There are numerous disadvantages with animal studies beyond the most obvious: expense and demand on time and space. The dose-response relationship between animals and humans has not been established, therefore it is not clear how the measured effect in animals relates to the potential for infectivity in humans (Jabubowski, 1996). The infective dose or ID_{50} varies between animals and is dependant on a number of host and oocyst factors (Dept. Envn., 1995). There is a lot of variability in results from one animal model to the next due to the use of different animals, the variability in dose preparation and delivery. Even the endpoint, or evidence of infectivity, can be defined differently among groups of researchers. Some studies use histological evidence of infection where others use proof of oocysts shedding. This lack of direct relationship and variability can make interpretation of *Cryptosporidium* analytical data difficult.

In vivo studies are limited in their application by the need to use large numbers of parasites in order to induce a detectable infection in animal models. Detection of low concentrations of parasites, typical of environmental samples, is therefore not consistent with animal infectivity studies.

In Vitro Excystation

Excystation provides an estimate of the oocyct viability based on its ability to release sporozoites following exposure to external physio-chemical stimuli. Calculation of excystation index requires high concentrations of clean oocysts and is based on the ratio of excysted vs unexcysted oocysts observed microscopically. The excystation index can be misleading if excystation results in destruction of sporozoites, which leaves empty shells that appear to be viable (Korich, 1990). Alternately, oocysts which fail to excyst, but are infectious, will be scored as nonviable. Inaccuracy in estimating oocyst viability and efficiency of treatment and disinfection is possible with this method. Korich (1990) reported that if sporozoites are counted, not empty shells, a better correlation with animal infectivity could be realized.

Evidence of excystation is not evidence for infectivity. Infection is predicated on

excystation but excystation does not imply infectivity of sporozoites. Nevertheless, excystation provides an inexpensive, easy way to estimate viability.

The correlation between viabiltiy and *in vivo* infectivity is an important issue. A correlation between excystation and animal infectivity in mice was reported by Blewett (1989) and Korich (1990) in inactivation studies, but later disputed by Finch (1993). Viability estimates based on this method should be regarded with caution, since the sensitivity depends on the age of the oocysts and subjective counting procedure.

Vital Dye Staining

The inclusion or exclusion of fluorescent vital dyes and Nomarski differential interference contrast microscopy (DIC) is often used for assessing viability of isolated oocysts. Although numerous dyes are available, dual labeling with 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) is most frequently used. The mixture contains a dye which penetrates the membrane and stains nuclear material in living oocysts (DAPI) and a dye which does not penetrate the oocysts unless there is a loss of membrane integrity (PI).

The use of vital dyes for detection of viable oocysts of *C. parvum* has been reported by numerous authors. Results from their studies show that estimates of viability from vital dye staining correlate highly with excystation (r^2=0.997). A good correlation is also noted between vital dye staining and excystation when oocysts were subjected to various environmental pressures (Robertson et al, 1992). In contrast, Finch (1993) reported that vital dyes overestimate oocysts infectivity when compared with animal infectivity; again illustrating the short comings of viability assays that do not measure infectivity directly. Studies which evaluate animal infectivity in conjunction with vital dyes staining are needed to determine the reliability of the method for predicting infectivity of oocysts.

Recently, in a study by Belosevis (1997) the use of a nuclear stains (SYTO-9, hexidium, SYTO-59) in the detection of viable oocysts was correlated with animal infectivity, but not excystation. This report provides an exciting new development in the use of vital dye staining. Vital dyes provide a convenient, reproducible, quick and easy viability method, yet variability in quality and consistency of staining have been reported (Jabubowski et al, 1996)). In an

inactivation experiment using environmental samples, the application of vital dye staining was found to be totally unreliable (Korich,1990). Disinfection and other environmental factors may affect the uptake or exclusion of dyes and oocyst membrane integrity. Parasites that show some metabolic potential may not necessarily cause infection in animal host, hence use of these indicators may lead to erroneous estimations of viability (Labatiuk, 1991).

PCR

Unlike other viability methods, PCR requires a small number of oocysts. PCR provides a sensitive (1 - 100 oocysts), highly specific method for the detection of oocysts, however it is plagued with inhibition by particulates found in environmental samples. Several studies have recently reported successful detection of viable oocysts using this method. Deng (1997) reported on detection of viable oocysts from environmental samples using PCR linked with excystation and immunomagnetic capture (IMC) to target sporozoites. In other studies, reverse transcriptase-PCR of heat induced mRNA, thought only to be present in viable cells (Lindley et al, 1988) along with 18s rRNA (Rochelle et al, 1997), was able to detect viable oocysts. PCR of mRNA in a study by Bej and Mahbubani (1991) showed correlation with the infectivity of *Legionella pneumophila*. mRNA provides a great target for molecular detection, because it is present in multiple copies thereby increasing the sensitivity of the assay (Johnson et al, 1995). Even though PCR has a potential for application with environmental samples, its sensitivity is linked to detection and recovery methods currently in use, therefore it can only be as good these methods which are inefficient and variable.

PCR has proven to be a sensitive method for the detection of oocysts, yet the quantitative nature has yet to be proven. Most PCR based viability assays involve in-vitro excystation and are therefore detecting viable but not necessarily infectious oocysts. Therefore results from these studies suffer from the same shortcomings as those mentioned previously. Further studies are needed which link animal infectivity with PCR results.

In vitro Cell Culture Assay

Current and Hayes (1984) were the first to describe the cultivation of a human isolate of *C.*

parvum in vitro using human fetal lung cells (HFL), primary chicken kidney (PCK) and porcine kidney cells (PPK). Since that time numerous papers have described the development of *C. parvum* in cell culture. A variety of different cell lines support the development and growth of *C. parvum* in vitro including: CaCo-2 (Burard et al, 1991), HT-29-77 (Flanigan et al, 1991), HFL (Current and Hayes, 1984), canine or bovine epithelial cells (Gut et al, 1993, Lamb, et al and Rosales et al, 1993), HCT-8 (Upton et al, 1994) and MDCK (Gut et al, 1991, Rosales et al, 1993, Hammer et al, 1994, Arrowood et al, 1996, Villacorta et al, 1996). The lack of host specificity supports the permissive nature of these numerous cell lines. Species specificity of cell culture lines has not been thoroughly addressed, but attempt to grow *C. baleyi* in cell culture have failed (Current & Lindsay 1988).

The ability to support the development through the complete life cycle has been reported in HFL, PCK, PK and BFTE and MDCK (Current and Hays, 1984, Yang et al, 1996), but only BFTE has confirmed cell culture-derived oocyst infectivity for animals (Yang et al, 1996). Development of the parasite in cell culture parallels that found in vivo; it develops extracytoplasmically at the surface of cells (Yang et al, 1996). However, Rosales (1993) reported that *C. parvum* developed in the cytoplasm of MDCK cells.

Cell culture of *C. parvum* is possible due to the development of procedures and techniques to produce large volumes of oocysts and the ability to purify and isolate them. *In vitro* cell culture assays allow the study of organisms in an environment most similar to the *in vivo* situation without the expense of using live animals. *In vitro* systems are advantageous for studying host-cell parasite interactions, metabolic pathways, parasite specific molecular targets for drug intervention and the basic biology of the organism. Application of the cell culture assay has been limited to these areas of research, but its use can also lead to a better understanding of disinfection and inactivation by processes used for *C. parvum*. Recently Slifko (1997) reported the use of HCT-8 cell cultures for development of a detection method for measuring the infectivity of oocysts from environmental samples. This method has been referred to as the foci detection method (FDM).

The first step in a cell culture assay is inoculation of the sample, or dilution of the sample, containing *Cryptosporidium* into the cell culture. Microbial contamination present in samples

requires decontamination to ensure sterility prior to cell culture inoculation. This is achieved by the addition of a cocktail of antibiotics at high concentrations (bactericidal and fungicidal). Both sporozoites and oocysts have been used as inoculum, but direct inoculation of cells with oocysts is preferred due to ease of use and direct access of sporozoites to cells (Upton, 1994). Treatment of oocysts with 10% sodium hypochlorite prior to inoculation is common among researchers to surface sterilize and promote excystation (Upton, 1994). Others have used sodium periodate treatment (You et al, 96) and bile extracts (Meloni et al, 1996). Incubations of 24 and 48 hours are typical, with 48 hours being preferred due to a peak in the number of endogenous stages observed in several cell lines (Meloni et al, 1996, Yang et al, 1996). Longer periods of incubation may lead to increased numbers of endogenous stages, but cell monolayer health begins to deteriorate after 48 hours.

The endpoints used for quantifying *C. parvum* infectivity are the asexual and sexual endogenous developmental stages. Parasite enumeration involves scanning a number of microscopic fields and counting the actual number of endogenous stages within the field of view. Detection of the infective stages of parasites is generally based on immunological reactions with antibodies, which target endogenous stages, conjugated to a flourescent dye. Both monoclonal and polyclonals that recognize epitopes of *C. parvum* are being used with IFA. Immunofluorescent assays (Arrowood et al, 1996, Slifko et al, 1997, Upton et al, 1994), enzyme linked immunoassays (ELISA) (Woods et al, 1995, Upton et al, 1994), *in situ* PCR (Rochelle et al, 1995) and chemiluminescence (CLIA) (You et al, 1996) detection methods have all been evaluated. Although the majority of detection methods are linked with microscopy, the ability to automate quantication such as with CLIA and flow cytometery, would represent an advantage over the more labor intensive, subjective methods.

Research toward optimizing cell culture parameters that favor the growth and development of *C. parvum* has been reported (Upton et al, 1994, Arrowood et al, 1996, Meloni and Thompson, 1996). Variables tested include type of media, culture supplements, and age of cells at time of inoculation. Reports supporting the use of a particular cell line due to better sensitivity are typical, yet there is conflicting evidence to support the claims. Differences in methodologies among investigators make comparisons among cell lines and between experiments difficult. Infection

rates ranging from 1% in MDCK to 39.4% in BFEC have been reported. Hammer (1994) reported 1% infection rates for MDCK, Rosales 3.6% (1993) and Yang 4.6% (1996). Such difference, even among similar cell lines illustrates the problem with such comparisons. The debate on which cell line is the most suitable for detecting oocysts infectivity with the best sensitivity will continue. It is therefore left to the researcher to select the most suitable cell line for their application.

Occurrence and Source of Oocysts in the Environment

Several investigations into the occurrence of *Cryptosporidium* oocysts in the environment have been conducted and are summarized in Table 2.1. Due to the differences in methodologies used in sampling, volumes sampled and the detection method applied, the data should be interpreted with caution. Variability in the recovery efficiency (5-59%) (Smith and Rose, 1990) and detection methods used can introduce uncertainty into the data since most of the results have not been corrected for these rates. Methods currently used for oocysts detection tend to underestimate the actual prevalence of these parasites in the environment. The oocysts detected represent a fraction of the total population - those that can be isolated and detected by methods known to be highly variable and insensitive.

Source Water

Despite sampling and diagnostic difficulties, oocysts are found widely distributed in various types of natural surface waters; both pristine waters and those subjected to impact from human activities. Data from occurrence surveys indicate that oocysts are ubiquitous in surface waters in low concentrations, and occasionally in high numbers in water subjected to fecal contamination. In a survey of 257 surface waters (80% pristine) conducted in the 17 states, Rose (1991) found oocysts present in 55% of the samples with an average concentration of 43 oocysts/100 L. In another survey of eastern surface waters (mostly impacted by sewage and agricultural discharges) oocysts were found in 87% of water supplies (LeChevallier et al, 1991). Examination of river waters in the western US showed oocysts in each of eleven rivers examined at a range of concentrations between 2 - 112 oocysts/L (Ongerth and Stibbs, 1987). A more recent and

thorough study conducted by LeChevallier and Norton (1995) of 262 surface waters, detected oocysts in 51.5% of samples with a geometric mean of 240 oocyst/100 L. These finding are in contrast with the previous study, and may suggest the possible cyclic variations of oocyst levels in the environment.

Oocyst concentrations in water samples are dependent upon hydrologic influences which affect water quality, watershed characteristics and seasonal trends (LeChevallier et al, 1991). Samples taken from rivers receiving effluents from industry and agriculture are reported to contain considerably higher levels of oocysts, up to 480/L (Dept. Envn., 1995). Oocyst densities tend to be higher in areas that are impacted by high sewage input and those that drain agricultural regions. Hansen and Ongerth (1991) reported oocyst levels highest downstream of dairy farms compared to upstream locations, with peaks during periods of high runoff. Lower levels of oocysts were found in areas of well managed watersheds. Watershed characteristics and the degree of protection were found to be important factors influencing oocyst levels in this as well as other studies (Ong et al, 1996, Crockett & Haas, 1995).

Seasonal peaks in oocysts incidence have been reported (Casemore, 1990, Rose, 1988). Highest oocysts concentrations are thought to coincide with peak rainfall and occurrence of infections in human and animal populations, especially among calves and lambs (Rose, 1988).

Three different studies (Madore et al, 1987, Rose, 1988, Ongerth and Stibbs, 1987) found that concentrations of oocysts were 1 1/2 to 12 times greater in samples taken from sources where agricultural pollution was a major contamination source. It has been suggested that agricultural sources of water pollution may be of as much or more concern as human waste (Madore et al, 1987, Rose, 1988). This hypothesis is supported by data implicating animals as major contributors based on the ratio of fecal coliforms to fecal streptococci and absence of human enteric viruses (Rose, 1988). The widespread practice of disposal of animal and human excreta to land may contribute indirectly to contamination of water courses. Oocysts from agricultural sources, from grazing or spraying of land with animal waste slurry, may find their way into surface waters along with runoff waters. Oocysts have been detected in runoff from animal feeding lots in concentrations as high as 100/ml (Dowd, et al, 1997).

Sources of oocyst contamination may originate from point or nonpoint reservoirs. Point

sources are known points of entry such as discharges from wastewater treatment facilities, whereas nonpoint sources include runoff from agricultural areas such as dairies and grazing land. The broad host range and high output of oocysts from infected hosts ensures a high level of oocyst contamination in the environment. Infected humans and animals contributed to this contamination through fecal material and sewage. The pathogen has numerous nonhuman reservoirs in the animal population. It is frequently found among feral and domestic animals. While it is clear that feral animals may contribute to the oocysts found in the environment, the most probable animal source for oocysts are dairy cattle and sheep (Casemore, 1988). Infected cattle and sheep can shed 10^{10} oocysts/day for up to fourteen days (Blewett, 1989) where infected humans shed 400 - 10^{6} oocyst/gram over the course of infection (Smith & Rose, 1990). The relative importance of oocyst sources has yet to be determined.

The information on the presence of oocysts in groundwater is sparse, yet oocysts have been found in shallow wells and boreholes. The occurrence of oocysts in groundwater is suspected to be rare, and their presence in wells has been related to contamination due to surface water influences. Even though most waterborne outbreaks have been associated with groundwater, all outbreaks are attributed to distribution deficiencies (Dept. Envn., 1995).

Drinking Water

The presence of oocysts in treated water is probably a more accurate indication of the potential health risk to the public than those found in source waters. Drinking water has been evaluated for *Cryptosporidium* oocysts, and reports confirm their presence, although at very low levels (0.02 - 0 009/L). The significance of these findings is unknown since increased waterborne illness has not been associated with the presence of low levels of oocysts (Jabubowski, 1996). Oocysts are detected in drinking water from plants employing conventional treatments operating within regulatory standards which meet the USEPA's Surface Water Treatment Rule for turbidity and coliform standards. Even through oocysts are recovered at low levels from treated drinking water, current methods do not indicate the viability or infectious nature of these isolates. Previous epidemics have been associated with suboptimal water treatment processes as well as with state of the art water treatment.

Table 2.2 summarizes the data available on oocysts concentrations in drinking water. It is evident from these data that even filtration does not present an effective barrier to the passage of oocysts into finished drinking water. The level of oocysts found in drinking water may be contributing to an endemic level of disease in populations which is not easily detected by epidemiological studies (Rose, 1995).

Wastewater

Few investigations have evaluated the occurrence of oocysts in either raw or treated wastewaters, sludges and slurries. Table 2.3 summarizes the available research findings. Concentration and isolation of oocysts from these samples is more difficult due to the presence of debris, therefore the data may be unreliable. Large numbers of oocysts can be excreted during the course of an infection and most enter the sewage system. Sewage systems may also receive slaughterhouse effluents that may contain *Cryptosporidium* oocysts. One study evaluated oocyst concentrations in slaughterhouse effluents and found as many as 14,000 oocysts/L (Madore et al,1984). In nine samples of activated sludge treated sewage effluents, oocysts were found at a range of 140 - 3,970/L (Madore et al, 87). Another study reported 91% of raw and treated sewage were positive for oocysts, with an average of 28.4 to 17 oocysts/L, respectively (Rose, 1988) In contrast with these findings, Parke and Smith (1993) found that 26 samples out of a total of 70 from 7 sewage works were positive for oocysts, with a range of 0.03 - 2.4/L. In a recent study by Wallis (1997), only 6.1% of samples were positive. The presence of oocysts in sewage is influenced by the size of the community and the rate of the infection in the population. Due to the high levels of oocysts found in sewage wastewater and the fecal oral route of transmission of this parasite, treatment processes are critical for reducing the number eventually discharged into the environment.

Table 2.1

Levels of Cryptosporidium Oocysts in Surface and Groundwaters

Source	Samples(n)	% Positive	Oocyst/L Range	Mean	Ref.
stream/river	6	100	0.8 - 5800	1920	1
river	11	100	2 - 112	25	2
stream	19	73.7	0 - 240	1.09	3
stream/river	58	77.6	0.04 - 18	0.94	4
stream/river	38	73.7	<0.001 - 44	0.66	3
river/lake	85	87.1	0.07 - 484	2.7	5
river/lake	262	51.5	0.065 - 65.1	2.4	6
river	22	31.8	0.01 - 75.7	0.58	7
river/lake	18	NR	7.1 - 28.5	17.8	3
lake	20	70.7	0 - 22	0.58	3
lake/resevoir	32	75	1.1 - 8.9	0.91	4
lake	24	58.3	<0.001 - 3.8	1.03	3
lake	44	27.3	0.11 - 251.7	4.74[2]	7
river, pris.	3	NR	NR	0.08	3
river, pris.	59	32.2	NR	0.29[2]	
lake, pris.	34	52.9	NR	0.093	3
sring, pris.	7	28.6	<0.003-0.13	0.04	3
well	18	5.6	NR	0.003[3]	

1 impact by human activities; 2 arithmatic mean; adj= adjusted for recovery efficiencies; 3 single value; 4 as cited by Butler, 1996; ref. 1. Madore et al, 1987, 2. Ongerth and Stibbs, 1987, 3. Rose et al, 1988, 4. Rose, 1988, 5. LeChevallier et al, 1991, 6. LeChevallier and Norton,1995, 7. Stetzenbach et al, 1988 NR = not reported

Table 2.2

Levels of Cryptosporidium Oocysts in Drinking Water

Source	Treatment	Samples	% Positive	Oocyst/L Range/Mean	Ref.
surface	filtration	262	13.4	0.29-57/0.03*	1
surface	filtration	83	27	0.13-48/0.015*	2
NR	+/- filtration	36	17	0.5 - 1.7/NR	3
NR	filtration	557	7	1 - 26/NR	4
NR	+/-filtration	1237	7.1	NR/NR	5

*geometric mean, ref. 1. LeChevallier et al, 1995, 2. LeChevallier et al, 1991, 3. Rose et al, 1988, 3. Dept. Envn., 1996, 5. Rose et al, 1996 NR = not reported

Table 2.3

Occurrence of Cryptosporidium in Sewage and Sludge[3]

Site	Type of Sample	(n)	Oocysts/L Range	Mean	Ref
AZ	raw sewage	4	850 -13,700	5180[1]	1
AZ	raw sewage	5	NR	521[2]	2
AZ	raw sewage	6	NR	4[2]	3
TX	activated sludge effluent	9	140 - 3,960	1297[1]	1
AZ	activated sludge effluent	14	NR	39.7[2]	2
CO	activated sludge effluent	2	NR	4[2]	3

1 Arithmetic mean (Madore et al., 1988); 2 geometric mean (DeLeon et al., 1988); 3 adapted from Rose, 1988 NR = not reported

Removal of Oocysts By Treatment Processes

While the evidence from outbreak investigations suggests that conventional water treatment maybe inadequate during periods of high numbers of *Cryptosporidium* oocysts, research data indicate that treatment processes are effective in removing oocysts from source water. Processes available for oocyst removal from water and wastewater are classified as those causing physical separation (sedimentation, coagulation, precipitation, filtration and adsorption) and those affecting inactivation (disinfection).

