Inactivation Responses of *Cryptosporidium parvum* to UV Radiation and Gamma Radiation

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ABSTRACT

Disinfection has always been a major barrier for control of microbial contaminants in water. However, during the spring of 1993 a massive outbreak of a waterborne disease took place in Milwaukee, Wisconsin. The contaminant was *Cryptosporidium parvum*, a protozoan parasite that is more resistant to conventional water treatment-disinfection processes than most waterborne bacteria or viruses. In this study, *Cryptosporidium parvum* was exposed to UV and gamma radiation. Inactivation responses were measured using the Foci Detection Method (FDM) and were enumerated using the Most Probable Number (MPN) procedure. Previous studies suggested that UV doses on the order of hundreds to thousands mW-s/cm^2 were required to inactivate *C. parvum*. Results from this study indicated that *C. parvum* was inactivated by both disinfectants over several orders of magnitude at doses comparable to those required for inactivation of bacteria and viruses. Based on these results, UV and gamma radiation appear to be effective physical disinfectants for the inactivation of *Cryptosporidium parvum*.

INTRODUCTION

Disinfection has been a major barrier for control of microbial contaminants in water for at least 100 years. In order to meet the 10^{-4} annual risk of *Cryptosporidium* infection goal, the average drinking water facility will need to provide 2.0 $\log_{10}$ (99%) disinfection (Rose *et al.* 1997). Current disinfection methods include chlorination, ultraviolet (UV) irradiation and ozonation. Ozonation has shown promise for effective disinfection of *Cryptosporidium* achieving

the 99% inactivation goal (Rennecker *et al.* 1999). Chlorine disinfection alone has not been effective for eliminating cryptosporidiosis in drinking water and recreational-water outbreaks (Korich *et al.* 1990). Also, previous research has shown that ultraviolet (UV) inactivation would not be cost effective because dosages on the order of hundreds to thousands of $mW\text{-}sec/cm^2$ would be required to achieve the desired inactivation goal (Campbell *et al.* 1995).

Much of the previous research regarding UV utilized *in vitro* excystation as the viability assay (Campbell *et al.* 1995). Excystation measures the ability of the oocyst to release sporozoites. However, it does not measure infectivity and has limitations regarding enumeration of sporozoites. Until recently, the only infectivity assay available required the use of animal hosts, which is costly and time-consuming. However, mouse infectivity studies indicate that low pressure and medium pressure mercury vapor UV lamp technology can achieve a 4 $\log_{10}$ inactivation of *Cryptosporidium* oocysts at a cost effective dose (Clancy *et al.* 1998; Bukhari *et al.* 1999).

Gamma irradiation is another possible physical disinfectant. Currently, the United States has large stockpiles of cesium-137, one source of gamma rays, in the form of spent nuclear fuel. Research has demonstrated it to be effective for inactivation of many bacterial and viral organisms, which are important in water processes. However, there had been only one published study at the time of this report to demonstrate inactivation of *Cryptosporidium parvum* by gamma irradiation (Thompson and Blatchley 2000).

Recently, cell culture technology has developed into a tool that can be used to study the organism in an environment most similar to the *in vivo* situation without using animal models. Using this new assay, inactivation of *Cryptosporidium* oocysts can be determined based on infectivity. The goal of this research is to determine the ability of gamma (γ) radiation and UV radiation to inactivate *Cryptosporidium parvum* using the novel cell culture assay and compare the findings to previous *in vitro* studies.

Drinking Water Regulations

The United States Environmental Protection Agency (USEPA) finalized the Interim Enhanced Surface Water Treatment Rule (IESWTR) on December 16, 1998. The purpose of the IESWTR is to improve control of microbial pathogens, specifically the protozoan *Cryptosporidium parvum*, in drinking water, and address risk trade-offs with disinfection byproducts. The following are some of the key provisions established in the IESWTR:

- Maximum Contaminant Level Goal (MCLG) of zero for *Cryptosporidium.*
- 2-$\log_{10}$ *Cryptosporidium* removal requirement using filtration only.
- Strengthened combined filter effluent turbidity performance standards and individual filter turbidity provisions.
- Disinfection benchmark provisions to assure continued levels of microbial protection while facilities take the necessary steps to comply with new disinfection byproducts standards.

The IESWTR applies to public water systems that use surface water or ground water under the direct influence of surface water (GWUNDI) and serve 10,000 or more people. The regulation was effective February 16, 1999.

The Final Stage 1 Disinfectant/Disinfection Byproduct (D/DBP) rule was promulgated December 16, 1998. In this rule, the USEPA finalized maximum residual disinfectant levels (MRDLs) for chlorine, chloramines, and chlorine dioxide:

- Free Chlorine – 4.0 mg/L as free chlorine
- Chloramines – 4.0 mg/L as total chlorine
- Chlorine Dioxide – 0.80 mg/L as chlorine dioxide

The implementation of these regulations will require water utilities to balance the disinfection process so as to minimize the formation of DBP's without compromising the inactivation of waterborne pathogens. Surface water and groundwater both contain a variety of naturally occurring organics. When these organics are exposed to chlorine, halogenated byproducts are produced. Therefore, chlorine as a disinfectant will probably be limited to utilities that use source waters with low levels of DBP precursors and utilities with high levels of DBP precursors will need to search for a suitable cost-effective alternative disinfectant.

Cryptosporidium parvum Biology and Life Cycle

Cryptosporidium is classified as an eukaryotic protozoan. The oocyst is spherical to ovoid and average size is 5 by 4.5μm. Sporulated oocysts each contain four sporozoites. The sporozoites are generally crescent shaped and average 4.9 by 1.2 μm in size (Fayer and Ungar 1986). Each sporozoite is unicellular and has a nucleus containing most of the cell's DNA enclosed by a double layer membrane. The sporozoite also contains dense granules, rhoptry, and micronemes. The machinery mediating the sporozoite invasion process is collectively housed in the anterior region of the sporozoite and is known as the apical complex. Rhoptry and micronemes are located in the apical complex and are therefore thought to be secretory organelles that facilitate penetration into the host cell (Tetley *et al.* 1998).

