University of Alberta

Infectivity of *Giardia lamblia* cysts in Municipal Wastewater after Ultraviolet Treatment

by

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DEDICATION

To my husband Yun, my son Andrew, and my parents

for their love and support

ABSTRACT

Giardia lamblia is a waterborne parasite that is commonly present in treated municipal wastewater; however, the public health risk associated with the cysts released to the environment is not clear. The objectives of this thesis were to investigate the ultraviolet inactivation of *G. lamblia* present in municipal wastewater effluent under field conditions and the factors that might impact parasite inactivation by UV, including differences in UV susceptibility between different strains, the reactivation potential after UV exposure, and the interaction with particulate matter. In laboratory experiments, two human-derived *G. lamblia* isolates (WB and H3) were found to have different susceptibilities to UV inactivation as measured by the Mongolian gerbil model. UV-exposed cysts produced different levels of infection in gerbils and these different levels of infections complicated the assessment of infectivity and inactivation by UV. Trophozoites exposed to low UV doses (< 20 mJ/cm^2) were found to be able to subsequently divide in *in vitro* culture, which suggests that the parasite is not as readily inactivated as previously believed or may be able to repair UV-induced damage. In a field study carried out at four municipal wastewater treatment plants, indigenous *G. lamblia* cysts present in primary effluent produced strong and moderate infections in gerbils; however, only low intensity infections were produced in gerbils inoculated with cysts obtained from secondary effluent both upstream and downstream of UV reactors. This suggests that the cysts survived the secondary treatment may be more resistant to UV irradiation and may reactivate after exposure to UV. There was no evidence that cysts survived due to association between *G. lamblia* cysts and particulates. Overall, the study

suggests that assessment of UV inactivation of *G. lamblia* cysts in wastewater effluents might not be as straightforward as indicated by previous studies. It is difficult to translate low intensity infections in gerbils to the potential for these parasites to cause disease in humans. The residual cysts in the wastewater effluents and the dilution factor in receiving waters suggest that these parasites would cause a relatively low public health risk provided that the hosts are immunocompetent.

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1 INTRODUCTION

1.1 Emerging Challenge of Water and Wastewater Microorganism Inactivation

Cryptosporidium parvum and *Giardia lamblia* (syn. *Giardia intestinalis*, or *Giardia duodenalis*) are intestinal parasites that can infect humans, domestic livestock and wildlife, as well as other animals, including birds. These parasites cause a disease in humans called cryptosporidiosis and giardiasis, respectively, with common symptoms including profuse watery diarrhea, fever and nausea. The infections with both parasites are self-limiting in immunocompetent individuals, while immunocompromised hosts such as those undergoing chemotherapy and HIV infected people can not eliminate the parasites and suffer from long lasting chronic infections. Cryptosporidiosis and giardiasis can be life threatening for people with poorly functioning immune systems, infants and the elderly. Consequently, these parasites represent a significant risk to public health. Fortunately, *Giardia* spp. infections (popularly called "beaver fever") are treatable with drugs such as metronidazole; however, there are no specific drugs for treatment of *Cryptosporidium* spp. infections.

Cryptosporidium spp. and *Giardia* spp. are transmitted by ingesting contaminated feces, drinking water or recreational water. The minimum infectious doses for both *Giardia* spp. and *Cryptosporidium* spp. are low. As little as 10 *Giardia lamblia* cysts (Rendtorff 1954) and 30 *Cryptosporidium parvum* oocysts (DuPont et al., 1995) can cause infection in humans. Both *Giardia* spp. and *Cryptosporidium* spp. can survive for months in the water environment, especially at low water temperatures. Temperature has an inverse relationship with the survival of microorganisms originating in fecal waste, with survival decreasing as temperature increases. Researchers found that the infectivity was lost when the oocysts were frozen, boiled, or heated to 60°C or more for 5 to 10 minutes or longer (Badenoch et al., 1990); or stored for 2 weeks at 15 to 20°C or stored for 5 days at 37°C (Sherwood et al., 1982). Oocysts of *Cryptosporidium parvum* have been observed to survive for up to 6 months in river water at ambient temperatures (Medema et al., 1997). *Giardia lamblia* cysts can survive more than 2 months at 4°C

(Adam 1991). Generally, *Giardia* spp. appears more sensitive to environmental condition than *Cryptosporidium* spp. (Sattar et al., 1999).

C. parvum and *G. lamblia* have been responsible for many outbreaks of waterborne disease worldwide from 1984 to 1996 (Rose et al., 2002). In Canada, cryptosporidiosis outbreaks have occurred in Collingwood, Ontario, Cranbrook and Kelowna, British Columbia, and more recently in North Battleford, Saskatchewan (Mitchell, 2002). Waterborne *giardiasis* outbreaks had been reported in Edmonton, Alberta in 1983 and Temagami, Ontario in 1994. Some outbreaks are traced to the contamination of drinking water intakes by upstream wastewater discharge. For example, the waterborne cryptosporidiosis outbreak at North Battleford, Saskatchewan in 2001, discharged wastewater located only 3.5 km upstream of drinking water intake was suspected as a source of the contamination and insufficient disinfection resulted in the outbreak (Hrudey and Hrudey, 2004).

Cryptosporidium spp. and *Giardia* spp. are common in the North Saskatchewan River basin because much of the North Saskatchewan River basin upstream of Edmonton is used for livestock production, and cattle are suggested as the main contributor to the high levels of these parasites in the river (Shepel 2000). A study conducted by a research team in Alberta found *Cryptosporidium* spp. and *Giardia* spp. were in feces from livestock and wildlife as well as in raw municipal sewage. Parasites were also present in streams draining non-agricultural and agricultural watersheds, in treated municipal sewage effluents, and in the raw source for drinking water (Mitchell 2002). The concentrations of *Cryptosporidium* spp. and *Giardia* spp. in raw water for Edmonton drinking water were between 0.7 to 90 oocysts/100 L and 15 to 2500 cysts/100 L with average of 13 oocysts/100 L and 420 cysts/100 L, respectively (EPCOR 2002).

Correctly operated water and wastewater treatment plants usually remove protozoan (oo)cysts with high efficiency. However, a sampling program found that 3.8% to 33.3% of 158 drinking water samples were positive for oocysts at concentration in the range from 0.001 to 0.048 oocysts/L (Fayer, 1997). Several studies have also found these parasites in treated sewage effluent and reclamation facilities (Armon et al., 2002; Bonadonna et al., 2002; Gennaccaro et al., 2003; Lowery et al., 2001; Quintero-Betancourt et al., 2003; Rose et al., 1996; Rose et al., 2001; Tsuchihashi et al., 2003).

Using Polymerase Chain Reaction (PCR) to amplify the β-giardin gene, both assemblage A or B type cysts of human infective *G. lamblia* were detected in untreated municipal wastewater (Robertson et al., 2006) and treated wastewater (Caccio et al., 2003). This stresses the potential risk associated with the reuse of municipal wastewater and the discharge of such water into surface waters. Thus, reclaimed water may not be pathogen-free and may contain infectious parasites (Gennaccaro et al., 2003).

C. parvum and *G. lamblia* remain a potential risk for drinking water consumers and recreational water consumers, not only due to the stormwater runoff, but also the wastewater discharge. Water quality laboratories face a major difficulty: detection of *Cryptosporidium* spp. and *Giardia* spp. cannot distinguish between live and dead (oo)cysts using the U.S. Environment Protection Agency Method 1623 only (US. EPA, 2001). At present, it must be assumed that all protozoan parasites detected in the raw water supply are potentially infective. Recent research combined the method 1623 with Foci detection method to determine the infectivity of *C. parvum* (Quintero-Betancourt et al., 2003). In reality, not all the parasites detected from wastewater or raw source water have the ability to infect humans since some species of *Cryptosporidium* spp. or *Giardia* spp. are not human-derived species (Bertrand et al., 2007). Recent genotyping studies have shown that human infective assemblages A and B were detected in urban wastewater with a predominance of assemblage A (Bertrand and Schwartzbrod, 2007; Robertson et al., 2006; Sulaiman et al., 2004). However, some of the *Cryptosporidium* spp. found in sewage may be non-infectious to humans (Hashimoto et al., 2006).

1.2 Ultraviolet as an Alternative Wastewater Microorganism Inactivation Process

UV radiation is commonly used as an alternative microorganism reduction technique in wastewater treatment to replace the chlorination. The main objective of UV in wastewater is to reduce the pathogens and their surrogate coliform bacteria below the regulated guidelines. The advantages of UV treatment over chlorination process are that no chemical residues are released to the receiving water body, no transportation or handling of toxic chemical is required, and no disinfection by-products (DBPs) are produced in the UV dose range used in the wastewater treatment. Another benefit of UV

treatment is the reduction of pathogenic parasites in wastewater to be discharged, based on laboratory study results (Clancy et al., 1998). Further, wastewater reclamation facilities often employ UV radiation as a microorganism reduction technique to protect the public from waterborne diseases. Initially, it was believed that UV radiation was not effective for the inactivation of *Giardia* spp. and *Cryptosporidium* spp. based on *in vitro* excystation assays and vital dye assays that were used to estimate the viability of UV exposed (oo)cysts. Recent research using animal infectivity assays has shown that UV radiation inactivates *G. lamblia* and *C. parvum* (oo)cysts effectively (Campbell et al., 2002; Clancy et al., 1998; Craik et al., 2000; Linden et al., 2002; Morita et al., 2002; Shin et al., 2005).

The actual performance of field UV reactors in wastewater treatment is affected by several factors including hydraulic conditions, the presence of UV absorbing chemical constituents in the wastewater matrix, the particulates in wastewater, and the characteristics of the microorganisms. This may cause a deviation of the log inactivation credits in full scale UV reactors from those in controlled collimated beam UV exposure experiments. It was found that some parasites survived after relatively high UV doses with significant inactivation (i.e. > 2 log) (Craik et al., 2001; Otaki et al., 2003). This situation may occur in full-scale UV reactors in wastewater treatment. In full scale UV reactors, this uncertainty of UV performance may also be caused by parasite strain variations, the potential of DNA repair, the particle interaction with parasites, and other unknown factors. Thus, the potential health risk for discharging UV treated wastewater posed by these phenomena is uncertain.

1.3 Thesis Scopes, Objectives and Overview

G. lamblia cysts tend to be present consistently and in higher concentrations in treated municipal wastewaters than *C. parvum oocysts* (Bukhari et al., 1997; Di Benedetto et al., 2005; Larin and Kashkarova, 2002). Therefore, in my research, I chose to focus on the assessment of potential infectivity of *G. lamblia* cysts present in UV-treated wastewater effluent.

The objectives of my thesis were: (1) to establish operational methods for purification

and quantification of *Giardia* spp. cysts in wastewater effluents and to carry out infectivity analyses of isolated parasites; (2) to investigate the inactivation of indigenous *Giardia* spp. cysts by field UV reactors and compare the inactivation results to those obtained in controlled laboratory experiments; (3) to investigate the susceptibility of different isolates of *G. lamblia* to inactivation by UV radiation; (4) to determine the potential reactivation/re-growth of *Giardia* cysts after UV exposure; and (5) to investigate the effect of particulate matter present in treated wastewater on inactivation of *Giardia* cysts spp. by UV radiation.

This thesis consists of six chapters. Chapter 1 is a review the literature on the pathogenic parasites *Giardia* spp. / *Cryptosporidium* spp. in wastewater, the detection method and viability assessments of protozoan pathogens in wastewater, and the potential for inactivation of protozoa by UV. Since both parasites are of public health significance and the methods of analysis are similar, the literature review included both parasites even though the experimental work concentrated on *G. lamblia* cysts. Chapter 2 was the study of the effect of different isolates of *G. lamblia* cysts on the inactivation by UV radiation. Chapter 3 examined the potential reactivation of *G. lamblia* trophozoites after UV exposure. Chapter 4 is a description of a two-year field study of the prevalence of *Giardia* spp. in wastewater and its inactivation by full-scale UV reactors at four Western Canadian wastewater treatment plants. Chapter 5 investigated the potential for association of *G. lamblia* cysts with particulate matter in wastewater effluent and the effect of this association on the inactivation by UV radiation using indigenous spores in wastewater as a surrogate. Chapter 6 is a general discussion, interpretation and synthesis of the results of the previous chapters and it focuses on the assessment of the public health risk due to *G. lamblia* cysts present in municipal wastewater effluents.

1.4 Literature Review

1.4.1. Biology of Giardia spp. and Cryptosporidium spp.

1.4.1.1 Giardia lamblia

Giardia lamblia (also known as *Giardia duodenalis or Giardia intestinalis*, the name of *Giardia lamblia* will be used in the rest of the thesis) has a simple and direct life cycle

and has two morphological stages: the cyst - the environmentally resistant stage, and the flagellated trophozoite - the intestinal dwelling stage, which reproduce asexually within a host. The cysts are excreted in feces from infected hosts and may survive for an extended period of time in a harsh environment. The cysts are elliptical in shape and are approximately 6 to 10 μm long (Feely et al., 1990). After the cysts are excysted, two trophozoites are released into the intestine of the new host, and then complete binary division resulting in mature trophozoites and establish the new infection. The trophozoite measures approximately 12 to 15 μm in length and 5 to 9 μm wide and resembles a pear shape with the flattened side representing the ventral disc (Feely et al., 1990). As the trophozoites pass through the intestine, they encyst and are released in the feces as cysts.

G. lamblia trophozoites were successfully grown in anoxic conditions in Diamond's TYI-S-33 medium containing the essential ingredient of L-cysteine and ascorbic acid (Radulescu and Meyer, 1990). The initials in TYI-S-33 stand for trypticase, yeast, iron and serum. Belosevic et al. (1982) reported successful establishment of *G. lamblia* cultures initiated using trophozoites from small intestine of naturally infected hosts using filtered TYI-S-33 medium. The initiation of trophozoite cultures has been done using an initial source of parasites either Mongolian gerbils or suckling mice infected with *G. lamblia* (Faubert et al., 1983; Wallis and Wallis, 1986). Cultured *G. lamblia* trophozoites are infectious to animals (Mongolian gerbils or suckling mice) and therefore *in vitro* cultivation can be used for the generation of large numbers of trophozoites and/or cysts.

Using molecular tools including polymerase chain reaction (PCR) technology, *Giardia* spp. are genotyped into six species: *G. agilis, G. muris, G. lamblia, G. ardeae, G. psittaci* and *G. microti* (Adam, 1991). Morphologically identical isolates of *G. lamblia* have been shown to be phenotypically and genetically heterogeneous, the majority of *G. lamblia* isolates can be separated into six subgenotypes (Table 1-1) with two distinct human infectious genetic assemblages A and assemblage B (Thompson et al., 2000; Caccio et al., 2005). Isolates belonging to these assemblages are found throughout the world, although Assemblage B isolates appear to be less widespread. There is growing evidence that differences exist between species and genotypes of *Giardia* spp. with respect to parasite development, growth rates, drug sensitivity and other phenotypic characteristics, as well as disease presentation (Caccio et al., 2005). The biological

differences between these two assemblages caused assemblage B (GS isolate) to be significantly more pathogenic in infections of human volunteers than assemblage A (WB isolate) (Nash et al., 1987). It is not known whether these biological differences will also result in differences on the UV response and inactivation by UV irradiation.

Table 1-1. Species of *Giardia lamblia*

Giardia lamblia species	Major host
Assemblage A	Human, livestock
Assemblage B	Humans
Assemblage C	Dogs
Assemblage E	Cattle, other hoofed livestock
Assemblage F	Cats
Assemblage G	Rats

1.4.1.2 Cryptosporidium spp.

Cryptosporidium spp. is a coccidian protozoan. *C. parvum* and *C. hominis* are the two major species responsible for clinical illness in humans (Fayer, 2004). However, cross transmission of *Cryptosporidium* spp. has been demonstrated between many mammalian isolates. For example, human isolates have been found to be infectious in cats, dogs, cattle and pigs; and vice versa (Fayer and Ungar, 1986). *Cryptosporidium* spp. completes its life cycle in the gastrointestinal tract and because they are obligate parasites, they can replicate only within the host. *Cryptosporidium* spp. oocysts are spherical in shape and between 3 and 5 μm in diameter (Fayer, 1997). The genus *Cryptosporidium* now comprised 14 species including *C. parvum* and *C. hominis* the two major species infecting humans (Adam, 1991). However, humans are also susceptible to other *Cryptosporidium* species, such as *C. felis, C. meleagridis, C. canis, C. suis,* and *C. muris,* especially if they are immunocompromised (Caccio et al., 2005).

1.4.2 Municipal Wastewater Treatment Process

1.4.2.1 Municipal wastewater treatment

A typical municipal wastewater treatment plant consists of (1) preliminary treatment

process to remove wastewater constituents such as rags, grit and grease that may cause maintenance or operational problems with the treatment operations, processes and ancillary systems, normally includes screening and grit removal; (2) primary treatment process to remove a portion of settable suspended solids and partial insoluble organics from the wastewater, includes primary clarifiers; (3) secondary treatment process specially to remove the biodegradable organic matters and suspended solids, normally achieved by biological methods such as activated sludge and incorporating secondary clarifiers. Nutrient removal (N and P) are often included in this section; (4) tertiary treatment or advanced treatment process - some wastewater treatment plants have to remove the nutrients and/or to further reduce the TSS by filtration, either sand filter or membrane filtration; and (5) treatment for microorganism reduction to reduce pathogenic microorganisms into the receiving water. The common wastewater effluent standard for secondary treatment is 25 mg/L total suspended solid (TSS) and 20 mg/L biochemical organic demand (BOD_5), and 200 cfu/100 mL fecal coliform for municipalities with populations than more 20,000 (AE, 2006). However, there are currently no requirements for monitoring parasites (i.e. *C. parvum* and *G. lamblia*) in municipal wastewater or meeting any regulatory limits. *C. parvum* and *G. lamblia* have been suggested as indicator microorganisms for monitoring wastewater reuse to improve current indicator organism and requirements (WERF, 2003).

1.4.2.2 UV reactors design

Ultraviolet disinfection guidelines for drinking water and wastewater reclamation, published by National Water Research Institute and American Water Works Research Foundation (2000) provide details regarding the determination of UV dose required, based on bioassay testing for adequate inactivation of the target microorganism(s). The recommended design UV doses are between 50 mJ/cm^2 and 100 mJ/cm^2 to reflect the dose requirements for different virus inactivation in reclaimed wastewater with a minimum design transmittance from 55% to 90% for the filtration process selected including granular filtration, membrane filtration and reverse osmosis. However, for wastewater discharge to the receiving water body, there are no specific guidelines for the

UV reactor design. The UV doses are normally designed to satisfy the permit limits of either fecal coliforms or total coliforms. The most widely used method to determine the required UV dose is bioassay.

The required UV dose is determined by the involvement of collimated beam UV device and a small reactor with known UV doses applied. A typical UV collimated beam apparatus is shown in Figure 1-1. A low pressure low intensity UV lamp in the collimated beam apparatus allows for accurate characterization of the applied UV intensity. The applied UV dose can be controlled by varying either the UV intensity or the exposure time. The UV irradiance at the surface will be measured by the radiometer and depth averaged UV intensity within the Petri dish sample can be calculated by the correction of reflection factor, divergence factor and water absorbance. The standardized inactivation curves are generated for target microorganisms such as fecal coliform, spores, MS2, and virus at a broad range of UV doses. The design UV dose is to be determined by the target microorganism and its required log reduction with a safety factor. The benefit of using collimated beam UV apparatus is the ideal measurement and control of parameters in the laboratory experiment, including the UV absorbance, mixing condition, and UV irradiation, etc. Bolton and Linden (2003) published a standardized protocol that includes specifications for the construction of a bench scale UV testing apparatus, methods for determination of the average irradiance in the water, details on UV radiometry, and considerations for microbiological testing. The use of this protocol helps in standardization of bench scale UV testing and provides increased confidence in data generated during such testing.

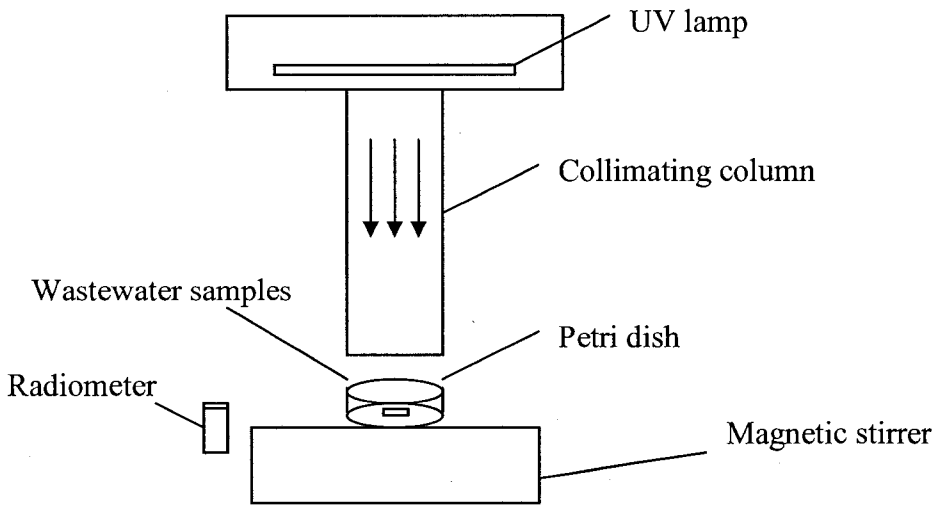

Figure 1-1. Schematic of collimated beam apparatus used to develop dose-response curves for UV disinfection

The effectiveness of UV disinfection is based on the UV dose to which the microorganisms are exposed. The UV dose F is defined as follows:

$$F = I \times t \tag{1-1}$$

Where F = UV dose, mJ/cm2

I = UV intensity, mW/cm^2

t = exposure time, sec

A validation test is required after the UV reactor design, which consists of quantifying the inactivation of a surrogate microorganism as a function of flow rate through the UV reactor. From the standardized curve, the reduction equivalent dose is to be determined based on the log reduction obtained from the validation test. In full scale UV reactor, the transmittance of water is monitored continuously and the UV dose is measured by a UV sensor mounted inside of the UV reactor. The actual inactivation performance of UV reactor is affected by the hydraulic condition, lamp aging and fouling, and wastewater quality.

1.4.3 Giardia/Cryptosporidium in Wastewater

Giardia spp. and *Cryptosporidium* spp. are commonly found in water and wastewater. They are also found in municipal wastewater effluents because they are not completely removed by physical-chemical and biological treatment processes. *Giardia spp.* cysts have been detected in municipal wastewater worldwide (Bertrand et al., 2004; Caccio et al., 2003; Menge et al., 2001; Rimhanen-Finne et al., 2001; Robertson et al., 2000; Wallis et al., 1996). *Giardia* spp. cysts have been detected more frequently than *Cryptosporidium* spp. oocysts in wastewater and are present at higher concentrations than *Cryptosporidium* spp. oocysts (Bukhari et al., 1997; Caccio et al., 2003; Di Benedetto et al., 2005; Larin and Kashkarova, 2002; Robertson et al., 2006). *Giardia* spp. cysts were found in 73% of raw sewage samples collected from 72 municipalities across Canada and 19 of 113 (17%) sewage samples resulted in positive *Giardia* infections on gerbils (Wallis et al., 1996). At the same time, *Cryptosporidium* spp. oocysts were found in 6.1% of raw sewage samples. Caccio et al. (2003) conducted an investigation of four wastewater treatment plants in Italy by sampling wastewater at each stage of the treatment process over one year. While *Cryptosporidium* spp. oocysts were rarely observed, *Giardia* spp. cysts were detected in all samples throughout the year, with peaks observed in autumn and winter. Menge et al. (2001) reported that *Giardia* spp. cysts were detected in all raw wastewater samples with immunofluorescence assay (IFA). Farias et al. (2002) investigated the presence of *Cryptosporidium* spp. oocysts in raw sewage and creek water in the City of Sao Paulo, Brazil and showed the occurrence of *Cryptosporidium* spp. oocysts in all wastewater samples analyzed.

Wastewater treatment processes remove most of *Giardia* spp. cysts and *Cryptosporidium* spp. cysts from the raw, untreated wastewater. The overall average removal efficiency in the treatment plants ranged from 69 to 98.4% for *Giardia* spp. cysts and 15 to 91% for *Cryptosporidium* spp. oocysts (Caccio et al., 2003; Robertson et al., 2000). It was observed that *Giardia* spp. cyst removal was significantly greater than *Cryptosporidium* spp. oocyst removal (Robertson et al., 2000), which may be caused by the higher settling velocity of *Giardia* spp. cysts than *Cryptosporidium* spp. Oocysts (Medema et al., 1998). Chauret et al. (1999) tested the extent of reduction in selected microorganisms during both aerobic wastewater treatment and anaerobic digestion of

sludge at the wastewater treatment plant in Ottawa and compared the removal of these two pathogenic protozoa with that of microbial indicators. All of the raw sewage samples were positive for *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts. During aerobic wastewater treatment, *Cryptosporidium* spp. and *Giardia* spp. were reduced by 2.96 $\log_{10}$ and 1.40 $\log_{10}$, respectively. During anaerobic sludge digestion, no statistically significant reduction was observed for *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts. These results demonstrated the relative persistence of the protozoa in sewage sludge during wastewater treatment. Another study showed that slow sand filtration was capable of reducing the number of *Giardia* spp. cysts, but not of *Cryptosporidium* spp. oocysts in wastewater (Brandonisio et al., 2000).

The mere presence of (oo)cysts in wastewaters does not necessarily imply a health risk to humans. However, the evidence from a few isolated studies suggests that (oo)cysts present in raw or treated wastewater are indeed infectious. One study determined that the concentrates from 22% of 135 raw wastewater samples caused giardiasis in laboratory gerbils (Wallis et al., 1996). Infectious *G. lamblia* cysts were also detected from primary effluent as measured by gerbil models (Garcia et al., 2002). Other researchers discovered that between 20% and 100% of *Cryptosporidium* spp. oocysts detected in the effluents from six municipal wastewater treatment plants in Scotland were viable by vital dye assay and further the viability was not affected by the treatment processes (Robertson et al., 2000). The vital dye assay measures the viability of the oocysts, not the actual ability of the parasite to infect a host, and therefore may overestimate the infectivity of the parasites (Craik et al., 2001). Araki et al. (2001) reported that the physicochemical conditions of high-rate algal ponds were responsible for a more than 97% reduction in the infectivity of *C. parvum* oocysts in neonatal mice. The use of semi-permeable bags of cellulose showed that pH, ammonia, and/or light seem to be major factors for the inactivation of oocysts in wastewater. The viability of Cryptosporidium spp. oocysts was reduced from 80% to 40% after exposure to 5 mg/L NH_3 concentration for 4 days (Reinoso et al., 2008).