Drinking Water Treatment

The role of effective water treatment in removing *Cryptospordium* oocysts and in minimizing this risk of waterborne cryptosporidiosis is well recognized. However, it is known that complete removal or inactivation of oocysts by treatment and disinfection processes is not possible. A small proportion of oocysts present in source water used for water supply may pass into the distribution system. Treatments for potable water range from disinfection of groundwater to comprehensive treatment for surface waters under the impact of sewage and agricultural

discharges. Conventional water treatment usually includes coagulation, flocculation, rapid granular media filtration and disinfection.

Physical Processes

Results from laboratory studies of pilot and full scale plants demonstrate that effective removals of oocysts are achieved by pretreatment for coagulation and sedimentation of solids followed by filtration and disinfection. Removals as high as 99.5 - 99.9 % (2.5 - 3 logs) of oocysts have been reported for well operating conventional systems prior to chlorination (Gregory, 1994). Hall (1994) conducted pilot-scale removal experiments at various oocyst doses and achieved consistently high removals (e.g. 3 log) with coagulation and filtration. Nieminski and Ongerth (1995) reported oocyst removal rates averaging 2.98 logs by pilot scale conventional treatment and 2.25 logs by full-scale test, with removals of 1.9 - 3 logs common. In plants with positive filtered effluents, reduction in oocyst levels average 2.4 logs, with negative filtered effluents, 2.2 logs.

Determining removal rates of oocysts by individual processes is difficult because most reports present results as overall efficiencies of sequential treatments. Oocyst removals of 0.6 - 1.5 logs are reported for well operated coagulation - clarification procedures (Plummer et al, 1995). Removal efficiencies by rapid and slow sand filtration following coagulation range from 0.5-4 logs, with higher removals reported with slow sand filtration (Rachwal, 96). Filtration without coagulation achieved removals of 1 log from river water (Rose, 1988).

Alternative filtration methods such as ultra-filtration and micro-filtration have been evaluated for oocyst removal from natural waters. Removal rates of at least 6 logs were reported using clean water (Rachwal, 96). Use of these filters in water treatment has been limited due to their expense and restriction to relatively clean waters.

Maintaining effective removal of oocysts is dependent on optimal operation of treatment processes and maintenance of low water turbidity (Nieminski and Ongerth, 1995 and Ongerth and Pecoraro, 1995). Coagulation control and filter flow rates are important parameters for efficient removal even though the actual choice of treatment parameters was not found to be associated with parasite detection (Butler and Mayfield, 1996). Periods of filter ripening are critical for control

of oocysts in finished water because particle breakthrough can occur during this time (Rachwal, 96). Prefiltration turbidity of raw water was found to be the greatest effector of plant performance.

Table2.4

Oocyst Removal Rates by Drinking Water Treatment Process

Treatment Process	Log Removals	Reference
Dissolved Air Flotation	>2	Plummer, 95
Slow sand filter	2 - 4	Rachwal, 96
Rapid sand filter(no coagulation)	0.5-1	Rachwal, 96
Rapid sand filter(chemicals, ozone)	1-2	Rachwal, 96
Sand filtration(no coagulation)	0.45	Gregory, 94
Sand filtration(coagulation)	1.0	Gregory, 94
Mixed Media filtration(coagulation)	2.4-2.7	Ongerth, 91
Membrane Filtration (0.2µM)		Ongerth, 91
San Jose	>6.9	Jacangelo, 95
Vigneaux	>6.3	Jacangelo, 95
Membrane Filtration (0.2µM)		
San Jose	>6.8	Jacangelo, 95
Vigneaux	>6.0	Jacangelo, 95
Membrane Filtration, 500 kD		
San Jose	>6.9	Jacangelo, 95
Vigneaux	>6.3	Jacangelo, 95
Conventional Treatment	93.3	Rose, 88
with coagulation	54.5	Rose, 88
without coagulation	1	Rose, 88
with coagulation	2.0-2.7	Gregory, 94
Coagulation and Filtration	2.5	Ongerth, 95

Inactivation Processes

Disinfection provides an effective barrier in conventional water treatment in preventing outbreaks of disease from most pathogens, but studies have shown oocysts have unusually high resistance to commonly used disinfectants, especially at normal working concentrations. A number of studies on the effectiveness of water disinfection on oocysts can be found in the literature, but comparative evaluation is difficult due to different experimental conditions and assessment methods used. To measure the effectiveness of disinfection oocyst viability is measured. Viability measurements are based on animal infectivity, in vitro excystation and vital dyes as previously discussed.

assessment methods used. To measure the effectiveness of disinfection oocyst viability is measured. Viability measurements are based on animal infectivity, in vitro excystation and vital dyes as previously discussed.

Disinfection studies show chlorine has little, if any, affect on the viability of *Cryptosporidium* oocysts at high concentrations as well as at levels normal in drinking water treatment. Fayer (1995) reports that oocysts treated with 5.25% sodium hypochlorite (50,000 ppm) for two hours retain their infectivity in neonatal mice. This result is in contrast to reports by Korich (1990) that claim complete elimination of infectivity at 80 ppm chlorine at 25°C for 2 hours, when tested with *in vitro* excystation. In his studies Korich calculated a chlorine CT value for 99% kill of 7200 mg. min/L for *Cryptosporidium*. Similarly high values are also reported by Smith (1989) and Sterling (1990). Monochloramine was found to have similar inactivation as free chlorine. Chlorine dioxide was found to more effectively inactivate oocysts than either chlorine or monochloramine (Korich et al, 1990, Peeters et al, 1989). CT values based on this data were 78 for chlorine dioxide, 7200 for chlorine and monocloramine for 99% inactivation at 25°C in buffered water (pH 7.0) (Korich et al, 1990).

Ozone has also been evaluated for its ability to inactivate oocysts. In the previously mentioned study, Korich approximated the CT value for ozone as between 5 and 10 mg. min/L. Using mouse infectivity and excystation, Finch (1993) determine CT values of 3.5 and 7 mg. min/L for 99% inactivation, at 22°C and 7°C. For 99.9% inactivation these values increased to 5 and 10 mg. min/L. In another study, Peeters (1989) was able to eliminate oocyst infectivity within six minutes at 1.11 ppm ozone. The levels required to inactivate oocysts are much higher than used in practice, and are not economically feasible.

Oocysts show relative resistance to UV, but reductions of 2-3 logs were obtained using a novel system were oocysts were embedded in fibers prior to UV exposure (Campbell et al, 1995).

Results from these inactivation experiments suggest that oocysts are capable of surviving typical disinfection treatment. Consequently, disinfection alone may not be sufficient to provide an adequate level of protection from *Cryptosporidium* oocysts. A few studies indicate that oocysts under stress may be more sensitive to disinfection. Parker and Smith (1993) reported that abrasion of oocysts by sand increased the efficiency of inactivation by chlorine. Other studies

have indicated that exposure to elevated temperatures, extremes of pH and time may increase the effect of disinfectants on oocysts (Dept. Envn., 1995).

Wastewater Treatment

Removal of oocysts by wastewater treatment takes place during primary and secondary treatment (activated sludge, trickling filters or other forms of biological treatment) processes. On the basis of laboratory simulations, primary sedimentation, secondary treatment and sedimentation resulted in overall oocyst reductions of 98.6% (Staderman et al, 1995) and 98.87% (Mayer and Palmer, 1996). Studies have shown that during primary sedimentation 80-90% of oocysts are removed from wastewater (Mayer and Palmer, 1996, Staderman et al, 1995, Villacorta et al, 1992). Poor removals during primary sedimentation occur because the sedimentation velocity of oocysts falls below the free fall design criteria of the tanks (Whitmore and Robertson, 1995). Although the activated-sludge process was not considered efficient for cysts removals (Bitton, 1980), reductions of 79-84% have been reported (Madore et al, 1987 and Villacorta et al, 1992). In a study to compare several secondary treatments Staderman (1995) reported removals of 92%, 50% and 42% by activated sludge, trickling filter, and biodisc filtration, after secondary sedimentation. The ability of treatment processes to remove oocysts from sewage is advantageous, but increases the hazard in the by-product of these processes, the sludge (Slade and Ford, 1983).

While removal rates in treatment processes are most often reported, it is what remains in the water that is important, not what is held back (Dept. Envn., 1995). Based on reported laboratory results approximately 2 logs of oocyst removal can be expected in wastewater treatment, but removals at this level would not satisfy the SWDR which calls for 99.9% removal of Giardia cysts (LeChevallier, 1991). Nor would this level of removal be sufficient in wastewater streams which contain high concentrations of oocysts. While treatment processes are effective at reducing the number of oocysts, they do not completely eliminate them, and those oocysts found remaining in sewage effluent have been shown to be infectious by animal assay (Villacorta et al, 1992). Since oocysts are capable of long term survival in the environment, additional treatment may be necessary to eliminate and reduce the public health risk posed by this organism (Mayer

and Palmer, 1996).

Table 2.6

Oocyst Reductions by Wastewater Treatment

Treatment	Removal	Ref
Primary Treatment		
Sedimentation	90%	Mayer, 96
	83.4%	Staderman,95
Secondary Treatment		
Activated Sludge	82 - 98%	Villacorta, 92
	92%	Staderman, 95
	99%	Mayer, 96
Trickling Filter	50	Staderman, 95
Biodisc Filter	42	Staderman, 95
Overal Removals	98.87%	Mayer, 96
	96.8%	Chauret, 95
	98.6%	Staderman, 96

Sludge Treatment

Sewage Sludge

Each year in the United States some 13,000 POTW's generate ~5.4 metric tons of sewage sludges, or 47 lbs/person (EPA FRD 94). The cost of managing these sludges represent about 50% of the operation cost of wastewater treatment. While sludge production is expected to double by the year 2000 (relative to 1994), options available for their disposal are decreasing. An option available for managing sewage sludges, and one encouraged by EPA in the Federal Water Control Act and the Clean Water Act, is direct application to agricultural lands. The reuse or reutilitzation of the products of sludge treatment, or biosolids, presents an attractive and economical option. Municipal biosolids are routinely utilized in agriculture through out the world. In the US as much as 33% of the biosolids produced is applied to agricultural lands (Pillai et al, 1996).

The use of municipal biosolids as fertilizers, soil conditioners and horticultural products are of value in a world of limited resources, but such use may present the hazard of introducing pathogens and chemical contaminants into the food chain and water supply. While possible routes of transmission from land applied biosolids exist, there have been no studies linking the practice to

reported an outbreak in 1988 of hepatitis A virus (HAV) by those eating shellfish contaminated by nightsoil in China.

While oocysts survival depends on a number of environmental conditions (i.e. predation sunlight, temperature, humidity), the soil-sludge matrix is capable of supporting the survival of pathogens for prolonged periods of time. In fact oocysts can persist in sludge treated soils for up to 44 days with only a decrease of 20-40% in viability (Whitmore and Robertson, 1995). Information on factors which affect their movement and transport in soil are scarce, but would help in the understanding of the hazard the presence of these pathogen presents. Dowd (1997) reported that oocysts were able to migrate through 10 cm of soil under simulated rainfall conditions. Clearly, through treatment of sewage sludges at the treatment facility the potential human health risk posed by oocysts in sludge destined for reutilization be eliminated.

During primary and secondary wastewater treatment oocysts along with other sewage solids are settled out and become concentrated in the sludge. Although methods used to treat sludge were originally directed toward sludge stabilization, it is serendipitous that pathogen inactivations are also realized by these treatment processes. Treatment options for sludge stabilization include anaerobic and aerobic digestion (mesophilic and thermophilic), lime stabilization and composting. The most common method of sludge stabilization in the US is anaerobic digestion.

Two studies investigating the effects of digestion of sludge on oocysts survival under mesophilic, thermophilic, aerobic and anaerobic conditions in laboratory stimulations were found in the literature. Whitmore and Robertson(1995) found oocysts recovered from aerobic thermophilic digesting sludge (55°) and pasteurized sludge (55°C) were completely inactivated at 24 and two hours respectively. They reported that while mesophilic (35°C) anaerobic digestion reduced overall viability, 10% of oocysts remained viable even after 18 days as determined by vital dye staining. Using mesophilic (37°C) anaerobic digestion, Staterman (1995) evaluated oocysts removal and inactivation using *in vitro* excystation. She reported 90% inactivation after four hours, and complete inactivation after 24 hours. She contributed the lack of concordance with Whitmore and Robertson's data to differences in oocyst preparations, but differences could also be due to assessment methods.

Robertson's data to differences in oocyst preparations, but differences could also be due to assessment methods.

The actual effect of temperature in oocysts inactivation has not been properly evaluated. While temperature is undoubtably the most important parameter in oocysts inactivation during these processes, other physical and chemical factors may contribute to reduction of microbes. It is possible that pH may affect inactivation rates, although this is unlikely since pH is generally maintained during both mesophilic and thermophilic processes. Robertson (1992) reported that oocysts are stable in pH ranges between 4 and 10. Other biological factors which may contribute to inactivation and degradation of oocysts include enzymatic and bacterial decomposition (Gavagham, 1993).

Laboratory simulations of digestion treatment processes are helpful in predicting the fate of oocysts during experimental treatments, but these results may not be consistent with actual sludge treatment processes. Possible differences in mixing dynamics and retention periods of full scale digesters may lead to different results.

Animal Waste

Animals are important reservoirs for *Cryptosporidium* oocysts in the environment, therefore management of livestock waste is an important public health issue. There is increasing circumstantial evidence that animal waste may be contributing to ground and surface water pollution, but estimates of these contributions have not be done. Little is known about the fate of oocysts in land applied waste. While animal waste was implicated in the Milwaukee and Carrollton outbreaks there was no direct evidence (MacKenzie et al, 1995).

Management of livestock waste does not come under the same level of federal regulatory control as municipal waste, but its potential as a source of oocysts, capable of contaminating the environment should not be underestimated. The prevalence rates in dairy cattle are reportedly as high as 80% (Scott et al, 1995). Infected livestock can excrete large numbers of oocysts in their feces, and because most animal wastes do not receive treatment of any kind, their disposal and reuse may pose an even greater hazard than human waste. Land application of animal waste is widely practiced and is an important waste management method. Application rates of these

manures are not governed by pathogen content, but by nitrogen loading rates. Increasing media coverage and greater public attention on Piefsteria have recently focused attention on animal waste management in North Carolina, especially piggery waste. As the role of animals in contributing to microbial contamination of the environment is elucidated it is possible that changes in animal waste management and runoff control, and watershed management practices will be realized.

There is increasing interest among livestock producers in the use of anaerobic thermophilic digestion as a way to manage animal waste. This interest is especially noticed in large systems where huge amounts of waste are generated. Successful full-scale treatment of animal waste by this method has been demonstrated (Monteith and Shannon, 1986). Although up-front expenses for this type of operation are significant, recovery of expense may be realized by successful reuse of products of digestion. Products of anaerobic thermophilic digestion include methane and single cell proteins. The methane can be used to maintain temperatures required for thermophilic digestion, deferring some of the operational cost and the proteins can be used as rations for livestock. As with municipal biosolids, animal biosolids can have use as agricultural products.

Oocyst Survival in the Environment

Most oocyst survival investigations have focused on oocyst resistance to disinfectants, while the effects of environmental pressures on oocyst survival have received little attention. Conclusions from the few studies that are available, indicate that oocysts are resistant to typical environmental pressures and are able to survive for long periods in cool moist conditions, but are vulnerable to temperature extremes and dessication. Table 2.6 summarizes results from these studies.

Early studies (Sherwood, et al, 1982) reported the loss of oocyst infectivity after 14 days in buffered saline at 20°C, and total destruction by freezing temperatures (Tzipori, 1983). In a more recent study, Robertson (1992) reported that oocysts from a variety of sources are persistent for long periods under a variety of environmental conditions, even freezing. Surviving oocysts are detected in samples subjected to typical environmental conditions after six months, yet a significantly higher proportion of oocysts are killed during warmer weather than during cooler times. While oocysts remain viable for extended periods in feces and seawater, they are completely killed

after brief dessication. The results from this extensive study suggest the importance of moisture and temperature in oocysts survival.

To better define the limits of survival at low temperatures, Fayer and Nerad (1996) subjected oocysts to temperatures ranging from 5° to -70°C. He found that oocysts are unable to survive exposure to -70°C, but are able to retain infectivity after freezing between -10 to -20°C for up to 24 hours.

The effects of elevated temperature on oocyst viability has been investigated by several laboratories. Fayer (1994) found that oocyst infectivity is destroyed after exposure to >/ 64.2°C for two minutes in water, but not after five minutes at 59.7°C. Blewett (1995) found a 92% decrease in viability after treatment at 55°C for five minutes, as determined by excystation. Table 2.7 summarizes the results from these studies. As with other studies, interpretation of these data is hampered by the differences in methodologies used to recovery and score oocyst viability between studies.

In a study by Parker and Smith (1993) shaking oocysts with sand for short periods of time was found to leave the oocysts more susceptible to disinfection with chlorine. The implication of this and other studies is that environmentally exposed oocysts are more readily inactivated than isolates from laboratory controlled conditions. This hypothesis is supported by an earlier study by LeChevallier (1991) that indicated oocysts exposed to the environment are more susceptible to inactivation by disinfectants. Work by Hall (1994) and Carrington and Ransome (1994) have also demonstrated the increased sensitivity of exposed oocysts to inactivation by disinfectants. Clearly more research in this area would lead to a better understanding in the role of environmental exposure on oocysts viability.

Table 2.6

Effect of Environmental Exposure on *Cryptosporidium* Oocyst Viability

Exposure Condition	%Dead or Infected/Uninfected
Dessication	
4 hr room temperature	>99
Freezing	
-22°C, 21 hr	67
-22°C, 152 hr	>90
-20°C, 24 hr	0/17*
-10°C, 168 hr	12/12*
-15°C, 168 hr	0/12*
-70°C, 1 hr	0/5*
Liquid Nitrogen	>99
River Water	
176 days, ambient T	94
Tap Water	
176 days, ambient T	96
Sea Water	
35 days, 4°C	38
Feces	
Human, 178 days, 4°C	78
Bovine, 176 days, ambient T	66

Adapted from Butler and Mayfield, 1996 which summarizes Robertson data and *Fayer, 96 data; viability determined by vital dye staining or animal infectivity

Table 2.7

Thermal Inactivation of *Cryptosporidium* Oocysts

Temperature, °C	Exposure time, mins.	Result	Ref.
54.4	1	+	Fayer, 94
59.9	1	+	Fayer, 94
59.7	5	+	Fayer, 94
64.2	2	-	Fayer, 94
67.5	1	+	Fayer, 94
72.4	1	-	Fayer, 94
71.4	0.05	-	Harp, 96
50	NA	+	Anderson, 81
55	NA	-	Anderson, 81
45	10 - 20	-[1]	Anderson, 81
55	5	92%[2]	Blewett, 89

1. oocysts suspended in gut homogenate, 2. excystation, all others assayed by animal infectivity, (+) infectious, (-) noninfectious

Standand Regulatory Control of Oocysts in the Environment

Water - Raw and Finished

At present no standards exist for the presence of *Cryptosporidium* in raw or finished water. There has been upheaval in the regulatory environment due to the concerns of microbial contaminants and chemical contaminant which has recently been addressed by the Enhanced Surface Water Treatment Rule (ESWTR) and the Disinfection & Disinfection by Product (D/DBP) rule. The USEPA submitted a plan to collect data regarding microbial and chemical contaminants in water and the ability of water treatment processes to remove these substances. The Information Collection Rule (ICR), an administrative order, resulted from regulatory negotiations of this proposal and requires utilities using surface water servicing >100,000 costumers or those using ground water servicing >50,000 too collect data regarding the occurrence of this parasite in source waters used as potable waters and in finished drinking water. This information will be provided to the EPA to assist in establishing new regulations and standards for this parasite. The rule is supposed to help provide information on how the oocysts are entering the system and identify potential source of oocysts. It will also provide information on the efficiency of water treatment processes as well as help estimate the cost impact for regulatory scenarios.