The *Cryptosporidium parvum* life cycle consists of two phases, the exogenous phase and endogenous phase (See Figure 1). The exogenous phase begins when an infected host excretes mature oocysts that can contaminate the environment, water or food. When a suitable host ingests the oocysts, the endogenous phase begins. The oocyst consists of a two-layered wall, with four sporozoites contained within. Once ingested, the sporozoites excyst from the oocyst and parasitize the epithelial cells of the intestine. Each sporozoite is capable of differentiating into a spherical trophozoite. The trophozoite nucleus then divides resulting in asexual multiplication, forming two types of meronts. Type I consists of 6 or 8 nuclei which will be incorporated into a merozoite. Each merozoite is able to infect a new host cell, and then develop into a new type I meront, or into a type II meront, which contains 4 merozoites when mature.

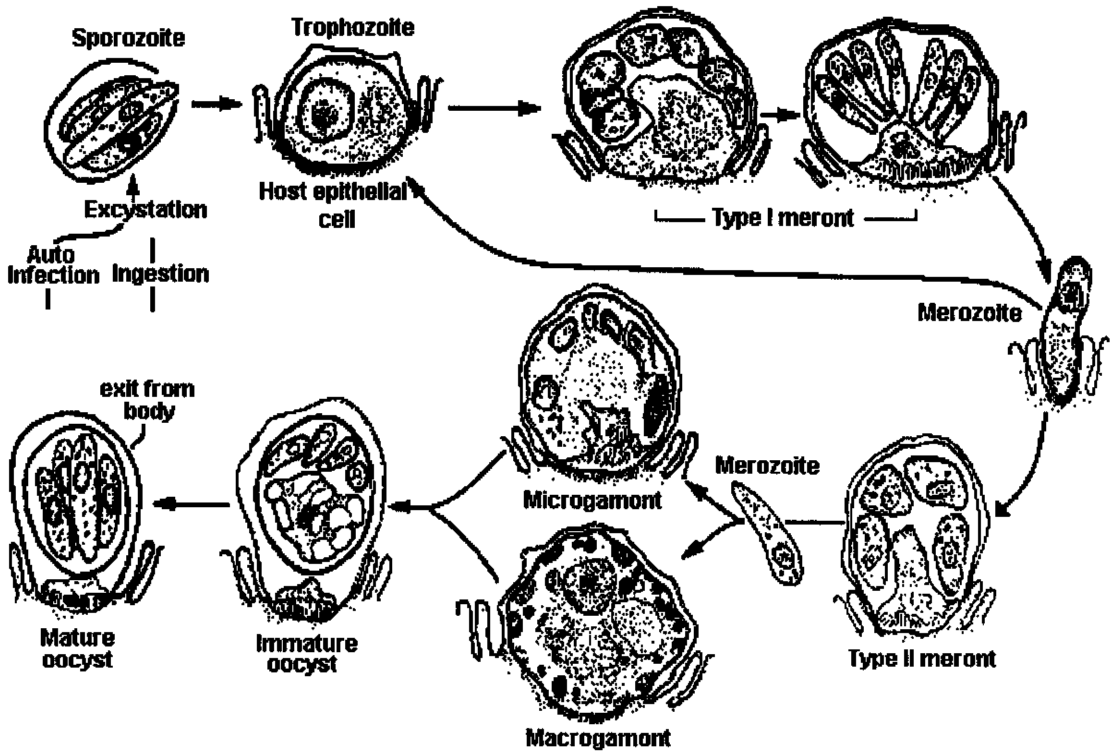

Figure 1: *Cryptosporidium parvum* Life Cycle (Fayer *et al.* 1997).

The merozoites from type II meronts also invade new host cells, but are able to initiate sexual multiplication by differentiating into either a microgamont (male) or a macrogamont (female). The macrogamont is fertilized by the microgamont and develops into oocysts that sporulate *in situ* and either release sporozoites for autoinfection, sequential asexual and sexual phases of the life cycle repeated within the same host, or pass from the body in the feces.

About 20% of the oocysts fail to form a wall. These oocysts are termed "thin-walled" oocysts since only a series of membranes surround the sporozoites. It is believed that these oocysts can excyst within the gut and infect new cells. The remaining "thick-walled" oocysts, which are released in the feces, are responsible for long term survival of the parasite in the environment (Upton 1999)

After ingestion by a human host, it takes 4 to 22 days for the oocyst to complete the endogenous life cycle and excrete newly developed oocysts. The duration of oocyst excretion ranges from 1 to 20 days.

Gamma Radiation

Gamma rays are electromagnetic waves emitted by radioactive nuclei, such as ^{60}Co and ^{137}Cs, with wavelengths in the range of 10^{-11} to 10^{-7} cm. Radiation that originates from atomic nuclei is termed gamma radiation while that originating from outside of the atomic nucleus are termed x-rays. Gamma rays are formed when an unstable atomic nucleus releases energy to gain stability. In the research described herein, the source of gamma rays was the ^{60}Co isotope, which, from the standpoint of radiation chemistry and disinfection, is likely to yield a similar behavior as

solution was removed from the dishes and transferred to sterile test tubes. Appropriate dilutions were made to achieve the desired concentration. The desired dilutions were then pipetted to the chamber well. Each slide, containing eight wells, was designated for one dilution. 500 μL of growth medium was then added to each well and the slides were incubated for 48 hours at 37° C with 5% CO_2.

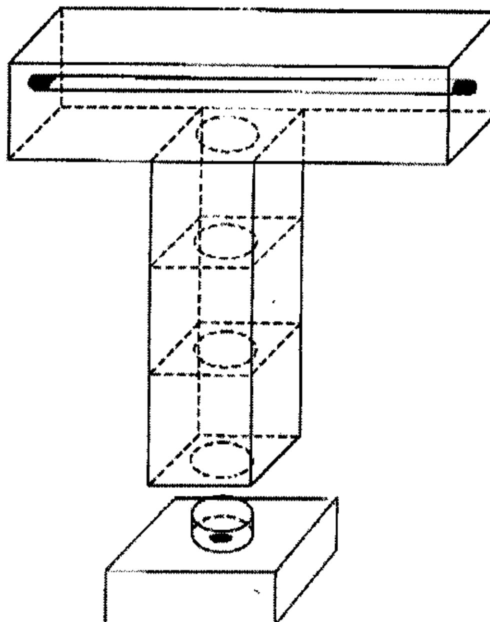

Figure 2. Schematic illustration of collimation apparatus for ultraviolet exposure experiments (WEF 1996).

Gamma Radiation

A Nordian International, Inc., Gammacell 220 (see Figure 3) was used for gamma irradiation. The Gammacell contains a ^{60}Co as the radiation source. The Purdue University Radiological and Environmental Management (REM) department supplied data regarding the radiation exposure rate. For this study, *C. parvum* was exposed to gamma irradiation in the presence/absence of dissolved oxygen.