Municipal wastewater discharges have been frequently implicated as the sources of *G. lamblia* cysts and *C. parvum* oocysts in waterborne disease outbreaks related to surface water supplies. For example, Temagami, Ontario experienced an outbreak of

waterborne giardiasis in 1994 and the contamination was traced to the leakage from the sanitary sewage system into the lake, which was the source of water supply for the community (Wallis et al., 1998). Another cryptosporidiosis outbreak occurred in the Town of North Battlefield, Saskatchewan, Canada in 2001. The raw water source for the North Battlefield surface water treatment plant is the North Saskatchewan River about 3.5 km downstream of the City's sewage outfall. Non-treated or poorly treated effluent from the Town's wastewater treatment plant was strongly suspected to be the source of *C. parvum* contamination (Health Canada, 2001), and *C. hominis* was identified as the parasite responsible for the outbreak (Hrudey and Hrudey, 2006). Tsushima et al. (2003) found that *Cryptosporidium* spp. oocysts detected in the river water in Tokachi, Japan were *C. parvum,* consistent with the available epidemiological data for the Tokachi area. At least 10 cryptosporidiosis outbreaks between 1984 and 1998 have been attributed to sewage contamination of drinking water (McCuin and Clancy, 2004). These incidents indicate that protozoan cysts and oocysts present in non-treated wastewater effluents are highly infectious to humans.

There is an interest as well as increased use of wastewater effluents for production of reused or reclaimed water for both non-potable uses, such as residential irrigation, agriculture irrigation or recreation, as well as potable use. However, reclaimed wastewater may not be pathogen free. In one study, *C. parvum* oocysts were detected in 25% of reclaimed water samples (Rose et al., 1996). *Giardia* spp. cyst levels decreased 100 fold after filtration, averaging 0.32/40L in a reclaimed water treatment plant (Rose and Gerba, 1991). Gennaccaro et al. (2003) reported that *Cryptosporidium* spp. oocysts were detected from several water reclamation facilities in the United States with an average of 7 infectious oocysts per 100 L. Another study reported that the samples collected from four reclaimed wastewater facilities were positive for *Cryptosporidium* spp. and *Giardia* spp. in concentrations ranging from 2 to 209 oocysts per 100 L of *Cryptosporidium* spp. and 13 to 118 cysts per 100 L of *Giardia* spp. (Quintero-Betancourt et al., 2003). However, further vital dye assay suggested that less than 5% of oocysts were viable.

1.4.4 Effects of Particles on UV Inactivation of Parasites

Suspended particles in unfiltered water can cause negative effects on the effectiveness of UV radiation for microbial disinfection because they may absorb, scatter, and block UV light (Qualls et al., 1983). Suspended particles block UV light by shading and shielding microorganisms. Particles in wastewater both absorb and scatter UV light, which results in decreasing the overall available UV radiation for disinfection. Previous research has shown that suspended particles in wastewater can increase microbial survival by shielding microorganisms in particles, reducing microorganism reduction (Emerick et al., 1999; Wu et al., 2005). Coliform bacteria can associate with particles to such a degree that they are fully shielded from UV radiation, resulting in a residual coliform bacteria concentration in the treated water (Emerick et al., 2000; Parker and Darby, 1995; Scheible et al., 1987; Severin et al., 1980). UV radiation can only penetrate particles because of high particle porosity, not by the transmission through solid materials (Loge et al., 1999). They pointed out that liquid media with similar bulk absorbance may contain wastewater solids with highly different absorbance. Obviously, the bulk liquid absorbance provides insufficient information regarding the ability of UV radiation to reach and inactivate organisms within particles. However, the parameter absorbance of bulk liquid medium is commonly used in both evaluating feasibility of UV disinfection and in design and operation of full scale UV reactors. The measurement of UV absorbance of bulk liquid is very critical to the determination of UV radiation reaching the microorganisms. Standard direct spectrophotometers measure accurately UV absorption coefficient in bulk liquid without suspended particles. However, for liquid with suspended particles, the standard spectrophotometric method tends to overestimate the absorbance of the bulk liquid for unfiltered samples and to underestimate the absorption of the bulk liquid for filtered samples (Linden and Darby, 1998; Scheible et al., 1986).

The light scattered by particles is out of the path of the detector and results in a high value of measured absorbance even though the light was not absorbed by the sample. Integrating sphere (IS) spectroscopy has been used for measuring the absorbance of scattering suspensions including wastewater and treated water in laboratory studies (Christensen and Linden, 2003; Linden and Darby, 1998; Nelson and Prezelin, 1993). IS

spectroscopy allows the absorbance of liquid with scattering particles to be measured accurately. Of the spectrophotometric absorbance caused by particles, about 75% was true absorbance and about 25% was actually scattering (Qualls et al., 1983). As the concentration of solids increases, the associated UV absorbance increases linearly (Linden and Darby, 1998). The direct method overestimated the UV absorbance of wastewater bulk liquid by about 25% with secondary effluent at average TSS of 14.3 mg/L, by 46% with primary effluent at average total suspended solids (TSS) of 80.9 mg/L (Scheible et al., 1986), and by 86% at TSS of 100 mg/L (Linden and Darby, 1998) by comparing filtered and unfiltered samples. Thus, the use of the direct method with unfiltered samples for predicting UV intensity may result in an overdesign of the UV disinfection system.

Although coliform bacteria have been demonstrated to associate with particles to varying degrees, little information is currently available regarding association of other pathogens with particles and its effect on UV inactivation of these microorganisms. Medema et al. (1998) reported that a significant proportion of *C. parvum* oocysts readily attached to the (biological) particles in a single spiked activated-sludge effluent sample. Approximately 30% of the oocysts attached to particles within the first 3 hours. This number increased to 75% after 24 hours.

The surface charge of parasites may govern the interaction between parasites and wastewater particles. *Cryptosporidium* spp. oocysts showed a pH-dependent surface charge, with zeta potentials becoming less negative as pH was reduced, starting at -35 mV for alkaline pH and reaching 0 at isoelectric points for pH 2.5 (Drozd and Schwartzbrod, 1996). *G. lamblia* showed a zeta potential of -12 mV at pH 7 (Dai and Boll, 2003). Dai and Boll (2003) tested the hypothesis that electrostatic forces govern the interaction between *C. parvum* oocysts and *G. lamblia* cysts and particles. They detected attached pairs of oppositely charged beads and (oo)cysts, while no attachment was observed between like charged beads, (oo)cysts, and soil particles. Another study found that *C. parvum* removal in a sand filter was more effective in the presence of added kaolin particles due to the electrostatic interactions between oppositely charged layers on the absorbing medium (Gitis et al., 2002). Humic materials inhibited the removal process, possibly by neutralizing the positive charge on the kaolin particles (Gitis et al., 2002),

which also contributed to electrostatic interactions between oppositely charged layers on the absorbing medium. The wastewater solids particles also exhibit a negative surface charge with zeta potentials of -35.5 mV for primary sludge solids and -29.6 ± 8.5 mV for activated sludge (Mikkelsen and Keiding, 2002). This may indicate the weak interaction between the negatively charged parasites and the negatively charged wastewater solids particles in wastewater effluent. However, Medema et al. (1998) analyzed the potency of (oo)cysts attached to the particulates in secondary effluent and the sedimentation velocity of *C. parvum* oocysts and *G. lamblia* cysts. When (oo)cysts were mixed with settled secondary effluent, (oo)cysts readily attached to the (biological) particles in effluent; 30% of both cysts and oocysts attached during the first hour of mixing, and this fraction increased to approximately 75% after 24 h. Therefore, since a significant proportion of both cysts and oocysts attached readily to organic biological particles in secondary effluent, sedimentation of attached (oo)cysts after discharge into surface water will probably be a significant factor in the environmental ecology of *C. parvum* and *G. lamblia*.

Recent research by Linden et al. (2002a) indicated that the UV dose-response of microorganisms added to filtered drinking waters is not altered by variation in turbidity of filtered water that met regulatory requirements. For unfiltered raw waters, source water turbidity up to 10 NTU does not impact the UV dose-response of separately added organisms (Passantino and Malley, 2001). In these experiments, however, organisms were added to waters containing various levels of treated or natural turbidity. Therefore, it was not possible to examine microorganisms associated directly with particles in their natural states. So, these studies can only suggest the impact of turbidity on dose-response as it relates to the impact of UV light scattering by particles rather than particle-association or clumping microorganisms in a real water matrix. Most recent studies have advanced the knowledge of the effects of particle-microbe aggregation on the UV inactivation by using coagulation and flocculation techniques to simulate the natural floc particles containing microbes. Uvbiama and Craik (2005) reported that some of the spores in the simulated dual-media filter effluent were aggregated with other spores or particulate matter and were shielded from UV. They spiked *B. subtilis* spores into untreated river water and the water was exposed to simulated conventional surface water

treatment conditions including alum-polymer coagulation, flocculation, sedimentation and sand-anthtracite filtration in a laboratory-scale apparatus. The homogenization step disrupted these aggregates, thus releasing the spores and increasing their exposure to UV. Further, using the same method to simulate the drinking water treatment processes, similar results for *C. parvum* were reported by Mahmud (2006) that if *C. parvum* oocysts are present in poorly filtered drinking water, they are likely to be aggregated to some extent and will exhibit a greater resistance to UV than oocysts seeded directly into clean water. Mamane-Gravetz and Linden (2006a) estimated 30% to 50 % of aggregated spores were protected from UV irradiation by inducing spore-spore aggregates and clay-spore aggregates using alum coagulant. Although the proper absorbance measurement (i.e. Integrating sphere) accounting for particle scattering light provides more precise pictures of UV dose that microbes absorb (Mamane and Linden, 2006b), these studies suggest that particles and particle-microbe association in water matrices will require an increased UV dose compared to a dispersed microbe system. The degree of protection from UV irradiation may be less than predicted based on seeding studies carried out using clean water.

Until now, very few studies reported the relationship between oocysts and particles in real-world wastewater effluent. Tsuchihashi et al. (2003) evaluated the degree of oocysts associated with wastewater particles using a most probable number-polymerase chain reaction (MPN-PCR) technique to detect *C. parvum* oocysts in secondary effluent samples collected from activated-sludge facilities. There were no detected particle-associated oocysts. But the association of spiked *C. parvum* oocysts with particles in secondary effluent drawn from wastewater plants with varying operating conditions indicated a weak correlation between the degree of association and the mean cell residence time of the system. In the PCR method, particles in samples were first separated into various sizes using serial filtration. The solids captured on the filters were eluted and centrifuged. Then, the nucleic acid was extracted and purified. The PCR method destroyed the structure of floc containing particles and oocysts.

1.4.5 UV Inactivation of Protozoan Parasites in Municipal Wastewater

Due to the drawbacks of conventional chlorination, improvements in UV technology

and advances in understanding of the UV process, UV treatment is being used increasingly for wastewater effluent microorganism reduction worldwide. Ultraviolet radiation adsorbed by microorganisms induces changes in DNA or other cellular materials that prevent cell growth or reproduction. It may also induce changes that result in toxic materials forming and being trapped in the cell, causing its death. The main advantages of UV radiation for inactivating microorganisms in wastewater is that there is no residual toxicity and no formation of disinfection by-products (DBPs) at dosages commonly used for treatment. In addition, there is no increase in the total dissolved solids (TDS) level of the treated effluent. This is very important in wastewater reuse.

Since 1998, it was demonstrated that UV could be appropriate for inactivation of *Cryptosporidium* spp. and *Giardia* spp. (Bukhari et al., 1999; Clancy et al., 1998; Craik et al., 2000). Before that time, UV was considered ineffective for inactivation of *Giardia* spp. and *Cryptosporidium* spp. (oo)cysts based on *in vitro* excystation assays (Carlson et al., 1985; Lorenzo-Lorenzo et al., 1993; Ransome et al., 1993; Rice and Hoff, 1981). Reported UV doses required for a 2-log reduction in infectivity of *C. parvum* oocysts were extremely low; around 2 mJ/cm^2 for cell infectivity assessed by cultured HCT-8 or MDCK cells (Linden et al., 2001; Shin et al., 1999). UV doses for 2-log reduction of WB strain *G. lamblia* cysts were 3 mJ/cm^2 (Mofidi et al., 2002). In that study, the infectivity of gerbils was determined by examining the presence of cysts in feces on day 10 and 14 post-infection and/or the presence of trophozoites in the small intestine on Day 14. Another researcher reported UV dose as low as 1 mJ/cm^2 for 4-log inactivation of CH3 strain *Giardia* cysts based in measurement if infectivity in the gerbil model (Linden et al., 2002b). Table 1-2 summarizes the UV disinfection studies on *Giardia lamblia*.

With increasing pressure to discontinue use of chlorine for the microorganism reduction in wastewater, UV radiation for wastewater treatment increased during early 1990s. This trend was aided by the development of new lamps, ballasts and ancillary equipment. In Western Canada alone, nine major wastewater treatment facilities have been commissioned using UV reactors as final effluent disinfection since 1993 and five more prairie cities are planning UV disinfection (Whalley, 2003). A survey in 2003 by Water Environment Federation showed that 24% of all respondents used UV disinfection in their wastewater treatment plants and 66% were planning to switch to UV (WEF, 2004). Even though the

intended goal of UV reactors in wastewater treatment plants is usually the reduction of coliform bacteria to less than the regulated discharge limits or reuse purpose, UV technology is in theory, beneficial for the reduction of other pathogens present in the wastewater effluent, such as *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts.

The effective UV dose received by the microorganisms in wastewater UV reactors is dependent on a number of variables, including the hydraulic condition and mixing in the reactor; the wastewater characteristics such as total suspended solids (TSS), iron and manganese, alkalinity and hardness; and the characteristics of microorganisms. TSS absorbs UV radiation and may shield embedded bacteria. Iron and manganese are strong adsorbers of UV radiation and can precipitate on quartz tubes. The recommended design UV dose for reclaimed water systems is 100 mJ/cm^2 for granular medium filtration effluent, 80 mJ/cm^2 for membrane filtration effluent and 50 mJ/cm^2 for reverse osmosis effluent (NWRI and AWWA, 2000). Currently, there are no guidelines for UV dose in wastewater treatment design for secondary treated wastewater discharge that is not used for water reuse. The UV doses applied in microorganism reduction in wastewater ranged between 20 and 50 mJ/cm^2.

Most of the UV exposure studies involving *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts have been done by exposing laboratory preparations of (oo)cysts suspended in relatively clean waters to control doses of UV radiation using a collimated beam apparatus. A number of factors may affect the scale up of laboratory results to full-scale wastewater treatment systems. Cysts and oocysts have been observed to attach readily to biological floc particles present in secondary municipal wastewater effluents (Medema et al., 1998). This may play a role in determining the effectiveness of UV exposure on these parasites in field UV reactors. Therefore, the efficacy of full-scale continuous flow UV reactors for (oo)cysts under field conditions is not known. Very few studies have measured the effectiveness of UV for parasite infectivity reduction in real-world situations. One recent study reported that after activated sludge process, *Giardia* spp. cysts still remained infectious after UV treatment measured when cyst infectivity was measured using an immuno-suppressed mouse model (Neto et al., 2006). Signs of trophozoites in intestinal scrapings were observed.

Table 1-2. Summary of UV inactivation studies of *Giardia lamblia* cysts

Reference	Strain	Assay used	Gender of animal	Age of gerbils	Experiment type	Lamp type	Water quality	Calculation method	Log reduction
Campbell and Wallis (2002)	WB	Gerbil infectivity	N/A	N/A	Batch	LP	PBS with 0.05% Tween 20	ID_{50}	< 2 at 10 mJ/cm^2 < 3 at 40 mJ/cm^2
Mofidi et al. (2002)	WB	Gerbil infectivity	Female	4 wks	Batch	LP	WTP filtered water	MPN	> 2 at 3 mJ/cm^2
Linden et al. (2002b)	CH3	Gerbil infectivity	Female	8 –10 wks	Batch	LP	De-ionized buffer water containing antibiotics	MPN	> 4 at 1 mJ/cm^2
Shin et al. (2005)	N/A	Gerbil infectivity	as (Linden et al. 2002b)	as (Linden et al. 2002b)	Batch	LP	Buffer solution containing antibiotics	MPN	> 4 at 1 mJ/cm^2
Rice and Hoff (1981)	Human donor	*In vitro* excystation	-	-	Batch	LP	Distilled water	Excystation ratio	< 1 between 1and 60 mJ/cm^2

1.4.6 Reactivation of Parasites after UV Exposure

It is well known that bacteria and other microorganisms are capable of repairing their DNA following damage by UV radiation. Harris et al. (1987) reported that a maximum 3.4 log photoreactivation of UV inactivated *Escherichia coli* was observed. Recent studies suggested that that *E. coli* underwent photorepair up to 5 log under fluorescent light conditions, which is more significant than dark repair up to 0.8 log in terms of bacterial count increase (Hu et al., 2005).

The principal UV inactivation mechanism is the formation of photoproducts in DNA. Pyrimidine dimers formed between adjacent pyrimidine molecules on the same strand of DNA interrupts both the transcription and the replication of the DNA. The formation of dimers can be reversed when the microorganism is subsequently exposed to either dark or light conditions. The two reactivation mechanisms are described as dark repair or photoreactivation. The method of reactivation varies significantly according to the level of biological organization of the microorganism and the kind of UV damage imposed. The photoreactivation capacity of stationary-phase or starved cells remained about the same as that of exponential-phase cells (Kadavy et al., 2000). Vegetative cells were capable of photoreactivation, but photo-induced repair of UV damage was absent in as seen on *B. anthracis* spores (Knudson, 1986). Sporulating cultures of *B. cereus* lose their photoreactivability at the same time that UV resistance increases (Romig and Wyss, 1957). However, *B. subtilis* spores do possess an enzymatic dark repair system which can monomerize the spore photoproducts in situ during germination (Harm, 1980).

Based on UV wavelength range, UV-A (320 to 400 nm) is known to affect cell membranes and membrane functions, while UV-B (260 to 320 nm) and UV-C (200 to 260 nm) have been shown to be absorbed by proteins (Jagger, 1985). It has been suggested that the broader wavelengths (200 to 300 nm) emitted by medium pressure lamps not only damages DNA but also causes damage to other molecules, making it more difficult for cells to repair their DNA (Oguma et al., 2002; Zimmer and Slawson, 2002). This self-repair ability poses problems when UV disinfection is used to treat wastewater for reuse. To overcome the DNA repair, it may be necessary to greatly increase the applied UV doses or to add chemical disinfectants post UV disinfection depending on the reuse of the wastewater.

The ability *Giardia* spp. and *Cryptosporidium* spp. to reactivate following UV exposure is unclear based on the studies that have been done. Most studies found that there was no detectable evidence of reactivation of *C. parvum* after UV exposure. No detectable evidence of repair of *C. parvum* oocysts was observed after incubation under light or dark conditions (up to 5 days) following either LP or MP UV lamp irradiation when infectivity was assessed using an HCT-8 cell culture with an antibody staining procedure (Zimmer et al., 2003). However, it was demonstrated that UV-exposed oocysts can repair pyrimidine dimers in the DNA of *C. parvum* continuously, while no recovery of animal infectivity was observed either by photoreactivation or by dark repair (Morita et al., 2002; Oguma et al., 2001). Belosevic et al. (2001) found that *G. muris* cysts and *C. parvum* oocysts exposed to medium-pressure UV doses of 60 mJ/cm^2 or higher did not exhibit resistance to and/or reactivation following storage in the dark at room temperature for 1 to 4 days (*G. muris*) or 1 to 17 days (*C. parvum*). These studies used a mouse infectivity assay to measure the infectivity of cysts. In contrast, they did observe *in vivo* reactivation of *G. muris* in three of seven independent animal infectivity experiments, when parasites were treated with relatively low doses of medium-pressure UV (<25 mJ/cm^2). Linden et al. (2002b) reported that the infectivity reduction of *G. lamblia* cysts at typical drinking water and wastewater doses of 16 and 40 mJ/cm^2 (LP UV irradiation) remained unchanged after exposure to either light or dark repair conditions. A recent study showed that *G. lamblia* cysts have the ability to repair their UV-damaged DNA after a dose of UV of 1 mJ/cm^2 irradiation when infectivity was measured using both animal assays and an Endonuclease Sensitive Site assay (ESS) (Shin et al., 2005). Hayes (2003) reported that at UV doses of 1.9 and 2.3 mJ/cm^2, 2.93 to 4.4 log reduction of *G. muris* cysts was observed in CF-1 mouse infectivity model. But it should be noted that in Hayes's study, 18.3% of individual animals fed by UV exposed cysts turned positive at week 2 after being negative at week 1 comparing with 7% of control animals. There was no standard experimental protocol for the studies of reactivation of infectivity. These studies used different repair times, temperatures, light conditions, and different assays to determine the reactivation of (oo)cysts. Table 1-3 summarizes the studies regarding to the repair of infectivity of these parasites after UV exposure.

Table 1-3. Comparative reactivation of protozoan parasites after UV exposure

Parasite	UV dose (mJ/cm^2)	Incubation period and temperature	Infectivity Assay	Reactivation	Reference
G. lamblia	16 and 40 (LP)	2 hr at 37°C 4 hr at 25°C	Animal	No evidence	Linden et al., (2002b)
G. lamblia	1 (LP)	2 hr at 25°C	Animal and ESS	Observed	Shin et al., (2005)
G. muris	< 25 (MP)	1 to 4 days at room temperature	Animal assay	Observed	Belosevic et al., (2001)
C. parvum	1 and 3 (LP and MP)	Up to 5days at 5°C and 25°C	Cell culture	No evidence	Zimmer et al., (2003)
C. parvum	2.2 for ESS 6.0 for Animal assay (LP)	2 hr at 20°C for light repair 24 hr at 20°C for dark repair	Animal and ESS	No evidence	Oguma et al., (2001)
C. parvum	> 60 (MP)	1 to 17 days at room temperature	Animal assay	No evidence	Belosevic et al., (2001)
C. parvum	0.9 to 8.0 (LP and MP) 5.0 to 6.3 (LP and MP)	Up to 7 days incubation Up to 10 days	Cell culture Animal assay	No evidence	Rochelle et al., (2004)

1.4.7 Detection Methods for Giardia and Cryptosporidium in Wastewater

1.4.7.1 Separation techniques

Since the concentrations of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in wastewater samples are normally low, a large volume of wastewater samples must be collected and concentrated to accumulate enough (oo)cysts for the measurement of parasite concentrations and infectivity studies.

Density centrifugation. Density flotation technique is based on the separating of microbes from background debris and concentrating them by means of a flotation fluid with an appropriate specific gravity by centrifugation at certain speeds. Zinc-sulphate flotation and sucrose gradient flotation methods are two commonly used methods to separate (oo)cysts from background debris of environmental samples and fecal materials. The Percoll-sucrose density gradient purification was the procedure of the Information Collection Requirements Rule (ICR) protozoan method and provided better recovery rate than other density based method (Chesnot and Schwartzbrod, 2004). Most of the early studies used density centrifugation method to detect parasites from water concentrates

(Gornik and Exner, 1991; Payment et al., 1989; Rose et al., 1987; Sykora et al., 1991). Skyora et al. (1991) used sucrose flotation method and detected *Giardia* spp. cysts from raw sewage samples in 11 wastewater treatment plants studied, but only about one half of the wastewater treatment plant effluents were positive for *Giardia* spp. cysts, with *Giardia* spp. cyst concentrations ranging up to 44 cysts/L. Based on sucrose flotation counts, the concentrations of cysts detected in the sludge ranged from 70 to 30,000 cysts/L. No matter what flotation method was used, several studies demonstrated that this procedure was inefficient for detecting protozoan parasites in water concentrates. Bukhari and Smith (1995) found that sucrose density flotation selectively concentrated viable oocysts. However, this method was much less expensive than immunomagnetic separation and flow cytometry methods, and was suitable for the purification of parasites from fecal materials in laboratory. Sucrose flotation method was used to concentrate and purify *Giardia* cysts from experimental gerbil feces in this research as it was effective to separate parasites at a large amount from fecal materials.

Immunomagnetic separation (IMS). IMS is an alternative to the use of density gradient flotation procedures and is increasingly being applied for the purification of parasites from wastewater matrix. IMS is also a procedure recommended in the U.S. Environmental Protection Agency's (EPA) Method 1623, which is the most widely used methods for the simultaneous detection of *Cryptosporidium* spp. and *Giardia* spp. in water (U.S. EPA, 2001). This method has also been adopted for the detection of (oo)cysts from environmental samples in most recent studies (Craik et al., 2004; LeChevallier et al., 2003; Quintero-Betancourt et al., 2003; Robertson and Gjerde, 2001; Ward et al., 2002). The protocol consists of filtration, IMS, staining with a fluorescent antibody, and microscopic analysis. Typically, 10 to 100 liter samples are filtered and the collected material is eluted with a detergent solution and the parasites and other debris are concentrated by centrifugation. The pellet is resuspended in phosphate buffer solution and mixed with specific monoclonal antibodies attached to magnetic beads. The (oo)cysts are then separated from the debris in a magnetic field. IMS had higher recovery efficiency for both *Giardia* spp. and *Cryptosporidium* spp. than the Percoll-sucrose density gradient purification (Hsu and Huang, 2000). Recovery efficiencies of IMS were 78.6% and 69.3% for cysts and oocysts, respectively, in contrast to the 22.7% for cysts

and 29.9% for oocysts by using Percoll-sucrose density gradient purification. Ware (2003) improved the average oocyst recovery from 41% to 71% in seeded reagent water, and from 10% to 51% in seeded river samples by using a new IMS dissociation procedure involving a 10-min incubation at 80°C. This heat dissociation method also improved the average 4',6-diamidino-2-phenylindole (DAPI) confirmation rate from 49% to 93% in reagent water, and from 48% to 73% in river samples. Myoda et al. (2001) designed a dual band filter set to allow the simultaneous viewing of two fluorochromes fluorescein isothiocyanate (FITC) and DAPI so that *Cryptosporidium* spp. oocysts and sporozoites can be viewed together, This increases accuracy and decreases examination time.