Currently the ESWTR requires removals or inactivations of *Giardia* and enteric viruses of 99.9 and 99.99% respectively. Removals and inactivation credits are given for specific treatment processes because it is not feasible to measure these contaminants in drinking water. It is expected that the new regulations would require removals of oocysts as well because of the inaccurate and variable methods for oocysts detection. Another possibility is the establishment of a maximum contaminant level, which would be set at zero (Plummer et al, 1995). It is expected that these new standards will establish removals and inactivations based on the concentrations believed to be present in the source water.

Based on current information regarding the occurrence of these oocysts in water courses a minimum of 3-6 logs of inactivation would be needed to attain the acceptable risk range currently adopted for microbial risk in drinking water. In a report by Rose and Haas (1994), the adoption of an action level for *Cryptosporidium* in source waters was presented. The authors suggest that 30

oocysts/L represents a level at which the probability of waterborne outbreaks would demand action on part of the water utilities. Implementation of a multiple barrier approach which includes watershed management represents the best control option.

Wastewater

Even though sewage treatment is effective in reducing the number of oocysts in wastewater, they are not completely eliminated after secondary treatment. Oocysts are known to survive for long periods in the environment, therefore it is important that treatment establishes a certain level of reduction of these pathogens through the treatment process. Reuse of wastewater for purposes of aquifer recharging and irrigation necessitates regulatory control. Currently no such standards are in place for *Cryptosporidium* oocysts. More information is needed on the levels of oocysts in raw and treated sewage to assist in determining the best approach.

Sludge

Sludges generated from wastewater treatment may contain large numbers of pathogens, therefore the safe disposal of these biosolids is of major importance to regulators in the US. In 1993 USEPA published the "503 Standards for the Use and Disposal of Sewage Sludge" (USEPA, 1992). These regulations establish standards for the beneficial land application of sewage sludge. Provision of these regulations which play a role in determining the environmental fate and health impacts of land applied sewage sludge are the specifications for pathogens in sludge. Performance based standards rather than treatment processes have been adapted for pathogens in sludge.

Animal Waste

In a 1994 survey by EPA of state water quality, farm runoff was identified as the biggest problem in 60% of rivers and streams included on the impaired list. Non-point pollution from farms is probably the most widespread source of water contamination in the US (Anderson, 1995). Yet only the largest operations with >1,000's of animals retained in pens require permits for the storage of manure. Currently only sixteen states have requirements for smaller farms which require farms to adhere to plans on how to manage manures and fertilizers, most states have purely voluntary

programs. The governments response to non-point pollution has been to recommend that farmers adapt programs of waste and nutrient management. The U.S. Department of Agriculture provides assistance, but neither they nor the EPA have authority to do anything more. Environmentalist have been calling for the Clean Water Act (CWA) to make nutrient and waste programs mandatory for all farmers. Data indicate that better animal waste handling practices, such as composting, anaerobic digestion, and wetland remediation can reduce the protozoan loading into the watershed.

CHAPTER THREE

RESEARCH OBJECTIVES

1. Evaluate the application of an in vitro cell culture infectivity assay to determine the reduction in infectivity of *Cryptosporidium parvum* oocysts following treatment processes.

2. Quantify *Cryptosporidium parvum* oocyst inactivation by thermal treatment in phosphate buffered saline within temperature ranges typical of mesophilic (35 - 49°C) and thermophilic (47 - 55°C) digesters.

3. Quantify *Cryptosporidium parvum* oocyst inactivation in cow manure at 23°C and after thermophilic anaerobicdigestion of cow manure at 55°C, and compare to oocyst inactivation in phosphate buffered saline.

CHAPTER FOUR

INITIAL RESEARCH ON A CELL CULTURE INFECTIVITY ASSAY METHOD

INTRODUCTION

In this chapter, the applicability of an *in vitro* cell culture infectivity assay for determining *Cryptosporidium* oocyst infectivity is evaluated. Three basic mechanisms have been widely used to assess the effectiveness of chemical and physical treatments: 1.) *in vivo* animal models, 2.) *in vitro* excystation of oocysts, and 3.) vital dye staining of oocysts (DAPI/PI). Currently, the "gold standard" for determining oocyst infectivity is the neonatal mouse model. Although this assay provides information on infectivity of the oocysts, it is limited by being both labor intensive and expensive. Excystation, while easy to perform, is based upon the assumption that oocysts which are capable of excysting are viable. This assumption may actually be erroneous and lead to underestimating oocysts viability, and overestimating the efficacy of treatment processes. Also, research has shown that animal infectivity and excystation results are not correlated (Finch et al, 1993). Vital dye staining predicts oocyst viability based on cellular membrane potential, but this may not accurately predict the infectivity of the oocyst. In this chapter, data are presented that evaluate the MDCK cell culture infectivity assay for determining the infectivity of oocysts.

MATERIAL AND METHODS

Source and production of *Cryptosporidium parvum* oocysts

The *Cryptosporidium parvum* isolate used for this study was the IOWA bovine strain. Oocysts generated in newborn Holstein bull calves were obtained from the laboratory of Mike Arrowood (Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA.). The original isolate was obtained from Harley Moon (USDA, Ames, Iowa).

The procedure used to propagate this isolate was previously described (Arrowood and Sterling, 1987). Briefly, three day old Holstein calves were orally inoculated with 10^6 cleaned oocysts. Upon the onset of oocyst shedding, oocysts were collected daily then purified from calf feces through discontinuous sucrose gradients (Arrowood and Sterling, 1987). Additional purification was achieved by including a cesium chloride gradient (1.15 g/ml, 1.15 specific gravity) following the differential sucrose gradients. Pellets from the secondary sucrose gradient were resuspended in a saline buffer (0.85% NaCl) and overlaid onto the cesium chloride solution. The tubes were centrifuged at 16,000 x g for three minutes. The top 1 ml of the sample containing the oocyst-enriched fraction was removed, washed in phosphate buffered saline (PBS, pH 7.2), then resuspended in 2.5% aqueous potassium dichromate. Oocysts were stored at 4°C and used for *in vitro* experimentation within three to four months of isolation.

Host cells - MDCK Cell Cultures

Madin-Darby canine kidney (MDCK) cell cultures (American Type Culture Collection CCL 34) were obtained from the laboratory of Mike Arrowood (DVC, CID, CDC). MDCK cells were maintained in Dulbelco's Modified Eagle's Minimum Medium/Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 15 mM HEPES buffer. Cells were passaged once a week and seeded in cell chambers at 7.5×10^4/1.5 ml. Assay cell cultures were propagated in double-chambered Lab Tech Chamber Slides (Nunc) using serum free Ultraculture™ medium (BioWhittaker, Inc., Walkersville, MD), supplemented with 250 µg kanamycin, 50 µg gentamycin, 150 µg mycostatin and 2 mM L-glutamine. Cultures were incubated at 37°C in a 5% CO_2 environment.

Monoclonal Antibody Preparation

OW50 (Arrowood, 1988), a monoclonal antibody specific for a *C. parvum* oocyst cell wall determinant was provided by Mike Arrowood. OW50 was labeled with fluorescein isothiocyanate (FITC) (Arrowood et al, 1995) for use in immunofluorescent assays. When used in the *Cryptosporidium* cell culture infectivity assay, this monoclonal antibody labeled only oocyst walls. Labeled oocysts fluoresced apple green under epifluorescence optics with blue excitation UV

illumination, using an I3 filter block (450 -490 nm).

C3-C3 (Arrowood, 1988), a monoclonal IgG antibody directed toward oocysts and sporozoites of *C. parvum* was generously provided by Mike Arrowood. This monoclonal antibody reacts with cytoplasmic antigens of sporozoites, as well as meront and gamont life cycle stages. C3-C3 antibody was labeled (You et al, 1996) with a rhodamine reactive dye, Fluorolink Cy3™ (Research Organics Inc., Cleveland, OH). Under epiflourescent microscopy with a N2.1 (515 - 560 nm) filter block, labeled proteins fluoresced bright red.

Oocyst Preparation and Enumeration For Experiments

C. parvum oocysts stored in potassium dichromate were prepared and enumerated for each experiment. Oocysts were washed free of potassium dichromate by three sequential washes in phosphate buffered saline (PBS, pH 7.2), then with PBS containing 0.1% BSA. Washed oocysts were centrifuged at 16,000 x g for three minutes. The supernatant was aspirated and the oocyst pellet was resuspended in 1 ml PBS. Oocysts were enumerated using a dual chamber hemocytometer and bright field microscopy. Duplicate 10 µl volumes of a diluted oocyst suspension were pipetted into the counting chamber and oocysts were allowed to settle for one to two minutes. Oocysts within the four large, corner squares, each containing 16 smaller squares, were counted under a 25X objective. This procedure was repeated for the other chamber on the hemocytometer. Counts for the total of eight squares were averaged. To calculate the number of cells per ml the following formula was used:

# of cells/ml = mean of 8 squares * 10^4 * dilution factor

Oocysts were diluted serially 10 fold from an initial concentration of 10^7 oocysts per ml with subsequent ten fold serial dilutions down to 10 oocysts per ml.

MDCK Infectivity Assay of *Cryptosporidium* Oocyst

Four-day-old MDCK cell monolayers were inoculated with 100 µl volumes of serially diluted oocysts with an estimated oocyst concentration ranging from 10^7 to 10^1 per ml. Duplicate wells were inoculated for each dilution including a negative control. Inoculated monolayers were incubated for three hours at 37°C to promote excystation and enhance cell infectivity. After this

incubation, the cell monolayer was washed with PBS, fresh Ultraculture medium was added to the chambers and the cells incubated for periods of 18, 24 or 48 hours. For incubations of 48 hours, the chamber medium was replaced after 24 hours.

Following incubation, monolayers were washed with PBS then fixed in Bouin's solution (Sigma, St. Louis, MO.). Fixed cells were decolorized in successive washes with 70% ethanol, followed by three PBS washes. Decolorized cells were blocked with 1% BSA-PBS for 30 minutes at room temperature with shaking to minimize nonspecific antibody binding. One hundred µl of diluted (1:500) monoclonal antibody C3-C3Cy3 and monoclonal antibody OW50-FITC (1:200) were added directly to cell cultures and incubated for 90 minutes in the dark at room temperature. Following incubations, the cell cultures were washed thoroughly with PBS and mounted under cover slips with polyvinyl alcohol-based mounting media (DABCO-PVA). Slides were stored at 4°C and microscopic observations were made within one week from staining.

Data Analysis

Microscope examination of *C. parvum* infected cells was conducted to determine the extent of infectivity based on the presence or number of living stages of parasites in microscopic fields. Labeled life stages were observed by UV illuminated epiflourescent microscopy using either a 25X or 40X objective. Both numeric and quantal approaches were assessed for quantifying parasitic infectivity. Oocysts were distinguished from parasites developmental stages based on fluorescein labeling. Oocysts fluoresced apple-green (FITC-OW50) and living stages fluoresced red (CY3-C3C3) (Figure 4.1, 4.2).

For quantal assays, sexual (gamonts) and asexual (meronts) developmental stages of *C. parvum* were scored in 100 randomly selected fields. No attempt was made to distinguish between asexual and sexual stages. Any field (a test unit) containing a rhodamine labeled fluorescent developmental stage of *Cryptosporidium*, a result of infection of an MDCK cell by an infectious oocyst, received a positive score. A negative score was assigned to those fields which contained no red flourescent life stages. The proportion of infected to non-infected test units was scored and recorded. Oocyst titer was calculated using the dilutions where only some of the 100 fields were infected, giving specific percentages. The percent positivity was converted to a probit

value, plotted against the $\log_{10}$ concentration of oocysts and then a best fit linear regression was calculated as a dose response.

For numeric assay, 20 microscopic fields were randomly selected for observation. In each random field the total number of individual asexual and sexual stages in the entire area was recorded per field. Up to 100 developmental stages per field were counted.

Statistical analysis was performed with INSTAT GraphPad Software (San Diego, CA) . Nonparametric tests were used (Kruskal-Wallis analysis of variance and rank and sum tests) to assess the significance of differences between variables.

RESULTS

Effect of Length of Incubation on Development

Preliminary studies to determine the optimal period for oocyst incubation with cell cultures indicated that the 48 hour incubations yielded appreciably more living stages than were observed for the 18 and 24 hour periods (figure 4.3 & table 4.1). The increased incubation period allowed for more proliferation thus increasing the absolute number of infectious stages detected. The increase in living stages is due to the recycling of type I meronts. Initially, meronts formations is evident after 24 hours. Kruskal-Wallis nonparametric ANOVA of test results comparing the number of living stages enumerated in the 18, 24 and 48 hour incubations indicated that there were statistically significant differences among column medians (table 4.2). A Dunn's Multiple Comparisons Test was performed and the results indicate that the difference in number of living stages between the 18 and 24 hour incubations was not quite significant ($p>0.05$) while the difference between 24 and 48 hour results were significantly different ($p<0.001$) (table 4.3). Increasing the incubation period and allowing more time for infection to occur improved the sensitivity, or the limit of detection, of the assay and increased detection of living stages of *Cryptosporidium*.

Table 4.1

***In Vitro* Culture of *C. parvum* in MDCK Cell Culture**

Number of Developmental Stages Counted/Microscopic Field

	Hours after incubation		
	18	**24**	**48**
mean	57.25	44.4	100
n	20	20	20
sd	13.396	12.659	NA
median	57	44.5	100
lower 95%CI	50.980	38.475	100
upper 95%CI	63.52	50.325	100

pvalue <0.0001, calculated by chi square approximation, n=1, 10^5 oocysts/well, NA=not applicable, a maximin of 100 living stages were enumerated

Table 4.2

Kruskal-Wallis Nonparametric ANOVA Test

Group	Number of Points	Sum of Ranks	Rank of Sums
18	20	528	26.4
24	20	292	14.6
48	20	1010	50.50

Table 4.3

Dunn's Multiple Comparisons Tests on the Mean Differences in Living Stages Between Different Incubations Periods

Comparison	Mean Difference	p value	signifigance
18 vs 24	11.8	p>0.05	no
18 vs 48	-24.1	p<0.001	yes
24 vs 48	-35.9	p<0.001	yes

Dose Response Relationship of Quantal In Vitro Infectivity Assay

To determine the quantitative characteristics of the *in vitro* assay and establish its limit of detection, tenfold serial dilutions of oocyst suspensions were prepared, inoculated onto cell monolayers, and the cell cultures were incubated as described previously. The living parasitic stages at each dilution were quantified by both quantal and enumerative methods and the sensitivities were compared between 24 and 48 hour incubations. The results from these experiments are shown in figures 4.4 & 4.5.

The data indicate a direct relationship between the number of oocysts inoculated into cell culture and the number of living stages present, both in absolute number and percentage of positive fields. The effect of oocyst concentration on production of living stages was evident for both the 24 and 48 hour data regardless of the quantification method applied. The quantal enumerative assay showed greater sensitivity and a more pronounced difference between concentrations than the enumerative assay for both incubation times investigated.

Regression analysis of oocyst concentrations vs. percent positivity of 100 fields was performed for the quantal data of several trials. The results indicated a strong relationship between the number of oocysts (dilution) and percentage of positive fields. While the dose-response pattern remained consistent, the sensitivity of the assay varied among trials. The variability in the sensitivity among trials resulted from the progressive loss of oocyst infectivity over time, as well as differences in infectivity between individual oocysts preparations. By expressing

infectivity of oocysts as the amount required to produce a 50% positive response in the quantal assay (50% tissue culture infectious dose or $TCID_{50}$, comparisons of relative or specific infectivity of oocysts between experiments can be made. Comparisons between $TCID_{50}$ titers of oocysts of various ages and of different preparations indicate differences in infectivity of the same number (dose) of oocysts (table 4.4).

Table 4.4

Infectivity of *Cryptosporidium* Oocysts of Different Age and Lot Number

Oocysts		
Lot	**Age, Days**	**$TCID_{50}$**
I	4	34
	19	101
	41	1,590
II	34	5,300
	46	7,590
	57	7,950
III	150	53,000
IV	19	8

DISCUSSION

In order to apply an *in vitro* cell culture assay technique for *C. parvum* oocysts to the study of disinfection and other treatment processes the quantitative nature and the reproducibility of the assay must be characterized. Initial experiments were directed toward characterization of the assay and optimization of assay conditions and parameters.

Previous applications of this cell culture infectivity assay for *C. parvum* oocysts concerned the evaluation of the inhibition of dinitroanline (Arrowood, 95) and muduramicin activity (Arrowood 94) on the development of living stages. In these experiments inhibition of infectivity was quantified based on number of living stages per mm^2. By comparing the number of living stages/mm for treated samples and controls it was possible to estimate the percentage reduction or inhibition of parasite infectivity. These data were suggestive of inhibition of infectivity of *C.*

parvum by chemicals, but they were not adequately quantitative for disinfection treatment studies.

To qualitatively measure *C. parvum* oocyst infectivity, it is necessary to obtain an estimate of the concentration or number for given number ofoocysts. Of the methods evaluated in this study, the quantal method proved to be the most reliable for expressing the infectivity titer of oocysts. Although quantal assays do not count the number of actual infectious units present in the inoculum, they provide an infectivity value per unit number of oocysts based upon an all or nothing infectivity response per microscopic field. Enumerative assays that rely on counting individual infective stages within a microscopic field are tedious, demanding and subjective. Because any one cell can be infected by more than one parasite (Rosales, 1993 and Yang, 1996) and the individual infective stages may not be randomly distributed on a cell monolayer, it is difficult to accurately count the number. This difficultly is further compounded with increasing oocyst inoculum per well. With quantal assays, quantifying oocyst concentrations over several orders of magnitude is possible. This range of detection allows for several orders of magnitude of sensitivity when used to assess oocyst inactivation by chemical, physical or biological processes. However, the sensitivity of this assay varies over time and among preparations. This infectivity titer comparison of treated samples and controls makes it possible to compute loss of infectivity.

The use of cell culture infectivity for detection of *Cryptosporidium* oocysts from environmental samples will depend on the ability to achieve reliable detection of low concentrations. Further improvements in the sensitivity of this assay may be possible with the application of alternative labeling or detection methods, such as chemiluminescence or PCR which have the capacity for greater signal amplification. This would allow for the detection of low numbers of oocysts, such is found in environmental samples. Alterations in protocol format could also improve detection sensitivity, such as the use of smaller inoculated cell monolayer areas, increases in the number of fields counted and longer incubation periods. The ability to detect low concentrations of infectious oocysts is needed so that a detection limit of complete inactivation in a sample can be realized. No assay currently in use allows for this level of detection sensitivity.

Another factor to consider in the applicability of this method to environmental samples is the ability to distinguish between species of *Cryptosporidium*. Because several species of *Cryptosporidium* may be present in environmental samples, it is important to distinguish those

which are relevant to public health risks from those that are not. The susceptibility of this cell culture system to other species of *Cryptosporidium* has not been explored.

In this study, cell culture infectivity assay was not evaluated in side by side experiments with animal infectivity. Initial results from experiments performed in the laboratory of Michael Arrowood suggest that cell culture infectivity is highly correlated with neonatal mouse infectivity (personal communication). In fact, cell culture may actually have an advantage not afforded by animal assays. Because there is no significant recycling of life cycle stages *in vitro*, the numbers of developing stages in cell culture correlate well with the original inoculum (oocyst number). In this cell culture system the complete development through the life cycle has not been reported, therefore there is no production of cell culture generated oocysts to initiate the infectious cycle.