For experiments that were in equilibrium with air, the stock oocyst solution was centrifuged and then transferred to a 15-mL centrifuge tube containing water that had been sparged with air for 30 minutes. The tubes were placed in a 1500-mL beaker and taped into place. Ice was then added to the beaker to prevent excystation during the irradiation process. Tubes were removed at the pre-designated times and placed in a refrigerator until all exposures were completed.

Experiments that were in the absence of dissolved oxygen were similar to those with dissolved oxygen. However, instead of sparging the water with air, the water was sparged with N_2 in order to remove any dissolved oxygen.

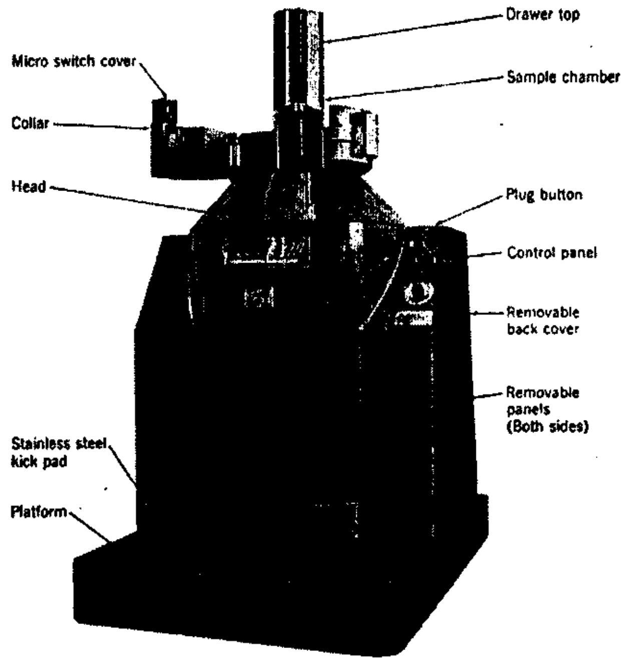

Figure 3. Gammacell 220 cobalt-60 radiation source (Spinks and Woods 1990).

RESULTS AND DISCUSSION

Figure 4 shows the inactivation results of *C. parvum* oocysts exposed to UV radiation as quantified by the FDM. The UV doses of 2, 4, 6, and 8 mW-s/cm^2 demonstrated little variation regarding inactivation. For a dose of 20 mW-s/cm^2, >3.59 $\log_{10}$ units of inactivation were measured. Public water systems that combine filtration with disinfection must achieve 2-$\log_{10}$ units of inactivation (99%) for *C. parvum*. According to the data presented in Figure 4 and Table 1, 3 $\log_{10}$ units of inactivation can be achieved at a dose of 10 mW-s/cm^2.

Table 1. UV Inactivation Responses *C. parvum*

UV Dose (mW-s/cm^2)	$\log_{10}$ Units of Inactivation
1	0.4
2	1.2
4	1.4
6	1.2
8	1.5
10	3.0
20	>3.6

When performing the FDM assay for the UV dose of 20 mW-s/cm^2, 10^5 oocysts were plated per well as well as lower dilutions. The slide containing 10^5 oocysts per well resulted in negative results for all wells. Since the well results were 0/8 (0 negative wells out of 8 possible positive wells), the MPN could not be determined. Therefore, the MPN was obtained for the UV dose of 20 mW-s/cm^2 by calculating the MPN if one well was positive out of 8 wells. This MPN translated to at least 3.6 $\log_{10}$ units of inactivation and therefore is greater than the true MPN for

the ^{137}Cs isotope. The ^{60}Co isotope undergoes β-decay with a 5.27 year half-life, producing an energetic electron and two γ-photons with energies of 1.173 and 1.332 MeV (million electron volts) (Spinks and Woods 1990).

Currently, in the United States and other countries, gamma radiation is used in the food irradiation process. Food is exposed to high levels of ionizing radiation resulting from ^{60}Co and ^{137}Cs, which penetrates into the food killing microorganisms without raising the temperature of the food significantly. Food is irradiated to extend shelf-life, eliminate insects, or inactivate microorganisms. The Food Safety and Inspection Services (FSIS) has recommended for meat food products be treated with ionizing radiation at dosages up to 4.5 kiloGrays (kGy), if refrigerated, and 7 kGy, if frozen.

However, many are skeptical of the safety of irradiated food, not only for consumption but also for the increased risk of accidents involving radioactive material and worker safety. The Food and Drug Administration (FDA) has examined numerous studies on the chemical effects of radiation, the impact of radiation on nutrient content of foods, potential toxicity concerns, and effects on microorganisms in or on irradiated products. The FDA concluded that irradiation is safe and does not compromise the nutritional quality of treated products. Also, the irradiation of food has been endorsed by the World Health Organization, the Food and Agriculture Organization, the American Medical Association, and the American Dietetic Association. Furthermore, the FSIS concluded that irradiation of meat food products would pose no significant risk to worker or transportation safety (Federal Register 1999).

In the area of water, a study was conducted by J.E. Thompson (1999) that determined the toxicity effects of gamma irradiated wastewater effluents. The study demonstrated that gamma irradiation had essentially no effect or actually improved effluent toxicity as compared to the undisinfected effluent. This is promising since wastewater contains a wider variety of organic and inorganic compounds, compared to drinking water, which could undergo a chemical change when subjected to gamma irradiation. Therefore, gamma irradiation should have no toxicity effect on drinking water.

UV Radiation

Ultraviolet radiation covers wavelengths ranging from 380 nm to 60 nm. The majority of UV disinfection applications and the research presented in this study use low-pressure mercury arc lamps, producing an output at a wavelength of 253.7 nm (Water Environment Federation (WEF) 1996). UV radiation does not transmit its energy to atoms or molecules by ionization like gamma radiation does, but by excitation, where an electron is raised to a higher energy level. Electronic excitation can result in breakage of a bond and/or formation of a new bond.

Viability vs. Infectivity

Cryptosporidium oocysts can be detected in the water supply by either using light microscopy, an enzyme-linked immunosorbent assay (ELISA) or immunofluorescence techniques. These methods successfully detect *Cryptosporidium*, but they do not provide information regarding the viability of the parasite and its ability to cause an infection. Several methods have been used to estimate the viability of parasites, including vital dye exclusion, *in vitro* excystation, parasite morphology, the uptake or exclusion of fluorogenic dyes, animal infectivity and most recently cell culture. Of these methods, only animal infectivity and cell

culture provide direct information about the ability of the organisms to cause an infection in the host.