The particles in water samples or the turbidity of water concentrates appear to be the most critical factors associated with the recovery efficiency of the immunomagnetic separation method. A study indicated that the suspended particles present in the water matrices contributed to the enhanced C. parvum oocyst recovery (Feng et al., 2003). The optimal particle size was determined to be in the range from 5 to 40 μm, and the corresponding optimal concentration of suspended particles was 1.42 g for 10 L of tap water (Feng et al., 2003). Rochelle et al. (1999) compared two commercial immunomagnetic separation kits for recovery of oocysts from environmental samples. Oocyst recovery efficiencies with the GC-Combo IMS kit (Dynal, Norway) and Crypto-Scan kits (Immucell Corporation, Portland, Maine) ranged from 62 to 100% and 34 to 74%, respectively, for seeded environmental water concentrates (turbidity of 210 to 11,480 NTU). Recovery efficiencies were also dependent on the mechanism of agitation during the magnetic capture procedure. McCuin et al. (2001) reported that mean recoveries of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in de-ionized water were 62% and 69% using Dynal's GC-Combo IMS kit, respectively. In turbid surface water matrices (turbidity ranged from 50 to 5000 NTU), mean recoveries of oocysts were between 55.9% and 83.1% while mean recoveries of cysts were between 61.1% and 89.6%. Campbell and Smith (1997) reported recoveries in excess of 90% when oocysts were seeded into relatively clean suspensions. McCuin et al. (2002) tested addition of detergent Tween 80 in the concentration step followed by IMS. The main purpose was to aid the dissolution of fecal fats, oils and grease that may present in wastewater to be exposed for facilitated IMS bead capture. Oocysts recoveries were generally below 20%,

which suggested that the oocysts were still embedded in the particles present in wastewater. McCuin and Clancy (2005) reported improved parasite recovery from raw wastewater and primary effluent by adding 0.75 g Kaolin clay into the concentrated samples before IMS method. High levels of iron may inhibit immunomagnetic separation and the recovery of (oo)cysts decreased sharply at concentrations of 4 mg/L for *Cryptosporidium* spp. and 40 mg/L for *Giardia* spp. (Yakub and Stadterman-Knauer, 2000). All these recovery experiments reported in the literature were conducted by spiking a certain number of (oo)cysts into different water matrices. The recovery of these seeded (oo)cysts may not necessarily represent the ability of the separation methods to recover indigenous (oo)cysts in wastewater, especially because the parasites are suspected to be hindered by the particulate bacterial floc present in wastewater matrices.

Detection of *parasites* in concentrated water samples depends on confirmation using immunofluorescence staining (IFA) and fluorescence microscopy. These methods require highly trained personnel for parasite identification and confirmation. The presence of autofluorescent particles such as algae, and particles binding nonspecifically with the monoclonal antibodies in water samples may mimic (oo)cysts and lead to incorrect results. Microscopic confirmation of cysts or oocysts by a skilled microscopist is still the accepted standard method and the method is approved by regulatory agencies such as the US EPA.

1.4.7.2 Detection methods

Immunofluorescence assay. The (oo)cysts are specifically stained with monoclonal antibodies which have been either labeled directly with FITC or indirectly labeled during staining with an FITC-labeled anti-mouse antibody. Several anti-*Cryptosporidium* spp. and anti-*Giardia* spp. antibodies are commercially available. The challenge is that they apparently cross-react with other species of the genera and therefore do not specifically identify *C. parvum* and *G. lamblia*. A further problem of identifying these parasites in environmental samples is that environmental pressure can alter the size and shape of both recently voided and aged organisms, making their appearance atypical. The monoclonal antibodies (mAbs) used with the magnetic beads in commercial IMS kits might have an

impact on the recovery of the number and species of parasites. The magnetic beads labeled with monoclonal antibodies are generally not species-specific. The author successfully used the Dynal G/C combo kit with anti-*Giardia* mAbs to separate *G. muris* cysts from the feces of mice. The mice were used for routine maintenance of *G. muris* in the laboratory. *G. muris* cysts were recovered by the IMS method, which demonstrates that the commercial IMS kits are indeed, not species specific (unpublished data).

Currently, immunofluorescence microscopy method is the only method accepted by the regulatory agencies in the UK and U.S. to identify the presence of *Cryptosporidium* spp. and *Giardia* spp. in water samples. Unfortunately, there are some algae that are very close in size and staining characteristics to those of (oo)cysts, and final identification often requires light, phase and differential interference microscopy in addition to immuno-fluorescence. Detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts by immunofluorescence requires microscopy specialized equipment and a high level of technical skill. Clancy et al. (1994) conducted a blind survey of 16 commercial laboratories in the United States and found that recovery ranged from 0.8 to 22.3% (average 9.3%) for *Giardia* spp. cysts and 1.5 to 5.5% (average 2.8%) for *Cryptosporidium* spp. oocysts. A similar study was commissioned by Health Canada to investigate eight laboratories including commercial, government and research levels in Canada. The recovery of *Giardia* spp. cysts ranged from 0 to 90% (average 21%) and of *Cryptosporidium* spp. oocysts recovery ranged from 0 to 43% (average 5.3%) for 10 unknown samples.

Polymerase chain reaction (PCR). The PCR is a biochemistry and molecular biology technique for exponentially amplifying a fragment of DNA via enzymatic replication, without using a living organism. Because the amount of product produced by PCR roughly correlates to the amount of starting DNA material, PCR can be used to estimate the amount of a given sequence that is present in a sample, and to determine the gene expression level. More than 20 PCR protocols for *Cryptosporidium* spp. and *Giardia* spp. detection in feces and water have been published with reported or calculated sensitivities ranging from 1 to 10 oocysts to approximately 5×10^7 oocysts (Bertrand and Schwartzbrod, 2007; Rochelle et al., 1997a; Straub et al., 2002). Most of the published PCR assays include primers amplifying a sequence of the 18rRNA gene (Lowery et al.,

2000; Sturbaum et al., 2002; Ward et al., 2002) or mRNA coding for heat stock proteins (i.e. reverse transcriptase PCR assay or real time PCR assay) (Caccio 2004; Griffin et al., 1999; Kaucner and Stinear, 1998; Rochelle et al., 1997b; Stinear et al., 1996).

The sensitivity of PCR detection method is not high. Rimhanen-Finne et al. (2001) developed a method based on immunomagnetic capture and polymerase chain reaction (IC-PCR assay) for detection of *C. parvum* and *G. lamblia* in sewage sludge and found that IC-PCR offers the possibility to distinguish between *Cryptosporidium* and *Giardia* genotypes. The detection limit of the IC-PCR assay for both organisms was 625 (oo)cysts per mL. By hybridization of PCR products the sensitivity could be increased to 125 oocysts and cysts per mL. The samples positive for *Giardia* (9 out of 44) were from eight wastewater plants and the *C. parvum* genotype 2 samples (3 out of 44) originated from different sewage works. Xiao et al. (2001) used a small-subunit rRNA-based PCR-restriction fragment length polymorphism (RFLP) technique to detect and characterize *Cryptosporidium* spp. oocysts. *Cryptosporidium* spp. oocysts were detected in 25 out of 55 surface water samples in the United States and 12 out of 49 raw wastewater samples collected from Milwaukee, Wisconsin. Mayer and Palmer (1996) evaluated the presence and reduction of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in sewage effluent by a combination of indirect fluorescent antibody (IFA) staining and PCR. The results indicated a 3-log reduction of *Giardia* spp. cysts and a 2-log reduction of *Cryptosporidium* spp. oocysts through the sewage treatment process as determined by IFA.

A nested PCR method was developed to detect *Cryptosporidium* spp. oocysts and a double PCR to detect *Giardia* spp. cysts. A 100% correlation was noted between IFA and PCR detection of *Giardia* spp. cysts while correlation for *Cryptosporidium* spp. oocysts was slightly less. Rochelle et al. (1997a) assessed the sensitivity of eight pairs of published PCR primers for the specific detection of *C. parvum* and G. *lamblia* in water. Maximum sensitivity was achieved with two successive rounds of amplification and hybridization, with oligonucleotide probes detected by chemiluminescence. Detection sensitivities ranged from 1 to 10 (oo)cysts for purified preparations and 5 to 50 (oo)cysts for seeded environmental water samples (<1.2 NTU). Multiplex PCR for the simultaneous detection of *Cryptosporidium* spp. and *Giardia* spp. was demonstrated with

primers amplifying 256- and 163-bp products from the 18S rRNA gene of *Cryptosporidium* and the heat shock protein gene of *Giardia*, respectively. A nested PCR was found to be the most sensitive for oocysts detection in backwash water (Gibbons et al., 1998). Bertrand and Schwartzbrod (2007) used a PCR assay targeting the triosephosphate isomerase (tpi) gene to compare the presence of various assemblages of *G. lamblia* in wastewater samples and detected assemblages A and B in municipal wastewater with a predominance of assemblage A.

Currently, detection of (oo)cysts is largely based on immunofluorescence assays (IFA) targeting cyst wall surface antigens. These assays lack specificity and will detect species other than *G. lamblia* and *C. parvum*. Moreover, IFA will detect nonviable (oo)cysts and (oo)cyst wall fragments that do not pose a public health risk., The detection of parasites from wastewater samples and the study of interaction between particles and parasites face the following challenges. First, the concentrations of parasites in water are relatively low, so a large amount of water has to be concentrated to accumulate sufficient materials, either by centrifugation or filtration onto a filter. The second challenge is that the background fluorescence created by organic and inorganic particles in wastewater makes it very difficult to distinguish a positive signal from auto-fluorescent particles. This may be reduced by choosing proper fluorescent labels and labeling technique. The third challenge is the physical differentiation between particle-associated and free-living microbes. Sonication has been used to detach microbes, such as viruses from wastewater sludge (Hejkal et al., 1981; Wellings et al., 1976); *Giardia* spp. and *Cryptosporidium* spp. from fruits and vegetables (Bier 1991; Robertson and Gjerde 2000); coliform bacteria from wastewater effluent (Parker and Darby 1995). The sonication times employed ranged from seconds to minutes. However, sonication can be used as a disinfection method to kill bacteria and other microorganisms. More than 90% of the dispersed *Cryptosporidium* spp. oocysts could be deactivated in about 1.5 min of continuous sonication at 20 kHz ultrasound (Ashokkumar et al., 2003). More than 99% of *E. coli* cells were inactivated within 180 seconds of sonication at 27.5 kHz ultrasound (Furuta et al., 2004). It is not certain that the effect of sonication as a separation technique to detach particle associated microbes will have on the viability and infectivity study of protozoa parasites. Other available methods of microbe-particle separation include homogenization

and chemical treatments with surfactants like Tween 20 or 80. Parker and Darby (1995) compared the blending and sonication techniques for extracting particle-associated coliforms from secondary effluent samples. They found that fragmentation of large flocs during homogenization, as evidenced by the change in particle size distribution, was most likely the responsible mechanism, and sonication proved ineffective.

1.4.8 Infectivity Assays

The U.S. Environmental Protection Agency (USEPA) developed Method 1622 for detection of *Cryptosporidium* spp. and method 1623 for detection of *Giardia* spp. and *Cryptosporidium* spp. in water (U.S. EPA, 1999; U.S. EPA, 2001). In these methods, the immunofluorescence assay (IFA) is used for detection of *Cryptosporidium* spp. in water samples, which includes filtration and elution of large volume of water sample, and immunomagnetic separation (IMS) prior to analysis by enumeration using epifluorescence microscopy. Even though the techniques generally have higher recovery efficiencies and greater precision than sucrose flotation procedures (Bukhari et al., 1998; Di Giovanni et al., 1999), they still suffer from the inability to assess the infectivity and public health significance of detected organisms (Mayer and Palmer, 1996; Simmons et al., 2001) and high variations have been reported between laboratories (Clancy et al., 1994).

There are three methods used to assess the infectivity of parasites: human studies, animal assay and *in vitro* surrogate assay. Obviously, human infectivity studies are not practical for routine use. Animal models are generally considered to be a "gold standard" for *Giardia* spp. and *Cryptosporidium* spp. infectivity. However, the reproducibility and quantitation as well as ethical issues have made researchers pursue other techniques to replace animal assay for water research. The application of cell culture for *Cryptosporidium* spp. infectivity began in the early 1990s (Upton et al., 1994a; Upton et al., 1994b; Woods et al., 1995). However, not like *Cryptosporidium* spp., there is no *in vitro* cell culture method available to measure the infectivity of *Giardia* spp. cysts.

The infectivity of parasites is either measured by an animal model or *in vitro* surrogate assay such as *in vitro* excystation and vital dye assay. It has been demonstrated that both *in vitro* excystation and vital dye assay methods significantly underestimate the

loss of infectivity of parasites after exposure to UV (Clancy et al., 1998; Craik et al., 2000). This can be explained by the germicidal action of UV light as the inactivation of microorganism by UV radiation is mainly caused by the damage of DNA, thus preventing the reproduction of organisms. *In vitro* excystation measures the ability of the parasite to complete only one part of its life cycle. Only parasites that do not excyst under *in vitro* conditions are considered to be dead. UV treatment does not affect the excystation of UV exposed cysts even though the DNA of the parasites had been sufficiently damaged by absorption of UV to preclude successful DNA replication, reproduction and establishment of infection in animal assay (Clancy et al., 1997; Craik et al., 2000). Vital dye assay normally use fluorescence stains such as 4',6-diamino-2-phenylindole (DAPI) with propidium iodide (PI) or SYTO 9 to stain the nuclei of parasites (Bukhari et al., 1999; Craik et al., 2000). The nuclei of viable parasites are stained dark green due to the SYTO 9, while the nuclei of dead parasites are stained orange/yellow or bright red due to PI. However, UV treatment does not appear to affect the integrity of the cell wall significantly, and, as a consequence the vital dye can not penetrate and combine with the organism to produce the fluorescence. Therefore, a portion of parasites after UV exposure are to be considered alive by vital dye assay, which is a poor indicator of inactivation of UV-exposed parasites when compared to infectivity. The animal assay is still the "gold standard" to determine the infectivity of *Giardia* spp. cysts after UV irradiation.

Initial studies determined that the parasite would replicate *in vitro* and could be detected microscopically using fluorescent antibody labeling after as little as 17 hr of incubation (Slifko et al., 1997; Slifko et al., 1999). Research has shown favorable results of *Cryptosporidium* cell culture in comparison with animal infectivity studies (Hijjawi et al., 2002; Jenkins et al., 2003; Rochelle et al., 2002; Slifko et al., 2002). The advantages of cell culture assay over the animal system are that it is more sensitive (i.e. shows infectivity more readily) and is considerably less expensive. It requires fewer parasites to produce an infection result, which is a significant advantage for environmental samples due to the low concentration of parasites. Slifko et al. (2002) compared data from a total of 31 dose-response trials using both tissue culture and mouse models to assess *C. parvum* infectivity. Average ID_{50}s developed using the logit dose-response method for tissue culture and mice were 8 and 107, respectively, suggesting that tissue culture was

more sensitive to infection. However, correlation (r) between tissue culture and mouse infectivity was statistically significant. Comparison of oocyst disinfection by UV and chlorine dioxide showed no significant difference between inactivation predicted by tissue culture and mouse models. Rochelle et al. (2002) found a good correlation between the average infectivity for HCT-8 cells and CD-1 mouse infectivity. High levels of *C. parvum* inactivation have been measured at relatively low UV dose (< 10 mJ/cm^2) using medium pressure (Mofidi et al., 1999) and low pressure UV systems (Shin et al., 1999). Jenkins et al. (2003) compared several viable and infectious tests for *C. parvum* oocysts and found that cell culture and mouse infection assay exhibited the best agreement.

Even though infectivity assays in live animals are costly and require specialized animal care facilities, the standard procedure for determining the infectivity of *G. lamblia* is still the Mongolian gerbil model. Currently, there is no suitable *in vitro* method to measure the infectivity of *G. lamblia*.

Animal model for human giardiasis are invaluable in studies dealing with the mechanisms of pathogenicity of human giardiasis. Belosevic et al. (1983) first proposed the Mongolian gerbil (*Meriones unguiculatus*) animal model to study human giardiasis. The authors reported that Mongolian gerbils are highly susceptible to infection with *Giardia* spp. cysts obtained from stool specimens of infected humans or cultured trophozoites. The trophozoites and cysts recovered from gerbils can be used to initiate new *in vitro* culture and the gerbils could be employed as a "live culture" to supply large number of cysts for use in laboratory and engineering studies (Swabby et al., 1988). The Mongolian gerbil model was used in this research to evaluate the infectivity of *G. lamblia* cysts obtained from wastewater samples and generation of fresh viable parasites.

1.4.9 Public Health Risk Associated with Wastewater Discharge and Reuse Containing Parasites

As discussed previously, *G. lamblia* cysts are common in raw sewage as well as treated wastewater (Wallis et al., 1996). The need to remove and inactivate protozoan cysts from wastewater for protection of public health is underscored by observation of high prevalence of the parasites in non treated wastewater (Garcia et al., 2002; Quintero-Betancourt et al., 2003; Robertson et al., 2000). Although current wastewater treatment

processes are reasonably effective in removing or inactivating most of the parasites (Caccio et al., 2003), there are reports indicating that the number of *G. lamblia* cysts present in effluents discharged from different wastewater treatment facilities may be high (Casson et al., 1990; Medema and Schijven, 2001). Municipal wastewater is usually the main source of *G. lamblia* cysts in waterborne giardiasis outbreaks (Wallis et al., 1998). The presence of cysts in wastewater discharges alone does not necessarily imply a human health risk. To cause disease, the parasites must be viable and infectious. Other factors, including the usage of the wastewater effluent, the characteristics of the receiving water body, background concentration of parasites in the receiving water, and precipitation and runoff in the watershed, may affect the risk of contact with *Giardia* cysts in wastewater effluent discharged into water bodies. Garcia et al. (2002) demonstrated that *G. lamblia* cysts were not infectious to gerbils after tertiary treatment with chlorination. However, *Giardia* spp. cysts did cause infection in the immunodeficient mice after UV treatment in full scale UV reactors (Neto et al., 2006). If an annual acceptable level of risk less than 10^{-4} for a yearly exposure were applied for microbial risk assessment, Rose et al. (1991) estimated a 4.8×10^{-3} yearly risk of infection after exposure to 2 L treated waters using a risk assessment model defined by a dose-response curve developed from human feeding studies for *Giardia* spp. and assumed 2 L of water consumption per day. Ryu et al. (2007) performed the quantitative risk assessment for *Giardia* and *Cryptosporidium* in non-potable tertiary treated reclaimed water and estimated that the risk of infection by *Giardia* resulting from accidental consumption of 100 mL reclaimed water could meet the annual acceptable risk of 1.0×10^{-4} at sites using a combination of chlorination and UV, but not at sites using chlorination only.

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2 COMPARISON OF *G. LAMBLIA* STRAINS ON UV INACTIVATION[1]

2.1 Introduction

Giardia lamblia (also referred to as *G. duodenalis*) is the most commonly identified protozoan parasite for worldwide outbreaks of waterborne disease and causes between 100,000 and 2.5 million cases a year in the US (Furness et al., 2000). Many of these giardiasis outbreaks were associated with municipal drinking water supplies which met regulatory standards for turbidity and coliforms and occurred in water systems using a surface water source (Craun, 1990).

Early studies suggest that *G. lamblia* cysts are resistant to ultraviolet (UV) irradiation at the doses commonly used by commercial ultraviolet treatment units when the viability of cysts was assessed by *in vitro* excystation surrogate assay (Rice and Hoff 1981). However, Craik *et al.* (2000) found that *in vitro* excystation and nucleic acid staining assays grossly underestimate inactivation of *G. muris* compared to infectivity reduction measured in a mouse model. Other researchers have since reported significant (i.e. > 99%) inactivation of *G. lamblia* cysts, measured by infectivity in Mongolian gerbils, at UV doses readily achievable in water treatment practice (Campbell and Wallis, 2002; Linden et al., 2002; Mofidi et al., 2002).

Clancy *et al.* (2004) concluded that five different isolates of *Cryptosporidium parvum* oocysts were equally sensitive to UV light. In contrast, no direct comparisons between the UV inactivation properties of different *Giardia* spp. isolates have been made. Marked differences in the biological behavior and host response in gerbils of antigenically distinct *Giardia* spp. isolates have been reported (Aggarwal and Nash, 1987). Faubert *et al.* (1983) compared the pattern of infection of human isolates and animal isolates of *G. lamblia* in Mongolian gerbils and found that the pattern of cyst release in these animals

[1] This chapter is a revised version of published paper "Comparison of inactivation of two isolates of *Giardia lamblia* cysts by UV light". Applied and Environmental Microbiology" 2007, 73: 2218-2223.

was a characteristic of the parasite and was independent of the host. There are no published studies that deal with the potential impact of biological differences between *Giardia* spp. isolates on the infectivity of UV irradiated cysts and the inactivation by UV irradiation. Campbell and Wallis (2002) reported up 2 $\log_{10}$ reduction of infectivity in Mongolian gerbils of human-derived WB isolate *G. lamblia* cysts that were exposed to a UV dose of 10 mJ/cm^2 from using a low pressure (LP) mercury lamp. However, Linden et al. (Linden et al., 2002) reported more than 4 $\log_{10}$ reduction of infectivity in Mongolian gerbils at a UV dose of 1 mJ/cm^2 using the CH3 isolate *G. lamblia* cysts. The apparent differences in UV sensitivity reported between these two studies may have been due to differences in the *G. lamblia* strains or due to other variables such as difference in cyst purification procedures, water matrix characteristics, UV exposure procedures and procedures for assessing infectivity in the gerbil. The objective of this research was to compare directly the UV inactivation of two human isolates of *G. lamblia* cysts using a Mongolian gerbil model.

2.2 Materials and Methods

2.2.1 Parasites

Two human isolates of *G. lamblia* cysts were used in this study. The WB strain *Giardia lamblia* cysts were obtained from ATCC and has been verified by ATCC using genotyping procedures. This strain was originally isolated from a patient infected in Afghanistan with chronic symptomatic giardiasis and belongs to the most representative assemblage A group of *Giardia lamblia* worldwide (Hopkins et al., 1999). Purified WB isolate *G. lamblia* cysts were obtained by infecting Mongolian gerbils with cultured trophozoites of the WB isolate *G. lamblia* as described elsewhere (Belosevic et al., 1982). Gerbils were inoculated orally with 0.2 mL of PBS containing ~1×10^5 *G. lamblia* trophozoites. Cysts in 2 hr collection from fecal samples of inoculated gerbils were purified by a sucrose flotation method and washed three times in deionized (DI) water by centrifugation at 500 × g for 10 min at 4°C as described elsewhere (Finch et al., 1993).

H3 isolate *G. lamblia* cysts (assemblage B group) (Guy et al. 2003) were obtained from Dr. Faubert at McGill University (Canada) and were maintained by passage through

Mongolian gerbils every two weeks. The H3 strain originated from Dr. H. Stibbs at Waterborne Inc., New Orleans, LA, and the stock was a kind gift for Dr. G. M. Faubert at McGill University. The cyst purification procedure was the same as described above for WB isolate cysts. All cysts collected daily were stored at 4°C and were used in experiments within 5 days of collection.

2.2.2 UV Exposure

Up to 2×10^6 *G. lamblia* cysts purified from gerbil fecal samples were suspended in 20 mL of DI water in 55 mm diameter glass Petri dishes. The suspensions were exposed to controlled doses of UV from a 10 W low-pressure mercury arc lamp using a collimated beam apparatus (Rayox®, Calgon Carbon Corporation). Suspensions were stirred continuously during the exposure. Irradiance at the surface of the liquid was measured using a calibrated radiometer (Optometer P-9710, Gigahertz Optik Inc., Newburyport, MA). The UV transmittance of suspension ranged from 11% to 89%. The depth averaged UV irradiance of 1.05 cm water path in the experimental suspensions was determined according to procedures described elsewhere (Craik et al., 2001). After UV exposure, the suspensions were centrifuged at 500 × g for 10 min at 4 °C to concentrate the cysts. The cyst concentration was determined by hemocytometer and serial dilutions were prepared for inoculation into Mongolian gerbils.

2.2.3 Animal Infectivity

Male Mongolian gerbils, 4 to 8 weeks of age were purchased from the Charles River Laboratories (Kingston, NY) and were maintained in the Biological Sciences Animal Care Facility at University of Alberta. All the procedures followed the protocols approved by University Animal Policy and Welfare Committee. Gerbils were inoculated with 0.2 mL of Milli-Q® water containing different numbers of cysts by gastric incubation using a blunt needle. Gerbils were housed in filter top cages, two animals per cage. Feces were collected from individual gerbils daily starting on day 6 post-infection and were examined for the presence of cysts using sucrose flotation technique. All gerbils were terminated and examined for the presence of trophozoites in the mucosal

layer of the small intestine between days 13 and 15 post-infection as described by Belosevic *et al.* (1983). The infectivity level was determined by the presence of cysts in the feces of inoculated gerbils and the presence of trophozoites in the small intestine of gerbils. For each infectivity experiment, two additional gerbils were used as negative controls and received no inoculum. The presence of trophozoites in intestinal samples was confirmed by nucleic acid stain using a Hema 3® stain kit (Fisher Scientific, Nepean Ontario, Canada).

2.2.4 Calculations

The dose-response of *G. lamblia* cysts in Mongolian gerbils was quantified using a logistic dose-response model developed by Finch *et al.* (1993).

$$\text{Logit} = \beta_1 + \beta_2 \log_{10} d \qquad (2\text{-}1)$$

where logit is $\ln[P/(1\text{-}P)]$, P is the proportion of animals infected and d is the number of infectious cysts in the oral inoculum. The logistic model parameters β_1 and β_2 were estimated by carrying out dose-response experiments in the gerbils and analyzing the outcome using the method of maximum likelihood as described by Gyürék et al. (1999). The parameters β_1 and β_2 determined for the WB and H3 isolates in the dose-response experiments were used to estimate the number of remaining infectious cysts following UV exposure. After a UV experiment, P was measured based on the gerbil infectivity result, d was calculated using Equation (1) and inactivation was determined as follows:

$$\text{Inactivation} = -\log_{10} (d / d_0) \qquad (2\text{-}2)$$

where d_0 was the total number of cysts in the inoculum estimated by hemocytometer count.

2.3 Results

2.3.1 Dose-Response Data for Mongolian Gerbils

To determine the infectivity of each isolate in the gerbils, several trials of dose-response experiments were carried out with inoculum sizes ranging from 10^1 to 10^4 cysts

per animal. Cohort sizes for each inoculum size in each experiment ranged between 4 and 8 gerbils. The pooled results of these dose-response experiments are summarized in Table 2-1. The logit dose-response model parameters and the dose required for 50% infection were calculated based the dose-response results of Table 2-1 and presented in Table 2-2 for each isolate. The confidence intervals for each parameter estimate were calculated based on the 95% joint confidence region (JCR) (Gyürék et al., 1999). The calculated ID_{50} for the WB isolate was 180 cysts, which is comparable to a previously reported ID_{50} for *G. lamblia* cysts infectivity in Mongolian gerbils of 248 cysts (Finch et al., 1993). The ID_{50} of 22 cysts for the H3 isolate was considerably lower. Based on the computed ID_{50}s, the H3 isolate was considerably more infectious to the gerbils than was the WB isolate. The 95% JCRs of the dose-response parameters (β_1, β_2) for the two isolates (not shown) did not overlap which indicates that the infectious properties for WB and H3 isolates were significantly different at the 95% confidence level.