Results of this study indicate the potential of *in vitro* cell culture techniques for use in disinfection and inactivation studies. Disinfection efficiency and inactivation results can be measured in terms of the degree of lost infectivity rather than loss of viability. Other methods being used to assess the effectiveness of treatment processes provide information on viability which is based upon the ability of oocysts to include or exclude dye or to excyst. While providing valuable information, these methods are not always correlated to the ability of the sporozoites to initiate infection (Finch). Animal studies do provide this type of information, but these assays are imprecise, tedious, time consuming and expensive. The *in vitro* method provides a useful, efficient and reproducible method for determining the infectivity of oocysts and their sporozoites. The ability of *Cryptosporidium* oocysts to initiate infection in cell culture provides valuable information on the potential to cause in vivo infection. By applying this assay to the studies on *Cryptosporidium* in treatment processes and disinfection, the risks of infection can be realistically assessed.

Figure 4.1 **OW-50-FITC labeled C. parvum oocysts, 40X magnification**

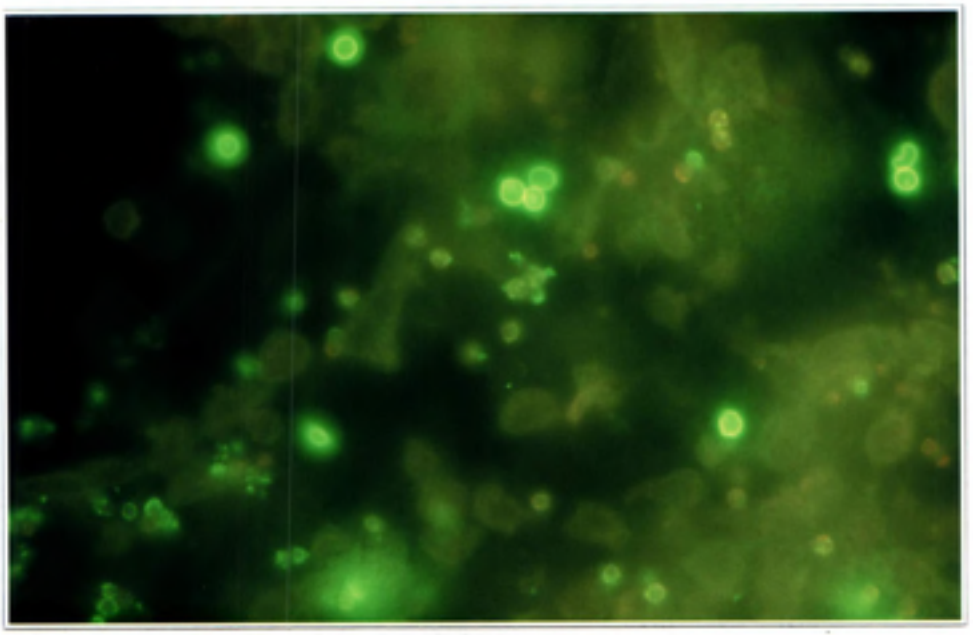


Figure 4.2 **CY3-C3C3 labeled living stages of C. parvum in MDCK cell cultures, 40X magnification**

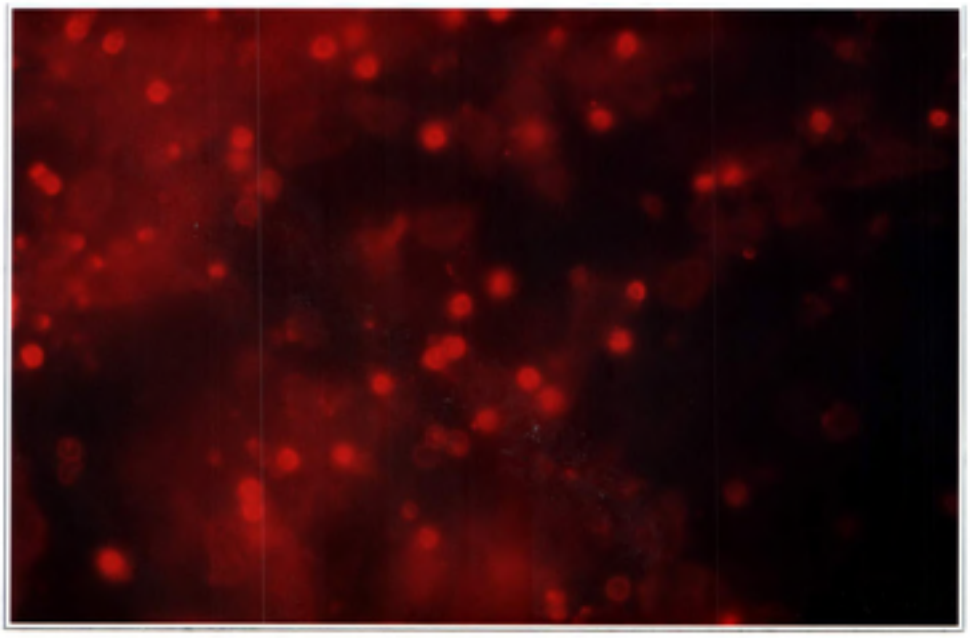
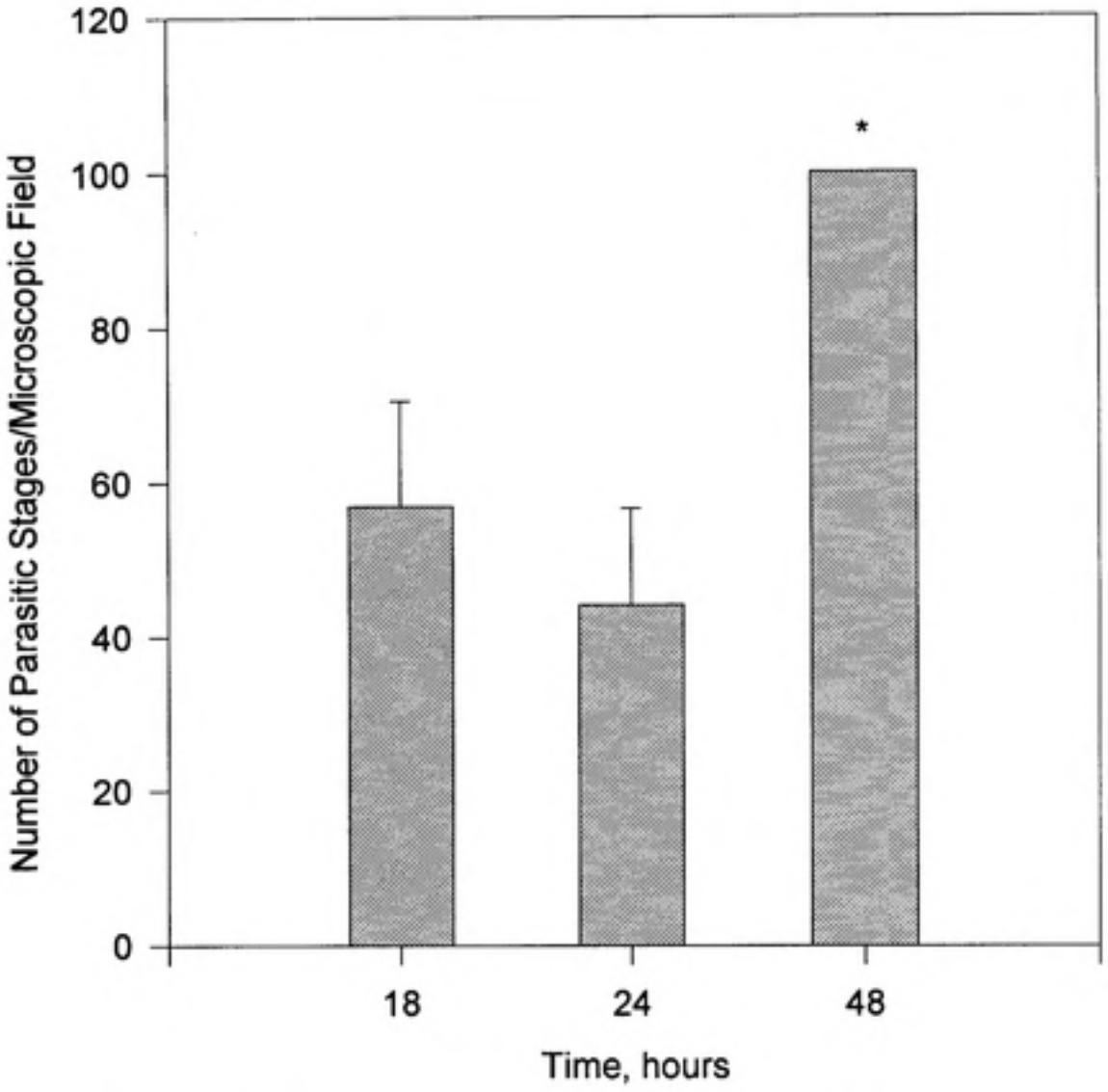


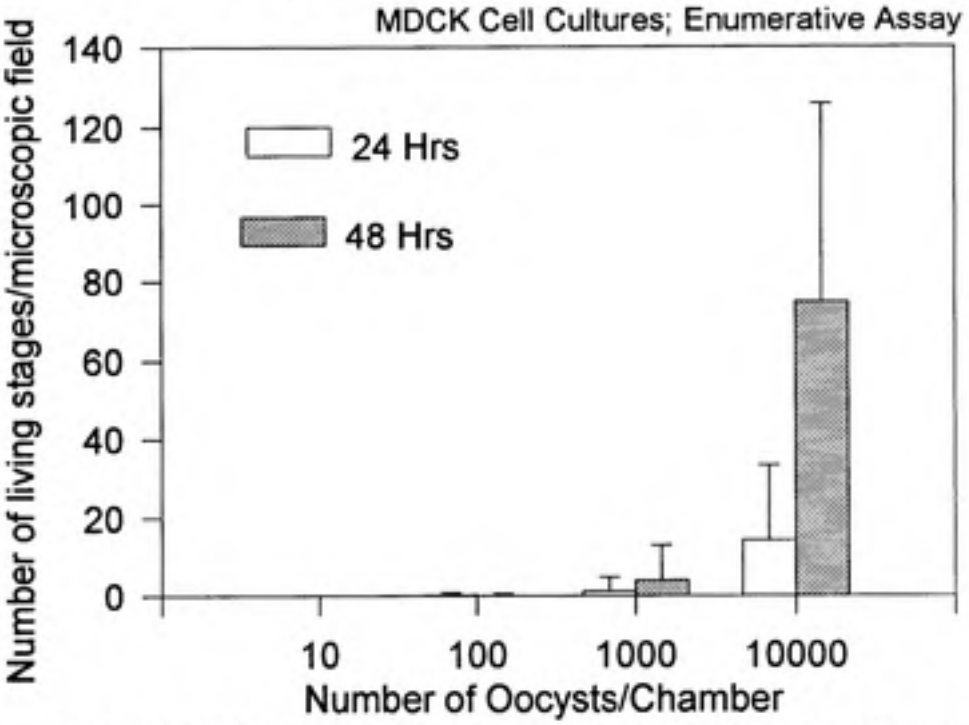
Figure 4.3

Effect of Incubation on Parasite Developement
18, 24, and 48 Hour Incubations



bars represent mean count of 20 microscopic fields,
*all fields positive (100%), n=1, error bars represent stand. dev.

Figure 4.4 **Effect of Increasing Oocyst Inoculum and Incubation Time on Development of Living Stages of *C. parvum* in MDCK Cell Cultures; Enumerative Assay**



Bars represent mean counts in twenty fields, error bars = sd

Figure 4.5 Effect of Incubation Time on Oocyst Infectivity of C. parvum in MDCK Cell Cultures Quantal Assay

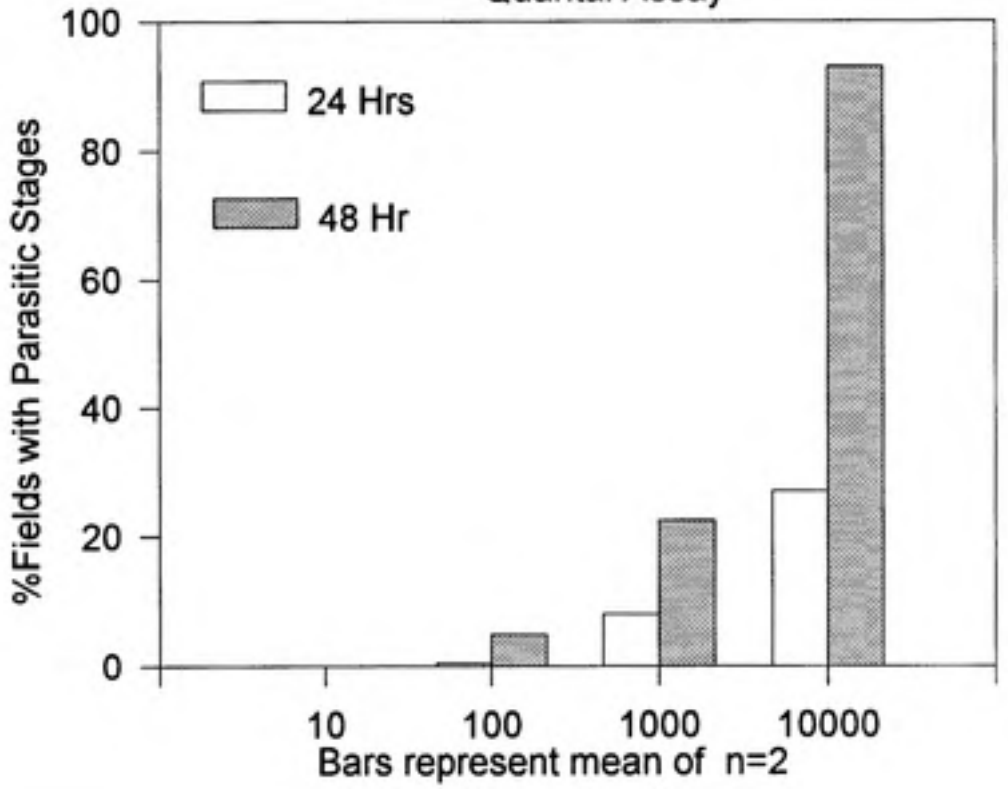
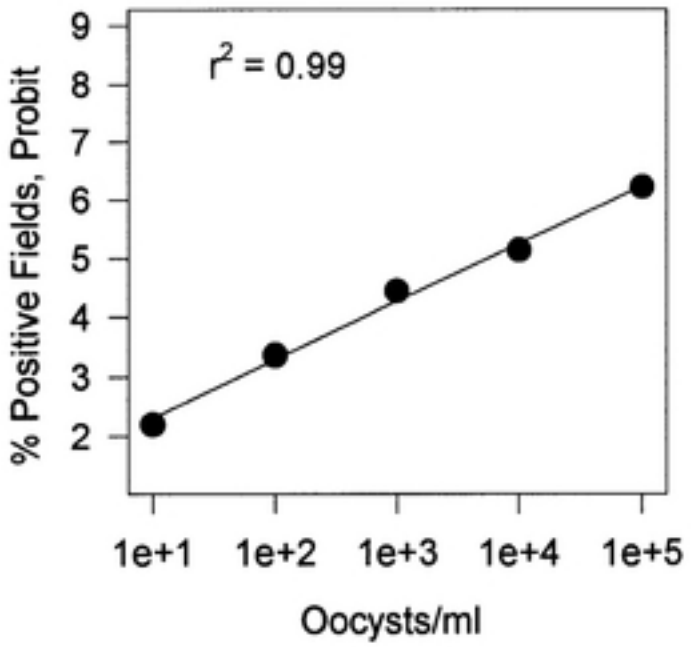


Figure 4.6

Linear Regression Analysis of Dose-Response Data for Cell Culture Infectivity of Untreated Control *Cryptosporidium* Oocysts



CHAPTER FIVE

KINETICS OF THERMAL INACTIVATION OF *C. PARVUM* OOCYSTS IN BUFFERED WATER

INTRODUCTION

This chapter focuses on the inactivation of *C. parvum* oocyst by thermal treatments. These studies were conducted to test the effects of elevated temperatures on oocyst viability/infectivity as determined by MDCK cell culture infectivity assay. Previous studies have reported on the inactivation of *C. parvum* oocysts by thermal processes, but this is the first study to apply a surrogate measure for the detection of oocyst infectivity to the determination of oocyst inactivation by heat treatment.

High temperature thermal treatment is commonly used in industry as a means to eliminate the potential risk of infectious disease transmitted by beverages. In addition, public health officials often recommend that water be boiled during periods of potential waterborne outbreak episodes. While somewhat conflicting, previous research has illustrated the heat sensitivity of oocysts at both high temperature and short exposure times (71°C for 5 - 15 seconds) (Harp et al, 1996), as well as at lower temperatures for longer exposure times (45° for 5 - 20 mins.) (Anderson, 1985) as determined by animal infectivity assay. However, there is still a need to provide more detailed information on the time-temperature relationship of *C. parvum* oocyst inactivation by thermal processes. Specifically, information on the potential inactivation of oocysts at treatment temperatures typical of mesophilic and thermophilic processes is needed. This study was undertaken to address these questions. Treatment temperatures were selected to represent the typical treatment temperatures in thermophilic and mesophilic sludge digesters and to approximate the lower temperature limit for pasteurization of aqueous fluids and beverages.

MATERIALS AND METHODS

Source, Preparation, Enumeration and Infectivity Assay of Cryptosporidium oocysts

C. parvum oocysts for these experiments were obtained from M. Arrowood (DPC, CID, CDC, Atlanta, GA.). The strain of *C. parvum*, oocyst purification methods, oocyst enumeration and related methods are identical to those previously described under Materials and Methods for a cell culture infectivity assay, Chapter 4.

Thermal Treatments

Exposures of *Cryptosporidium* oocyst suspension to heat were done as batch experiments. For each experiment, 0.9 ml aliquots of PBS were distributed into sterile, siliconized 1.5 ml polypropylene microfuge tubes. Duplicate samples were prepared for each time and temperature condition tested. Sample tubes were incubated in a thermostatically controlled water bath preset at a target temperature for different periods of time. The temperature of the sample buffer was monitored using a standard thermometer. Upon reaching target temperature, a 100 µl volume containing 1 x 10^6 washed oocysts was added to each sample tube. The sample was incubated at the target temperature for a specific time interval, after which the sample tube was transferred to a wet ice bath and held until completion of the experiment. At completion of a final time interval, the sample tubes were centrifuged at 16,000 x g, the supernatants were aspirated and the oocyst pellets were resuspended in 0.1 ml PBS. In each thermal inactivation experiment an untreated control consisting of tenfold serial dilutions of *Cryptosporidium* oocysts in PBS at concentrations of 10^7 per ml to 10^1 per ml was prepared from the same oocyst suspension used for treated samples (Figure 5.1).

MDCK Infectivity Assay

Thermally treated and control oocysts were assayed for infectivity in MDCK cell cultures immediately following treatment. Sample volumes of 100 µl of a treated sample were inoculated

onto four day cell monolayers and processed according to protocol described in the Materials and Methods section of Chapter 4. Cell monolayers were incubated for 48 hours, fixed and stained according to the previously described protocol. Fully processed slides were stored at four °C and observed microscopically within one week of staining.

Assessment of Treated Oocysts

Microscopic examinations of inoculated cell monolayers were conducted to evaluate the extent of infectivity based on the frequency of viewed fields containing live parasite stages as previously outlined in section Chapter 4. A best fit linear regression was calculated as a dose response relationship for the untreated oocyst controls. The infectivity of the thermally treated samples as percentage of positive microscope fields out of 100 was matched to the percentage positivity of the untreated controls. The difference in oocyst concentrations between untreated and treated oocysts giving the same percentage of cell culture infectivity was considered the concentration of oocysts inactivated by the treatment. That is, the loss of infectivity, or the $\log_{10}$ reduction in infectivity, based on the equivalence of infectivity observed in treated and untreated control samples having different oocysts concentrations. If there was no inactivation of treated oocysts the untreated oocysts would be the same for suspensions having the same oocyst concentration.