Viability is the ability of the sporozoite to excyst. Excystation is the process by which sporozoites are released from the oocyst after ingestion by a suitable host. *In vitro* excystation can be performed in the laboratory by providing conditions similar to those found in the gut of the host, which include appropriate levels of carbon dioxide, acid, pancreatic enzymes, bile, and proper temperature. Quantification of oocyst viability can be made microscopically by determining the ratio of totally and partially excysted oocysts to the total number of oocysts enumerated according to Robertson *et al.*(1993).

Infectivity is the ability of the oocyst to reproduce. The Foci Detection Method (FDM), a cell culture assay, also provides conditions similar to those found in the human gut by using human epithelial cells. The oocyst stock solution is applied to the cells and allowed to infect over a period of forty-eight hours. The developmental stages of *Cryptosporidium parvum* are detected by an enzyme-linked immunosorbent assay that involves a polyclonal antibody developed against *C. parvum* sporozoites. Quantification of oocyst infectivity is made microscopically by determining the number of infectious oocysts per mL in a sample using the most probable number (MPN) method.

MATERIALS AND METHODS

Foci Detection Method

The following procedure for the Foci Detection Method (FDM) assay was adapted from the procedure developed by Slifko *et al.* (1997). The cells required in this experiment are Human Illeoccal Adenocarcinoma Cells (HCT-8) and were purchased from American Type Culture Collection (ATCC) (CCL-244). The HCT-8 cells have been found to best support *Cryptosporidium parvum* growth in a 5% CO_2 environment (Upton *et al.* 1995) and since they are human epithelial cells they best represent the conditions of the human intestine. The maintenance medium, which is used during cell growth, consisted of RPMI 1640 (Mediatech Cellgro 15-040-CV) supplemented with 5% Fetal Bovine Serum (FBS) purchased from Hyclone (catalog # SH30071.03), 1% 200 mM L-glutamine (Sigma G-7513) and 2% 1M HEPES (Sigma H-0887). During oocyst infection, growth medium was added to the cells. The growth medium is the same as the maintenance medium, but the FBS was increased to from 5% to 10% to promote *C. parvum* growth (Upton *et al.* 1995).

The cells were grown in 75-cm^2 vented Costar tissue culture flasks (Fisher 07-200-68) at 37° C, with a 5% CO_2 atmosphere, and 100% humidity. The cells were passaged every 2 or 3 days depending on when the cells reached a 70 – 80% cell confluence. The medium was aspirated from the flasks and 2 mL of a trypsin solution was added to wash the cells of any debris. The trypsin solution contained 0.25 % trypsin + 0.1% EDTA in Hanks Balanced Salt Solution (HBSS) without calcium, magnesium, or sodium bicarbonate (Mediatech Cellgro 25-053-CI). The flasks were gently rotated to ensure even coverage of the solution and then the solution was immediately aspirated off.

Trypsinization begins when the 5 mL of the trypsin solution is added to the flask. The flask is quickly rotated to ensure even coverage of the solution, and then incubated for 10 minutes

at 37° C in the CO_2 atmosphere to assist in the disruption of the monolayer. The cells are then pelleted by a 10 minute centrifugation (100 x g), resuspended in maintenance medium and vigorously pipetted through a 1000 - µL pipette tip repetitively until the cells were separated (about 30 times). The concentration of the cells was determined by a direct hemacytometer count. Nunc Lab Tek II chamber 8 well slides, (Fisher 12-565-8) were plated with 2.5×10^5 cells per well, grown to 70 to 85% confluence (24 to 48 hrs) and then infected.

C. parvum oocysts were obtained (live in PBS) from Waterborne Inc. (New Orleans, LA) in batches of 1×10^7 and 1×10^8 oocysts. Maintenance media was aspirated from the cell monolayer and washed once with sterile 0.01M phosphate buffered saline (PBS) (Sigma P4417). Between 100 and 125 µL of oocysts were added to each chamber well and 500 µL of the growth medium were added to each well chamber. The cells were incubated at 37°C under 5% CO_2 for 48 hours. After incubation, the medium was aspirated and the infected monolayers were fixed with 100% methanol at room temperature for 10 minutes. The chamber wells were removed from the slides and the monolayers were rehydrated at room temperature for 30 minutes with a blocking buffer containing 1% normal rabbit serum (Sigma R-4505) and 0.02% Tween 20 (Fisher BP337-100) in 0.01M PBS. The blocking buffer inhibits nonspecific binding of the primary antibody to the cell monolayer. The slides were then stained using the direct antibody method.

Sporo-Glo (Waterborne, A600-FL), a fluorescein-labeled polyclonal rat IgG antibody made against sporozoites of *C. parvum*, was applied to the cell monolayers. Stock primary antibody was diluted (1:80) in a Dilution/Blocking Buffer (Waterborne, B100) to the desired working volume. 200µL of the diluent was added to each slide and then incubated for 30 minutes at room temperature in the dark to prevent fluorescein bleaching. The slides were then washed 4 times with 0.01M PBS. No Fade mounting medium (Waterborne, M-100) was applied to each slide and the coverslips were mounted with clear nail polish.

Slides were examined under epifluorescence and Normarski differential interference contrast (DIC) microscopy at 200x, 400x and 1000x magnification. The infective oocysts fluoresced an apple green color under fluorescein isothiocyanate (FITC), 480-nm excitation and 550-nm emission, with a dark background of HCT-8 cells. Each well was scanned to determine if infection occurred. In order for a well to be scored as positive, it must have satisfied two criteria. The first criterion was the presence of an "infection focus" caused by sporozoite invasion. Meronts, microgametocytes, or macrogametocytes are possible infection foci that may be found. Confirmation of possible infection foci was made under DIC. Foci will possess sporozoites that are evident under DIC.

The second criterion is clustering, which is a result of secondary infection. A cluster must be comprised of three or more foci (*i.e.* meronts). If invasion and clustering were present in a well, then that well was scored as positive. However, if both invasion and clustering were not present, then the well was scored as negative (Slifko *et al.* 1999).