Table 2-1. Dose-response of *G. lamblia* cyst in Mongolian gerbils indicating the intensity of infection

Strain	Inoculum (cysts/gerbil)	Total No. of trials	Gerbils per dose	Total gerbils infected	Intensity of infection			Total Proportion infected
					Strong	Moderate	Weak	
H3	10	3	16	4	1	0	3	0.25
	100	5	18	16	14	1	1	0.89
	1,000	3	10	10	10	0	0	1
	10,000	1	5	5	5	0	0	1
WB	100	4	16	7	1	0	6	0.44
	1,000	4	16	11	1	1	9	0.69
	10,000	4	16	15	13	1	1	0.94

In Table 2-1, the gerbils were scored positive for infection based on the presence of either cysts in the feces or trophozoites in the intestine or both. However, the intensity of infection, as indicated by the observation of cysts or trophozoites in gerbils scoring positive varied considerably, even between gerbils in the same cohort. For example, in some gerbils scoring positive for infection, cysts were observed in the feces and many

actively motile trophozoites were observed in the intestinal mucosal scrapings. In others, no cysts were observed in the feces and only a few non-active or non-motile trophozoites were observed in the intestinal scrapings. These non-active trophozoites were confirmed based on size, shape and nucleic acid staining and were considered as evidence of infection in this study. To investigate the levels of infection further, gerbils that scored positive for infection in Table 2-1 were sub-divided into three categories: gerbils in which cysts were observed in the feces and many active trophozoites were observed in the small intestine were scored as positive for strong infection; gerbils in which no cysts were observed in the feces, but live and active trophozoites were observed in the small intestine were scored as positive for moderate infection; gerbils in which no cysts were observed in the feces but non-active trophozoites were observed in the small intestine were scored as positive for weak infection. All other gerbils were scored as negative for infection. About 89 percent of the gerbils inoculated with 100 H3 isolate *Giardia* cysts were infected and most these were scored as strong infections. In contrast, only 44 percent of the gerbils inoculated with 100 WB isolate cysts were infected and most these were scored as weak infections. All gerbils inoculated with 1000 H3 isolate cysts were strongly infected while only 69 percent of gerbils inoculated with 1000 WB cysts were infected and of these, most of the infections were weak. The H3 isolate cysts, therefore, were more infectious in the Mongolian gerbil host both in terms of total numbers of gerbils scored positive for infections and in terms of the intensity of infection.

Table 2-2. Mongolian gerbil logit dose response model parameters with upper and lower 95% confidence intervals for *G. lamblia* cysts.

Isolate	β_1	β_2	ID_{50}†	Source
WB	-3.06 (-3.89.-2.18)	1.36 (1.07, 1.69)	180	(this study)
H3	-4.36 (-5.48, -3.24)	3.25 (2.57, 4.11)	22	(this study)
WB, CDC:0284:1 and 10 other isolates	-3.69 (-5.04, -2.37)	1.54 (0.89, 2.20)	248	(Finch et al. 1993)

† ID_{50} = - antilog($-\beta_1 / \beta_2$)

2.3.2 UV Dose-Response

WB and H3 isolates *G. lamblia* cysts were exposed to a constant UV dose of 40 mJ/cm^2 and inoculated into gerbils within 2 hours of exposure. Results of the UV exposure experiments are presented in Table 2-3. Following UV exposure, the H3 isolate was less infectious in the gerbils than the WB isolate both in terms of total infections and in terms of the intensity of infections. Only weak infections were observed in the gerbils inoculated with up to 100,000 UV-exposed H3 cysts while some strong infections were observed in gerbils inoculated with 100,000 WB cysts and moderate infections were observed in gerbils inoculated with 10,000 WB cysts. Strong and medium infections, characterized by active trophozoites and/or the presence of cysts in the feces were also observed in those gerbils inoculated with 20,000 WB isolate cysts exposed to an UV dose of 0.5 mJ/cm^2 and 10,000 WB isolate cysts exposed to an UV dose of 1 mJ/cm^2 (data not shown here). This indicates that the H3 isolate cysts, though more infectious in the gerbils prior to UV exposure, were more readily inactivated by UV radiation.

Table 2-3. UV- dose response of *G. lamblia* cysts in the Mongolian gerbils at 40 mJ/cm^2 indicating intensity of infection.

Strain	UV dose (mJ/cm^2)	Inoculum (cysts/gerbil)	No. of gerbils infected	Gerbils per dose	Intensity of infection			Proportion infected
					Strong	Moderate	Weak	
H3	40	1,000	1	4	0	0	1	0.25
		10,000	4	24	0	0	4	0.17
		100,000	4	18	0	0	4	0.22
WB	40	10,000	8	16	0	1	7	0.5
		100,000	11	16	4	0	7	0.69

Using the logistic dose-response models for H3 and WB isolates estimated in this study (Table 2-2), the inactivation of *G. lamblia* cysts was calculated and is shown in Table 2-4. The mean inactivation at a UV dose 40 mJ/cm^2 was calculated to be 2.0 log for the WB isolate and 3.6 log for the H3 isolate. The difference in computed mean UV inactivation between the two isolates was 1.6 log. The infectivity of the WB isolate and

H3 isolates was significantly different at the 95% confidence level (p = 0.03). Cyst inactivation measured in this study was compared to UV inactivation of *G. lamblia* cysts measured in other published studies in Table 2-5.

Table 2-4. Reduction of *G. lamblia* infectivity in Mongolian gerbils by UV at 40 mJ/cm^2

Isolate	Trial No.	Inoculated cysts per gerbil, d_0	Total no. of gerbils inoculated	Total no. of gerbils infected	Proportion infected, P	Infectious cysts, d	Inactivation, -log d/d_0	Mean Inactivation†
WB	1	10000	4	1	0.25	34	2.5	2.2
		100000	4	3	0.75	1027	2.0	
	2	10000	4	2	0.5	186	1.7	1.9
		100000	4	3	0.75	1027	2.0	
	3	10000	4	2	0.5	186	1.7	1.9
		100000	4	3	0.75	1027	2.0	
	4	10000	4	3	0.75	1027	1.0	1.9
		100000	4	2	0.5	186	2.7	
H3	2	1000	6	0	0	< 7	> 2.2	4.2
		10000	6	0	0	< 7	> 3.2	
		100000	6	1	0.17	7	4.2	
	3	1000	4	1	0.25	10	2.0	2.4
		10000	8	3	0.38	15	2.8	
	4	10000	4	0	0.00	<10	> 3.0	4.2
		100000	6	1	0.17	7	4.2	
	5	10000	6	1	0.17	7	3.2	3.5
		100000	6	2	0.33	13	3.9	

† the arithmetic mean inactivation computed for each inoculum

Table 2-5. Summary of UV inactivation studies on *G. lamblia* cysts using the Mongolian gerbil model.

Isolate	Host age	Gender of host	Water matrix	Method used to estimate no. of viable cysts	Log_{10} reduction and UV dose	Reference
WB	n/a	n/a	PBS with 0.05% Tween 20	ID_{50}	< 2 at 10 mJ/cm^2 < 3 at 40 mJ/cm^2	Campbell et al. (2002)
WB	4 wks	Female	WTP filtered water	MPN	> 2 at 3 mJ/cm^2	Mofidi et al. (2002)
CH-3	8 to 10 wks	Female	Buffered DI water containing antibiotics	MPN	> 4 at 1 mJ/cm^2	Linden et al. (2002)
n/a	8 to 10 wks	Female	Buffered DI water containing antibiotics	MPN	> 4 at 1 mJ/cm^2	Shin et al. (2005)
WB	4 to 8 wks	Male	DI water	Logistic model	1.9 at 40 mJ/cm^2	This study
H3	4 to 8 wks	Male	DI water	Logistic model	3.5 at 40 mJ/cm^2	This study

2.4 Discussion

In this study, the intensity of giardiasis infections in the Mongolian gerbils were divided into three categories based on the presence of cysts in the feces of inoculated gerbils and the presence and activity of trophozoites purified from the small intestine of gerbils inoculated with *G. lamblia* cysts. The infections, characterized by the presence of only non-active trophozoites in the gerbil intestinal samples, have not been reported by previous researchers who have carried out UV inactivation studies and are difficult to interpret in terms of infection within the host. Similar signs of weak infection were absent in the un-inoculated control gerbils. One hypothesis for the presence of these inactive trophozoites is that they originated with the original cyst inoculum and were not the result of trophozoite division within the host. That is, the parasites were able to exist in the stomach upon ingestion by the gerbil; the trophozoites that emerged from the cysts were able to attach to the intestinal wall, however, they were not able to thrive and divide. A previous study showed that *G. muris* cysts that were exposed to UV were able

to excyst but were not able to establish infectivity in mice (Craik et al., 2000). Given that the time between inoculation and examination of the intestines was 13 to 15 days and the detection limit for trophozoites in the intestine by microscopy (i.e. approx 10,000), it does not seem plausible that the inactive trophozoites detected in the intestinal samples could have arisen without at least some low level of trophozoite division within the host. Another hypothesis is that some of the cysts exposed to UV were able to repair their damaged DNA after ingestion by the host. Belosevic *et al.* (2001) reported evidence of *in vivo* reactivation of *G. muris* in three of seven independent animal infectivity experiments, when cysts were exposed to relatively low doses of medium pressure UV (< 25 mJ/cm^2). One recent study showed that *G. lamblia* cysts have the ability to repair their UV-damaged DNA after being exposed to a UV dose of 1 mJ/cm^2 from a low pressure (LP) lamp (Shin et al., 2005). The weakness with these arguments is that the weak infections were found not only in gerbils inoculated with UV-exposed cysts , but also in gerbils inoculated with fresh cysts.

This study directly compared two human isolates of *G. lamblia* cysts and their inactivation by UV radiation. The results indicated that the cysts of the two isolates, WB and H3, produced different levels of infections in the Mongolian gerbils both for fresh cysts and UV-exposed cysts. Isolate variation has been previously reported not only with respect to infectivity in gerbils (Visvesvara et al., 1988), but also in the clinical signs of giardiasis infections in humans (Nash et al., 1987). It should be considered that the Mongolian gerbil, though it is the model of preference for use in microorganism reduction studies, is not the natural host for human-derived strains of *G. lamblia* and the zoonotic potential of the various *G. lamblia* assemblages has been controversial for many years (Caccio et al., 2005). The differential infectivity of WB and H3 isolates, therefore, is not surprising. Studies have shown that isolates of *G. lamblia* from different geographic locations and/or hosts can be divided into 2 major groups based on the genetic characteristics: Group 1/2 and Group 3 (Nash, 1992), or assemblages A and B (Mayrhofer et al., 1995). The WB isolate falls into assemblage A (Nash, 1992); whereas the H3 isolate (Guy et al., 2003) and CH-3 isolate (Lu et al., 2002) fall into assemblage B. These different isolates possess different surface antigens, have different patterns of infection and induce qualitatively and quantitatively different host immune responses

(Aggarwal and Nash, 1987). The unique surface antigens could differentially protect the isolates from digestion by intestinal protease. Nash *et al.* (1991) found trophozoites of different isolates differ in their ability to survive after exposure to intestinal proteases, which implies that different isolates will survive and thrive to different degrees within the small intestine of the host. This may explain the number of low intensity infections observed in the Mongolian gerbils inoculated with the fresh WB cysts.

The comparison in Table 2-5 indicates that there are differences between the levels of UV inactivation reported in this study and other published studies that have used the Mongolian gerbil as an infectivity model. The differences reported between studies may be related to a number of variables including methods of cyst preparation and purification, the sex of the gerbils (male or female), the analytical methods and criteria used to define infection endpoints, and the mathematical methods used to interpret the infectivity results. UV inactivation of *Giardia* spp. may be influenced partly by the method used for cyst purification. The average inactivation level of *G. muris* purified by an additional Percoll-sucrose flotation step was more than 1 $\log_{10}$ greater than the inactivation of *G. muris* purified by single sucrose flotation alone at an UV dose 40 mJ/cm^2, possibly due to a reduction in residual fecal matter in the water matrix (Amoah et al., 2005).

In our study, male Mongolian gerbils were used as hosts, while other researchers have used female gerbils. Distinct differences exist between male and female animals in their ability to harbor and eliminate this intestinal parasite. The trophozoite burden in males was reported to be significantly higher in male mice infected with *G. muris* than in female mice (Daniels and Belosevic, 1995). Infected male mice released cysts in their feces and harbored trophozoites in their intestines for a longer period than females. Therefore, giardiasis infections may be more readily detected in male gerbils than in females.

In this study, gerbils in which the only evidence of infection was the presence of non-active trophozoites in intestinal samples were considered as positive for infection. If these animals had been considered negative for infection, then no infections would have been scored for the UV-exposed H3 isolate cysts at all inoculum levels and the reported inactivation would have been > 4 $\log_{10}$. This level of inactivation is more consistent with

the findings of other researchers who studied CH-3 cysts (Linden et al., 2002; Shin et al., 2005). Although they indicated that infections were based on the presence of cysts in the feces or trophozoites in the intestine, these researchers did not provide details on the intensity of the observed infections.

Both most probable number (MPN) and logistic model methods have been used to estimate the number of infectious cysts administered to gerbils. The MPN technique was originally developed to quantify bacteria using broth culture tubes and has been adapted for infectivity assays by some researchers (Linden et al., 2002; Mofidi et al., 2002; Shin et al., 2005). Even though animal models are recognized as the most appropriate method for testing the infectivity of *Giardia* spp. following UV exposure, data generated with infectivity models vary considerably (Rochelle et al., 2002). The effect of variability in the animal response on the uncertainty in MPN technique is unknown. Large differences (0.3 to 1.9 $\log_{10}$) have been reported between direct hemocytometer counts of fresh cysts and the MPN estimated from infections in Mongolian gerbils (Mofidi et al., 2002). The number of infectious cysts may be significantly underestimated by the MPN method.

Inactivation of the WB and H3 cyst isolates exposed to the same level of UV radiation using identical procedures and assessed using the same animal model was found to be considerably different. Even if the gerbils that were positive for non-active trophozoites in the intestine were scored negative, the inactivation of WB strain cysts would still be limited to 2 $\log_{10}$ at 40 mJ/cm^2. This difference between isolates may help to explain apparent differences in *G. lamblia* inactivation levels that have been observed in previous studies (see Table 2-5). It has been reported that different isolates of *E. coli* have different UV sensitivity, as demonstrated by Sommer *et al.* (2000). In contrast, multiple isolates of *C. parvum* were found to be equally sensitive to ultraviolet disinfection (Clancy et al., 2004; Rochelle et al., 2004). This research provides the first evidence that different isolates of *G. lamblia* may not necessarily be equally sensitive to ultraviolet disinfection. Since the dataset is still limited, more isolates of *Giardia* spp. need to be tested to support this finding. In addition, it is not clear if the difference in sensitivity to UV radiation of these human-derived strains of *G. lamblia* is real, or whether it is due to an artifact related to the Mongolian gerbil host model. Although the different isolates responded differently to UV when assessed using the Mongolian gerbil

model, UV radiation appears to be very effective for inactivation of human *G. lamblia* isolates in water.

2.5 References

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3 *G. LAMBLIA* TROPHOZOITES REGROWTH AFTER UV RADIATION[2]

3.1 Introduction

The use of ultraviolet (UV) radiation as a microorganism reduction technology for drinking water and municipal wastewater treatment is increasing in North America. Several laboratory studies have shown that waterborne protozoan parasites, such as *Giardia* species (spp.) and *Cryptosporidium* spp. can be readily inactivated at germicidal UV doses commonly used in water and wastewater treatment (Bolton et al., 1998; Craik et al., 2001). However, it is well established that some bacteria are capable of repairing DNA lesions induced by exposure to germicidal UV in the wavelength range of 200 to 300 nm (Hillebrandt and Mueller, 1991; Knudson, 1986; Lindenauer and Darby, 1994; Schoenen and Kolch, 1992). Inactivation of *Escherichia coli* by monochromatic UV radiation (254 nm) decreased from 4 $\log_{10}$ to 1 $\log_{10}$ after subsequent exposure to long wavelength UV or visible light. This was attributed to a photocatalyzed DNA repair mechanism (Schoenen and Kolch, 1992). The ability of encysted protozoans to repair UV-induced DNA damaged is not well understood. Hillebrandt and Mueller (1991) demonstrated photocataytic repair of DNA in cysts of the amoeba *Naegleria gruiberi* exposed to germicidal UV radiation. Although photorepair of UV-induced pyrimidine dimers in the DNA of *Cryptosporidium* spp. oocysts exposed to germicidal UV has been demonstrated (Oguma et al., 2001), studies so far have shown no evidence that the UV-exposed oocysts regain their ability to infect a host following incubation under either dark or light conditions (Oguma *et al.*, 2001; Rochelle *et al.*, 2004).

Linden *et al.* (2002) reported complete (> 4 $\log_{10}$) infectivity reduction of *G. lamblia* cysts exposed to UV doses of 16 and 40 mJ/cm^2 with no evidence of DNA repair after subsequent *ex vivo* exposure to either light or dark conditions. In contrast, Belosevic *et al.* (2001) reported evidence that *G. muris* cysts exposed to a UV dose of 25 mJ/cm^2 may

[2] This chapter is a revised version of the published paper "Survival of *Giardia lamblia* trophozoites after exposure to UV light". FEMS Microbiology Letters" 2008, 278: 56-61.

be able to repair UV-induced DNA damage under dark conditions given a sufficiently long period of incubation within a mouse host, as observed in mice in 3 out of 7 independent experiments. These findings suggest that actively metabolizing *Giardia* spp. trophozoites may be better able to repair UV-induced DNA lesions than the dormant encysted form. A recent study has reported that *G. lamblia* cysts have the ability to repair their UV-damaged DNA *ex vivo* after exposure to a UV dose of 1 mJ/cm^2 radiation based on both a gerbil infectivity assay and measurement of pyrimidine dimer formation by an endonuclease sensitive site assay (Shin et al., 2005).

The standard procedure for determining the infectivity of *G. lamblia* is the Mongolian gerbil model. Infectivity assays in live animals, however, are costly and require specialized animal care facilities. The purpose of this study was to determine the survival and reactivation potential of *G. lamblia* trophozoites exposed to different doses of germicidal UV radiation using an *in vitro* culture method. Although the cyst is the environmental form of the parasite of relevance to water and wastewater treatment, the study of the trophozoites in *in vitro* culture may provide useful insight into the survival and reactivation potential under dark conditions after cysts exposed to UV within a host.

3.2 Materials and Methods

3.2.1 Parasites

The WB strain of *G. lamblia* trophozoites used in this study was obtained from American Type Culture Collection (ATCC 30957) and were routinely maintained *in vitro* in Diamond's TYI-S-33 medium (Belosevic et al., 1982). Actively growing trophozoites (48 to 96 hr culture) were harvested as described previously (Belosevic et al., 1982). Trophozoites were diluted with sterilized 0.05 M phosphate buffered deionized (PBDI) water (pH 7.2) to a final concentration of 10^5 trophozoites per mL for use in UV exposure experiments.

3.2.2 Ultraviolet Exposure

Twenty mL suspensions of trophozoites in glass Petri dishes were exposed to UV radiation produced by a low pressure mercury lamp (Model G12T6L, Atlantic Ultraviolet

Corp, Haupauge, NY) using a collimated beam apparatus (Calgon Carbon Corporation). The Petri dishes were fitted with quartz covers to maintain sterile conditions. Irradiance was measured at a distance from the lamp equivalent to the surface of the liquid in the Petri dish using a calibrated radiometer (Optometer P-9710, Gigahertz Optik Inc., Newburyport, MA). Reflection of UV light was accounted for by placing the radiometer beneath the cover during the irradiance measurement. Suspensions were stirred continuously during the exposure to ensure that the parasites received a uniform UV dose. The UV absorbance at 254 nm of each suspension was measured using a spectrophotometer (UV-2401 PC, Shimadzu Corp., Columbia, MD) and the depth-averaged UV dose was determined using procedures described elsewhere (Craik et al., 2000). The average germicidal UV irradiance throughout the water volume was approximately 0.03 mW/cm^2 and the exposure time ranged from 30 s to 60 min for UV doses ranging from 1 to100 mJ/cm^2. All experiments were performed at 20 ± 2°C.

3.2.3 In vitro Culture Procedure

Aliquots (500 to 700 µL) of suspension containing UV-exposed trophozoites were transferred to 25 cm^2 canted-neck tissue flasks (Corning, Cat no. 430168). For each UV exposure, up to 30 identical flasks were prepared and incubated at 37°C in the dark. Fresh Diamond's TYI-S-33 medium was added to the flasks every 3 to 5 days to supply nutrients required for trophozoite growth. Two or three flasks were removed from the incubator on day zero (within 3 h of UV exposure) and on each day following UV exposure. The flasks were placed on ice for 15 min to detach the parasites from the bottom of the flask. The contents of each flask were transferred to a 50 mL conical centrifuge tube and were centrifuged at 500 × g for 10 min at 4°C. The pellets were re-suspended in sterile PBDI water and the number of live trophozoites was counted by light microscopy at 200 × magnification. The pellets were kept at 4°C before enumeration in order to prevent the trophozoites from attaching to the surface of the tubes. As observed during the experiment, the chilling did not reduce the motility of trophozoites under microscopic examination. Trophozoites that displayed either swimming or spinning motility or flagella movement were considered alive.

3.2.4 Gerbil Infectivity Assay

Male Mongolian gerbils, 4 to 8 weeks of age were purchased from the Charles River Laboratories (Kingston, NY) and were housed in filter top cages, two animals per cage, in the Biological Sciences Animal Care Facility at University of Alberta. All gerbil procedures followed protocols approved by University of Alberta Biological Sciences Animal Policy and Welfare Committee and were consistent with guidelines of the Canadian Council of Animal Care.

Actively growing trophozoites were centrifuged at 500 × g for 10 min at 4°C. The pellet was re-suspended in PBDI and split into two portion for the gerbil infectivity assay. One portion was not exposed to UV and served as a positive control. The other portion was exposed to a UV dose of 1 mJ/cm^2. Each suspension was concentrated by centrifugation (500 × g for 10 min at 4°C). Dilutions were prepared in PBDI water and trophozoites were counted by hemocytometer. Gerbils were inoculated orally with 0.2 mL dilutions containing 2.1×10^5 *G. lamblia* trophozoites using a blunt needle. Feces were collected from the gerbils for a 2 hr period on each day following inoculation, purified using sucrose flotation, and enumerated by hemocytometer as described elsewhere (Finch *et al.*, 1993).

3.3 Results

3.3.1 Effect of Exposure to Air on In vitro Trophozoite Reproduction

Trophozoites are aerotolerant anaerobes and the trophozoites were exposed to air in the covered Petri dishes for up to 60 min during the UV exposure trials. An experiment was conducted to investigate the effect of exposure to air on trophozoite growth during *in vitro* culture. A PBDI water suspension containing approximately 10^5 trophozoites/mL was divided into two equal portions: The first portion was transferred into a covered Petri dish and was stirred for 60 min with exposure to atmospheric air but without exposure to UV; the second portion was transferred to a sealed plastic centrifuge tube and served as a control. After 60 min at room temperature, 1.0 mL aliquots were removed from the Petri dish and the control culture, and transferred into separate series of culture flasks. The flasks were then incubated at 37°C under a 5% CO_2 as described above. On days 0, 1, 3

and 5 after the exposure to air, three flasks were removed from each series and the trophozoites were enumerated. The results of the enumeration, shown in Figure 3-1, indicated that there was no statistical difference (2-Factor ANOVA, $p = 0.24$) in the reproduction of trophozoites that were exposed to air in the Petri dish for 60 minutes when compared to the trophozoites that were not exposed to air (control). It was concluded that exposure to air under the conditions of this study did not result in significant trophozoite inactivation.

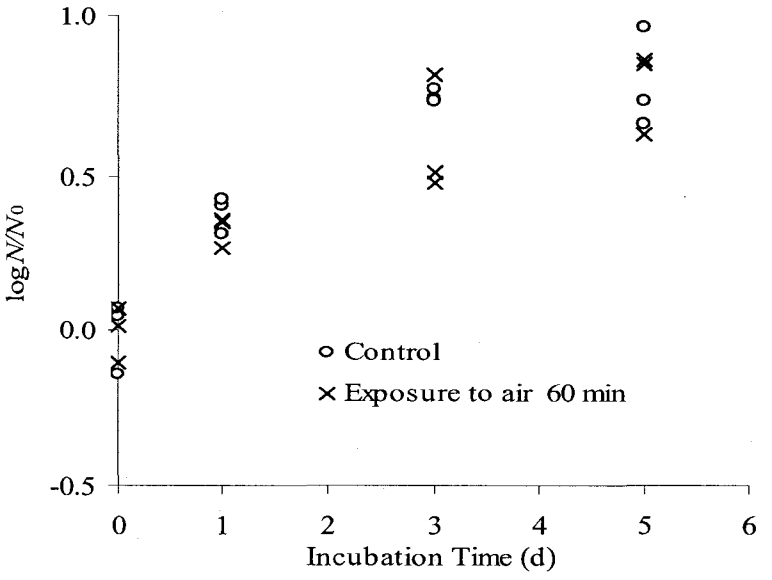

Figure 3-1. Effect of exposure of trophozoites in Petri dishes to air for 60 min on the subsequent *in vitro* reproduction. Different symbols represent results from independent experiments.

3.3.2 Reproduction of Trophozoites after UV Exposure

The reproduction rate of *G. lamblia* trophozoites was measured *in vitro* after exposure to UV doses of 0, 1, 10, 20, 40 and 100 mJ/cm^2. For each UV dose, two or three independent trials were carried out and the results are shown in Figures 3-2 to 3-7. The initial number of live trophozoites in the flasks (N_0) on day zero varied between 1×10^4 and 2.5×10^5. The results in Figures 3-2 to 3-7 were normalized and expressed in terms of $\log (N/N_0)$, where N represents the number of live trophozoites on each subsequent

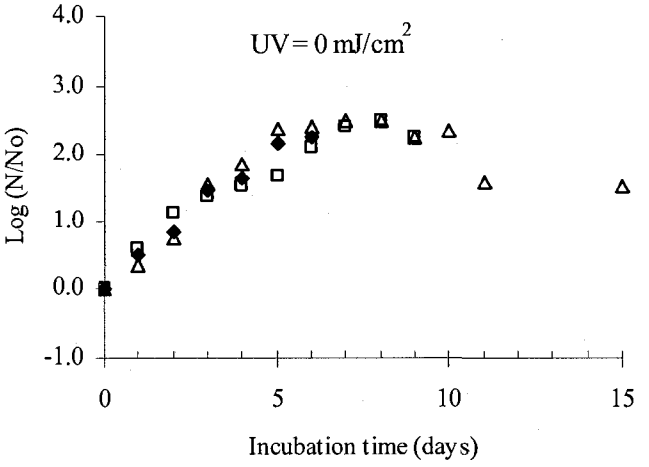

Figure 3-2. In vivo reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 0 mJ/cm^2 (control). Different symbols represent results from independent experiments.