RESULTS

Thermal Inactivation of *Cryptosporidium parvum*

Inactivation kinetics of *Cryptosporidium* oocysts in PBS exposed to temperatures of 23, 35, 45, 55 and 60°C are summarized in table 5.1 and figures 5.2 - 5.5, respectively. Microscopic examination of cell monolayers infected with untreated *C. parvum* oocysts showed living stages at concentrations as low as 10 - 1,000 oocysts/well. The absence or a lower concentration of living stages of *Cryptosporidium* in samples treated at higher temperatures, indicated loss of oocyst infectivity. Comparisons of infectious oocyst concentrations based on percent positive microscopic fields per inoculated cell culture well between untreated controls and thermally treated samples

indicated appreciable loss of oocyst infectivity after only short incubation periods from exposure at higher temperature. Due to the detection limits of this assay as performed in these experiment, it is not possible to discern small losses of oocysts infectivity at lower temperatures. For the lowest exposure temperature of 23°C, all microscope fields (100%) were positive for living stages of *Cryptosporidium* at oocysts concentrations of 10^5 or 10^6 per culture well. Because of the lack of negative fields, an infectivity titer can not be reliably estimated. Untreated controls gave 100% positivity for living stages in microscopic fields at an oocyst concentration of 10^5 per culture well and <100% positivity oocysts concentration of 10^4 or less per culture well. Based on these data it is estimated that the titer reduction of oocysts treated at 23°C for up to 24 hours was < 1 $\log_{10}$. At a temperature of 35°C, there was little or no reduction in *Cryptosproidium* oocyst infectivity after 1 hour, but measurable reductions of about 0.6 and 1.4 $\log_{10}$ after 2 hours and 24 hours respectively. This loss of oocysts infectivity at the modest temperature approximating body temperature suggest that oocysts may have excysted during exposure in PBS.

Temperatures of 55° and 49°C were very effective at reducing oocyst infectivity, even after brief incubations. A 30 minute incubation at 49°C resulted in reductions in oocyst infectivity of >2 $\log_{10}$, and a reduction of >3 $\log_{10}$ (99.9%) after one hour. Longer incubations of 2 and 24 hours at 49°C both resulted in >4$\log_{10}$ reductions (the maximum detection limit of assay), and possibly complete overall inactivation of oocysts. At 55°C, oocyst infectivity was rapidly reduced. After five and 15 minute incubations at 55°C, oocysts infectivity was reduced by 2.75 $\log_{10}$ and 3 $\log_{10}$ respectively. Incubations at 55°C for more than 1 hour resulted in >4 $\log_{10}$ reductions in infectivity (detection maximum). At 60°C, infectivity of oocysts was reduced by ~99.99% (4 $\log_{10}$) within five minutes, but not all of them were rendered noninfectious. However after 15 minutes at 60°C inactivations of >5 $\log_{10}$ (>99.99%) in oocysts infectivity was observed. Due to limitations of this assay as performed, it is not possible to claim that complete inactivation of oocyst infectivity has occurred. While it is probable that temperatures above 49°C, at incubations greater than 1 hour result in complete destruction of oocyst infectivity we are unable to establish this in this assay system.

DISCUSSION

Determining the inactivation of *C. parvum* oocysts by thermal treatment is important because this process is used for emergency treatment of water, pasteurization of milk and other foods, and treatment of biosolids. Therefore, the inactivation of oocysts by thermal processes was studied initially in buffered water using a quantal cell culture assay to assess the infectivity of treated oocysts. The conditions were selected to model the temperatures typical of mesophilic and thermophilic digesters and were conducted to provide baseline data for further thermal inactivation experiments in manure.

Results of this study indicate that modest thermal treatments produce inactivation of *C. parvum* oocysts. As expected, inactivation is more rapid and extensive at higher temperatures. *Cryptosporidium* oocysts appear to be heat labile, as a temperature as low as 49°C is quite lethal (>/99.9% inactivation by 1 hour).

The results of this study are consistent with those of previous studies. Fayer (94) exposed oocysts to temperatures between 54.4 to 71.4°C for short incubation periods and used mice for infectivity assay. Oocyst infectivity was completely destroyed by short incubations (<2 minutes) at temperatures >60°C, but not eliminated at temperatures below 59.7°C. Other investigators have reported similiar results (Anderson,1985 & Harp et al, 1990). Comparison of results among studies is hampered by differences in the method used to assess infectivity/viability and in the preparation of oocysts. Despite these experimental differences it is evident that *Cryptosporidium* oocysts are susceptible to thermal inactivation by temperatures as low as 49°C and typical of pasteurization temperatures are lethal within minutes.

The detection limit of the infectivity assay dictate the maximum infectivity reductions that can be observed. At a temperature of 60°C oocysts are inactivated to a detection limit of 5 $\log_{10}$ after 15 minutes. A limitation of the cell culture infectivity assay as applied in this study is that small changes in infectivity (<1 log) are difficult to measure. This is because only one or two relatively high concentrations of treated ooycsts were assayed for infectivity. At the other extreme of complete loss of infectivity, inactivation is reported as being greater than a particular value (i.e. >4 log). Animal infectivity, vital dye staining and excystation assays also suffer from the same

quantitative limitations. In animal studies, generally only small numbers of animals are used at each temperature and time point. Therefore sensitivity is dictated by the oocyst doses per animal and the number of animals per dose. For excystation and vital dye staining counting is usually limited to 100 oocysts, therefore 2 logs of reduction can be followed unless the oocysts are further concentrated befoe microscopic counting.

The precise mechanisms of thermal inactivation of *C. parvum* oocyst infectivity were not addressed in these experiments. It is likely that temperature effects on the oocyst integrity were the dominant parameter. At temperatures in the 50 - 60°C range, protein denaturation occurs rapidly, as does membrane disintegration and loss of cellular integrity. Temperatures of 37°C are known to promote oocyst excystation (Fayer & Ungar, 1995) which may explain the observed reduction in oocysts infectivity at 35°C. The released sporozoites are probably more susceptible to destruction by heat than the hardy oocysts or they may be lost during sample processing. Once free, the naked sporozoites may not be able to survive environmental conditions or initiate infection in hosts. Therefore even mesophilic heat treatment may be capable of reducing infectious oocysts if excystation can be induced. However, temperatures in the thermophilic range of 49 - 60°C are clearly capable of achieving extensive inactivation of *C. parvum* oocysts.

In order to follow the inactivation of *C. parvum* oocysts over several orders of magnitude samples were seeded at concentrations far exceeding those found naturally in the aquatic environment. Inactivation of *C. parvum* in environmental waters may be different due to the presence of other constituents. The effects of inorganic solutes were not specifically studied, although the test water was buffered saline. The findings of this study may not be applicable to other waters containing turbidity, organic solutes and low sodium chloride concentrations

Use of purified *C. parvum* oocysts also differs from what is found in nature. In environmental samples oocyst populations are likely to be more heterogeneous. It is possible that the response of oocysts to inactivation by chemical or physical treatment would also differ due to heterogenicity of oocyst strains and physical states in the sample. Oocysts exposed to environmentally stress (ie. age, temperature condition, disinfection treatment, etc.) prior to treatment might be more susceptible to thermal inactivation than carefully maintained laboratory preparations. The generalization of the results to different *C. parvum* oocyst strains and their

different chemical and physical conditions must be made with caution. The numerous factors that may affect the thermal resistance or susceptibility of oocysts needs further study.

Figure 5.1

Exposure and Recovery of *Cryptosporidium parvum* Oocysts in PBS

Preheat 0.9 ml PBS to

23°, 35°, 49°, 55° and 60°C

↓

Seed with 10^6 *C. parvum* oocysts

↓

Incubate at target temperatures for specific time interval

↓

Transfer samples to wet-ice (4°C)

↓

Centrifuge at 16000xg, 3 minutes

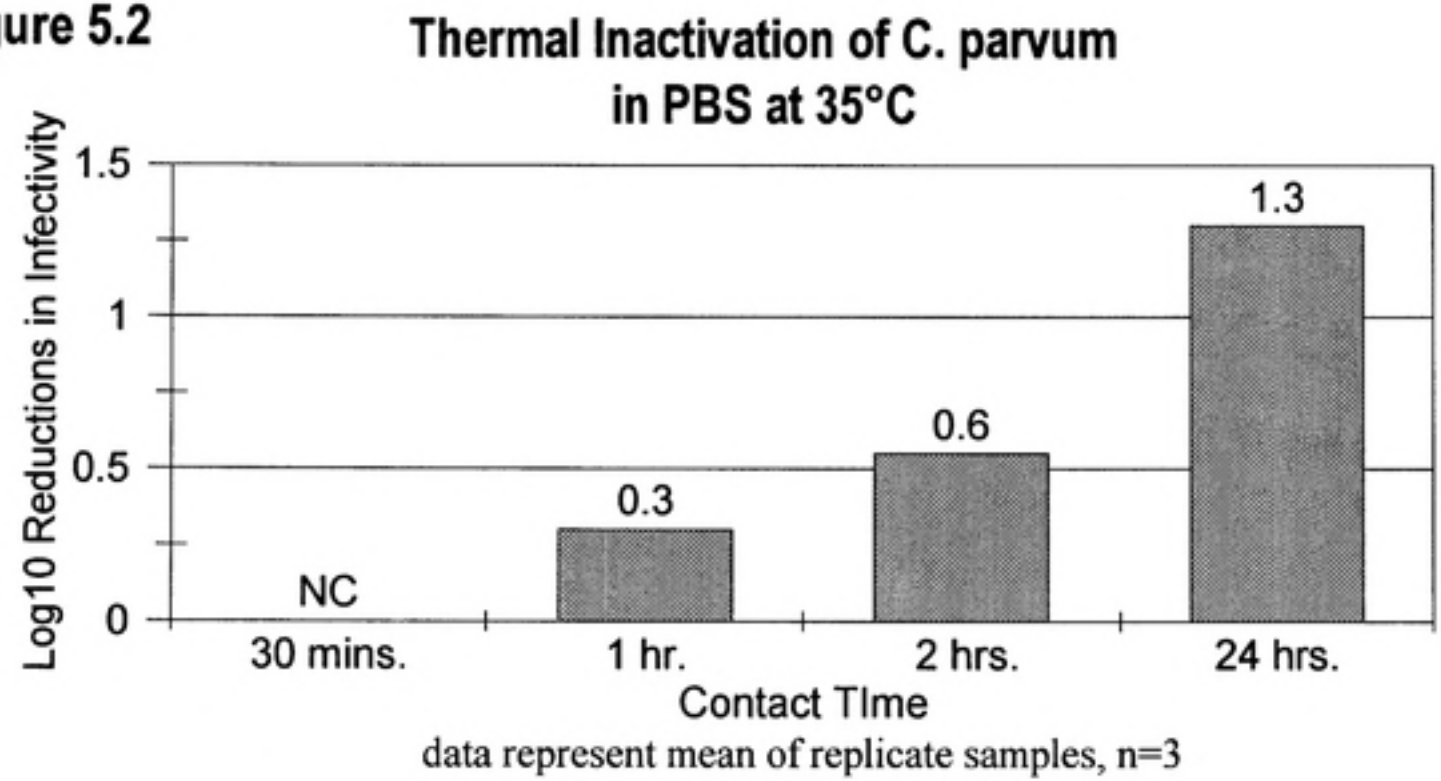
↓

Resuspend oocysts pellet in 0.1 ml PBS

↓

Inoculate 4 day old MDCK cell cultures

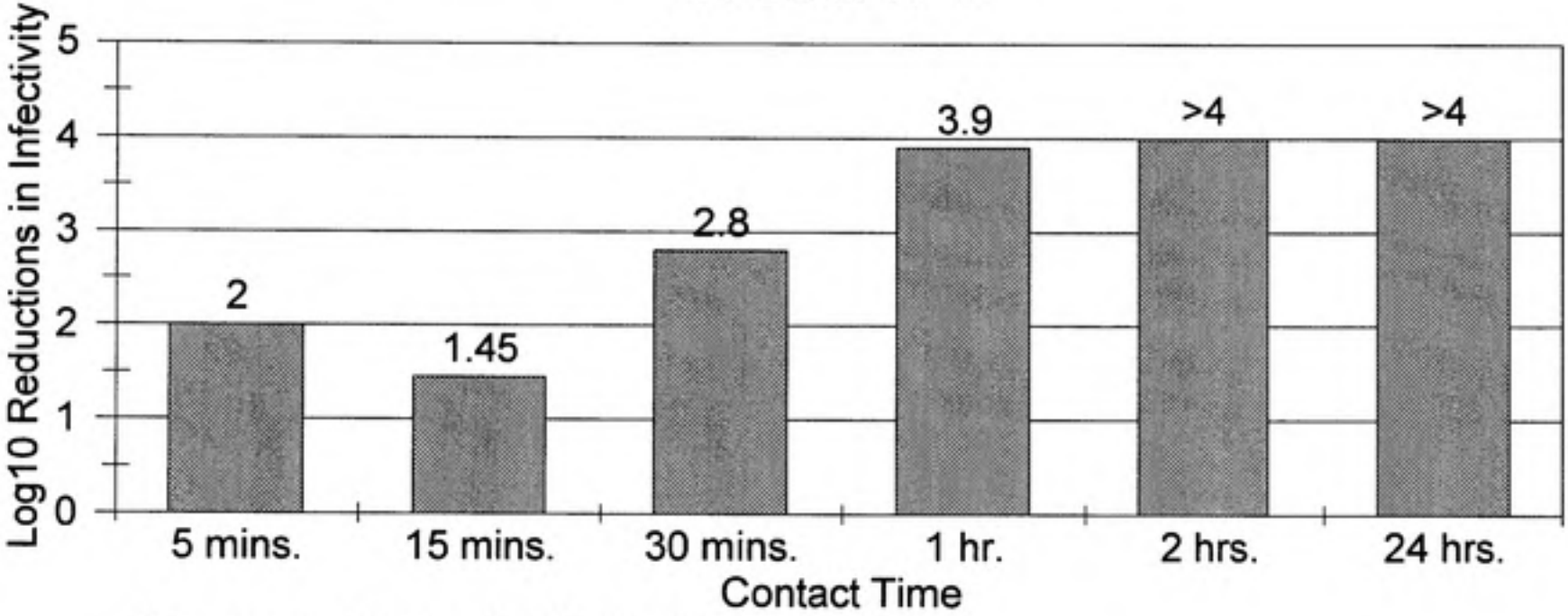
Figure 5.2



data represent mean of replicate samples, n=3

Figure 5.3

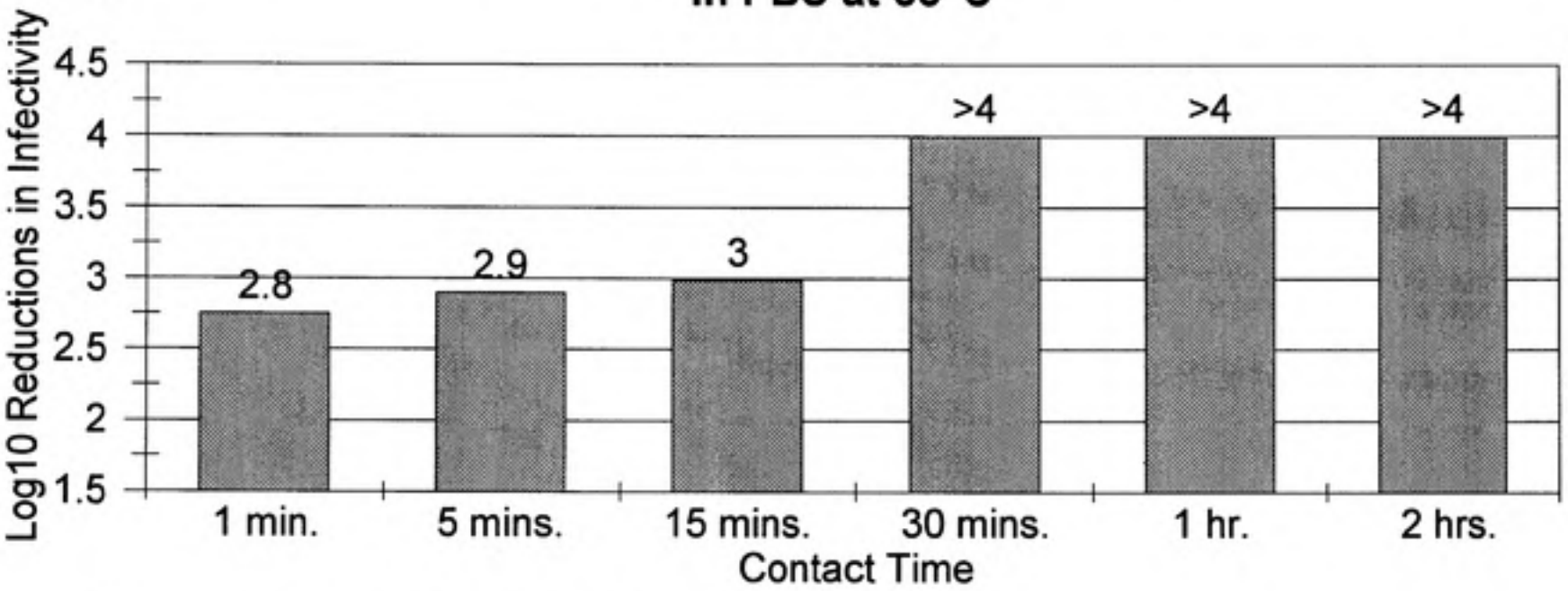
Thermal Inactivation of C. parvum in PBS at 49°C



data represent mean of replicate samples, n=1

Figure 5.4

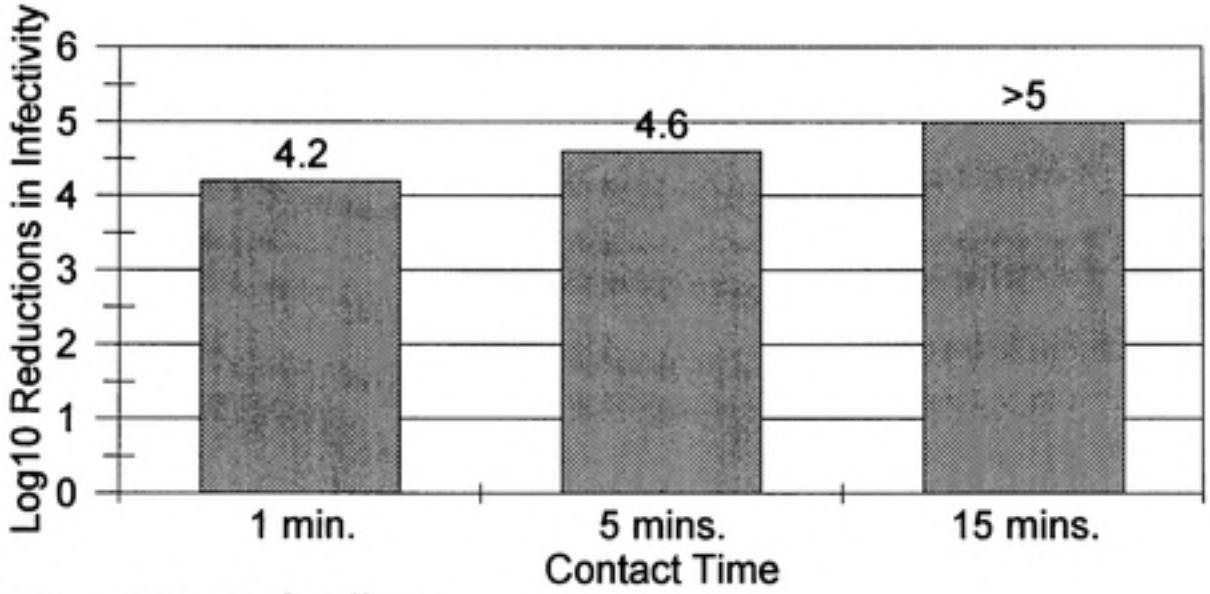
Thermal Inactivation of C. parvum in PBS at 55°C



data represent mean of replicate samples,

Figure 5.5

Thermal Inactivation of C. parvum in PBS at 60°C



data represent mean of replicates samples

Table 5.1
Thermal Inactivation of C. parvum Oocysts in PBS (Log_{10} Reduction)

Experiment Number	No. oocysts/ culture well	Temperature °C	Incubation time	Mean %Positive Fields	log_{10} Reductions
SBXV	10^6	49	5 mins.	100	<2
			15 mins.	97.5	~2.5
			30 mins.	100	<2
			1 hour	100	<2
	10^6	55	1 mins.	96	2.75
			5 mins.	89	2.9
			15 mins.	78.5	3.0
	10^6	60	1 mins.	15	4.2
			5 mins.	10	4.6
			15 mins.	0	>5
	10^6	23	5 mins	100	<1
			15 mins.	100	<1
			30 mins.	100	<1
			1 hour	100	<1
SBXIX	10^6	49	1 hour	39	3.9
			2 hours	0	>4
			24 hours	0	>4
	10^6	55	1 hour	0	>4
			2 hours	0	>4
			24 hours	0	>4
	10^6	23	1 hour	100	<1
			2 hours	100	<1
			24 hours	100	<1
SBXXII	10^5	35	30	100	<1
			1 hour	100	<1
			2 hour	100	<1
			24 hour	100	<1
SBXXIII	10^6	35	30 mins.	100	no change
			1 hour	100	no change
			2 hour	95	0.8
			24 hours	55	1.7
	10^5	35	30 mins.	83	no change
			1 hour	72	0.3
			2 hour	67	0.3
			24 hours	40.5	0.9
	Averaged Overall Reductions for 10^5& 10^6	35	30 mins.		no change
			1 hour		0.3
			2 hour		0.55
			24 hours		1.3
	10^6	49	15 mins.	68.5	0.4
			30 mins.	12	2.8
			1 hour	0	>3.8
			2 hours	0	>3.8
	10^5	49	15 mins.	0	>2.8
			30 mins.	0	>2.8
			1 hour	0	>2.8
			2 hours	0	>2.8
	Averaged Overall Reductions for 10^5& 10^6	49	15 mins.		0.4
			30 mins.		2.8
			1 hour		>2.8
			2 hours		>2.8

CHAPTER SIX

RECOVERY OF *C. PARVUM* OOCYSTS FROM FECAL WASTE SAMPLES AND INACTIVATION BY THERMOPHILICALLY DIGESTED SLUDGE

INTRODUCTION

Cryptosporidium oocysts have been detected in high numbers in raw sewage, treated effluent, and animal wastes (Madore et al, 1987, Ongerth & Stibbs, 1987 and Scott et al, 1995). In fact, as much as 91% of raw and treated sewage samples (Rose, 1988) and 62.4% of cattle fecal samples (Scott et al, 1995) are positive for *Cryptosporidium* oocysts. Because these protozoan parasites are present in wastewater streams and animal waste, treatment processes are critical for reducing the number or inactivating the oocysts which are eventually discharged into the environment.