In order to determine the most probable number (MPN) concentration of infective oocysts in a sample, the Most Probable Number Calculator, Version 1.00 was used. The calculator enables the user to input the number of replicates, dilutions and volumes used to generate a MPN with confidence intervals. The MPN calculator can be found at the EPA's Internet website: http://www.epa.gov/microbes.

In Vitro Excystation

The following procedure was performed by J.E. Thompson (Thompson and Blatchley 2000). Fresh bovine isolates of *Cryptosporidium parvum* oocysts were obtained from Waterborne, Inc. (New Orleans, LA) and stored in a 4°C constant temperature room for up to ten days while conducting disinfection experiments. Oocysts were purchased in numbers of 5 or 10 million per shipment and were sent overnight by Waterborne and immediately placed in the constant temperature room for storage.

Disinfection experiments were performed using batch reactors placed in the Nordian Gammacell Irradiator described in the following section. Oocysts were purified by centrifugation and resuspension of the *Cryptosporidium* pellet in Nanopure water. For each experiment, approximately 250,000 oocysts (each) were placed into 15 mL screw-cap plastic centrifuge tubes containing 5 mL of sterile Nanopure water. One centrifuge tube was used for each dose in each experiment. In addition, an identical control tube was prepared which was not exposed to γ radiation, and was kept on ice throughout the experiment.

After samples were placed in the irradiator, each tube was removed at the appropriate time to achieve the desired dose. Samples were then returned to the lab for viability studies using a slight modification of the *in-vitro* excystation protocol performed successfully by Robertson *et al.* (1993). Samples were centrifuged for 4 minutes at 1000 g in a swinging bucket centrifuge (IEC clinical centrifuge). Supernatant was removed to a volume of 0.1 mL and the pellet was resuspended in 3 mL of Nanopure water. Samples were mixed and recentrifuged as above and supernatant removed to 0.1 mL of water.

One mL of Acidified Hanks Balanced Salt Solution (HBSS - Sigma H2513, acidified by addition of 1% by weight of 1.0 N hydrochloric acid) was added to each of the samples. Each tube was then mixed and incubated for 30 minutes at 37°C. After incubation, each sample was centrifuged (1000 g for 4 minutes) and aspirated to 0.1 mL. Concentrated oocysts were then resuspended in 3 mL of Nanopure water, centrifuged, and aspirated to 0.1 mL to fully remove the acidified HBSS. Each concentrated sample (0.1 mL) was then mixed with 0.2 mL of 1% Bovine Bile in Eagles' Minimal Essential Medium (MEM - Sigma M5775). Additionally, 50 µL of 0.44% sodium bicarbonate solution was added. All solutions were prepared for these experiments within 20 minutes of use. Samples were then mixed and incubated for 45 minutes at 37°C. Once again, samples were centrifuged and supernatant removed as described above.

Samples were then observed using a Nikon Optishot Microscope at 1000X magnification (100X objective lens). Each oocyst that was counted was classified as excysted (EO), partially excysted (PEO), or intact (IO). Excysted oocysts, or ghosts, were observed as relatively flat, with a prominent cell wall. Partially excysted oocysts were observed with sporozoites breaking the outer wall of the oocyst. Intact cysts were highly granular with an intact cell wall.

The control sample was used to determine the fraction of oocysts that would excyst without the application of a disinfectant. The control excystation percentage was calculated using the following equation (subscript c denotes control samples).

$$\% \, Control \, Excystation = \frac{EO_c + PEO_c}{EO_c + PEO_c + IO_c} * 100\% \qquad (1)$$

The remaining excystation data were then analyzed assuming that the control excystation represented (initially) viable organisms for all samples. Thus, the excysting (viable) fraction for a given dose was calculated as follows.

$$Excysting \, Fraction = \frac{EO + PEO}{EO + PEO + IO} * \frac{100\%}{\% Control \, Excystation} \qquad (2)$$

UV Radiation

For these experiments, a bench scale, collimated beam system was used (see Figure 2). The apparatus contained two low-pressure mercury arc lamps. UV intensity was measured using an International Light, Inc., Radiometer/Photometer (Model IL 1400A) with detector attachment (SEL 240). The dose calculations were determined using the following equations:

$$\frac{I_{avg}}{I_o} = \frac{1 - \exp(-\alpha \cdot h)}{\alpha \cdot h} \qquad (3)$$

and

$$D_{avg} = I_{avg} \cdot t \qquad (4)$$

The Radiometer/Photometer measured the intensity at the height of the solution in the quartz dish resulting in I_o. The height of the solution in the quartz dish, h, was also measured. Alpha (α) was determined by the following equation:

$$T = \frac{I}{I_o} = e^{-\alpha l} \qquad (5)$$

where T = transmittance at 253.7 nm
l = path length of the cuvette (1 cm)

Once the intensity average (I_{avg}) was determined by using equation 3, equation 4 was used to determine the time required achieving the desired average dose.

One-mL samples of oocyst stock solution were placed in sterile quartz dishes containing miniature stir bars. The dishes were placed in the freezer overnight to prevent the stock solution from warming during irradiation. For most coccidia, excystation of sporozoites requires exposures to reducing conditions followed by exposure to pancreatic enzymes and/or bile salts. For *C. parvum*, such exposure may enhance excystation, but sporozoites can excyst in warm aqueous solutions alone (Fayer *et al.* 1997).

The dishes were placed on a magnetic stirrer located directly beneath the collimated beam to ensure a completely mixed system. After exposure to the UV radiation, the oocyst

the UV dose of 20 mW-s/cm^2. Therefore, the dose of 20 mW-s/cm^2 results was not used in developing the model fit. In order to determine a more accurate MPN for UV dose 20 mW-s/cm^2, more wells or oocysts should be used.

The data for *C. parvum* inactivation by gamma irradiation in air-saturated and nitrogen-saturated solutions are presented in Figure 5. Air and nitrogen saturation was used to vary the dissolved oxygen concentration in the samples. For the air-saturated solutions, a dose of 150 Gy resulted in 2.3 $\log_{10}$ units of inactivation and 2.6 $\log_{10}$ units of inactivation were obtained at a dose of 200 Gy. At doses of 300 and 400 Gy, the assay was beyond the limit of detection, so >2.9 $\log_{10}$ units of inactivation were achieved for both doses.

For the nitrogen-saturated solution, a dose of 100 Gy resulted in a 2.4 $\log_{10}$ units of inactivation and the doses of 150 Gy and 200 Gy produced a 2.9 $\log_{10}$ units of inactivation. At doses of 300 and 400, the assay was beyond the limit of detection, so Gy > 2.9 $\log_{10}$ units of inactivation were achieved.