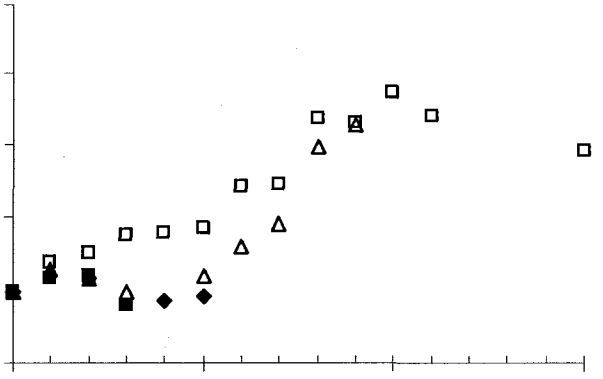

Figure 3-3. *In vivo* reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 1 mJ/cm^2. Different symbols represent results from independent experiments.

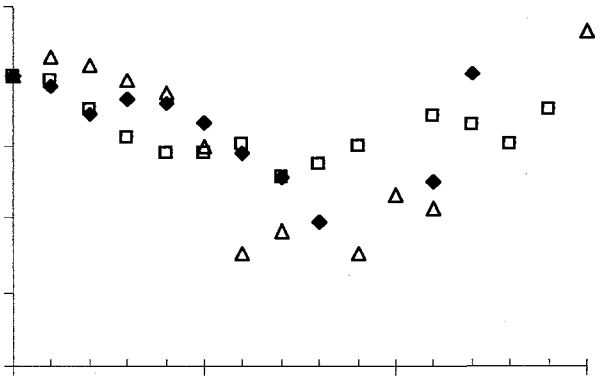

Figure 3-4. *In vivo* reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 10 mJ/cm^2. Different symbols represent results from independent experiments.

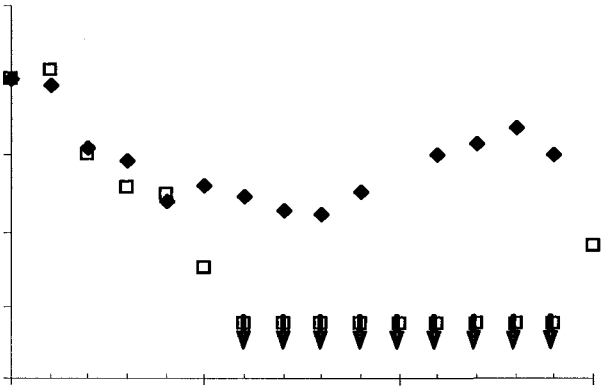

Figure 3-5. *In vivo* reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 20 mJ/cm^2. Different symbols represent results from independent experiments.

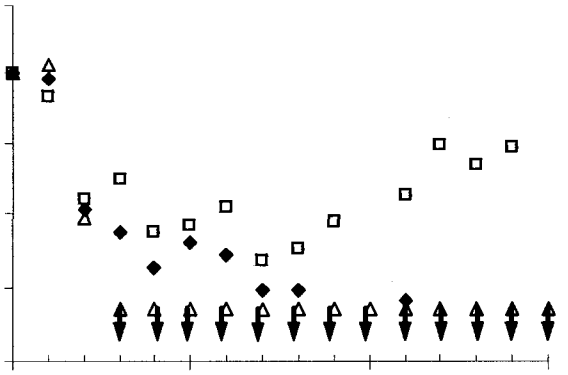

Figure 3-6. *In vivo* reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 40 mJ/cm^2. Different symbols represent results from independent experiments.

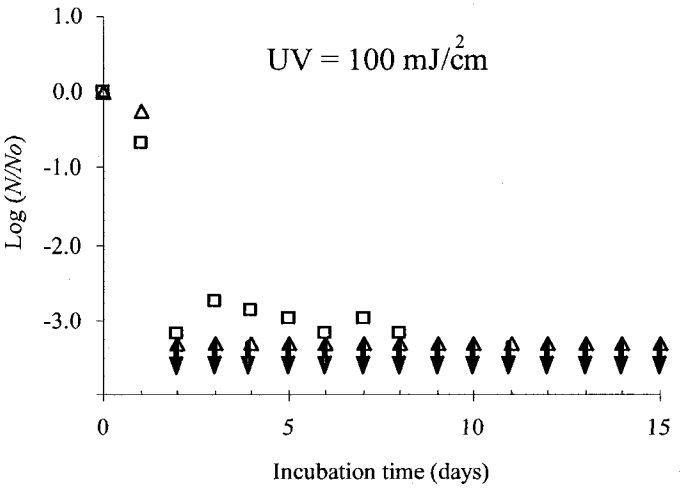

Figure 3-7. *In vivo* reproduction of *G. lamblia* trophozoites in Diamond's TYI-S-33 medium after exposure to UV dose of 100 mJ/cm^2. Different symbols represent results from independent experiments.

day after UV exposure. The different sets of symbols represent results from independent experiments. The downward arrows indicate that the number of trophozoites were below the indicated detection limit. For non-UV exposed parasites (Figure 3-2), the number of trophozoites in the culture medium increased exponentially starting at day 1 and stabilized at about 3 log and at 6 to 7 days incubation. For the UV dose of 1 mJ/cm^2, there was a delay in the onset of exponential trophozoite reproduction until approximately day 5 (Figure 3-3) when the trophozoite numbers began to increase exponentially at a rate similar to that of the non-UV exposed parasites. For the UV dose of 10 mJ/cm^2, the trophozoite numbers declined initially and stabilized at - 1.5 to - 2.5 log at day 7 to 8 (Figure 3-4). Thereafter, however, the trophozoite numbers in all three trials began to increase. A linear regression analysis indicated that the increase in trophozoite numbers after 7 days was statistically significant ($p = 0.002$). The trophozoite numbers decreased initially in both of the two trials conducted at 20 mJ/cm^2 (Figure 3-5). In one trial, the trophozoite numbers decreased to below detection limit and did not increase again, while in the other trial the trophozoite numbers stabilized at -1.5 to 2.0 log and began to increase again at day 7 to 8. At 40 mJ/cm^2, the trophozoite numbers decreased to less than detection limit in two trials and did not increase further (Figure 3-6). In the third trial, however, the numbers decreased initially but began to increase after day 7 to 8. The variation observed among different trials of experiments at 20 and 40 mJ/cm^2 may have been caused by the variation between each experimental batch of trophozoites. At 100 mJ/cm^2, the trophozoite numbers decreased rapidly to less than detection limit in both trials and did not increase thereafter (Figure 3-7). In addition, microscopic observations of the culture flasks revealed that the trophozoites exposed to UV dose of 100 mJ/cm^2 were unable to attach to the bottom of the culture flasks.

3.2.3 Infectivity of Trophozoites in Mongolian Gerbils

The effect of exposure of trophozoites to a UV dose of 1 mJ/cm^2 on infectivity in Mongolian gerbils is shown in Figure 3-8. Trophozoites exposed to 1 mJ/cm^2 resulted in infections, as determined by the presence of cysts in the feces in 3 of 4 inoculated gerbils,

while non-UV exposed trophozoites produced infections in 3 of 3 gerbils. The pattern of cysts shedding in the gerbils produced by UV-exposed and non-UV-exposed trophozoites was similar (Figure 3-8). The average cyst output per gram of feces collected from gerbils inoculated with non-UV exposed trophozoites was not statistically different than that of gerbils infected by UV-exposed trophozoites ($p = 0.85$).

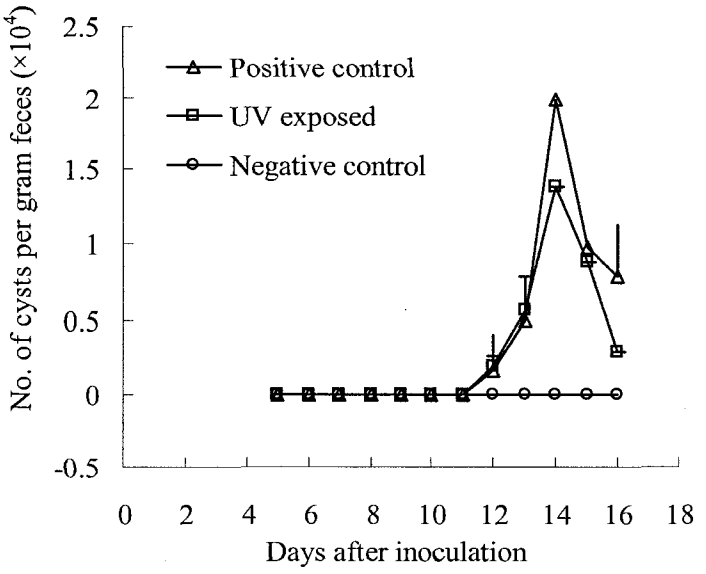

Figure 3-8. Cyst release in the gerbils after inoculation with *G. lamblia* trophozoites exposed to 1 mJ/cm^2 UV dose. Each point represents the mean number of cysts shed from three or four gerbils in a 2-hr collection period.

3.2.4 Modeling of Trophozoite Reproduction

The initial reduction in trophozoite numbers within the first few days following UV exposure is consistent with the germicidal effect of UV on the parasites. Presumably, the trophozoites that did not divide and multiply successfully due to UV-induced DNA lesions died and lysed. There are two potential explanations for increase in trophozoite numbers in the culture flasks that began several days after UV exposure that was observed for the UV doses up to 40 mJ/cm^2. The increase may have been due to division of a small number of survivors that somehow escaped full UV exposure or were part of a more UV resistant sub-population. Alternatively, the increase in trophozoite numbers

may have been the result of delayed reactivation of parasites that were temporarily inactivated by UV exposure arising from DNA repair. Reactivation due to a DNA repair mechanism is likely to be associated with a delay in trophozoite division while simple survival is not. To investigate this question, a simple mathematical model was used to predict the pattern of trophozoites reproduction that would be observed after UV exposure assuming that the increase in trophozoite numbers was solely due to survival and not due to repair. The model is based on a trophozoite number balance and is given by:

$$\frac{dN_T}{dt} = k_a N_a - k_d N_d \tag{3-1}$$

In Equation 3-1, N_T is the total number of active trophozoites in the flask; N_a is the number of active trophozoites that escaped UV exposure and their progeny; N_d is the number of trophozoites inactivated by UV radiation that are still active; k_a and k_d represent the first-order kinetic rate constants for the division rate of active trophozoites and the death rate of inactivated trophozoites, respectively. Values of k_a and k_d were estimated from the observed reproduction curves of non-UV exposed trophozoites and trophozoites exposed to a UV dose of 100 mJ/cm^2, respectively, and using a least-squares regression fit to the data of Figure 3-2 and 3-7. The estimated values of k_a and k_d were 0.356 d^{-1} and 1.388 d^{-1}, respectively.

Model-predicted trophozoites reproduction curves are shown in Figure 3-9. Curves were generated for scenarios of 10^5 active trophozoites prior to UV exposure, $N_{T,0}$, and 10^1, 10^3, 10^4 and 10^5 survivors immediately following UV exposure, $N_{a,0}$. While the overall shapes of the model-predicted reproduction curves (Figure 3-9) and the observed reproduction curves (Figures 3-2 to 3-7) are similar, there are distinct differences between the two sets of curves that should be noted. For non-UV exposed trophozoites (i.e. $N_{a,0} = 10^5$), the trophozoite numbers in the model-predicted reproduction curves increased exponentially. For the UV-exposed trophozoites, the model-predicted curves are characterized by an initial rapid decrease in numbers as the death rate of UV inactivated trophozoites exceeds the rate of reproduction rate of the survivors. The rate of this initial decrease is the same regardless of the number of initial survivors, $N_{a,0}$. The

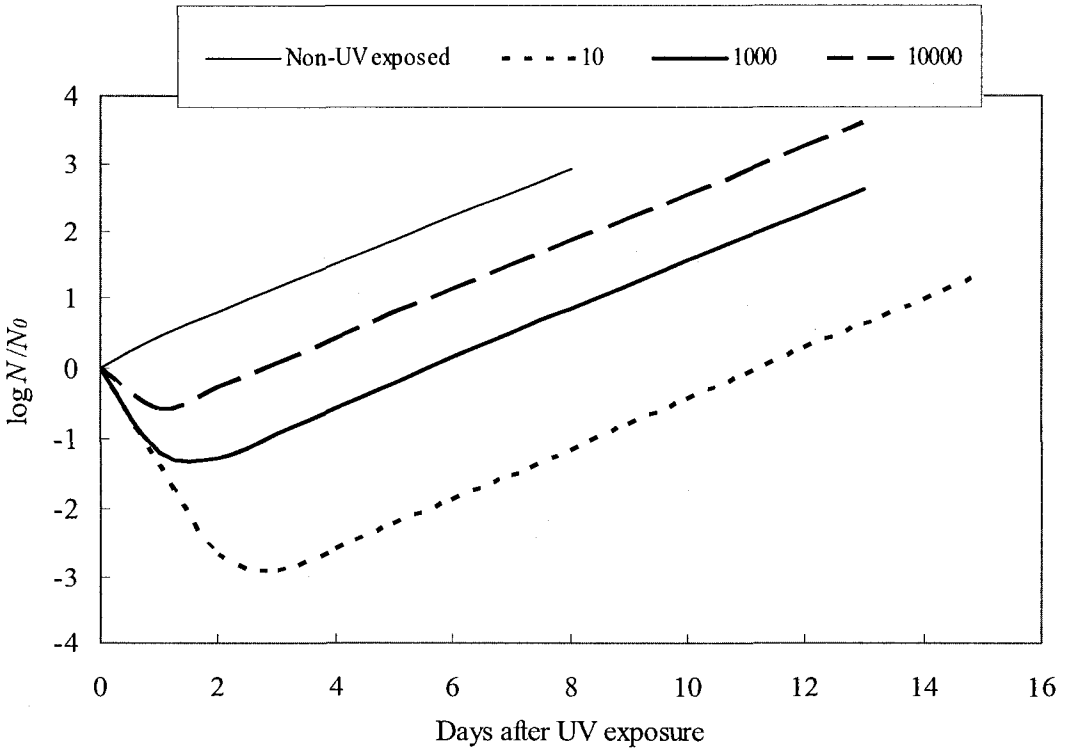

Figure 3-9. Model-predicted trophozoite reproduction after UV exposure assuming no reactivation due to DNA repair. The number of live trophozoites before UV exposure was 10^5. The legend numbers (10, 10^3 and 10^4) represent the number of active trophozoites that were assumed to remain after UV exposure.

trophozoite numbers then increase sharply as the reproduction rate of the surviving trophozoites exceeds the death rate of the inactivated trophozoites. The rate of increase is again, the same for all numbers of survivors.

In contrast, the rate of decline in trophozoite numbers in the first few days after UV exposure was different in the observed reproduction curves and was inversely proportional to the UV dose. In addition, the observed increase in trophozoite numbers after several days was much more gradual than the model-predicted increase. In the observed reproduction curves there was a delay period before the onset of the increase in trophozoite numbers during which the parasite numbers were relatively constant. This delay period was not present in the model-predicted curves. Finally, the observed trophozoite reproduction rate following recovery was inversely proportional to the UV

doses applied. This suggests that the shape of the observed trophozoite reproduction curves cannot be explained in terms of survivors alone, but, rather suggests involvement of a mechanism characterized by a lag phase such as reactivation by DNA repair.

3.4 Discussion

The cyst is the environmental stage of the *G. lamblia* parasite that is present in contaminated water and wastewater, while the trophozoite is primarily restricted to the intestinal tract of an infected host. Exposure of trophozoites to UV radiation during water and wastewater treatment, therefore, is unlikely to occur. This study presumes that the ability of the parasite to reactivate following exposure of the cyst to UV can be assessed by measuring the ability of the trophozoites to reproduce *in vitro.* When a mature cyst is ingested by a suitable host, excystation occurs and the trophozoite that is released divides into two binucleate trophozoites. A previously published study reported that exposure to the UV doses less than 80 mJ/cm^2 had little impact on *in vitro* excystation of *G. muris,* however, infectivity of the cysts in mice was reduced (Craik et al., 2000). The assumption of this study, therefore, is that exposure of *G. lamblia* cysts to the relatively low UV doses used in water and wastewater treatment (i.e. typically less than 100 mJ/cm^2) prior to ingestion will have little impact on excystation and subsequent division of the quadrinucleate cysts into binucleate trophozoites. The effect of UV is rather on the ability of the binucleate trophozoites to reproduce and cause infection since this phase involves DNA replication. At the doses typical of water and wastewater treatment, the germicidal effect of UV on microorganisms is believed to be the formation or pyrimidine dimers on the DNA resulting in inhibition of DNA replication (Oguma et al., 2001). If *G. lamblia* trophozoites are capable of multiplying under *in vitro* culture conditions following UV exposure, therefore, it was assumed that UV-exposed cysts will be able to multiply *in vivo* after ingestion by a susceptible host.

When cysts derived from fecal specimens are exposed to UV in Petri dish experiments, some parasites may receive a lower UV dose than others due to variations in the cyst wall thickness or adsorption of colloidal fecal material onto the surface of the cyst wall. Because trophozoites lack the protection of the cyst wall and are relatively

free of extraneous debris, the potential protective effect of the cyst wall is eliminated and the UV exposure is more uniform in trophozoite experiments.

After exposure to UV doses of 1 and 10 mJ/cm^2, the trophozoites were able to survive and reproduce in *in vitro* culture in all trials; however, there was a delay in the onset of reproduction. The initial reduction in trophozoite numbers within the first few days following UV exposure is consistent with a germicidal effect of UV on the parasites. Presumably, the trophozoites that did not divide and multiply successfully due to UV-induced DNA lesions died and lysed. The increase in trophozoite numbers that began 5 to 7 days after exposure to UV doses of 1 and 10 mJ/cm^2 may have been due to the division of a small number of survivors that somehow escaped full UV exposure or were part of a more UV resistant sub-population that were not inactivated. Or, the increase in trophozoite numbers may have been the result of dark repair of DNA lesions and reactivation of parasites that were temporarily inactivated by UV exposure. The delay in the increase in trophozoite numbers observed in these experiments suggests the involvement of a mechanism characterized by a lag phase, such as reactivation by DNA repair. This finding is consistent with the reactivation measured by animal infectivity assays for *G. muris* (Belosevic et al., 2001) and for *G. lamblia* (Shin et al., 2005). Shin *et al.* (2005) reported that *G. lamblia* cysts may repair their DNA and reactivate after exposure 1 mJ/cm^2 of UV radiation. In the present study, trophozoites exposed to 1 mJ/cm^2 of UV reproduced *in vitro* after a delay period of about 5 days.

The experimental findings of this study indicate that *G. lamblia* trophozoites may survive and potentially reactivate after exposure to UV doses as high as 10 mJ/cm^2. At UV doses of 20 mJ/cm^2 and 40 mJ/cm^2, on the other hand, there was an increase in trophozoite numbers in some of the trials but not in others. At 20 and 40 mJ/cm^2, the results were ambiguous and could not be statistically tested. At the highest UV dose of 100 mJ/cm^2, trophozoite numbers decreased rapidly to less than detection limits and there was no evidence of survival or reactivation after 15 days.

If there is a lag period in the division as shown in Figure 3-3 to Figure 3-7, trophozoites that emerge from the cysts after UV exposure may remain in the intestine or may be flushed out before they have an opportunity to reactivate. However, the pattern of cyst shedding in gerbils inoculated with trophozoites exposed to 1 mJ/cm^2 UV dose

was statistically identical to that of gerbils inoculated with trophozoites that were not exposed to UV (Figure 3-8). This suggests that trophozoites exposed to this relatively low UV dose will likely remain in the intestine during the lag phase, perhaps by attachment to enterocytes, and following recovery may be able to establish infection in the host. Although this experiment was limited to a single UV dose, trophozoite reproduction using the *in vitro* model seems to be a reasonable predictor of the ability of UV-exposed trophozoites to cause infection in a host. It also suggests that the survival and reproduction of UV-exposed trophozoites *in vitro* is consistent with infection and may have public health significance, at least at low UV doses.

In conclusion, the results of this study suggest that, while the ability of *G. lamblia* trophozoites to reproduce *in vitro* is clearly impaired, UV radiation may not completely eliminate the ability of the trophozoite stage of the parasites to reproduce *in vitro* at UV doses up to 10 mJ/cm^2. Reactivation *G. lamblia* after UV exposure was reported in previous studies and the ability of *G. lamblia* trophozoites to survive and divide *in vitro* after exposure to UV doses of up to 10 mJ/cm^2 was demonstrated statistically in this study. *G. lamblia* cysts exposed to UV doses of up to 10 mJ/cm^2 may still be able to cause infection in humans. At UV doses of 20 and 40 mJ/cm^2, evidence of survival or reactivation was ambiguous and statistically inconclusive, while at 100 mJ/cm^2 there was no evidence of survival or reactivation.

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4 INFECTIVITY OF CYSTS FROM UV TREATED WASTEWATER[3]

4.1 Introduction

*Giardia lamblia i*s a protozoan parasite that is known to cause waterborne outbreaks (Craun, 1990). Studies have shown that *G. lamblia* cysts, the environmentally resistant form of the parasite, are common in raw sewage as well as treated wastewater (Wallis et al., 1996). The need to remove and inactivate protozoan cysts from wastewater for protection of public health is underscored by observation of high prevalence of the parasites in non treated wastewater (Garcia et al., 2002; Quintero-Betancourt et al., 2003; Robertson et al., 2000). Although current wastewater treatment processes are reasonably effective in removing or inactivating most of the parasites (Caccio et al., 2003), there are reports indicating that the number of *G. lamblia cysts* present in effluents discharged from different wastewater treatment facilities may be high (Casson et al., 1990; Medema and Schijven, 2001). Municipal wastewater is usually the main source of *G. lamblia* cysts in waterborne outbreaks of this infection (Wallis et al., 1998).

Ultraviolet (UV) treatment is rapidly becoming the predominant method of wastewater disinfection in North America. It has now been established in laboratory studies, using collimated beam UV reactors and animal infectivity assays, that UV is effective against protozoan parasites such as *Giardia* spp. at UV doses that are readily achievable in wastewater treatment systems (Craik et al., 2000; Mofidi et al., 2002). The UV dose response curve is generally characterized by two regions depending on the target microorganism: a first order inactivation region at relatively low UV doses that represents the inactivation of dispersed organisms and a tailing region at high UV doses that attributes to the association of microorganisms with particles (Emerick et al., 1999; Qualls et al., 1983). The tailing region typically occurred after about 3 to 4 log inactivation of coliform bacteria (Qualls et al., 1983) and *Giardia muris* (Craik et al.,

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[3] A version of this chapter "Infectivity of *Giardia lamblia* cysts obtained from wastewater treated with ultraviolet light" has been submitted for publication in *Water Research* and is currently in revision.

2000). To date, the effectiveness of full-scale wastewater UV reactors for inactivation of protozoan parasites under field conditions has not been measured directly. The inactivation efficiency under field conditions may be limited by factors such as the presence of suspended solids in wastewater effluent, UV reactor hydraulic conditions, and strain-to-strain variation in UV sensitivity. Furthermore, *G. lamblia* cysts are exposed to environmental stressors during wastewater treatment processes, which may influence their susceptibility to UV inactivation.

The objective of this study was to assess the infectivity of *G. lamblia* cysts in wastewater treatment processes and the effectiveness of UV inactivation under field conditions. The approach was to concentrate and purify *G. lamblia* cysts from four separate wastewater effluents immediately before and after UV treatment in full-scale UV reactors and in primary effluent. The infectivity of the cysts recovered from wastewater effluents was assessed using the Mongolian gerbil model (Belosevic et al., 1983). The main premise was that cysts are present in sufficient concentration in secondary wastewater to permit a direct determination of infectivity reduction using established infectivity assays.

4.2 Materials and Methods

4.2.1 Wastewater Treatment Plants

The field study was carried out at four municipal wastewater treatment plants (WWTP) in Western Canada: the Gold Bar WWTP in Edmonton, Alberta, the Raymer Avenue WWTP in Kelowna, British Columbia, the South End WWTP in Winnipeg, Manitoba, and the Alberta Capital Region WWTP located in Strathcona County outside Edmonton, Alberta. All the secondary effluent samples were obtained during spring and summer of 2004 and 2005 except for one winter sampling event for Edmonton in 2004. The primary effluent samples were taken during spring and summer in 2006. Samples from the Edmonton and Alberta Capital Region facilities were transported directly to the University of Alberta Environmental Engineering laboratory and were processed on the same day. Samples taken from Kelowna and Winnipeg were placed on icepacks and were shipped to University of Alberta by overnight courier and processed within 48 to 72

hours. The UV dose of the field reactors and the UV transmittance of wastewater (@ 254 nm) were recorded from the UV reactor control panel during sampling. Information on the total suspended solids (TSS), fecal coliform (FC) and total coliform (TC) concentrations in the secondary wastewater for each sampling day were provided by the respective WWTP laboratories. Table 4-1 summarizes the treatment processes and the characteristics of the treated wastewater at the four sites. The UV reactor at Winnipeg was operated seasonally between May and September; while the UV reactors at the other three WWTPs were operated year-round. The total suspended solids (TSS) in the treated wastewater varied considerably between study sites, ranging from as low as 0.1 mg/L at the Kelowna WWTP, a biological nutrient removal (BNR) plant equipped with sand filtration, to as high as 19 mg/L at Winnipeg WWTP, a conventional activated sludge plant. The fecal coliform and total coliform concentrations in the wastewater prior to UV treatment also varied from site to site and were correlated with TSS. The highest coliform concentrations in this study were observed downstream of the UV reactor at Winnipeg and were related to a relatively high wastewater effluent flow rate during the sampling event. During this sampling event, the wastewater flow rate was 126,000 m^3/d which was considerably greater than the UV reactor design flow rate of 70,000 to 100,000 m^3/d.

4.2.2 Cyst Sampling and Purification

Between 90 and 1200 L of secondary wastewater effluent samples were pumped (1 to 4 L/min) directly from the wastewater channels at points both immediately upstream and downstream of the UV reactors according to U.S. EPA method 1623 (U.S. Environmental Protection Agency, 2001) with the following modifications. The wastewater was filtered through FiltaMax™ foam filters (IDEXX Laboratories, Westbrook, Maine). For each sampling event, two filters were used to collect and concentrate the samples from both upstream and downstream of the UV reactors. The particulate material captured on the filters was eluted and concentrated in the laboratory.