Laboratory studies have demonstrated that oocyst removals of ~98% occur during wastewater treatment processes, and that the oocysts are concentrated in the sewage sludges (Staderman et al, 1995, Mayer & Palmer, 1996 and Villacorta et al, 1992). Therefore processes which treat sludge and animal waste are important for inactivating *Cryptosporidium* oocysts since the ultimate fate of digested sludge and animal waste may pose a public health risk. Very few studies have evaluated the effect of sewage sludge digestion processes on the viability/infectivity of *C. parvum* oocysts and no studies have evaluated this process using animal waste. Results from one study indicate that exposure to aerobic, thermophilic digested sludge at 55° for two hours completely inactivates oocysts, but that viable oocysts remain after anaerobic treatment at 35°C after 18 days as determined by vital dye staining (Whitmore & Robertson, 1995). These results

were challenged when Staderman (1995) reported that oocysts treated at 37°C for 24 hours were completely reduced or inactivated, as determined by excystation.

In this chapter the reduction in infectivity of *C. parvum* oocysts by thermophilic anaerobic digestion using the MDCK cell culture infectivity assay was determined. We specifically evaluated the loss of oocysts infectivity due to exposure to anaerobic, thermophilically digested cow manure. For these experiments we operated a model digester using cow manure as feed. The effluent from this digester was used for batch-exposure experiments with seeded oocysts.

MATERIALS AND METHODS

Source, Strain, Preparation and Enumeration of *Cryptosporidium*

C. parvum oocysts for these experiments were obtained from M. J. Arrowood (DPD, DID,CDC, Atlanta, Ga.). *C. parvum* purification and preparation methods and enumerations and dilutions are identical to those previously described under Materials and Methods for Cell Infectivity Assay in Chapter 4.

Waste Sample Collection and Processing

Bovine fecal samples were collected from the North Carolina State University Research Farm (Raleigh, N.C.). Feces were collected off a concrete floor in a free stall barn which houses both Holstein and Jersey dairy cows ranging in age from two to ten years. Because animals are housed together, collected fecal specimens contained stools representative of all animals in the group. The samples were placed in 1 L sterile plastic bottles or 5 L containers and immediately placed on ice for transport to the laboratory, where they were stored at 4°C.

Collected fecal samples were tested in triplicate to determine the percent total solids by the methods described in Standard Methods 209A (Anon., 1995). The fecal samples were diluted to 12% - 15% total solids with dechlorinated tap water then retested to verify that the solids percentage fell within desired range. These samples were homogenized in a Waring Blender, stored at 4°C and used as seed for a laboratory anaerobic digester within 2-3 months.

A representative sample of feces was analyzed in triplicate to detect indigenous

Cryptosporidium oocysts by a slide assay using an indirect monoclonal fluorescent-antibody kit specific for *Cryptosporidium spp.* and *Giaridia spp.* [Hydrofluor Combo Kit, EnSys Environmental Products, Inc., Research Triangle Park, N.C.]. Samples (1g) were resuspended in 1 ml of PBS, and 100 µl of suspension was smeared onto a glass microscope slide. Slides were air dried at room temperature then heat fixed by passing through a flame. Slides were sequentially washed in PBS, fixed for ten minutes in methanol then washed in PBS/BSA. Samples were stained according to kit procedures. Slides were observed for the presence of FITC-labeled *Cryptosporidium* oocysts with epifluorescence optics and blue excitation (450 - 490 nm), using an I3 filter block under 40X magnification. Only samples negative for *Cryptosporidium* oocysts were used for seeded studies on recovery and treatment.

Seeding Studies on *Cryptosporidium* Oocyst Recovery from Wastes

Ten ml aliquots of cow feces or PBS (pH 7.5) controls were placed into 50 ml siliconized disposable conical tubes. Then, 20 ml of eluting solution (PBS containing 0.1% Tween 80, 0.1% sodium dodecyl sulfate [PTS]) was added to each sample. Samples were seeded with 100 µl suspensions containing either $1x\ 10^6$, $2x10^6$ or $5x10^6$ *C. parvum* oocysts and vigorously mixed by hand. Samples were transferred to stainless steel containers and blended for two minutes in an Omni-blender at setting #2 while being held on ice. Following homogenization the samples were sieved through Para-Pak Macro-Con stool concentration filtration units (Meridian, Cincinnati, OH). The filtrate was collected and washed three times in equal volumes of PTS and centrifuged at 1500 x g and 4°C for twenty minutes. Pellets were washed three times in 0.85% saline containing 0.1% Tween80 (ST), centrifuged as above and resuspended to a final volume of 5 mls in ST.

A modified discontinuous sucrose gradient procedure (Arrowood & Steriling, 1987, Arrowood & Donaldson, 1996) was used to isolate oocysts from cow feces. For this procedure gradients were prepared in sterile, 50 ml disposable siliconized tubes by overlaying ten ml of a 1:4 dilution of Sheather's Solution over ten ml of a 1:2 dilution of Sheather's Solution (specific gravity 1.064 and 1.103 g/L respectively) (Arrowood & Steriling, 1987). Then, 5 ml of washed fecal samples or PBS controls were layered over the 1:4 solution and the tubes were centrifuged at 1000 x g and 4°C for 25 minutes. The upper ten ml was aspirated and discarded. The middle ten

ml containing the oocysts was collected and washed twice in 25 ml of ST. Following centrifugation at 1500 x g and 4°C for ten minutes, the oocyst pellet was resuspended in five ml ST, subjected to another discontinuous sucrose gradient, washed and resuspended in 500 µl saline. Samples were layered over one ml of cesium chloride (1.15 g/L specific gravity), centrifuged at 16,000 x g for three minutes at room temperature. The upper 500 µl of the gradient was collected, diluted with one ml of saline, then centrifuged as above. The recovered oocysts were resuspended in 100 µl PBS.

To estimate recovery efficiency of this isolation protocol, the recovered oocysts were stained by immunofluorescence with the HydroFluor Combo Kit. A modified procedure was used to stain oocysts in an aqueous solution rather than on a solid phase. Briefly, 100 µl of gradient purified samples were centrifuged at 16,000 x g for three minutes, resuspended in 100 µl PBS containing primary antibody (1:10) and goat antisera (1:10) then incubated for 30 minutes at room temperature. Primary antibody labeled oocysts were washed three times in PBS, resuspended in 100 µl of PBS containing labeling reagent (1:10 FITC-antimouse antibody) and incubated for 30 minutes at room temperature in the dark. FITC labeled oocysts were washed sequentially in PBS, centrifuged as above and resuspended in PBS. Samples were observed and enumerated for the presence of FITC labeled oocysts in a hemocytometer by epifluorescent optics with a 25X objective lens. Oocysts were identified based on size (5-7 µm), apple green fluorescence, spherical shape and distinctly stained walls. To determine oocyst concentrations, duplicate 10 µl volumes of each sample were counted as described in Chapter 4. Recovery estimates were based on the proportion of labeled oocysts isolated from cow feces relative to the number seeded into samples and expressed as a percentage of the initial number.

Operation of a Bench-Scale, Model Thermophilic Anaerobic Digester

Thermophilic digested sludge was obtained from the South Durham (N.C.) Water Reclamation Plant. The sludge was collected in a 1L sterile, polypropylene bottle and transported to the laboratory in an insulated container. The total solids concentration of collected sludge was measured in duplicate and ranged from 2.5 - 3.5%. On the day of collection a 3:1 volume ratio of sludge and cow feces was combined, manually mixed and placed into a one liter water-jacketed

glass reactor. A circulating waterbath set at 57°C was used to maintain a constant sludge temperature of 55°C within the reactor. The reactor head contained three outlets: one was capped with a rubber stopper which could be removed for feedings, one contained a rubber stopper with a glass tube to allow for gas collection and one held an adapter for an electric mixer. Gas produced from the digester was trapped in a 12 L glass carboy and the volume was measured by displacement of an acidified, saturated sodium chloride solution (pH 4.3). The hydraulic residence time (HRT) of the digester was ten days at the beginning of the run, but was later increased to twelve days in order to achieve stable reactor conditions. The hydraulic residence time is the average time that the entire contents of the digester are turned over.

Every other day, at approximately the same time, the following procedures were performed. The digester mixer was turned on for ten minutes and the temperatures of the waterbath and digester sludge were recorded. Gas production was measured by first equilibrating pressure within the reactor system then recording the volume of liquid displaced in the gas collection system. One hundred and sixty six ml of digested sludge (1/6th of the total sludge volume) was removed from the reactor and replaced with an equal volume of prewarmed (55°C) diluted cow feces. The reactor was purged with nitrogen gas to displace any air that might have entered during this draw-fill procedure, the gas collection system was reset, and then the outlet recapped. Duplicate five ml aliquots of the treated manure were analyzed for total solids (TS), fixed solids (FS), and volatile solids (VS). Measurements of pH, TS, FS and VS followed the procedures in Standard Methods (Anon., 1995). The remaining manure withdrawn from the reactor was immediately used in batch reaction experiments on *Cryptosporidium* survival or discarded.

Effects of Thermophilic Exposures on Survival of *Cryptosporidium* Oocysts

Cryptosporidium oocysts were exposed under batch conditions to anaerobic thermophilic digested sludge in order to simulate semi-continuous exposures. For these exposures, 10 ml volumes of the digested manure from semi-daily draws were dispensed into airtight 12 ml amber serum bottles and capped. Equal volumes of PBS were prepared as controls. Bottles were placed in a thermophilically controlled waterbath pre-equilibrated to 25°C and 57°C. The temperature of

the manure and PBS within the bottles was monitored with a thermometer to verify that they reached target temperatures of 23* and 55°C.

Temperature equilibrated samples were seeded with 100 µl of suspension containing 2 x 10^6 oocysts. Negative controls consisted of uninoculated sludge and PBS containing no added oocysts. The bottles were purged with nitrogen, recapped and held at the specific target temperature for timed incubation intervals of up to three hours. Duplicate samples were removed from the waterbath at set intervals and stored on wet ice. Samples cooled down to below 35°C within three minutes on wet ice. At completion of the time course of exposure, treated oocysts were recovered and purified by discontinuous sucrose and cesium chloride gradients. The oocyst samples were assayed for infectivity by inoculation of cell cultures on the day of isolation.

MDCK Cell Culture Infectivity Assay of *Cryptosporidium* Oocysts

Thermally treated, gradient purified oocysts were sanitized for three hours at 37°C in 100 µl of an antibiotic mixture containing 50 µg Gentamycin, 250 µg Kanamycin and 150 µg Nystatin prior to assay. Decontaminated, treated oocysts along with titer controls consisting of ten-fold dilutions of oocysts suspensions used to seed treatment samples were immediately inoculated onto four day-old MDCK cell monolayers and processed as described in Chapter 4.

To monitor for cytotoxicity or bacterial contamination of inoculated cell cultures, oocysts isolated from cow feces and negative controls were tested in the MDCK infectivity assay prior to experiments using treated oocyst samples. 100 µl of gradient purified samples were inoculated in duplicate onto MDCK cells cultures and observed over three days for evidence of cytotoxic affects or contamination as evidenced by cell death, lack of cell division and presence of bacterial or fungal growth.

Cryptosporidium Oocyst Infectivity Determination

Microscopic examination of inoculated cell monolayers was conducted to evaluate the extent of infectivity of treated and control oocysts. *C. parvum* oocyst infectivity was based on the frequency of the presence of live stages of the parasites in microscope fields as previously outlined in Chapter 4 under Materials and Methods. To account for oocyst losses during recovery

from treated samples, the initial oocyst concentrations ($2x10^6$) were adjusted for overall recovery efficiencies. The initial concentration of $2x10^6$ oocysts was multiplied by 0.289 for PBS and 0.039 for sludge samples, resulting in starting concentrations of $5.9x10^5$ for PBS and $7.8x10^4$ for manure.

RESULTS

Initial Characterization of Cow Feces

The total solids concentration of the collected cow manure samples ranged from 12.8 - 18.9%. Samples were adjusted when necessary to give total solids concentrations between 12 and 15%. Analysis of fecal samples for the presence of indigenous oocysts yielded negative results. All samples were free of indigenous *C. parvum* oocysts at the detection limit of the analysis (<100/g).

Operation Characteristics of a Semi-Continuous, Model Anaerobic, Thermophilic Digester

The results for the operational characteristics of the bench-scale digester in terms of conventional parameters are summarized in table 6.1. The relatively low coefficients of variation for these manure parameters indicate stable reactor performance for the duration of experimental period. Gas production volume was monitored during startup of the digester, but was discontinued after 49 days due to the development of a leak in the reactor gasket. Performance of the reactor (VS destruction) and pH remained stable after this leak occurred.

Table 6.1

Operational Conditions of Bench-Scale Digester

Parameter Measured	Mean	Coeff. Var.
pH	7.36%	+/- 0.14%
Volatile Solids		
Influent	11.1%	+/- 0.57%
Effluent	7.69%	+/-1.9%
Volatile Solids Reductions	59%	+/- 11.9%
Total Solids		
Influent	12.7%	+/- 0.67%
Effluent	9.24%	+/- 0.24%
Total Solids Reductions	72.8%	+/-4.8%

Recovery of Oocysts From Waste and Control Samples

The recovery efficiencies of the gradient procedure determined for five trials with seeded PBS and manure are summarized in table 6.2. For samples seeded with $1x10^6$ oocysts, the average percent recoveries and their standard deviation were 33% (+/-23%) for PBS and 3.9% (+/-2.2%) for feces. Mann Whitney unpaired, nonparametric statistical analysis of oocyst recoveries from feces and PBS indicate a marginally significant difference between the two media ($p=0.0759$), if the criterion for significance is set at $p<0.1$. The standard deviattion in oocysts recovery were high in both PBS (23%) and feces (2.2%). The very high CV in PBS could be the result of evaluation of only five replicates. There was overlap in the recoveries in both media and the 95% confidence intervals of the sludge recoveries were within those of PBS recoveries. Statistical analysis of the data for oocyst recoveries from feces at two different initial oocyst concentrations ($1x10^7$ and $2x10^6$) averaged 7.6 and 3.9% respectively, and showed no significant differences ($p=0.31$). Because considerable losses of oocysts resulted from purification and isolation procedures, experiments aimed at determining loss of oocyst infectivity due to thermal processes and other treatments would require seeding at high levels in order to follow the inactivation kinetics over several orders of magnitude.

Results from the cytotoxicity and bacterial contamination study for samples of processed oocysts assayed in cell cultures revealed that neither of these concerns was a problem. Antibiotic treatment suppressed bacterial contamination of cell monolayers for the 48 hour incubations used in the assay. Background fluorescence was extremely high in cell culture assays, but infective stages could be distinguished above this background. Overall, the isolation procedure yielded oocyst preparations that were suitable for infectivity assays.

Assessment of Treated Oocysts

Data summarizing the reduction in infectivity of the oocyst seeded into sludge and PBS exposed to temperatures of 23 and 55°C are listed in Table 6.3 and Figures 6.1 & 6.2 respectively. The data represented in this table are the result of two consecutive experimental trials conducted during August and September, 1997.

The data indicate that oocyst infectivity is significantly reduced in both the PBS and digested manure at 55°C after only short incubations. At 55°C, oocysts infectivity was reduced 3.1 (0.49) $\log_{10}$ in PBS and 2.3 (1.8) $\log_{10}$ in sludge after a 15 minute exposure. A sixty minute incubation at 55°C in PBS and sludge resulted in reductions in oocysts infectivity of 3.7 (0.71) and >/3.3 $\log_{10}$ (the maximum detection limit of the assay), respectively. Microscopic examination of cell monolayers infected with oocysts treated at 55°C in PBS showed one living stage out of 100 observed, in both experimental trials. This result suggests that while most ooycsts are effectively inactivated at this temperature, at least one oocyst was able to maintain its infectivity at this time-temperature. In the previous chapter, we reported that oocysts exposed for 60 minutes at 55°C in PBS did not retain their infectivity (>4 log10). This time-temperature combination is probably close to the critical point of complete oocysts inactivation, and the difference we see in the sensitivities of assays is probably due to variation in oocyst infectivity between oocyst preparations. Incubations longer than 60 minutes resulted in >/3.99 $\log_{10}$ reductions in PBS, the detection limit of the assay. The infectivity of the treated oocysts in both the PBS and manure declined over time to below the detection limit of the assay (10 oocysts). The rate of loss of infectivity of the control oocysts in PBS was similar to the rate seen for oocysts incubated in treated manure. The overall trends in inactivation appear to be similar and there appears to be no significant difference between inactivation kinetics of the two suspending media. Thus it appears that temperature alone is the main factor influencing oocyst inactivation.

At 23°C no significant changes in oocyst infectivity were observed over the time course studied. Oocyst infectivity remained consistent over the time course; the infectivity of oocysts at the zero time point were similar to oocyst infectivities at fifteen minutes, one and three hours. Based on these results it is estimated that the reduction in oocysts infectivity in PBS or sludge at 23°C over three hours was less than 1 $\log_{10}$

DISCUSSION

Because of the accumulation of *C. parvum* oocysts in sewage and animal sludges the efficacy of treatment procedures for these waste is important to public health. Thermophilic anaerobic digestion, a method sometimes used for treatment of municipal sludge and for large

dairy facilities, was investigated to determine its efficacy of oocyst inactivation during exposures ranging from 15 minutes to 3 hours.