Only one study has been published (Thompson and Blatchley 2000), at the time of this report, which measured inactivation of *C. parvum* by gamma irradiation. The method of detection was *in vitro* excystation. The data from this study is presented in Figure 6. A dose of approximately 10,000 Gy for the air-saturated solution resulted in 2 $\log_{10}$ units of inactivation. This dose is 100x greater than the dose of 100 Gy for a N_2 solution using the FDM.

As shown in this study and by Bukhari *et al.* 1999, the *in vitro* excystation assays are inadequate in predicting the infectivity of oocysts. The assays are a good indicator of viability (*i.e.* the ability of the oocyst to excyst) but cannot produce reliable results regarding infectivity (*i.e.* the ability of the oocyst to reproduce) that the mouse and cell culture assays can. The assay also limits the degree of inactivation that can be measured. Thompson (2000) reported that only two $\log_{10}$ units of inactivation could be measured since a limited amount of cysts (ca. 500) were counted for each dose.

The Thompson (2000) study also evaluated the oxygen effect. Because *C. parvum* displayed a non-linear inactivation response, the difference in the response of the organism to irradiation in the nitrogen- and air-saturated solutions varied with the degree of inactivation. In general terms, the dose required to achieve a given inactivation response for *C. parvum* in the nitrogen-purged solutions was 1.5-2 times as large as that required in the air-saturated solution.

According to the complied gamma data presented in Table 2 for this study, the difference between the air-saturated solution and the nitrogen-saturated solution do not appear to be significant.

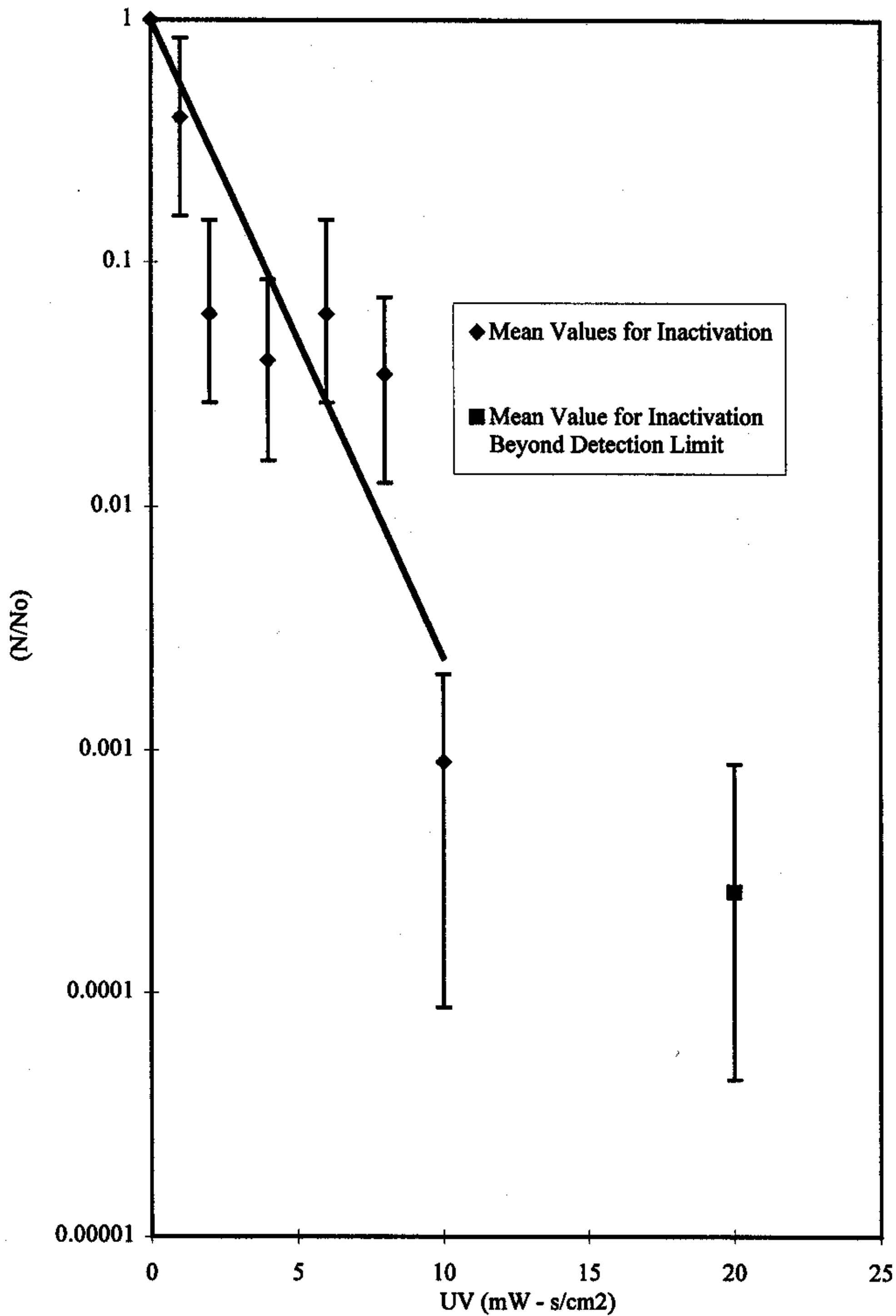

Figure 4. Dose-response behavior of *Cryptosporidium parvum* aqeuous solution based on exposure to UV radiation from a collimated-beam reactor.