Primary effluent grab samples (4 to 8 L) were collected from each WWTP. Tween 80 solution was added to each 800 mL sub-sample to yield a concentration of 1% and the sample was then homogenized using a tissue homogenizer (Powergen 700, Fisher

Table 4-1. Wastewater treatment plants characteristics and average wastewater quality

Sampling sites		Edmonton, AB	Kelowna, BC	Winnipeg, MB	Alberta Capital Region, AB
Secondary treatment process		BNR and Clarifier	BNR, Clarifier and Sand filtration	Activated sludge and Clarifier	BNR and Clarifier
UV system		Trojan UV4000^{TM} MP lamp system	Trojan UV3000^{TM} LP lamp system	Trojan UV4000^{TM} MP lamp system	Wedeco LP high intensity lamp system
Number of sampling events		5	6	2	2
UV dose recorded on-site (mJ/cm^2)		23 to 33	50	18.6 to 50	35 to 57
TSS (mg/L)		4 to 5	0.3 to 1.2	8 to 19	3 to 4
UV transmittance (@254 nm, 1cm)		58% to 64%	63% to 65%	51% to 65%	51% to 58%
FC	U/S of UV	8,600 (5,900 to 20,000*)	1,500 (0 to 3,000[&,#])	230,000[&]	30,000 (16,000 to 56,000*)
	D/S of UV	28 (12 to 52*)	1 (0 to 1[&,#])	4,300[&]	250 (210 to 320*)
	Log reduction	2.5±0.3	3. 6±0.4	1.7	2.1±0.3
TC	U/S of UV	128,000 (110,000 to 300,000)	25,700 (8,600 to 110,600)	1,500,000[$]	193,000 (120,000 to 310,000)
	D/S of UV	490 (380 to 1,770)	1 (0 to 5.4)	9,300[$]	220 (20 to 2,300)
	Log reduction	2.4±0.4	4.4±0.4	2.2	3.0±1.3

LP = low-pressure; MP = medium-pressure; LP -HI = low pressure-high intensity; * CFU/100 mL; [&] MPN/100 mL;

[#] Measured as *E. coli*; [$]There was a laboratory error with this TC test and the results are considered presumptive only.

Scientific, Nepean, Ontario) at a speed of 13,500 rpm for 3 min. Samples were concentrated by centrifugation at 1500 × g for 15 min @ 4°C in a 200 mL conical centrifuge tubes. The pellets from two tubes were combined and transferred to a flat-sided Dynal® L10 tube (Cat. 740-03, Dynal).

The samples were further purified using immunomagnetic separation (IMS) techniques and the anti-*Giardia* Dynabeads® GC-Combo kit (Cat.730-12, Dynal, Brown Deer, WI). For primary effluent concentrate, before adding 100 µL of anti-*Giardia* Dynabeads into each Dynal® L10 tube containing wastewater concentrates, 0.75 g kaolin (Sigma Chemical, Inc., catalog no. K7375) was added to each tube to improve cyst recovery as described by McCuin and Clancy (2005). The flat-sided tubes were rotated for 2 hours on a Dynal Sample Mixer (Cat. 947-01, Dynal, Inc., Lake Success, NY), and two acid dissociation steps with 100 µL of 0.1 N HCl were performed to maximize the recovery of cysts from the wastewater. The final volume (440 µL) was diluted to 2.5 mL using de-ionized water produced from a Millipore™ Academic system (Fisher Scientific, Whitby, ON) for microscopic enumeration of the cysts and for inoculation of gerbils. The concentration of *Giardia* spp. cysts in the diluted pellet was determined by immunofluorescence assay (IFA) using a direct antibody (Aqua-Glo G/C direct, Waterborne Inc., New Orleans, LA) and enumeration using epifluorescent microscopy at 200X magnification (Microphot-FXA, Nikon).

4.2.3 Cyst Recovery

The recovery of the analytical method was determined by spike tests using known numbers of formalin fixed and preserved *G. lamblia* cysts (Waterborne Inc., New Orleans, LA) as stated in Method 1623 (U.S. Environmental Protection Agency, 2001). For recovery tests using wastewater matrices, the spiked cysts were stained with Texas Red dye (Sigma Chemical, Inc., catalog no. 86185) in order to differentiate them from indigenous cysts. The average recovery was 75% (ranging from 71 to 83%) for *G. lamblia* cysts seeded into de-ionized water by the IFA procedure alone. The average cyst recovery was 39% for cysts spiked to DI-water and 23% for cysts spiked to wastewater effluent.

4.2.4 Measurement of Cyst Infectivity

The infectivity of cysts recovered from wastewater concentrates was determined using the Mongolian gerbil model described by Belosevic et al. (1983). Briefly, male gerbils, 4 to 6 weeks old were purchased from the Charles River Laboratories (Kingston, NY) and were housed in filter top cages, two gerbils per cage. Cohorts of 10 gerbils were inoculated with 0.2 mL of the purified wastewater concentrate containing *Giardia* spp. cysts by gastric incubation using a blunt needle. Feces were collected from individual gerbils daily starting on day 6 post-infection and were examined for the presence of cysts using microscopy. The total number of cysts released in a 2-hr fecal collection (8 to 10 AM) by individual gerbils was determined using the sucrose flotation method as described by Belosevic et al. (1983).

All gerbils were terminated and examined for the presence of trophozoites in the mucosal layer of the small intestine between day 13 and 15 post inoculation. Briefly, the small intestine was divided into four equal segments and cut longitudinally. The mucosal layer was removed by scraping and was placed in 3 mL of phosphate-buffered saline (pH 7.2). After incubation for 30 min in an ice bath, the contents were mixed using a vortex mixer for 30 seconds and filtered through gauze. The filtrate was collected and centrifuged at 1,000 x g for 15 min @ 4°C. The supernatant was removed to 1 mL and the pellet was resuspended. The presence and activity of trophozoites were determined by examining the final suspension at 200X magnification using bright light microscope (Microphot-FXA, Nikon). Infection in the gerbils was determined by the either the presence of cysts in the feces, or the presence of trophozoites in the small intestine. It was previously shown that different levels of infections were observed in Mongolian gerbils infected with either fresh or UV-exposed *G. lamblia* cysts (Li et al., 2007). According to the scheme proposed by Li et al. (2007), the infections in individual gerbils inoculated with cysts isolated from wastewater were scored as strong, moderate, or weak. Strong infections were characterized as the presence of cysts in the feces. Moderate infections were characterized by the absence of cysts in the feces, but the presence of active trophozoites (flagellar movement), and weak infections were characterized by the absence of cysts in the feces, and the presence of only non-active trophozoites in the intestinal samples. No infections either strong, moderate or weak, were observed in any

of the negative control gerbils (not inoculated with cysts) in any of the experiments.

4.2.5 Genotyping of Giardia spp. Cysts

Giardia spp. cysts concentrated from primary effluents and cysts collected from the feces of gerbils that were previously inoculated with cysts concentrated from wastewater were genotyped by Dr. Norman F. Neumann, at Alberta Provincial Laboratory for Public Health, Calgary, Alberta, Canada. *Giardia* cysts isolated from wastewater were shipped on dry ice to Calgary by overnight courier services. The methods used to genotype the parasites were described by Anderson et al. (2006).

4.3 Results

4.3.1 Giardia spp. Cyst Concentrations

Cyst concentrations measured in the primary effluents at the four WWTPs were shown in Figure 4-1. The concentrations of *Giardia* spp. cysts detected in the secondary effluent, both upstream and downstream of the UV reactors at the four sites was summarized in Figure 4-2. By comparing the cyst concentrations measured in the primary and secondary effluents, it can be deduced that greater than 90% of the *Giardia* cysts present in the primary effluent were removed by subsequent treatments at the Edmonton, Capital Region and the Kelowna WWTPs. At the Winnipeg WWTP with conventional activated sludge (AS) treatment, the removal was approximately 80%. It should be noted that the cyst concentrations in the primary effluents and secondary effluents were not measured at the same time; therefore, these removal efficiencies can only be considered to be rough estimates. Similar variation in cyst removal efficiency for different WWTPs was also observed by Casson *et al.* (1990) and Robertson et al. (2000).

From May 2004 to June 2005, a total of 30 secondary effluent wastewater samples were collected from the four study sites for *Giardia* spp. cyst enumeration and infectivity determination. The cyst concentrations in samples collected from upstream and downstream of the field UV reactors were essentially the same based on a paired Student's t-test (p = 0.20). This finding was expected since UV treatment, at the doses typical for UV inactivation in wastewater treatment practice, only damages the DNA of

exposed cysts and does not alter the physical appearance of cysts.

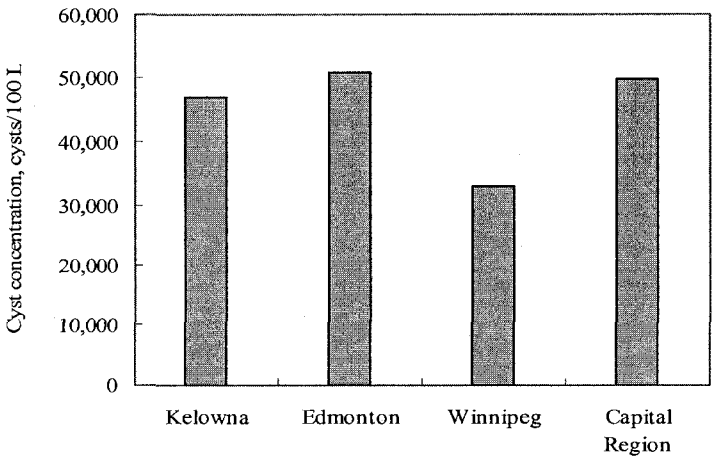

Figure 4-1. The number of *Giardia* spp. cysts in primary wastewater effluent.

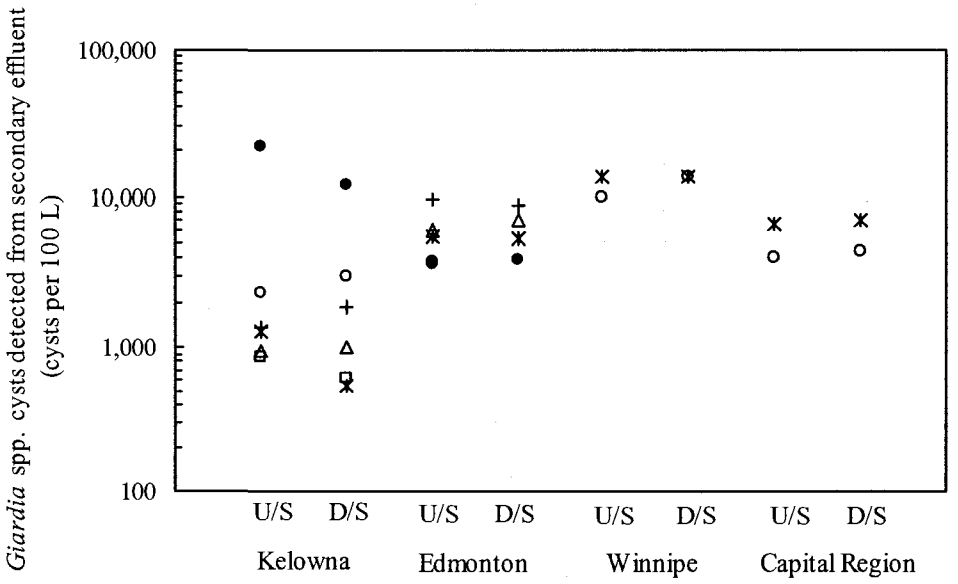

Figure 4-2. The number of *Giardia* spp. cysts in wastewater samples collected from upstream and downstream of full-scale UV reactors. For each study site, pairs of identical symbols (i.e. ● for U/S and ● for D/S) indicate that the samples were collected during the same sampling event. The numbers of cysts indicated in the figure were corrected for the method recovery efficiency of 39% measured in reagent-grade water. U/S donates upstream of the U/V reactor and D/S donates downstream of the UV reactor.

4.3.2 Inactivation of Giardia by Field UV Reactors

Giardia spp. cysts recovered from upstream and downstream of full-scale UV reactors were administered to cohorts of 10 gerbils each. The results of 15 sets of infectivity tests carried at the four WWTPs were summarized in Table 4-2. There were no cysts detected in the feces of gerbils inoculated by parasites obtained from the secondary effluent concentrates from any of the four WWTPs. Cyst production in infected gerbils is known to be intermittent (Belosevic et al. 1983); therefore, the lack of cyst production by itself does not ensure the absence of an infection in the gerbils. For this reason, the small intestines of the exposed gerbils were examined for the presence of trophozoites. Trophozoites were detected in the intestinal samples of most gerbils inoculated with cysts obtained from upstream of the operating UV reactors indicating the presence of infectious cysts in the wastewater. The infection intensity, however, was consistently low. For example, none of the trophozoites detected in the intestinal samples appeared to be viable or active upon microscopic examination. This was true for samples collected from both upstream and downstream of the UV reactors. The weak infections produced by cysts obtained from upstream of UV reactors indicated that some of the cysts in primary effluent survived the secondary wastewater treatment processes.

The overall infectivity of the UV treated cysts obtained from all of the wastewater samples collected from downstream of the UV reactors at the four WWTPs was only slightly lower than those collected from upstream of the UV reactors. Figure 4-3 depicts the overall infectivity ratio of cysts obtained from upstream and downstream of the UV reactors for each of the study sites. As shown in Figure 4-3, 86% of the 44 gerbils inoculated with cysts isolated upstream of the UV reactors at the Edmonton WWTP during five sampling events were scored positive for weak infection, whereas 76% of the gerbils inoculated with cysts collected from downstream of the UV reactors at Edmonton were scored for positive weak infections. Similar results were obtained at all four wastewater plants.

Table 4-2. Infectivity of *G. lamblia* cysts obtained from wastewater samples collected from upstream and downstream of full-scale UV reactors as measured by infectivity in Mongolian gerbils.

Wastewater Treatment Plant	Sampling date	Sampling location relative to UV reactor	Sample Volume (L)	Inoculum (cysts per gerbil)	Equivalent wastewater volume (L)	Number of gerbils infected/total inoculated
Edmonton	27/05/2004	Upstream	200	260	16	10/10
		Downstream	140	145	11	9/10
	14/06/2004	Upstream	200	260	16	10/11
		Downstream	120	80	10	8/11
	14/07/2004	Upstream	200	180	16	10/10
		Downstream	200	190	16	10/10
	05/08/2004	Upstream	190	460	15	9/10
		Downstream	190	420	15	6/10
	01/12/2005	Upstream	175	255	14	5/10
		Downstream	165	260	13	3/10
Kelowna	7/19/2004	Upstream	1135	270	91	9/10
		Downstream	1151	110	92	9/10
	3/14/2005	Upstream	365	1430	29	4/8
		Downstream	645	1410	52	3/8
	4/25/2005	Upstream	550	1840	44	10/10
		Downstream	510	240	41	10/10
	5/16/2005	Upstream	630	170	50	9/10
		Downstream	790	230	63	5/10
	6/6/2005	Upstream	836	40 / 100 *	67	8/10
		Downstream	897	40 / 130 *	72	4/10
	6/21/2005	Upstream	459	250	37	6/10
		Downstream	826	356	66	9/10
Winnipeg	09/08/2004	Upstream	190	485	15	9/10
		Downstream	160	670	13	4/10
	6/13/2005	Upstream	110	360	9	7/10
		Downstream	110	365	9	5/10
Alberta Capital Region	5/25/2005	Upstream	90	85	7	8/10
		Downstream	90	90	7	7/10
	6/01/2005	Upstream	120	205	10	7/10
		Downstream	120	190	10	7/10

Note: * cysts purified by sucrose flotation method / cysts purified by IMS method.

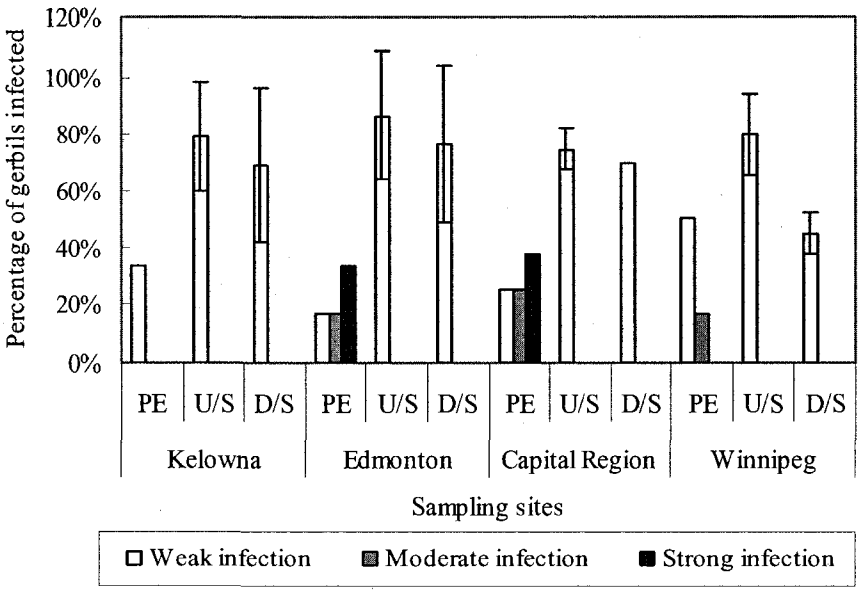

Figure 4-1. Summary of *G. lamblia* cysts infectivity in the Mongolian gerbils obtained from primary effluent, and from secondary effluent upstream and downstream of full-scale UV reactors. PE represents the primary effluent; U/S represents secondary effluent upstream of the UV reactors; D/S represents the secondary effluent downstream of the UV reactors. The error bar represents the standard deviation. No standard deviation was calculated for primary effluents because only one sample was collected for each site.

4.3.3 Infectivity of Cysts in Primary Effluent

To further investigate the weak infections of *Giardia* spp. cysts collected from upstream of the UV reactor, one grab sample of primary effluent was taken at each study site in 2006. The infectivity of cysts purified from primary effluents at four sites was also summarized in Table 4-3. *Giardia* spp. cysts collected from primary effluents at Edmonton and Alberta Capital Region were infectious to gerbils with strong and moderate intensities. *Giardia* cysts collected from Winnipeg and Kelowna were infectious to gerbils with moderate and weak intensities. This suggests that *Giardia* cysts in primary effluent are viable and capable of infecting gerbils. Figure 4-3 compares the infectivity of cysts obtained from primary effluent and secondary effluent.

Table 4-3. Infectivity of *Giardia* spp. cysts obtained from primary wastewater effluents measured using the Mongolian gerbil model.

Wastewater Treatment Plant	Number of gerbils inoculated	Inoculum (cysts per gerbil)	Infectious intensity [1]			Percentage of gerbils infected
			Strong infection	Moderate infection	Weak infection	
Edmonton	6	330 to 385	2	1	1	67%
Kelowna	6	340 to 570	0	0	2	33%
Winnipeg	6	210 to 360	0	1	3	67%
Capital Region	8	300 to 500	3	2	2	88%

[1] Intensities of infection were as described by Li et al. (2007)

4.3.4 Genotypes of Giardia Cysts

Genotype analysis of cysts concentrated from the primary effluents, and of cysts collected from the feces of gerbils inoculated with primary effluent cysts, demonstrated that *Giardia* spp. cysts detected in wastewater effluent were of the *G. lamblia* species (Table 4-4). *G. lamblia* assemblage A cysts were identified in the primary effluents from all four WWTPs; but assemblage B cysts were detected only in the feces of infected gerbils. This implies that cysts from both assemblage A and B were present in the primary effluent. Further, assemblage A cysts may have been present in larger numbers than assemblage B cysts, but the assemblage B cysts that were present were more infectious to the gerbils.

Table 4-4. Genotype analysis of *Giardia* spp. cysts obtained from primary effluents and from the feces of gerbils inoculated with cysts obtained from primary effluents.

Sampling Date	Facilities	Origin of the genotyped cysts	Genotype
03/23/06	Alberta Capital Region	Primary effluent (PE)	Assemblage A
		Feces of gerbil inoculated with cysts from PE	Assemblage B
		Feces of gerbil inoculated with cysts from PE	Assemblage A
04/27/06	Edmonton	Primary effluent	Assemblage A
		Feces of gerbil inoculated with cysts from PE	Assemblage B
05/02/06	Kelowna	Primary effluent	Assemblage A
05/11/06	Winnipeg	Primary effluent	Assemblage A

4.4 Discussion

The weak infections observed in gerbils exposed to cysts obtained from UV treated secondary wastewater may have been attributed to the survival cysts following UV exposure, the potential reactivation of cysts after UV exposure, or differential susceptibility of gerbils to different *Giardia* spp. present in wastewater. Alternatively, the sampling and processing procedures, particularly the IMS acid dissociation step, may have resulted in the damage of cysts prior to inoculation into gerbils. In a separate experiment, however, the IMS acid dissociation step was found to have a statistically insignificant effect on the infectivity of *G. lamblia* cysts in the Mongolian gerbils. At an inoculum of 10,000 cysts, 6 out of 8 gerbils inoculated with cysts concentrated using the IMS method were infected, whereas 7 out of 8 gerbils inoculated with cysts concentrated using the sucrose method were infectious. The difference was statistically insignificant at the 95% level based on a Student's *t-test* ($p = 0.49$).

Germicidal UV light (UVC and UVB, 200-320 nm) light inactivates microorganisms by damaging their DNA due to the formation of pyrimidine dimers between adjacent thymine molecules. These dimer lesions inhibit the normal replication of the genome and result in inactivation of the microorganisms (Harm, 1980). In addition to DNA, proteins and enzymes with unsaturated bonds are also known to absorb UVC and UVB, which may result in significant damage to the organisms (Kalisvaart, 2001). The effectiveness of UV radiation on inactivating microorganisms depends on three factors: the penetration of UV radiation through the cell wall, the efficiency of producing DNA damage that could cause inactivation, and the DNA repair capacity of organisms. In wastewater effluent, UV radiation may not reach and penetrate into the cyst wall to cause the DNA damage due to the shielding effect of wastewater particles and this may result in the survival of *Giardia* spp. cysts in UV reactor effluent. *Giardia* spp. cysts in wastewater may exist either as individual, mono-dispersed cysts or in association with wastewater flocs. Based on previous laboratory UV exposure studies of *Giardia* spp. cysts (i.e. Craik et al., 2000, Linden et al. 2002), most of the mono-dispersed cysts present in the secondary effluent wastewater should have been readily inactivated at the UV doses recorded on the UV reactor control panels during the sampling events (Table 4-1). These doses, however, may not have been sufficient to inactivate *Giardia* spp. cysts protected

by particles or sub-populations of cysts with higher resistance to UV radiation. During the cyst purification procedure, cysts attached to or contained within wastewater particles were likely separated from those particles and this may have accounted for the increase in the number of survivors in the inoculums to gerbils. Alternatively, bacteria and/or colloidal organic material present in the wastewater may have attached to the surface of cysts such that the cysts were covered by a thin layer of biofilm (Luchtel et al., 1980). This film may have partially hindered the penetration of UV radiation to the cyst wall and resulted in increased resistance of the cysts to UV. It is also possible that some cysts present in wastewater were intrinsically more resistant to UV reactors due to a thicker cell wall.

Reactivation, where some of the cysts exposed to UV were able to repair their damaged DNA after ingestion by the host, is another potential explanation for the infections in gerbils inoculated with cysts collected from downstream of the UV reactors.. The results of previous studies on reactivation have differed, some showing that *Giardia* spp. can reactivate *in vivo* (Belosevic et al., 2001; Shin et al., 2005), while others showed no evidence of reactivation (Linden et al., 2002). In one study, *G. lamblia* cysts were able to repair their UV-damaged DNA after being exposed to a UV dose of as low as 1 mJ/cm^2 (Shin et al., 2005). In another study, evidence of *in vivo* reactivation of *Giardia muris* cysts exposed to a UV dose of 25 mJ/cm^2 was observed in three out of seven individual experiments, as measured by infectivity in mice (Belosevic et al., 2001). This suggests that the cysts have the ability to reactivate; however, they may not be able to complete their life cycle in a healthy host. A recent study reported that *Giardia* spp. cysts, collected from an activated sludge treatment plant either before or after UV treatment, were infectious to immunodeficient BABL/c nude mice (Neto et al., 2006).

In a classic study using volunteers, as few as ten *G. lamblia* cysts was reported to cause infection in two healthy humans (Rendtorff and Holt, 1954). The infectious dose causing 50% host infected (ID_{50}s) for Mongolian gerbils was variable depending on the studies and isolates used. The ID_{50} in gerbils was reported to be 250 cysts based on 10 different *G. lamblia* isolates including WB isolate (Finch et al., 1993). Similar results were obtained for WB isolate cysts by Campbell and Wallis (Campbell and Wallis, 2002). However, 50% infectious doses were estimated to be less than ten for H3 isolate

cysts (Garcia et al. 2002) and as few as five CH3 isolate cysts caused infection in gerbils (Linden et al. 2002). In the present study, the average number of cysts in the gerbil inoculum was approximately 320 $\pm$ 150 cysts for wastewater effluent both upstream and downstream of UV reactors. If these were fresh cysts produced from gerbils, at least 50% of gerbil would be expected to be infected with either strong or moderate infections. However, this level of infection was not observed in the gerbils inoculated with cysts concentrated from the wastewaters. Different levels of intensity of infection, based on the presence of cysts in the feces and the observation of trophozoites detected from the intestine of infected gerbils, was previously reported (Li et al., 2007). The infections were scored into three levels: strong infection (cysts and free swimming trophozoites detected), moderate infection (only free swimming trophozoites detected), and low intensity infection (non-active trophozoites detected). Generally, previous *G. lamblia* UV studies assessed infections in gerbils based only on the presence of cysts in feces and free swimming trophozoites in the intestinal samples. As long as one live swimming trophozoite was observed, there was no need to examine further and the gerbils were considered as positive for infection. However, the detection of non-active trophozoites is difficult and time-consuming, and requires careful examination of the complete grid of a hemocytometer slide. Weak infections have been observed in the gerbils inoculated with low numbers of freshly prepared cysts (Li et al. 2007), which may suggest that the weak infections depend more on the biological differences between cyst isolates and differences in the response of the gerbil immune system to the infection. As expected, the indigenous *G. lamblia* cysts in wastewater effluent were comprised of different isolates. Different genotypes of *G. lamblia* isolates have different surface-antigens (Nash and Keister, 1985). *Giardia* spp. cysts possessing different surface antigens have different patterns of infection and induce qualitatively and quantitatively different immune responses in the host (Aggarwal and Nash, 1987; Nash et al., 1987).