Recovery of *C. parvum* oocysts from environmental samples is generally low and highly variable, and sewage and fecal materials present an even greater challenge than aqueous samples because the oocysts are associated with the organic solids material. High levels of solids and contaminating microorganisms interfere with recovery and result in poor recovery efficiency.

Several methods are available for isolation of oocysts from fecal material. Most procedures first employ differential sucrose gradient isolation with additional purification steps. Production of a relatively free sample, clean of contamination, is achieved at the expensive of poor quantitative recoveries. For this study, differential sucrose gradients were followed by cesium chloride gradients isolation because they are relatively easy to use, inexpensive and do not generate chemical hazardous wastes. Previous use of this method in other laboratories demonstrated compatibility of the recovered oocysts with the cell culture infectivity assay (You, et al, 1996).

When high numbers of oocysts were seeded into bovine feces and PBS and recovered by differential sucrose and cesium chloride gradients for further analysis by cell culture infectivity assay, poor and highly variable recovery rates were obtained. However, the application of this recovery method was useful in these seeded sample studies on thermal treatments. For environmental samples this recovery method would be undesirable due to its low recovery efficiency. *C. parvum* concentrations in most environmental samples are rarely as high as the levels used in this study. An important advantage of the recovery method is its applicability to large sample volumes. By using this method in conjunction with a detection method that is highly sensitive and specific (i.e. Magnetic capture and PCR), detection of oocysts from fecal waste samples is possible.

The selective recovery of viable oocysts was not addressed by this study. It is possible that recovery of oocysts from environmental samples could vary from our results. Our recovery rates were significantly lower than those reported previously using this method (Arrowwood & Donaldson, 1996). Differences in recoveries may be due to the concentration of oocysts in seeded samples vs those found in feces of infected animals. Another possibility is that in our procedure

procedure we did not use potassium dichromate to suspend the oocysts. Possible differences in suspending media could lead to variable recovery rates. The effect of pH on the surface charge of oocysts has not been fully evaluated (Anquish,1997), so it is possible that with increased understanding of this phenomenon we can can achieve better recoveries.

To investigate the reduction of oocyst infectivity during thermophilic anaerobic digestion we employed a batch mode of operation. Reductions of *C. parvum* oocysts during sewage sludge digestion have been investigated in recent years (Whitmore & Robetson, 1995, and Staderman et al, 1995). Previous studies used semi-continuous systems for their analysis and focused on municipal sludges. Determination of oocysts viability after digestion was measured using in vitro excystation (Staderman et al, 1995) and vital dyes (Whitmore & Robertson, 1995). The batch method was chosen for this study due to previous results which indicated that thermal inactivation occurred rapidly in the thermophilic range. By using a batch system, more than one temperature could be investigated at a time, and replicate samples could be included. Another advantage was that the number of oocysts needed to conduct the study was significantly less than would have been required in a semi-continuous experiment. The dominant, active parameter contributing to oocysts inactivation in our study appeared to be temperature. This result is in agreement with the known thermotolerance of this organism (Blewett, 1989, Fayer, 1995, and Anderson, 1981). Only a limited number of parameters were evaluated in this study, but other factors may contribute to inactivation over time by sludge digestion, especially in the mesophilic range. Other factors that may contribute to the decrease in number of oocysts over time in digesters include enzymatic and bacterial decomposition. These effects may be associated with the temperature effects of the sludge.

In this study we did not address the reduction in numbers of oocysts, only the inactivation of infectivity of those oocysts which were isolated relative to a control. Even though oocysts were inactivated below the levels of detection of our assay, they may not be completely killed or eliminated by digestion. With the method we used to score infectivity, we are unable to achieve the sensitivity which allows for this level of detection. One would expect that in typical digesters, the inactivations would probably be at least as great or greater than those realized in this study. Furthermore, the time scale over which inactivation was observed was two orders of magnitude

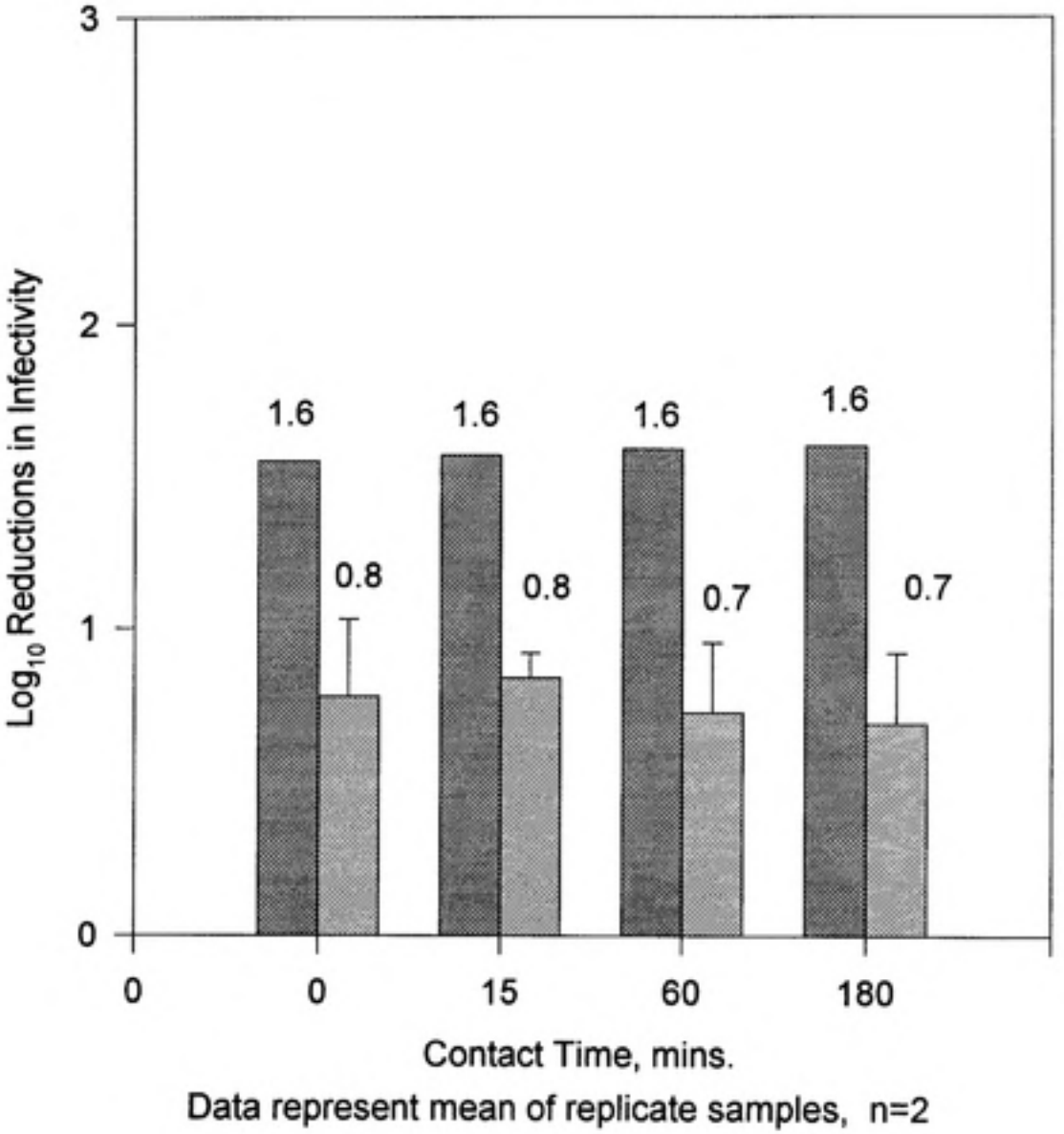
lower than the reactor HRT. Although in theory one would expect complete inactivation, it is possible that short-circuiting and mixing problems within a full scale digester could lead to some oocysts surviving. Post treatment of digested sludges often includes a drying or dewatering phase. Evidence from other studies suggest that oocysts are sensitive to water availability, and that desiccation, even for as little as 4 hours, can be lethal (Robertson et al, 1992 and Barbee et al., unpublished).

Anaerobic digestion under thermophilic conditions was an effective method for inactivating oocyst infectivity in both milieu tested. The observation of inactivation or loss of infectivity following sludge digestion is in agreement with previous studies (Whimore & Robertson, 1995, and Staderman et al, 1995). Whitmore and Robertson (1995) reported that oocysts were completely inactivated by thermophilic aerobic digestion at 55°C after two hours, but 10% remained infective after mesophilic (37°C), anaerobic digestion. Staderman (1995) reported 99.98% inactivation after 24 hours by anaerobic mesophilic digestion. While these studies provide valuable information, they fail to provide information on the infectivity of treated oocysts since they use methods which score for viability alone. Our data indicate that *C. parvum* oocysts infectivity is reduced by at least 3 $\log_{10}$ in both PBS and sludge after one hour.

USEPA standards for treated sludges intended for land application were developed based on target reductions in pathogens. Although not documented, transmission via land applied sludge (animal or human) could occur by direct or indirect routes. Unfortunately only limited data are available regarding the contribution from various sources and the relative survival in the environment. Since inactivation at 55°C was rapid, treatment of sludge or cow manure at or above this temperature should be considered a safe procedure. Safe utilization of treated sludge would therefore be expected. The regulations contain no specific information or guidelines for the destruction of *C. parvum* oocysts.

Figure 6.1

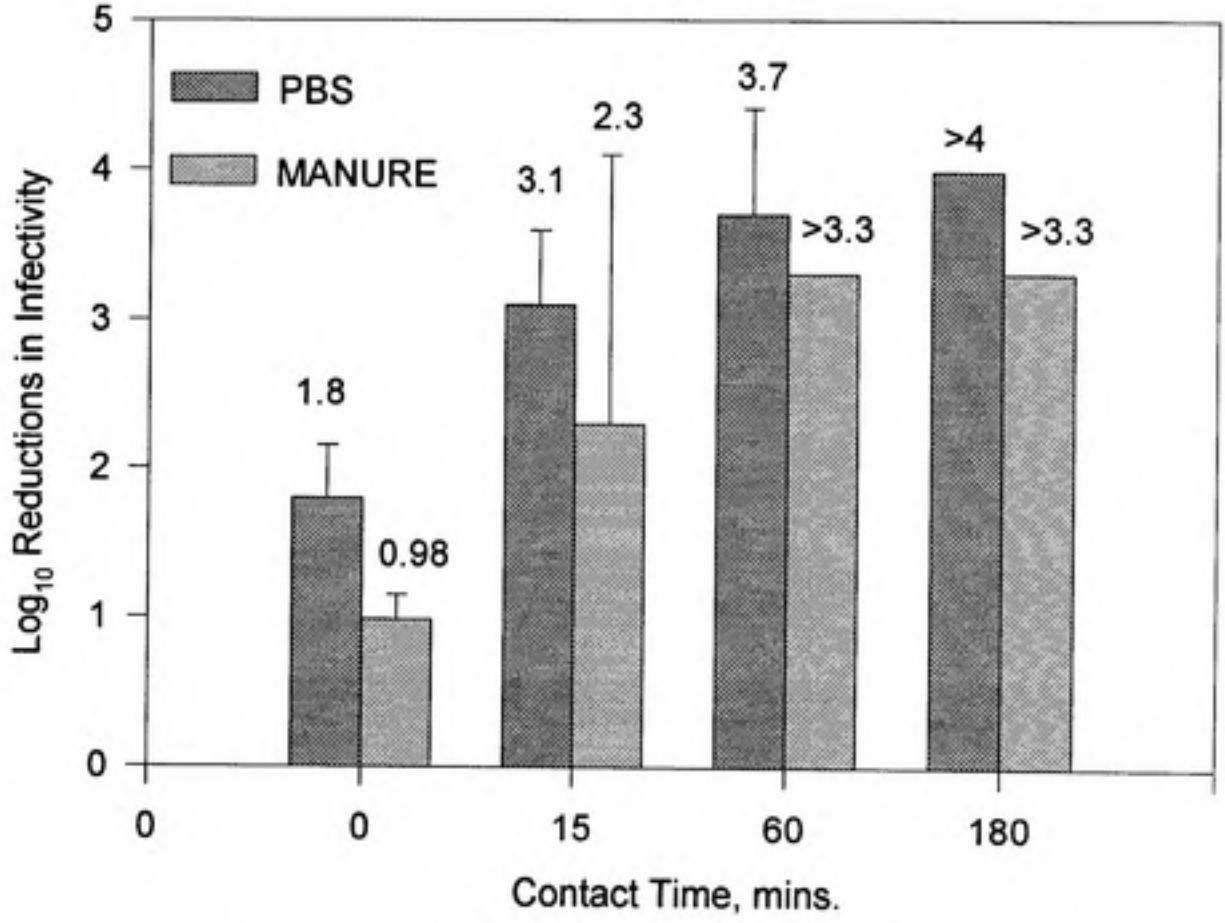
Inactivation of C. parvum in PBS and Manure at 23°C



Data represent mean of replicate samples, n=2

Figure 6.2

Inactivation of *C. parvum* in PBS and Manure at 55°C



Data represent mean of replicate samples, n=2
errror bars = standard deviations

Table 6.2

Thermophilic Anaerobic Digestion Inactivation of C. parvu

Experiment	Temperature °C	Incubation time, mins.	Mean %Positivity	log Reductions
Trial #1		0	73	2.2
		15	12	3.49
PBS	55	60	0.5	4.2
		180	0	>4.48
		0	93.5	0.54
		15	85	0.9
SLUDGE	23	60	90	0.56
		180	87.5	0.53
		0	83	1.1
		15	2	3.6
SLUDGE	55	60	0	>3.7
		180	0	>3.7

Experiment	Temperature °C	Incubation time, mins.	Mean %Positivity	log Reductions
Trial #2		0	83.5	1.1
	23	15	80.5	1.06
PBS		60	75	1.16
		180	76.5	1.13
		0	76.5	1.6
		15	11.5	2.8
PBS	55	60	0.5	3.2
		180	0	>3.5
		0	73	0.9
		15	77	0.78
SLUDGE	23	60	72	0.89
		180	71	0.85
		0	81	0.86
		15	6.5	1
SLUDGE	55	60	0	>3.6
		180	0	>3.6

Temp.	Incubation time, mins.	Mean/sd	
		PBS	Sludge
	0	1.55	0.78/0.25
	15	1.57	0.84/0.08
23 °C	60	1.59	0.725/0.23
	180	1.6	0.69/0.23
	0	1.8/0.35	0.98/0.17
55°C	15	3.1/0.49	2.3/1.8
	60	3.7/0.71	>3.3*
	180	3.99/0.7*	>3.3*

*both data points included censored data
n=2 except for PBS at 23°C

CHAPTER SEVEN

CONCLUSIONS

1. The results of this study indicate the potential of *in vitro* cell culture infectivity assay techniques for use in disinfectiona and inactivation studies. This method provides a useful, efficient and reproducable tool for determining the infectivity of *Cyptosporidium parvum* oocyst and their sporozoites.

2. The results of these studies suggest that high temperature (>49°C) thermal treatment rapidly reduces the infectivity of *Cryptosporidium parvum* oocysts as determined by an *in vitro* cell culture infectivity.

3. Anaerobic digestion of cow manure under thermophilic conditions is an effective method for inactivating *Cryptosporidium parvum* oocysts infectivity.

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APPENDIX

Raw Data

Appendix A

Notes

Experiment		Oocyst/well			
SBI		**1E+05**	**1E+04**	**1E+03**	**1E+02**
Serial	+/-fields	18/2	3/17	0/20	0/20
Dilution	%positive	90	15	0	0
Study	mean	7.3	0.15	0	0
	sd	8.5	0.37	0	0
	range	-36	1	0	0
	n	20	20	20	20

24 hour incubation

	Number Foci/Field oocyst/well			
Field	**1E+05**	**1E+04**	**1E+03**	**1E+02**
1	7	0	0	0
2	0	0	0	0
3	8	0	0	0
4	0	0	0	0
5	16	0	0	0
6	5	1	0	0
7	6	0	0	0
8	1	0	0	0
9	9	1	0	0
10	3	0	0	0
11	4	0	0	0
12	9	0	0	0
13	4	0	0	0
14	3	1	0	0
15	1	0	0	0
16	5	0	0	0
17	36	0	0	0
18	10	0	0	0
19	16	0	0	0
20	3	0	0	0

Time Course Study

Experiment		Incubation, Hours		
SBV	**Field #**	**18**	**24**	**48**
	1	60	55	>100
	2	59	45	>100
	3	95	32	>100
Number of Foci/Field	4	62	28	>100
	5	70	42	>100
	6	64	41	>100
	7	55	39	>100
	8	54	86	>100
	9	38	41	>100
	10	38	47	>100
	11	53	49	>100
	12	53	51	>100
	13	45	48	>100
	14	51	50	>100
	15	74	39	>100
	16	65	47	>100
	17	58	31	>100
	18	45	25	>100

1e5 oocyst/well

19	41	44	>100
20	62	48	>100

1e5oocyst/well

	18 hr	**24 hr**	**48 hr**
+/-fields	20/0	20/0	20/0
%positive	100	100	100
mean	57.1	44.4	>100
sd	13.4	12.6	
range	-57	-61	
n	20	20	20

Experiment Serial Dilutions/ 24 hour
SBVIII

	1E+05	**1E+04**	**1E+03**	**1E+02**
+/-fields	15/20	19/1	1/19	1/19
%positive	100	95	5	5
mean	52.86	25.5	0.4472	0.4472
sd	34.83	20.36	0.1	0.1
range	10-138	2-70	0-2	0-2
n	20	20	20	20

Quantal Counts

Pos/Neg (100 Fields)	100/0	33/67	14/86	1/99
%positive	100	33	14	1

Number Foci/Field
oocyst/well

Field	**1E+05**	**1E+04**	**1E+03**	**1E+02**
1	71	18	0	0
2	82	19	0	0
3	39	0	0	2
4	34	57	0	0
5	10	28	0	0
6	32	2	0	0
7	52	14	0	0
8	70	8	0	0
9	67	2	2	0
10	48	17	0	0
11	40	35	0	0
12	148	38	0	0
13	11	45	0	0
14	>100	41	0	0
15	>100	70	0	0
16	>100	52	0	0
17	>100	4	0	0
18	>100	33	0	0
19	>100	3	0	0
20	>100	28	0	0

Serial Dilutions/ 24 hour

Experiment		**1E+04**	**1E+03**	**1E+02**	**1E+01**
SBIX	+/-fields	18/5	4/16	2/18	0/20
	%positive	78	20	10	0
	mean	13.3	1.2	0.2	0

sd	18.1	3.5	0.6	0
range	0-77	0-15	0-2	0
n	20	20	20	20
(100 Fields)	21/79	2/98	0/100	0/100
%positive	21	2	0	0

Number Foci/Field
oocyst/well

Field	1E+04	1E+03	1E+02	1E+01
1	0	0	0	0
2	7	0	0	0
3	17	0	0	2
4	10	0	0	0
5	2	2	2	0
6	18	0	0	0
7	30	0	0	0
8	12	15	0	0
9	0	0	2	0
10	2	0	0	0
11	77	2	0	0
12	0	0	0	0
13	0	0	0	0
14	15	5	0	0
15	2	0	0	0
16	19	0	0	0
17	0	0	2	0
18	4	0	0	0
19	32	0	0	0
20	40	0	0	0

SBXI Serial Dilutions/ 48 hour

	1E+04	1E+03	1E+02
+/-fields	20/20	5/20	1/20
%positive	100	25	5
mean	74.57	3.95	0.0952
sd	51.11	8.9	0.4364
range	3-166	0-35	0-2
n	20	20	20