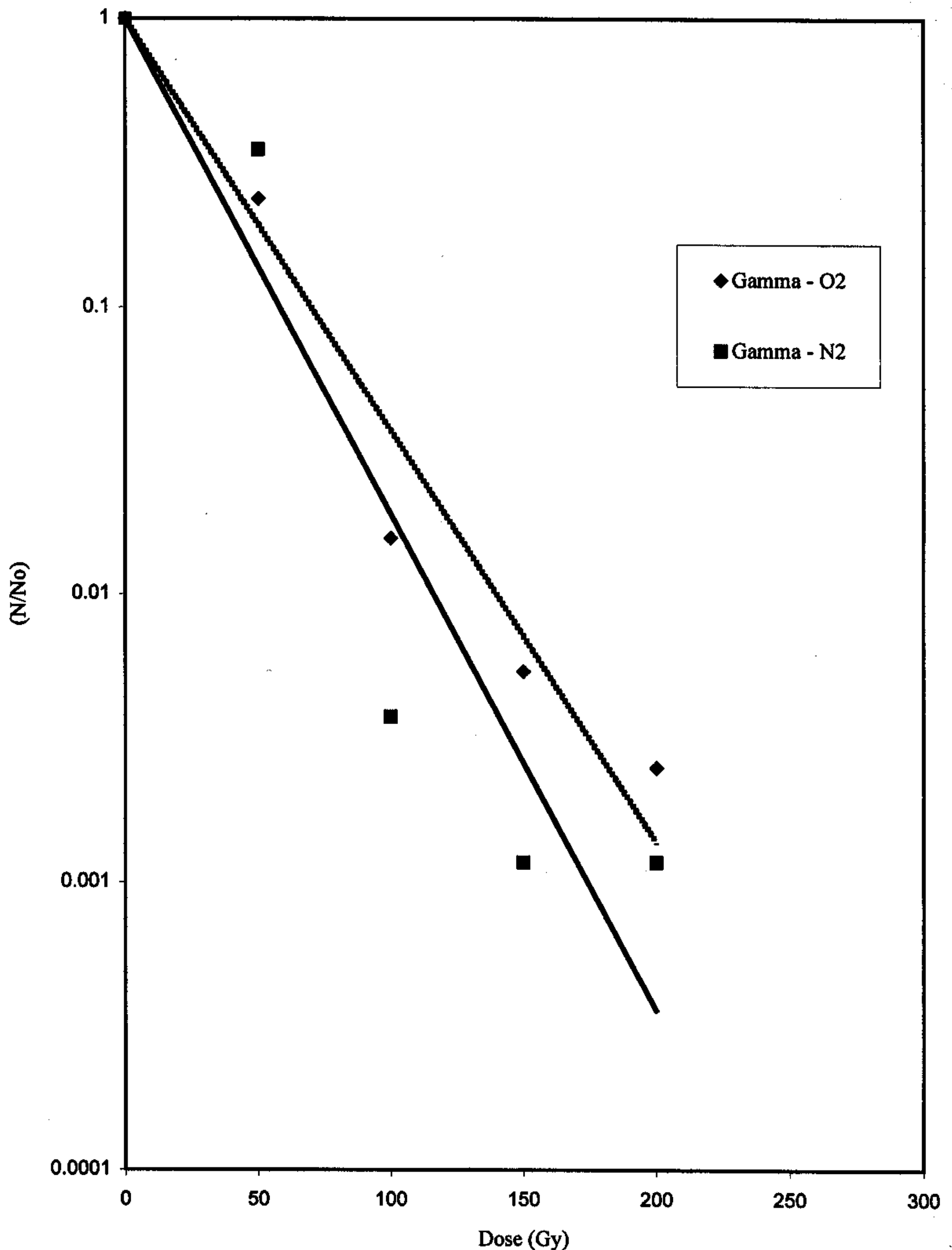

Figure 5. Dose-response behavior of Cryptosporidium parvum in air and nitrogen aqueous solutions based on exposure to g radiation from a ^{60}Co source.

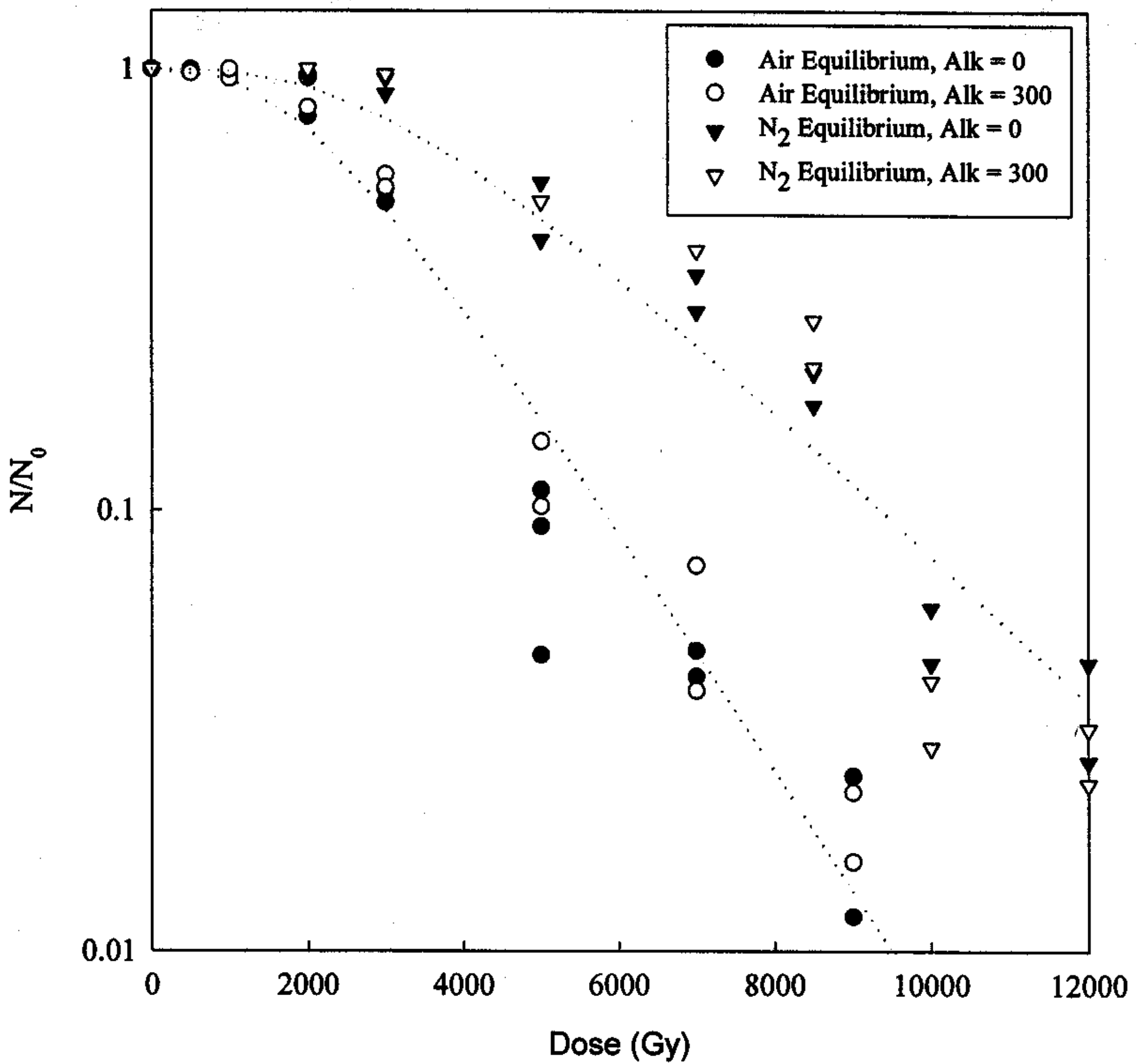

Figure 6. Dose-response behavior of *Cryptosporidium parvum* suspensions subjected to γ irradiation from ^{60}Co source. Dotted lines represent best-fit of multi-target model (n=4) to data.