It is also possible that the non-active trophozoites detected in the intestines may have originated from the original inoculum and may not have been the result of division occurring post-inoculation – that is, they were not the result of true infection. However, given the sensitivity of microscopic determination of the presence of trophozoites in the small intestines (~ 1.25 x 10^3 trophozoites), and the numbers of trophozoites observed in

the intestines higher than the number of trophozoites excysted from cysts after fed to the gerbils, it is highly unlikely that the trophozoites detected in the intestinal sample were derived from the initial inoculum. Thus, the non-active trophozoites more likely originated from the division of trophozoites excysted from inoculated cysts (Li et al., 2007). UV exposed cysts are known to have the ability to excyst after relatively low doses of UV. Live *G. muris* trophozoites that were able to excyst in an *in vitro* excystation assay, were unable to reproduce in a mouse assay (Craik et al., 2000). However, the more recent research found that the trophozoites exposed to low UV doses had the ability to reproduce in *in vitro* culture and also caused infection in gerbils (Li et al., 2008). The observation of trophozoites in the intestines suggests that excystation occurred and some of the released trophozoites had the ability to reproduce *in vivo* after UV exposure.

The infectivity of cysts from Winnipeg and Kelowna might have been affected by the shipping and storage conditions. The cysts from Edmonton and Capital Region were processed and inoculated into gerbils within 24 hrs after collection. However, the cysts from Kelowna and Winnipeg were inoculated to gerbils between 72 and 96 hrs after collection. Method 1623 (U.S. Environmental Protection Agency, 2001) states that bulk samples or samples filtered in the field must be shipped at 0 to 8°C and eluted within 96 hours. However, it was practically impossible to comply with the sample holding conditions specified in the EPA methods, particularly those specified in the description of sample collection and storage. Samples from Kelowna and Winnipeg frequently failed to reach the laboratory within 24 h of collection, and the temperature at which the sample was held could not be controlled. Further, the U.S. EPA Method 1623 procedure only considers the effect of the sample holding time on the recovery of enumerated *Giardia* and *Cryptosporidium*, and does not account for possible effect on the infectivity of parasites. As measured *in vitro*, *G. lamblia* cysts can survive up to 77 days in tap water at 8°C (Bingham et al., 1979) and *G. muris* cysts remained viable for approximately 1 month at 15 to 20°C in lake water (deRegnier et al., 1989). However, these studies are not related to the effect of extended sample holding time on the infectivity of detected cysts.

Somehow, *Giardia* spp. cysts were damaged by the environmental stresses in secondary wastewater treatment process, such as sludge recycle, exposure to aerobic or anaerobic condition, or temperature. Only decreased water temperature (<10° C) was consistent with prolonged survival of *Giardia muris* in different types of environmental water (deRegnier et al., 1989). *G. muris* showed no sign of viability after 14 days suspended in tap water and 28 days in river water, measured by mouse infectivity. Considering the temperature of wastewater samples during the experiments (average 18°C), and the average time it might take for a single cyst to pass through the biological nutrient removal (BNR) process and activated sludge (AS) processes (for example, the solid retention time for the Kelowna BNR is 22 days in summer and 35 days in winter), some *Giardia* cysts may lose their infectivity during the biological treatment process. Thus, it is not surprising to see that the infectivity of cysts in primary effluent was reduced by BNR or AS processes.

Previous studies showed that not all *G. lamblia* cysts have the same ability to establish infection in gerbil hosts and in humans (Nash et al., 1987); and the infectious levels were different between 12 strains of *G. lamblia* cysts (Visvesvara et al., 1988). As discussed earlier, the reported ID_{50}s were different for various isolates, and ranged from 250 cysts for assemblage A to as low as 5 cysts for assemblage B. Two commonly used human-derived *G. lamblia* cysts, the WB and H3 isolates, had different susceptibility in gerbils after exposed to UV irradiation (Li et al., 2007). This might be explained by the innate biological differences in gene sequence between these two isolates, as the WB isolate belongs to assemblage A and the H3 isolate belongs to assemblage B. Selectivity may also occur when using the gerbil model to assess infectivity (Visvesvara et al., 1988). However, these genotyping results might explain the weakly infectious cysts in the Kelowna and Winnipeg primary effluents. It was recently reported (Li et al., 2007) that the proportion of strong , moderate and weak infections are different for different strains of *Giardia* cysts, as observed in laboratory studies. The WB strain of *G. lamblia* cysts (assemblage A) was less infectious than the H3 strain (assemblage B) cysts as measured by the Mongolian gerbil model. Differential infectivity was also reported when human volunteers were infected with cysts from different genotype groups of *G. lamblia*, and assemblage A (WB isolate) cysts were less infectious than assemblage B

(GS/M isolate) cysts (Nash et al., 1987). The genotyping results suggest that geographic differences in cyst genotype make-up may have existed between the four municipalities studied. As shown in Table 4-4, Edmonton and Capital Region primary effluents contained both assemblage A and assemblage B in the wastewater and these two wastewater treatment plants served populations that are in adjacent geographic areas. Only assemblage A *G. lamblia* cysts were detected in Kelowna and Winnipeg primary effluents, and no cysts were detected in the feces of inoculated gerbils. This may be another reason for the detection of low intensity infection cysts from Kelowna and Winnipeg primary effluent. The primary effluents at Edmonton and Capital Region contained a mixture of assemblage A and B and resulted in stronger infections in the gerbils than cysts from Kelowna and Winnipeg primary effluents. It is not known if a synergic effect exists on the infection pattern of gerbils when more than one strain of *Giardia* spp. cysts are inoculated to the gerbils simultaneously.

Comparing the total infectivity of *G. lamblia* cysts in primary effluent and secondary effluent before UV disinfection (Table 4-2 and Table 4-3), the infection detected in gerbils inoculated with cysts concentrated from secondary effluent was only weak with the absence of cysts in feces and/or active trophozoites in the small intestine of gerbils. However, the cysts collected from Edmonton, Alberta Capital Region and Winnipeg primary effluents were strongly or moderately infectious to gerbils with the presence of cysts in the feces and/or active trophozoites in the small intestine of gerbils. As seen in Table 4-2 and Table 4-3, the numbers of cysts present in the inoculums were similar for both primary effluent and secondary effluent cysts. This suggests that at three out of four wastewater treatment plants, secondary wastewater treatment processes not only reduced the number of cysts in wastewater (Casson et al., 1990), but also damaged the cysts and reduced the intensity of infectivity of *Giardia* spp. cysts in gerbils. However, the cysts that survived the secondary wastewater treatment may have had a greater resistance to UV irradiation and/or a superior reactivation capability. The results of previous studies on reactivation differed, some showing that *Giardia* spp. can reactivate *in vivo* (Belosevic et al., 2001; Shin et al., 2005), while others showed no reactivation (Linden et al., 2002). The commonly recognized mechanism is that UV irradiation damages the DNA of cysts and the parasites lose the ability to replicate within the host. When these surviving cysts

were ingested by the host, they excysted (Craik et al., 2000) as UV doses have little impact on excystation and subsequent division of the quadrinucleate trophozoites into two trophozoites. Furthermore, *G. lamblia* trophozoites are capable of multiplying under *in vitro* culture conditions following UV exposure (Li et al., 2008). This suggests that the UV exposed cysts were capable of multiplying *in vivo* after ingestion by a host. This conclusion was consistent with a previous publication (Garcia et al., 2002) that the infectivity of *Giardia* spp. cysts was reduced by the secondary wastewater treatment processes. These researchers reported that gerbils were infected with 300 cysts from primary effluent; however, gerbils were not infected by 200 and 1000 cysts from final chlorine disinfected effluent.

The presence of cysts in wastewater discharges alone does not necessarily imply a human health risk. To cause disease, the parasites must be viable and infectious. Other factors, including the usage of the wastewater effluent, the characteristics of the receiving water body, background concentration of parasites in the receiving water, and precipitation and runoff in the watershed, may affect the risk of contact with *Giardia* cysts in wastewater effluent discharged into water bodies. Rose et al. (1991) estimated a 4.8×10^{-3} yearly risk of infection after ingestion of treated drinking water using a risk assessment model defined by a dose-response curve developed from human feeding studies for *Giardia* and assuming 2 L of water consumption per day. Ryu et al. (2007) performed a quantitative risk assessment for *Giardia* and *Cryptosporidium* in non-potable tertiary treated reclaimed water and estimated that the risk of infection for *Giardia* resulting from accidental consumption of 100 mL reclaimed water could meet the annual acceptable risk of 1.0×10^{-4} at sites that used a combination of chlorination and UV treatment, but not at sites that used only chlorination treatment. The weak infections detected in the gerbils inoculated with *Giardia* cysts concentrated from secondary effluent at the four wastewater treatment plants in the present study does not necessarily mean that these cysts would infect human hosts. Considering the equivalent volumes of wastewater administered to the gerbils (i.e. 7 to 92 L), it seems unlikely that exposure to the UV treated wastewater would produce giardiasis in a healthy human host. However, the risk for immunodeficient individuals may not be eliminated completely.

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5 EFFECT OF PARTICLE ASSOCIATION ON UV INACTIVATION USING SPORE-FORMING BACTERIA AS A SURROGATE[4]

5.1 Introduction

Particles in water and wastewater matrices have been shown to influence the inactivation of bacteria by ultraviolet (UV) irradiation (Emerick et al., 1999; Mamane and Linden, 2006; Qualls et al., 1983; Uvbiama and Craik, 2005). Based on laboratory studies, UV treatment is known to effectively inactivate pathogenic protozoan parasites, such as *Giardia* spp. and *Cryptosporidium* spp. at UV doses commonly used in disinfection of secondary municipal wastewater (Clancy et al., 1997; Craik et al., 2000). Microorganisms often aggregate or are associated with particles in wastewater, which may reduce the effectiveness of the UV treatment. The extent of aggregation and particle association of *C. parvum* and *G. lamblia* (oo)cysts in municipal wastewater and the effect of this aggregation on UV inactivation are not known.

It has been shown that coliform bacteria and virus are associated significantly with wastewater particles (Hejkal et al., 1981; Loge et al., 2002; Parker and Darby, 1995). Particle-associated organisms are less easily inactivated by UV radiation than are dispersed organisms in wastewater (Darby et al., 1993; Emerick et al., 1999; Madge and Jensen, 2006; Qualls et al., 1983). One important difference between coliform bacteria and protozoan parasites *C. parvum* and *G. lamblia* present in wastewater is that coliform bacteria are in a vegetative state and may actively attach to and colonize the surfaces and interior of wastewater particles. Protozoan parasites, in contrast, are present in a dormant encysted state and may be less likely to attach to particles during treatment.

Őrmeci and Linden (2003) suggested that *C. parvum* oocysts are associated with particles in significant numbers in wastewater effluent. However, another study reported that particle-associated *C. parvum* oocysts were not detected in municipal secondary

[4] A version of this chapter "The Assessment of Particle Association and UV Disinfection of Wastewater Using Indigenous Spore-forming Bacteria" was submitted to *Water Research*.

wastewater effluent (Tsuchihashi et al., 2003). Direct measurement of the association between particles and parasites present in wastewater was limited by the detection limit of the methods used and the relatively low concentrations of indigenous *G. lamblia* and *C. parvum*. To overcome these limitations, the potential for parasite-particle association has been studied by seeding wastewater samples with parasites. Medema et al. (1998) reported that seeded (oo)cysts readily attached to biological particles present in wastewater effluent. Amoah et al. (2005) seeded *C. parvum* oocysts and *G. muris* cysts into suspensions of particulate matter concentrated from a lake. They observed a small but statistically significant reduction in UV inactivation of the parasite when the particulate matter concentration was high even after the UV dose was adjusted for increased absorbance due to the presence of the particles. Other studies have indicated that turbidity up to 10 NTU does not impact the effectiveness of UV on the inactivation of spiked organisms in filtered drinking water or unfiltered raw water (Batch et al., 2004; Craik et al., 2002; Linden et al., 2002; Passantino and Malley, 2001). Seeding artificially high numbers of parasites into water or wastewater matrices, followed by simple mixing, however, may not accurately simulate the state of indigenous parasites and the interaction with particle matter that occurs in treatment processes.

More recent studies have attempted to simulate the interaction between microorganisms and particulate matter in drinking water treatment processes. One set of studies, *Bacillus subtilis* spores (Uvbiama and Craik 2005) and *C. parvum* oocysts (Mahmud, 2006) were added to raw river water, which was subsequently coagulated with alum, settled and then filtered. The microorganisms in the filtered water were found to be associated with particulate matter, and consequently, the level of inactivation by UV was reduced. In another study, viral phage particles coagulated with alum in the presence of humic substances were found to have increased resistance to UV (Templeton et al., 2005).

The objective of this research was to investigate the potential effect of parasite-particle association in municipal wastewater effluent on UV inactivation by using indigenous aerobic spore-forming (ASF) bacteria as surrogate microorganisms. The concentration of live indigenous spore-forming bacteria present in samples of wastewater collected upstream and downstream of operating UV reactors in two municipal

wastewater treatment plants was measured. The ASF bacteria are more appropriate surrogates for *G. lamblia* and *C. parvum* cysts than are coliform bacteria, because, unlike coliforms, they are present in a dormant inactive state.

5.2 Methods and Materials

5.2.1 Wastewater Treatment Plants

Wastewater effluents samples were collected from points both upstream and downstream of operating UV reactors from the Edmonton Gold Bar (EGB) wastewater treatment plant in Edmonton, Alberta, Canada and the Alberta Capital Region (ACR) wastewater treatment plant in Strathcona County, Alberta, Canada between June and August 2006. All samples were stored at 4°C before analysis. The wastewater treatment processes in these two plants are: screening and grit removal, primary clarification, biological nutrient removal (BNR), secondary clarification and final UV disinfection. The UV reactor system used in EGB was a Trojan 4000^{TM} medium pressure lamp system, with a design UV dose of 23 mJ/cm^2. A Wedeco TAK55M 9-5(6)/143 x 2i2W low pressure high intensity lamp system was used in ACR with a designed UV dose of 36 mJ/cm^2.

5.2.2 Enumeration of Aerobic Spore-Forming Bacteria

Isolation and enumeration of ASF bacteria was done according to a procedure described by Barbeau et al. (1997). One hundred mL samples collected in sterile glass bottles were pasteurized in a water bath for 15 min at 75°C. Serial dilutions were prepared and the samples were filtered through a 0.45 μm cellulose nitrate membranes (Millipore, cat no. HABG04700, Bedford, MA). The membrane filters were then placed on pads soaked with 1.65 mL tryptic soy broth in sterile plastic Petri dishes. Colony counts were determined within 22 to 24 h of incubation at 35°C and recorded as colony forming units per 100 mL (CFU/100 mL). Each counted colony was assumed to arise from a single ASF bacterium. The same procedure was used to enumerate *Bacillus subtilis* (ATCC 6633) spores for all the test conditions throughout the experiment for comparison purposes.

Stock solutions of *B. subtilis* (ATCC 3633, American Type Culture Collection, Manassas, VA.) were obtained, cultured and stored for use in experiments as described by Uvbiama and Craik (2005). The stock solution containing ~ 1.0×10^9 CFU/mL *B. subtilis* was spiked into 1 L of secondary wastewater samples to make a final concentration of ~ 10^6 CFU/mL spores. The wastewater samples were then mixed by a magnetic stirrer for 15 min. The same procedure used to enumerate indigenous ASF spores was used to enumerate *B. subtilis* spores.

5.2.3 Homogenization

To extract ASF bacteria from particles, a dispersal buffer described by Camper et al. (1985) was added to the samples. The resulting concentration of the dispersal buffer after addition to the sample was 10^{-6} M Zwittergent 3-12 (Calbiochem, La Jolla, CA), 10^{-3} M EGTA (Fisher Scientific, Pittsburgh, PA), 0.01M Tris buffer (Sigma, St. Louis, MO) and 0.1% peptone (Difco, Detroit, MI). The samples were stirred continuously on a magnetic stirrer for 10 min to mix and disperse the buffer thoroughly. Then, a tissue homogenizer (Powergen 700D, Fisher scientific, Pittsburgh, PA) was used to disaggregate and disperse the particles and the ASF bacteria. In preliminary experiments, two homogenizing speeds (13,500 rpm and 25,000 rpm) and four homogenization times (0.5, 1, 1.5 and 2 min) were tested with and without addition of the dispersal buffer. The combination of speed, time and buffer resulting in the largest increase in colony counts relative to the colony count in the starting sample (as control) was considered to be the optimized procedure for disaggregating and dispersal of the spores.

Wastewater samples were collected from upstream and downstream of the operating UV reactors at the two WWTPs. The samples were homogenized using the optimized procedure and incubated at 75°C for 15 min before enumeration of ASF bacteria. For laboratory UV exposure experiments, the effect of particle association on the inactivation of the ASF bacteria by UV radiation was evaluated under three test conditions: (1) samples were not homogenized before UV exposure; (2) samples were homogenized before UV exposure; (3) samples were first exposed to UV followed by homogenization. All samples were incubated at 75°C for 15 min before enumeration of ASF bacteria.

5.2.4 Absorbance Measurements

The absorbance of unfiltered wastewater samples was measured by both standard spectrophotometry on a UV-vis spectrophotometer (Shimadzu Corporation, model UV-2401PC, Kyoto, Japan) equipped with a 10 mm path length quartz cell, and on the same spectrophotometer equipped with an integrating sphere attachment (Model ISR-2000, Shimadzu Corporation, Kyoto, Japan). The depth-averaged UV dose for the laboratory UV exposure experiments was calculated based on duplicate absorbance measurements for each sample.

5.2.5 Collimated Beam UV Exposures

For each UV exposure, twenty-five mL of wastewater sample was placed in a 500 mm diameter glass Petri dish and exposed to UV radiation from a 10 W low pressure mercury lamp (Ster-L-Ray, model G12T6L, Atlantic Ultraviolet Corp, Haupauge, NY) using a collimated beam apparatus (Rayox® Advanced Oxidation System, model PSI-1-120, Calgon Carbon Corporation). The wastewater sample was stirred continuously during exposure using a 10 mm x 3 mm Teflon^{TM} magnetic stir bar. The Petri dish was covered with a quartz lid during the exposure to reduce the potential exposure of the researchers to wastewater aerosols. For each test condition, a total of 100 mL of sample was UV exposed (four exposures with 25 mL each) and the exposed suspensions were combined and incubated at 75°C for 15 min to inactivate vegetative bacteria. Serial dilutions were prepared and each dilution was enumerated in triplicate. The irradiance at the liquid surface and at the center of the Petri dish surface was measured using a calibrated radiometer (Optometer P-9710, Gigahertz Optik Inc., Newburyport, MA) and was between 0.052 to 0.055 mW/cm^2. This measurement accounted for the quartz lid. The depth averaged UV dose in the liquid was determined by correcting the radiometer measurement for reflection and the liquid surface, radial variation, and attenuation with depth according to procedures described elsewhere (Bolton and Linden, 2003). The duration of exposure to UV radiation was controlled by a pneumatic shutter located beneath the UV lamp.

The log inactivation of ASF bacteria was calculated as $-\log N/N_0$, where N_0 is the arithmetic mean concentration of ASF bacteria without UV exposure (CFU/mL); N is the

arithmetic mean concentration of spores after UV exposure (CFU/mL). The first-order UV inactivation rate constant k in cm^2/mJ, was determined by linear regression of a plot of inactivation versus dose, F, in mJ/cm^2,

$$-\log_{10}\frac{N}{N_0} = kF \tag{5-1}$$

Regression calculations and statistical analyses were performed using least square linear regression analysis on Microsoft Excel™ 2003.

5.3 Results

5.3.1 Dispersion of Particle Associated ASF Bacteria

The effect of homogenization time, speed and presence of dispersal buffer on the number of ASF bacteria enumerated in a sample of secondary wastewater effluent are shown in Figure. 5-1. At 13,500 rpm with dispersal buffer, the measured ASF bacteria concentration increased with homogenization time up to 1.5 min and then decreased at a longer homogenization time. At 25,000 rpm with dispersal buffer, the concentration of ASF bacteria was lower. This suggests that the higher homogenization speed may have resulted in damage to some of the spores. Other researchers have observed this (Parker and Darby, 1995). The measured concentration of ASF bacteria was lowest when no dispersal buffer was used, indicating the importance of dispersal buffer in optimizing spore elution. The ASF bacteria concentration was greatest at a homogenization speed of 13,500 rpm for 1.5 min in the presence of dispersal buffer. Therefore, these conditions were selected for further work.

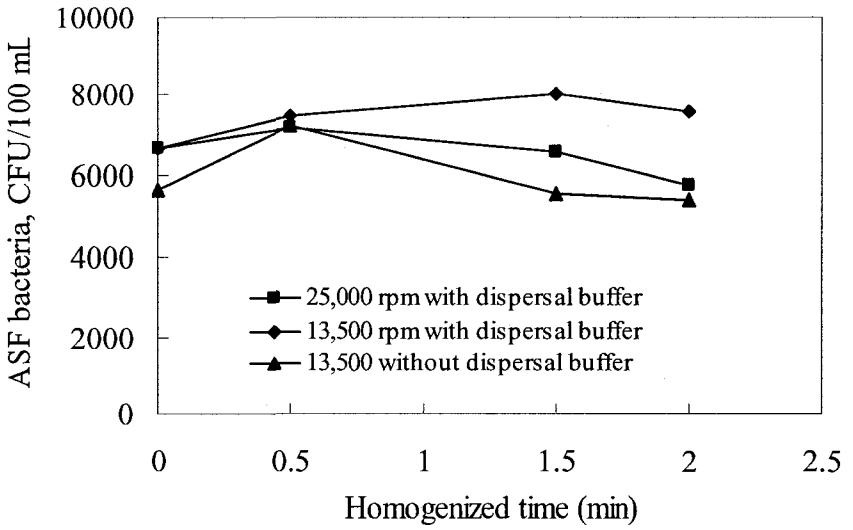

Figure 5-1. The concentrations of indigenous aerobic spore-forming bacteria in wastewater effluent after homogenization with or without disperse buffer.

A comparison of particle counts before and after sample homogenization indicated that the homogenization procedure was effective at disaggregating larger particles (Figure 5-2). The number of particles larger than 20 μm was substantially less in the homogenized samples than in the non-homogenized samples. The number of enumerated ASF bacteria increased by approximately 20% with 1.5 min homogenization at 13,500 rpm in the presence of a dispersal buffer relative to non-homogenized samples. The concentration of indigenous ASF bacteria examined in wastewater effluent was in the range of 10^4 CFU/100 mL for both EGB and ACR wastewater treatment plants.

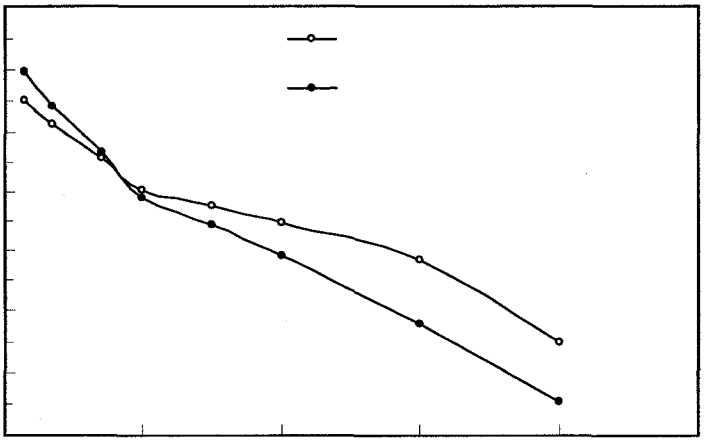

Figure 5-2. Cumulative particle distribution in EGB wastewater effluent before and after homogenization (13,500 rpm with dispersal buffer).

5.3.2 Absorbance Measurements and UV Dose

Particles present in wastewater both absorb and scatter UV light (Qualls et al., 1983). Linden and Darby (1998) observed that integrating absorbance measurements accounted for both absorbance and scattering and provided the most accurate measure of the UV irradiance in wastewaters when compared to standard spectrophotometry on filtered or unfiltered samples. The absorbance of each wastewater sample in this study was measured using conventional transmission spectrophotometry on unfiltered samples and spectrophotometry with an integrating sphere attachment (Figure. 5-3). Absorbance measured by standard spectrophotometry was 10% lower than absorbance measured using the integrating sphere. In addition, sample homogenization resulted in an increase in absorbance measured using both techniques, probably due to the breaking of larger particles into smaller sizes. The effect of homogenization and absorbance measurement method on measured absorbance were both statistically significant at the 95% confidence level ($p = 0.005$). For UV exposure experiments in this study, the absorbance measured using the integrating sphere was used to determine the depth-averaged UV irradiance.

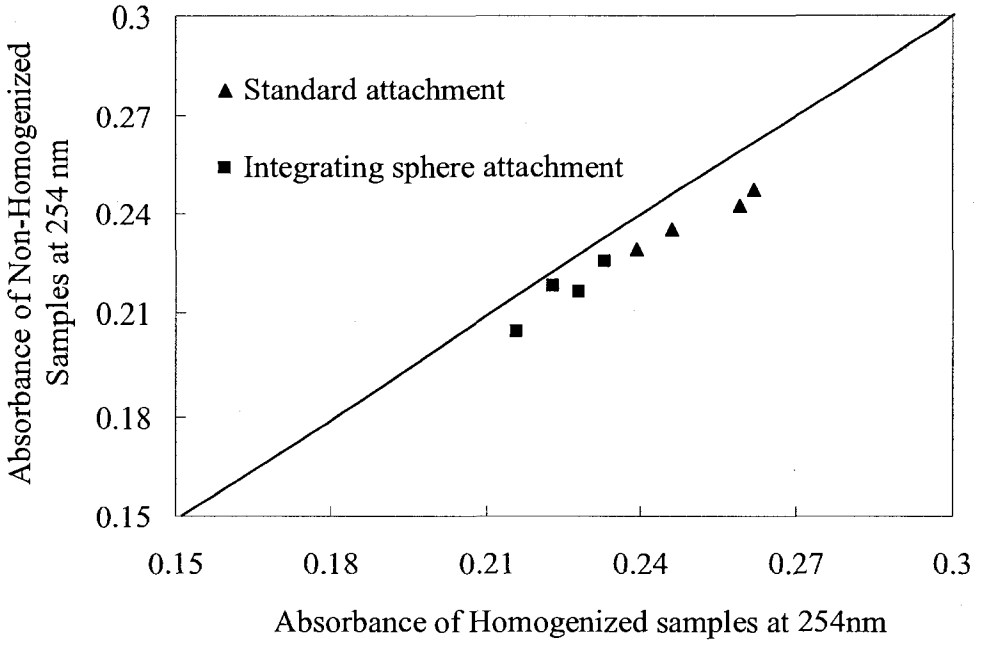

Figure 5-3. UV Absorbance of wastewater samples at 254 nm measured by standard spectrophotometry and spectrophotometry with an integrating sphere attachment for both homogenized and non-homogenized samples.