Quantal Counts	**Pos/Neg**	93/100	23/100	5/100
	%positive	93	23	5

Number Foci/Field
oocyst/well

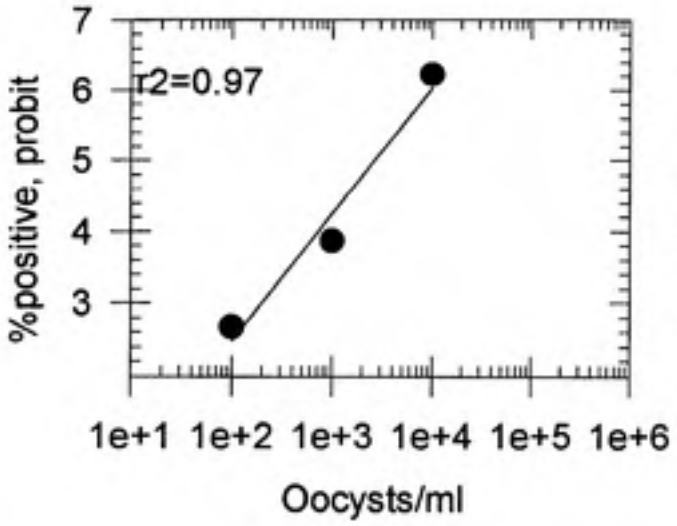
Field	1E+04	1E+03	1E+02
1	125	0	0
2	166	0	0
3	129	0	0
4	71	0	0
5	7	6	0
6	41	0	0
7	43	0	0
8	11	21	0
9	40	0	0

10	147	14	0
11	91	2	0
12	130	0	0
13	130	0	2
14	20	5	0
15	98	35	0
16	67	0	0
17	120	0	0
18	3	0	0
19	18	7	0
20	56	0	0
21	53	0	0

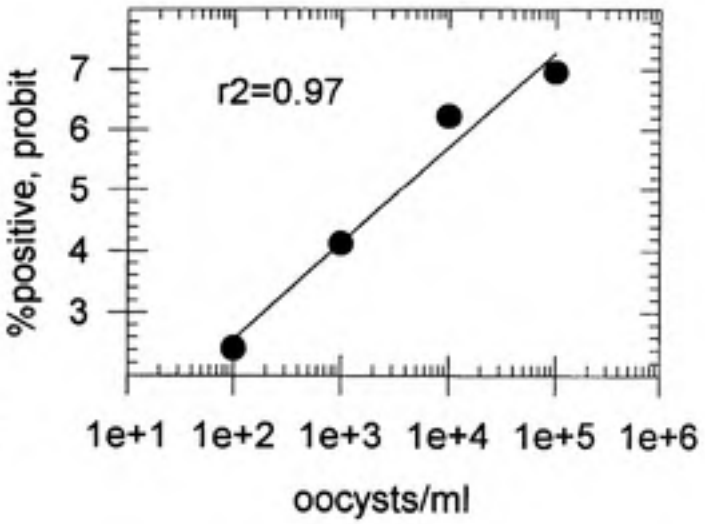
Thermal Inactivation Studies

Linear Regression Analysis/Dose-Response Curves

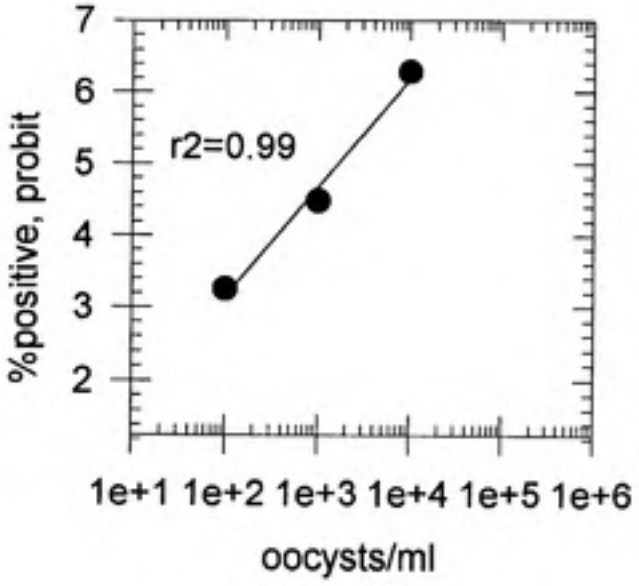
SBXIX



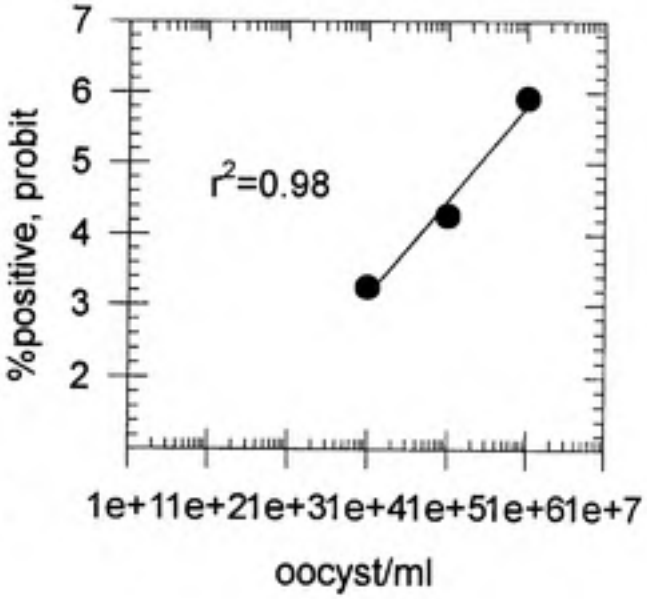
SBXV



SBXXII



SBXXIII



Appendix E
Raw Data Thermophilic Anaerobic Digestion in Sludge and PBS

Trial #1

Temperature °C	Incubation time (mins.)	%Positive Fields for 10^6 treated oocysts per culture well			Mean Probit Value
		#1	#2	Mean	
55 PBS	0	76	70	73	5.61
	15	12	12	12	3.82
	60	0	1	0.5	3.36
	180	0	0	0	</2.67
23 SLUDGE	0	95	92	93.5	6.52
	15	80	90	85	6.04
	60	90	90	90	6.28
	180	93	82	87.5	6.15
55 SLUDGE	0	90	76	83	5.95
	15	2	2	2	2.95
	60	0	0	0	</2.67
	180	0	0	0	</2.67

Control Dilution Series

#oocyst/well	%positive fields			Mean
	#1	#2	Average	Probit
1E+06	100	100	100	>/8.09
1E+05	100	100	100	>/8.09
1E+04	87	75	81*	5.88
1E+03	62	60	61.25*	5.29
1E+02	6	9	7.5*	3.55
1E+01	0	0	0	</2.67

*used for linear regression analysis

Trial #2

Temperature °C	Incubation time (mins.)	%Positive Fields for $2x10^6$ treated oocysts per culture well			Mean Probit Value
		#1	#2	Mean	
23 PBS	0	85	82	83.5	5.97
	15	81	80	80.5	5.86
	60	72	78	75	5.67
	180	82	71	76.5	5.69
23 SLUDGE	0	76	70	73	5.61
	15	78	76	77	5.74
	60	73	70	72	5.58
	180	70	72	71	5.55
55 PBS	0	72	81	76.5	5.69
	15	15	8	11.5	3.79
	60	3	0	0.5	3.36
	180	0	0	0	</2.67
55 SLUDGE	0	80	82	81	5.88
	15	8	5	6.5	5.39
	60	0	0	0	</2.67
	180	0	0	0	</2.67

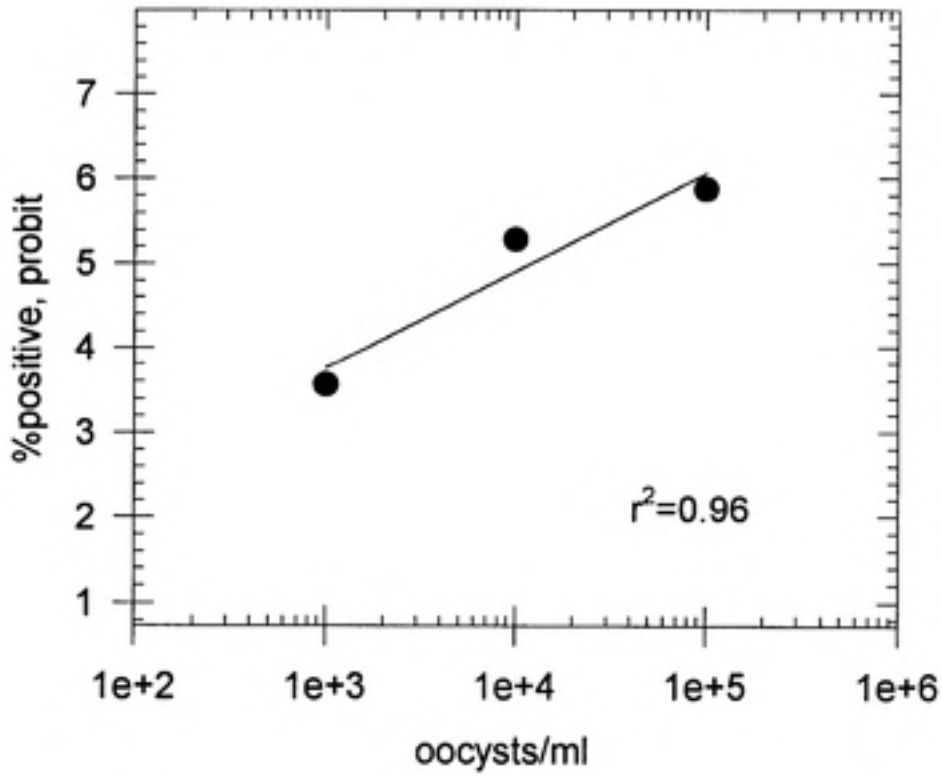
Control Dilution Series

#oocyst/well	%positive fields			Mean
	#1	#2	Average	Probit
1E+06	100	100	100	>/8.09
1E+05	98	100	99*	8.09
1E+04	82	75	78.5*	5.79
1E+03	5	7	6*	3.45
1E+02	1	0	0.5*	</2.67
1E+01	0	0	0	</2.67

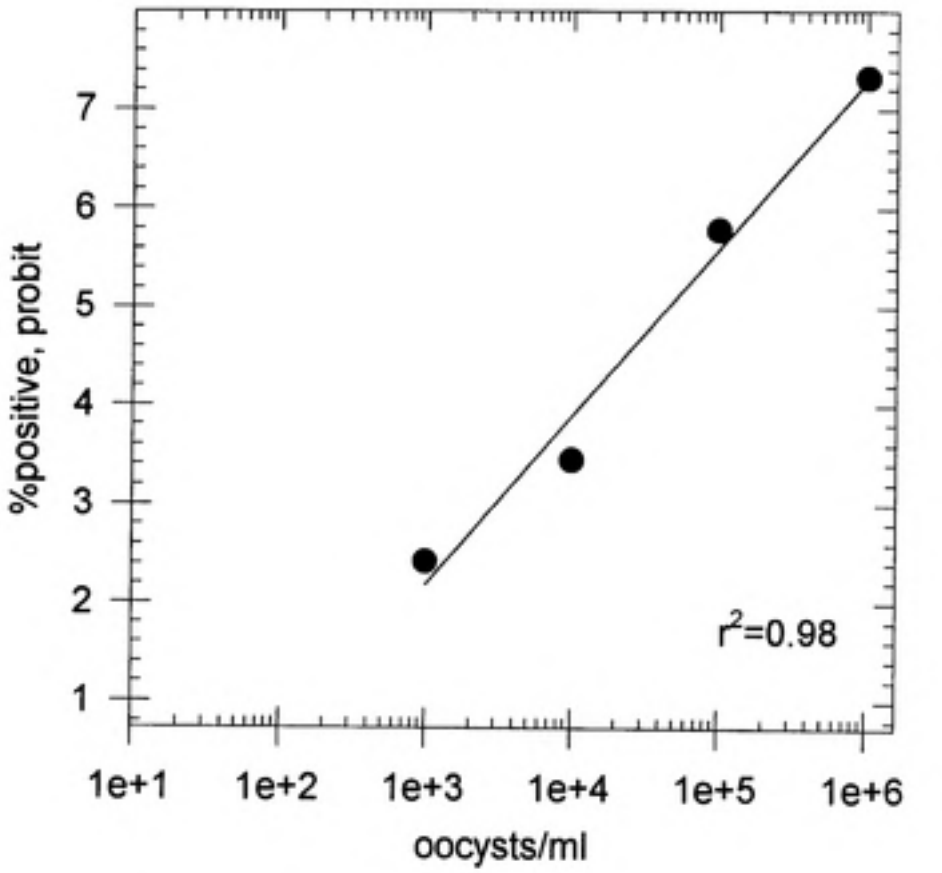
*used for linear regression analysis

Linear Regression Analysis/Dose-Response Curves

Thermophilic Anaerobic Digestion



Trial #1



Trial #2

TRAY,G	W/SAMPLE	105C	500C	%TS	%FS	%VOC	VOS RED
0.983	4.258	1.11	1.021	3.88	1.16	2.72	22.00
0.985	6.692	1.46	1.09	8.32	1.84	6.48	52.50
0.985	5.556	1.38	1.066	8.64	1.77	6.87	55.82
0.98	6.71	1.44	1.08	8.03	1.75	6.28	50.87
1	7.3	1.52	1.109	8.25	1.73	6.52	52.82
1	7.54	1.4	1.094	6.12	1.44	4.68	37.89
1	6.4	1.33	1.075	6.11	1.39	4.72	38.24
1	7.46	1.29	1.115	4.49	1.78	2.71	21.94
1	6.58	1.26	1.08	4.66	1.43	3.23	26.12
1	6.18	1.53	1.11	10.23	2.12	8.11	65.65
1	5.07	1.45	1.07	11.06	1.72	9.34	75.60
1.003	5.853	1.511	1.107	10.47	2.14	8.33	67.45
1.002	5.323	1.462	1.045	10.65	1.00	9.65	78.14
0.999	5.695	1.534	1.1	11.39	2.15	9.24	74.83
1.001	5.47	1.492	1.1	10.99	2.22	8.77	71.02
1.002	11.58	2.463	1.247	13.81	2.32	11.50	
1.003	4.227	1.362	1.076	11.14	2.26	8.87	71.83
1	4.923	1.446	1.087	11.37	2.22	9.15	74.10
0.993	4.038	1.357	1.072	11.95	2.59	9.36	75.79
1.006	3.554	1.299	1.056	11.50	1.96	9.54	77.22
1.003	4.329	1.378	1.09	11.27	2.62	8.66	70.11
0.995	5.616	1.534	1.11	11.66	2.49	9.18	74.30
1.011	4.525	1.392	1.082	10.84	2.02	8.82	71.43
1.002	4.02	1.351	1.069	11.56	2.22	9.34	75.66
0.998	7.966	1.563	1.123	8.11	1.79	6.31	51.13
0.999	6.846	1.48	1.093	8.23	1.61	6.62	53.59
1.022	3.381	1.199	1.032	7.50	0.42	7.08	57.32
1.004	3.565	1.235	1.052	9.02	1.87	7.15	57.86
1.004	4.44	1.32	1.078	9.20	2.15	7.04	57.03
1.004	4.16	1.302	1.065	9.44	1.93	7.51	60.81
0.992	3.998	1.268	1.047	9.18	1.83	7.35	59.53
1.008	3.257	1.195	1.045	8.31	1.65	6.67	54.01
1.002	4.004	1.258	1.048	8.53	1.53	7.00	56.64
1.003	6.534	1.48	1.114	8.62	2.01	6.62	53.56
1.008	4.468	1.34	1.092	9.60	2.43	7.17	58.04
1.007	4.985	1.38	1.098	9.38	2.29	7.09	57.40
1.003	5.091	1.377	1.074	9.15	1.74	7.41	60.02
1.006	4.653	1.346	1.071	9.32	1.78	7.54	61.06
1.003	4.576	1.308	1.073	8.54	1.96	6.58	53.26
1.003	3.909	1.251	1.077	8.53	2.55	5.99	48.48
1.001	4.883	1.31	1.094	7.96	2.40	5.56	45.05
1.006	5.842	1.386	1.073	7.86	1.39	6.47	52.41
1.003	5	1.317	1.083	7.86	2.00	5.85	47.40
1.003	5.921	1.429	1.051	8.66	0.98	7.69	62.24
0.997	6.512	1.424	1.045	7.74	0.87	6.87	55.65
1.008	3.932	1.22	1.062	7.25	1.85	5.40	43.75
1.004	5.894	1.411	1.081	8.32	1.57	6.75	54.64
1.001	6.047	1.432	1.089	8.54	1.74	6.80	55.04
1.015	6.469	1.401					
1.105	6	1.392	1.105	5.86	0.00	5.86	47.47
0.999	4.788	1.265	1.088	7.02	2.35	4.67	37.83
1.002	4.022	1.305	1.055	10.03	1.75	8.28	67.03
1.002	6.485	1.389	1.074	7.06	1.31	5.75	46.52

1.002	5.861	1.352	1.067	7.20	1.34	5.87	47.49
0.997	4.16	1.233	1.053	7.46	1.77	5.69	46.08
1.004	6.08	1.345	1.032	6.72	0.55	6.17	49.93
1.008	6.412	1.473	1.082	8.60	1.37	7.24	58.59
1.003	6.672	1.516	1.083	9.05	1.41	7.64	61.85
1.009	3.319	1.172	1.036	7.06	1.17	5.89	47.67
1.004	4.683	1.272	1.045	7.28	1.11	6.17	49.96
1.008	6.795	1.599	1.112	10.21	1.80	8.42	68.14
1.008	6.553	1.591	1.108	10.51	1.80	8.71	70.53
1.012	5.802	1.468	1.075	9.52	1.32	8.20	66.43
1.009	5.933	1.474					0.00
1.003	3.318	1.209	1.028	8.90	1.08	7.82	63.31
1.007	5.1	1.414	1.054	9.94	1.15	8.80	71.22
1.006	6.071	1.453	1.057	8.83	1.01	7.82	63.31
1.004	5.353	1.365					
1.009	5.356	1.426					
1.005	7.194	1.608					
1.001	5.134	1.439					
1.001	5.893	1.522					
0.993	3.62	1.301	1.022	11.72	1.10	10.62	86.00
0.998	5.995	1.483	1.067	9.71	1.38	8.32	67.41
1.001	5.89	1.47	1.067	9.59	1.35	8.24	66.74
1.004	6.237	1.513	1.078	9.73	1.41	8.31	67.31
0.997	6.476	1.542	1.078	9.95	1.48	8.47	68.57
1.005	3.898	1.189	1.032	6.36	0.93	5.43	43.94
1.003	5.424	1.371	1.072	8.32	1.56	6.76	54.76
1.001	4.059	1.393	1.048	12.82	1.54	11.28	91.35
0.998	3.28	1.318	1.028	14.02	1.31	12.71	102.90
1	5.099	1.405	1.062	9.88	1.51	8.37	67.76
1.004	4.101	1.451	1.051	14.43	1.52	12.92	104.58
0.997	4.85	1.31	1.056	8.12	1.53	6.59	53.38
1	4.688	1.258					
1.001	3.31	1.401					
1.013	4.93	1.333	1.048	8.17	0.89	7.28	58.91
1.011	4.354	1.271	1.04	7.78	0.87	6.91	55.95
0.995	3.916	1.283	1.048	9.86	1.81	8.05	65.14
1.003	4.377	1.324	1.054	9.51	1.51	8.00	64.80
1.013	4.733	1.358	1.059	9.27	1.24	8.04	65.08
1.005	3.541	1.225	1.033	8.68	1.10	7.57	61.30
1.003	3.216	1.308	1.037	13.78	1.54	12.25	99.16
1.013	3.712	1.254	1.05	8.93	1.37	7.56	61.20
0.996	5.312	1.474	1.083	11.08	2.02	9.06	73.35
1.001	2.849	1.21	1.036	11.31	1.89	9.42	76.24
1.002	4.014	1.364	1.064	12.02	2.06	9.96	80.65
1.006	4.398	1.314	1.079	9.08	2.15	6.93	56.10
1.001	5.086	1.379	1.058	9.25	1.40	7.86	63.63
1.002	3.901	1.274	1.04	9.38	1.31	8.07	65.36
0.996	4.59	1.434	1.054	12.19	1.61	10.57	
0.998	3.168	1.263	1.032	12.21	1.57	10.65	
1	6.69	1.708	1.043	12.44	0.76	11.69	