Table 2. *Cryptosporidium parvum* Inactivation Results for Gamma Irradiation - Air and Nitrogen-saturated Solutions

Dose (Gy)	Air-saturated Solution Log_{10} Extent of Inactivation	Nitrogen-saturated Solution Log_{10} Extent of Inactivation
50	0.6	0.5
100	1.8	2.4
150	2.3	2.9
200	2.6	2.9
300	>2.9	>2.9

Previous research has suggested that microbial inactivation is enhanced in the presence of oxygen by gamma irradiation (Farooq *et al.* 1993, Thompson and Blatchley 2000). However, the results from this study suggest that the oxygen effect does not hold true for *C. parvum* infectivity.

An oxygenated solution will form reactive radicals, including HO• during irradiation. Kuipers and Lafleur (1998) characterized DNA damage induced by gamma irradiation derived water radicals using DNA repair enzymes. The results in the study found that the •OH is much more effective than •H atoms in producing single strand breaks (ssb) in DNA. But, there is evidence of a contribution by the •H atom towards DNA damage in the absence of oxygen (Kuipers and Lafleur 1998; Farooq *et al.* 1993).

In this study, the nitrogen-saturated solution irradiated at a dose of 100 Gy produced 2.42 $\log_{10}$ units of inactivation, but the oxygen-saturated solution irradiated at a dose of 100 Gy only produced 1.81 $\log_{10}$ units of inactivation. Similarly, a dose of 150 Gy for the nitrogen-saturated solution produced 2.93 $\log_{10}$ units of inactivation, but the oxygen-saturated solution produced only 2.26 $\log_{10}$ units of inactivation. Singh and Vadasz (1983) reported similar findings for *E. coli* exposed to gamma radiation. Solutions sparged with N_2 showed a slight enhancement of *E. coli* inactivation at varying doses compared to aerated solutions. Based on their findings, Singh and Vadasz (1983) concluded that hydroxyl radicals, and hydrogen atoms are both involved in the inactivation process. Therefore, since the hydrogen atom appears to play an important role in the inactivation process, an aerated solution might not produce better inactivation results compared to a non-aerated solution.

Cryptosporidium parvum responses to UV and gamma irradiation followed the Chick-Watson disinfection kinetics model with considerable scatter. The scatter could be a result of several factors. One possible cause for variability was the age of the oocyst. The infectivity of oocysts has been shown to decrease as the oocysts age (Slifko *et al.* 1999). The oocyst stock should be analyzed before being used in a disinfection study by using a viability assay such as nucleic acid staining or vital dye staining. Oocyst viability
was not analyzed prior to the disinfection studies presented in this study. The gamma irradiation experiments with nitrogen and air-saturated solutions were conducted at the same time. Therefore, oocyst age is not an explanation for the observed behavior of air-saturated and nitrogen-saturated solutions.

Another possible source of error is the MPN method for microbial populations. The MPN method has low precision. For a more precise estimate, a larger number of wells must be inoculated at each dilution. Confidence intervals at 95% can be narrowed by increasing the number of wells at each dilution or by narrowing the dilution ratio. The MPN program used in this study uses either Loyer and Hamilton limits or Cornish and Fisher limits. The Loyer and

Hamilton limits are more rigorous than the Cornish and Fisher limits, which therefore leads to a narrower confidence level (Klee 1995). The Loyer and Hamilton method involves assuming an MPN and then calculating the probability of all possible results for a given number of wells and dilutions. If the criterion of this method is not met, then a another MPN is selected and new calculations are made. This iteration can be very lengthy for a large number of wells and dilutions. Therefore, the MPN program defaults to the approximate method, Cornish and Fisher, which has wider, more conservative limits (Klee 1995).

The cell culture assay can also be a source of variability in the inactivation results level. The cell culture system must be capable of supporting viable parasite growth. There are several variables in the assay including growth medium and Fetal Bovine Serum (FBS) that could affect *C. parvum* growth. As these solutions age, the nutrient content decreases which can hinder *C. parvum* development. The growth medium's pH is another factor in *C. parvum* development. A pH in the range of 7.0 to 8.0 can result in *C. parvum* sporozoites binding to the host cells more effectively (Upton *et al.* 1995). Another limitation of the FDM assay is the non-specificity of the antibody. The antibody binds to the reproductive stages of *C. parvum*, but it will also bind to cellular debris, a result of the cell culture assay. The non-specific binding makes enumeration subjective.

Conclusion

The results of this study indicate that both UV and gamma irradiation effectively inactivate *C. parvum* at doses similar to those required for inactivation of bacteria and viruses. Previous studies indicated that extremely high doses of UV and gamma radiation were required to inactivate *C. parvum*. These studies used *in vitro* assays that determined viability but not infectivity. Nucleic acids represent one of the major cellular targets for both UV and gamma radiation (Cadet *et al.* 1991). One possible explanation for the observed behavior is that the UV and gamma rays alter the DNA of the sporozoite but the sporozoite is still capable of excysting. Sporozoites exposed to UV radiation have been observed to excyst, initiate invasion of the host cells, but not complete the reproductive cycle even after 48 hours of incubation (Slifko *et al.* 1999). This observation was also found to be true in the UV and gamma irradiation experiments conducted for this study. Whether or not this is a result of DNA damage is unknown. However, these results suggest that viability assays are not an effective method for determining *C. parvum* infectivity when UV and gamma radiation are the disinfectants.

The results of this study and previous studies using the mouse infectivity assay indicate that >3 $\log_{10}$ units of inactivation by UV and gamma irradiation can be achieved for *C. parvum* at doses comparable to the doses required for bacterial and viral inactivation. Therefore, *C. parvum* will probably not limit UV system design.

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