5.3.3 Comparison of Inactivation of Indigenous ASF in Field UV Reactors and in Laboratory UV Exposure Experiments

Spore concentration in samples collected from directly upstream and downstream of the UV reactor was measured for two wastewater treatment plants. The samples were split and one sub-sample was homogenized in dispersal buffer before spore enumeration while the other sub-sample was not homogenized. The computed log inactivation (-log N/N_0) is plotted as function of the UV dose reported by the reactor control system at each WWTP in Figure 5-4. According to an ANOVA, there was no significant statistical difference at the 95% confidence level between inactivation determined based on samples that were homogenized and inactivation determined based on samples that were not homogenized. ($p = 0.26$ for the ACR WWTP and $p = 0.84$ for the EGB WWTP). This finding suggests that the indigenous spores present in wastewater effluents were not associated with particles or this association did not significantly affect spore inactivation

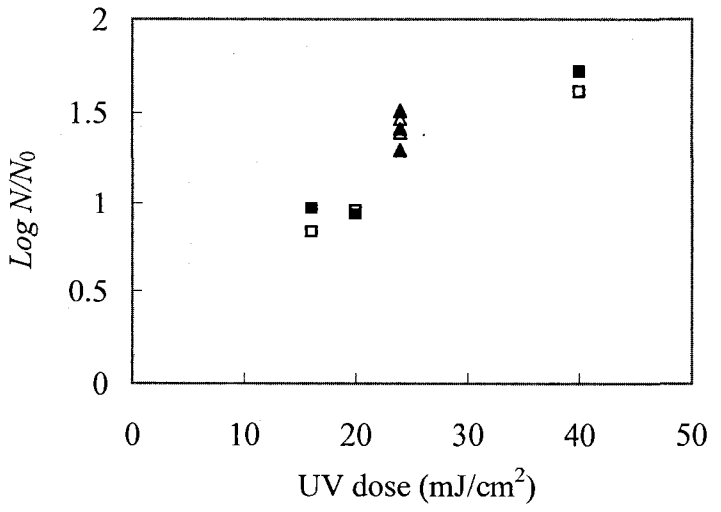

Figure 5-4. Log inactivation of indigenous aerobic spores by the UV reactor at two wastewater treatment plants. Solid symbol represents inactivation based on samples that were not homogenized. Open symbol represents inactivation based on samples that were homogenized. Sample site: Alberta Capital Region (□, ■); City of Edmonton (Δ, ▲). UV dose is based on the dose reported by the on-site UV reactor control system.

by UV radiation. If significant particle association were occurring the homogenization step would have disrupted the particles and dispersed any ASF spores that were harbored within the spores and protected from UV exposure. The inactivation determined using homogenized samples, therefore, would be expected to be lower than inactivation determined using un-homogenized samples.

Samples collected from upstream of the UV reactor were also exposed to controlled UV doses in laboratory collimated beam experiments. The samples were homogenized in the presence of dispersal buffer before UV exposure (homogenization + UV) or after UV exposure (UV + homogenization) or were not homogenized at all (UV - no homogenization). For both wastewater treatment plants, average inactivation was greater in samples that were homogenized before UV exposure in comparison to samples that were homogenized after UV exposure and samples that were not homogenized at all (Figure 5-5). However, ANOVA revealed that the difference in inactivation was not significant at the 95% confidence level (p = 0.10). Inactivation rate constants were

determined accordingly by regressing Equation 5-1 to the data and are compared to literature values in Table 5-1. There was no apparent tailing effect in the UV inactivation curves of the indigenous ASF bacteria at the UV doses used in this study (up to 40 mJ/cm^2).

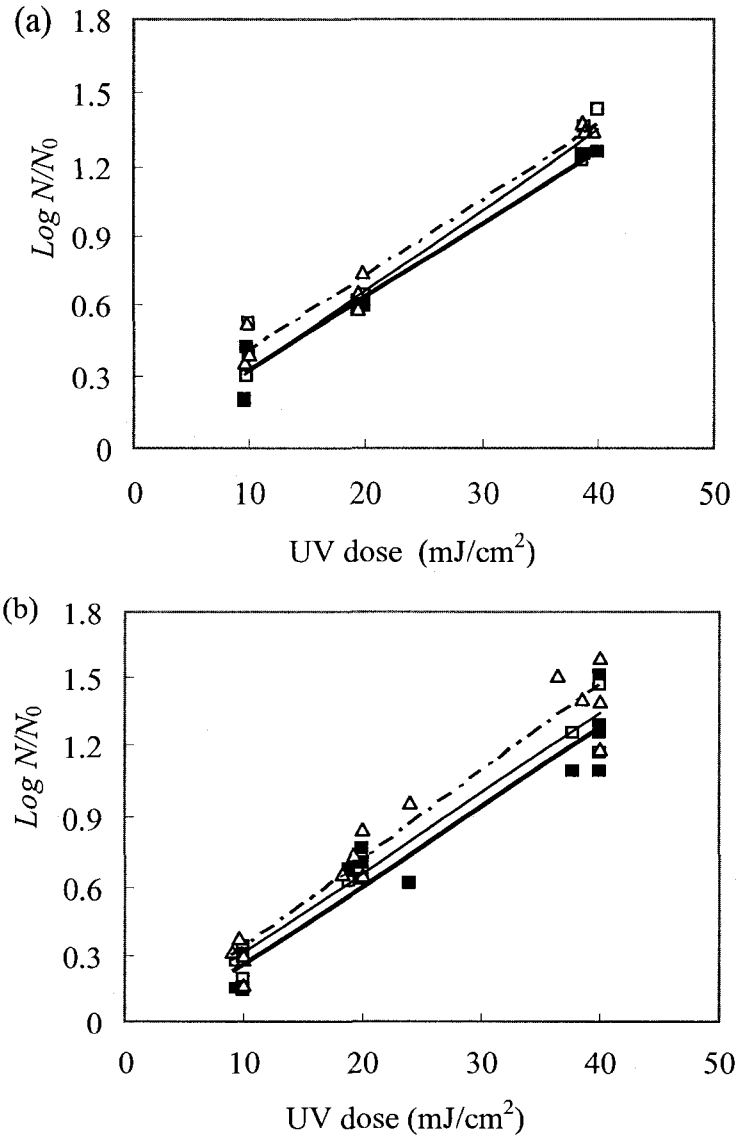

Figure 5-5. Log inactivation of indigenous aerobic spores determined in batch UV exposure experiments using a collimated beam UV apparatus. (a) Alberta Capital Region WWTP and (b) City of Edmonton WWTP. Symbols: UV – no homogenization (■); UV + Homogenization (□); Homogenization + UV (Δ).

Table 5-1. Inactivation rate coefficient of indigenous spores for field UV reactors and collimated beam UV apparatus.

Spores	UV exposure	Inactivation kinetic coefficient (k), cm^2/mJ [a]			Note
		UV (no Homogenization)	UV + Homogenization	Homogenization + UV	
Indigenous spores	Field UV reactors	0.0456 (0.84)	0.0428 (0.89)		ACR
	Collimated beam	0.0319 (0.98)	0.0337 (0.95)	0.035 (0.96)	ACR
	Collimated beam	0.0342 (0.93)	0.0344 (0.96)	0.038 (0.94)	EGB
ATCC 6633	Collimated beam	0.066 (0.81)	-	0.075 (0.91)	Spiked into ACR wastewater effluent
Indigenous spores [b]	Collimated beam	0.013 to 0.022	-	-	Raw surface water
ATCC 6633 [c]	Collimated beam	0.085 (0.98)	-	-	Buffered DI water

[a] Data in brackets are the R^2 for the regression.

[b] Mamane-Gravetz and Linden, 2004

[c] Uvbiama and Craik, 2005

5.3.4 Inactivation of Cultured Spores in Laboratory Collimated Beam UV Experiments

Cultured *B. subtilis* spores (ATCC 6633) were added to samples taken from upstream of the field UV reactor at ACR to a concentration of 10^5 CFU/mL. At this concentration, the impact of indigenous ASF bacteria was considered negligible because these are present at a much lower concentration (<100 CFU/mL). The overall log inactivation of *B. subtilis* spores in samples homogenized before UV exposure was slightly higher than inactivation of *B. subtilis* spores in samples were not homogenized (Figure 5-6). An ANOVA revealed that the difference was not statistically significant at the 95% confidence level (p = 0.09). These results indicate that the spores added into the wastewater did not interact with particulate matter in such a way that UV inactivation was affected significantly. Either the spores did not attach to wastewater particles, or the spores attached only to the surface of the particles where they were fully exposed to the effects of UV irradiation.

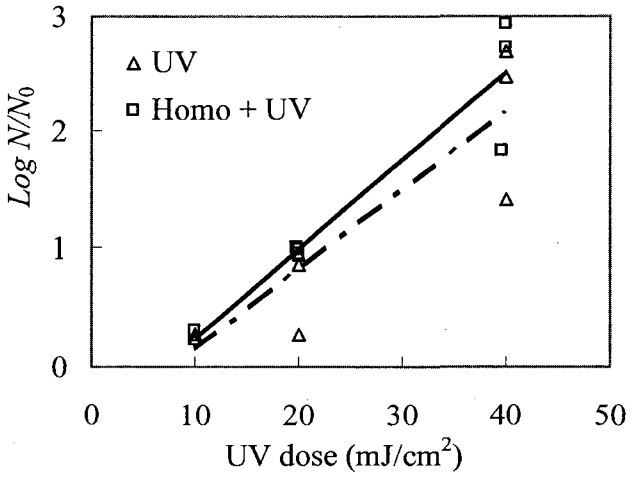

Figure 5-6. Log inactivation of *B. subtilis* ATCC 6633 spores added to ACR wastewater. Samples were either homogenized before UV exposure (homo + UV) or were not homogenized (UV)

5.4 Discussion

Particle association has been demonstrated to limit UV inactivation of coliform bacteria in secondary treated municipal wastewater (Emerick et al. 1999). No studies have examined the effect of particulate matter on UV inactivation of *G. lamblia* cysts present in secondary wastewater. At present, there are no suitable methods to determine the degree of association between cysts and wastewater particles directly. The concentration of cysts in wastewater is relatively low and the methods used to concentrate the cysts for enumeration tend to change the nature of the interaction between the particles and the cysts. Indigenous aerobic spore formers, which are relatively plentiful in wastewater, therefore, were used as surrogates to study the potential for a particle association effect on *G. lamblia* cysts. It was reasoned that if no particle association effect was measured using the indigenous spores, then the probability of particle association effect with *G. lamblia* cysts would be low since spores are

considerably smaller than cysts.

In this study, it was found that indigenous ASF spores were not associated with wastewater particles to a degree that the UV inactivation characteristics were significantly affected. Most coliform bacteria present in secondary treated municipal wastewater are dispersed and are readily inactivated by UV radiation; however, a small fraction may be associated with particles and completely shielded and protected from UV radiation (Parker and Darby, 1995). The number of particle-associated coliform is directly correlated to the concentrations of live coliform bacteria remaining in the wastewater after high applied UV doses. These survivors result in a characteristic tailing effect on the inactivation versus UV dose curve (Emerick et al., 1999). The behavior of ASF bacteria, however, may be different from coliform bacteria in wastewater treatment processes. Coliform bacteria can propagate in wastewater and produce extracellular polymeric substances which play an important role in cell aggregation and cell adhesion (Burdman et al., 2000; Frank and Belfort, 2003). However, spores tend to behave as inert particles, and may attach only to the surface of particles. *G. lamblia* cysts and *C. parvum* oocysts are similar to bacterial spores in that they are present in a dormant encysted state in wastewater and will behave like inert particles. It may be hypothesized that *G. lamblia* cysts and *C. parvum* oocysts will also be unlikely to associate with particulate matter in secondary wastewater.

G. lamblia cysts are less likely to be sheltered from the effects of UV by wastewater particulate matter due to the larger size of cysts (size 7 to 14 μm) than aerobic spores (~ 1μm). The association between particles and microorganisms and its effect on UV inactivation in wastewater depends on the size of microorganisms and the size of particles. Hejkal et al. (1981) reported that virus in wastewater effluents are mainly associated with particles of 0.22 μm and larger. Particles less than 2 μm in diameter are large enough to protect viruses from UV radiation (Templeton et al., 2005). Emerick et al. (1999) found that all particles greater than 10 μm in diameter shield the coliform bacteria from UV exposure. Domenec et al. (2001) further reported suspended particle sizes of 7 μm or larger present in tertiary effluent will decrease the UV inactivation rate of coliform bacteria. In one study, there were no particle-associated indigenous *C. parvum* oocysts detected in secondary effluents of five activated sludge wastewater

treatment plants (Tsuchihashi et al., 2003); however, the researchers found that spiked *C. parvum* oocysts readily associated with particulate matter of diameter greater than 80 μm. Medema et al. (1998) found that *Giardia* spp. cysts added to wastewater readily attached to the wastewater particles and were settled in secondary clarifiers. This suggests that particles must be at least 10 times larger than the microorganism in order to shield them from UV radiation. Applying this factor to *G. lamblia* cysts (8-16 μm), particles with diameter greater than 80 μm would be required to shield them from UV radiation. In the two wastewater treatment plants examined in the present study, 89% of the particles present in the secondary wastewater samples were smaller than typical *G. lamblia* cysts (7 to 14 μm), whereas only less than 0.001% of secondary effluent particles were larger than 80 μm, comparing with about 30% of particles between 7 and 14 μm measured from Edmonton WWTP (Figure 5-2). Therefore, *Giardia* spp. cysts remaining in wastewater effluent were not likely attached to the wastewater particles and be completely shielded from UV exposure.

The physico-chemical characteristics of spores and cysts are similar with respect to surface charge and hydrophobicity. Both cysts (Dai et al., 2004) and spores have a negative surface charge as do the biological flocs in municipal activated sludge wastewater treatment reactors (Mikkelsen and Keiding, 2002). As reported by Dai et al. (2004), the hydrophobicity of the solid surface played a more important role in the adhesion of *G. lamblia* cysts than the surface charge when the contact angle was larger than 90° (hydrophobic). However, the municipal activated sludge flocs have a mean contact angle of 7° to 9° (Sponza, 2003). This also supports the hypothesis that the cysts in wastewater are not likely attached to the wastewater flocs.

The measured log inactivation of indigenous ASF bacteria in the field UV reactors was slightly higher when the samples were homogenized compared to when the samples were not homogenized (Figure 5-4). If the ASF bacteria were attached to or embedded within floc particles, the homogenization step would have presumably released the ASF bacteria from the particles resulting in an increase in the count of ASF bacteria in the homogenized samples. However, an ANOVA showed that the difference was not statistically significant at the 95% confidence level ($p = 0.26$). This finding suggests that ASF bacteria are not significantly associated with particles in wastewater effluent, and

also the association of ASF bacteria with particles in the wastewater effluent does not have a statistically significant impact on inactivation by UV radiation in the field reactors. This was not the same as observed for UV reduction of coliform bacteria in wastewater effluent. The coliform bacteria in wastewater tend to associate with wastewater particles and are protected from UV radiation (Emerick et al., 1999; Loge et al., 1999). For samples homogenized before collimated beam UV exposure in the laboratory, it was assumed that the ASF bacteria spores were mostly dispersed and fully exposed to UV radiation. For samples homogenized after UV exposure, it was expected that the number of survivors following UV exposure would be higher. Some of the ASF bacteria spores would have been shielded from UV exposure and then released from particles by the homogenization step following UV exposure. This was not observed; therefore, the results of this study indicate that particle association does not have a significant effect on UV inactivation of ASF bacteria. The collimated beam experimental results were consistent with the results from the field UV reactors.

The relationship between ASF bacteria inactivation and UV dose observed in the collimated beam experiments was approximately first order with respect to UV dose. There was no evidence of a tailing effect for indigenous ASF bacteria at the UV doses used in this study (up to 40 mJ/cm^2). In another study, there was not tailing effect observed for UV exposure of ASF bacteria spores in lake water at UV doses up to 110 mJ/cm^2 (Mamane-Gravetz and Linden, 2004). The tailing region typical of coliform UV dose-inactivation curves is observed because some of the coliform bacteria are fully protected from UV exposure by the particles or particle-bacteria association in wastewater (Emerick et al., 2000; Jolis et al., 2001; Loge et al., 1996; Qualls et al., 1983). These researchers observed that the effects of particle association started to limit the inactivation of coliform bacteria at the 3 to 4 log inactivation level. The level of inactivation of coliform bacteria, and the tailing, was observed at doses applied in wastewater treatment UV reactors (<40 mJ/cm^2). In my research, the inactivation of indigenous spores was less than 1.5 log at UV 40 mJ/cm^2 because ASF bacteria spores are more intrinsically resistant to UV than are coliform bacteria. Mamane-Gravetz and Linden (2004) similarly reported that there was no tailing effect for indigenous spores isolated from surface water and exposed to UV doses up to 100 mJ/cm^2 and at 2 log

inactivation. Therefore, there was no significant effect of particle association on the inactivation of indigenous spores up to the 2 log inactivation level in wastewater effluent and surface water. Perhaps the effects of particles would only become evident at higher log inactivation levels. At low inactivation levels, the measured inactivation is dominated mainly by fully dispersed microorganisms and the UV dose-inactivation curve has a steep slope because the observed rate of inactivation is high. At higher doses and inactivation levels, once the dispersed microorganisms are inactivated completely, the inactivation process is dominated by particle associated microorganisms that have higher apparent resistance to UV. The slope of the inactivation curve decreases and tailing is observed. No matter how high the UV dose is, the UV photons will not reach the particle-protected spores. The interaction between spores and wastewater particles implies that *G. lamblia* cysts may be more likely dispersed in wastewater effluent and the inactivation of cysts by UV radiation may not be affected by the particle shielding effect.

The inactivation coefficients of wastewater ASF bacteria in this study were somewhat higher than those reported by Mamane-Gravetz and Linden (2004) for the ASF spores isolated from surface water, in which indigenous spores from surface water were treated by UV irradiation followed by UV exposure experiments. The resistance of those spores to UV irradiation might have been higher due to the acquired resistance by repeated environmental UV exposure and DNA repair (Ewing, 1997).

The observed difference in UV sensitivity for indigenous spores and cultured *B subtilis* spores (Figure 5-5 and Figure 5-6) may be attributed to the physiological state of the microorganisms, initial concentration of microbes, and spore type diversity. The cultured *B. subtilis* spores were more sensitive to UV inactivation than the indigenous ASF bacteria. B. subtilis spore inactivation was 2.5 log at a UV dose of 40 mJ/cm^2 compared to less than 1.5 log inactivation for the indigenous spores at the same UV dose (Nicholson and Law, 1999). Indigenous spores purified from soil exhibited 2-3 fold higher resistance to UV than both the same strain spores after cultivation and *B. subtilis* strain 168 (Nicholson and Law, 1999). The initial concentration of microorganisms may also have an impact on the inactivation by UV radiation. Hass and Haymak (2003) reported that there is an apparent decrease in disinfection efficiency of ozone against *G muris* (at pH 8 and 15°C) as the initial microorganism concentration decreases. Pham et

al. (1997) also found that inactivation of viable spores in aqueous TiO_2 suspension increased with an increase in spore density. The concentrations of indigenous spores in the wastewater samples used in this study were in the range of 10^4 CFU/100 mL, whereas the cultured spores used in laboratory studies are normally 3 to 4 magnitudes higher, in the range 10^5 to 10^7 CFU/mL. Different species and strains of *Bacillus* spores have different susceptibility to UV irradiation (Myasnik et al., 2001) possibly due to the differences in the penetration depth of UV photons into the spore, the efficiency of producing DNA damage that could cause inactivation, and the repair capacity of each type of spore (Munakata et al., 1991).

Indigenous ASF bacteria spores may be used as an internal biodosimeter to assess dose delivery in full-scale UV reactors in wastewater treatment. The conventional validation test of full-scale UV treatment reactors requires spiking surrogate microorganisms into the water or wastewater and using biodosimetry to determine the actual UV dose delivered to achieve certain design target. There are several advantages to the use of indigenous microorganisms for biodosimetry over spiked surrogate microorganisms for the biodosimeteric validation of UV reactors. These include lower costs because culturing of large numbers of surrogate microorganisms is not required, no requirement for pumping and mixing the surrogate microorganisms, reduced likelihood of a negative public reaction to the addition of foreign microorganisms to the environment, and no need to dispose of water that contains spiked microorganisms, etc. The results of this study showed that indigenous spores are not significantly associated with wastewater particulate matter in wastewater and they are present in sufficient concentrations and constantly detected. This is another key feature of ASF spores that make them potentially usefully as organisms for UV reactor dose validation studies.

In conclusion, particle association and its effect on the inactivation by UV radiation were studied in this research using indigenous spores as a surrogate in wastewater. It was concluded that indigenous ASF bacteria are not associated with wastewater particles in secondary wastewater effluent to the extent that the UV inactivation of the spores was affected significantly. The indigenous ASF bacteria may be useful microorganisms for assessing dose delivery in full-scale wastewater treatment UV reactors. They are present in sufficient concentrations, and fairly easily measured. Most of all, ASF bacteria do not

associate with particles (at least in a way that significantly affects UV inactivation) and have appropriate resistance to UV in the UV dose used in the reactors. The indigenous ASF bacteria may be used as a surrogate to infer parasite behavior in wastewater treatment processes since both ASF bacteria and parasites are inert particles in wastewater.

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6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

The main purpose of this thesis was to assess the public health implications of *G. lamblia* cysts present in UV-treated secondary municipal wastewater. This was done by investigating the effects of UV irradiation on the inactivation of *G. lamblia* in field UV reactors, strain-to-strain variation in UV resistance, the potential reactivation of the parasite after UV irradiation, and particle effects in wastewater matrices. The UV inactivation of WB and H3 isolates of *G. lamblia* was assessed using identical UV exposure procedures and animal infectivity. The results showed that the inactivation of the WB and H3 isolates exposed to the same level of UV radiation was remarkably different, suggesting that these two isolates might have differential susceptibility to UV irradiation. Based on the genotyping analysis, the WB isolate is a member of assemblage A (Nash, 1992); whereas the H3 isolate (Guy et al., 2003) and CH-3 isolate (Lu et al., 2002) are members of assemblage B of *G. lamblia* cysts. The observed differences in UV inactivation between these two isolates may be due to genetic differences between isolates since it was established that they possess different surface antigens, have different patterns of infection, and induce qualitatively and quantitatively different host immune responses (Aggarwal and Nash, 1987). Nash *et al.* (1991) found that trophozoites of different isolates differed in their ability to survive after exposure to intestinal proteases, suggesting that the survival of different isolates within the small intestine of the host may not be comparable. Similar to my results, previous studies reported that the UV inactivation of assemblage A (WB isolate) (Campbell and Wallis, 2002; Mofidi et al., 2002) and assemblage B (CH3 isolate) (Linden et al., 2002) differed.

The cysts obtained from City of Edmonton and Alberta Capital Region waste water treatment plant primary effluents were more infectious to gerbils than the cysts obtained from City of Kelowna and City of Winnipeg primary effluents. It is interesting to note that the Edmonton and Alberta Capital Region wastewater treatment plants serve populations that are in adjacent geographic areas. This may account for some of the similarities and differences in *G. lamblia* isolates that were observed. Assemblage A was detected at all four wastewater facilities, while assemblage B was only detected from

the Edmonton and Alberta Capital Region wastewater facilities. The infectivity of cysts from primary effluents and secondary effluents matched the genotyping results. WB isolate (assemblage A) was less infectious to gerbils than H3 isolate (assemblage B) before UV exposure, while WB isolate (assemblage A) was more infectious to gerbils than H3 isolate (assemblage B) after UV treatment (Li et al., 2007a).

One interesting finding in this study was the relatively low intensity of *G. lamblia* infection observed in the inoculated gerbils. As indicated in Chapter 2 of this thesis, the observed low intensity infection was believed to be evidence of trophozoite division within the intestine of the host, rather than trophozoites produced from excystation of the original cysts inoculum. These low intensity infections were not only detected in gerbils inoculated with the UV exposed cysts, but also in gerbils inoculated with fresh, non-UV exposed cysts. The low intensity infections were evident in gerbils inoculated with cysts obtained upstream and downstream of UV reactors. The observed low intensity infections may be explained by the biological difference between *G. lamblia* isolates (Li et al., 2007a), the DNA repair potential of UV exposed trophozoites as discussed in Chapter 3, or environmental stress during the wastewater treatment processes, as discussed in Chapter 4. It was not likely to be the manifestation of protection of the cysts from UV exposure due to particles or the particle association in wastewater as indicated by the findings of Chapter 5. Strong and moderate infections were detected from 3 out of 4 primary effluents of the four study sites. However, only low intensity infections were detected from either upstream or downstream of the UV reactors, suggesting that the infectivity of cysts was reduced during the secondary biological wastewater treatment process (Garcia et al., 2002). The cysts that survived the secondary wastewater treatment may have had a strong resistance to UV irradiation and/or a higher reactivation capability.

The results of previous studies on reactivation differed, some showing that *Giardia* spp. can reactivate *in vivo* (Belosevic et al., 2001; Shin et al., 2005), while others showed no reactivation (Linden et al., 2002). The commonly recognized mechanism is that UV irradiation damages the DNA of cysts and the parasites lose the ability to replicate within the host. When these survived cysts are ingested by the host, they excyst (Craik et al., 2000) because the UV doses used in wastewater treatment have little impact on

excystation and subsequent division of the quadrinucleate trophozoites into two trophozoites. Furthermore, *G. lamblia* trophozoites are capable of multiplying under *in vitro* culture conditions following UV exposure (Li et al., 2007b). This suggests that the UV exposed cysts were capable of multiplying *in vivo* after ingestion by a host. Moreover, some of cysts in wastewater UV reactors may have received a lower UV dose than others, due to the variations in the cyst wall thickness or adsorption of colloidal material onto the surface of the cyst wall.

The low intensity *Giardia* spp. infections reported here were also a feature in the gerbils inoculated with cysts obtained from downstream of the UV reactors, after UV treatment. Non-active trophozoites, detected in the intestinal samples of most of the gerbils inoculated with cysts collected from both upstream and downstream of the field UV reactors, indicated the presence of at least a small number of infectious cysts in the wastewater. Considering the calculated UV doses delivered by the UV reactors, the sensitivity of *Giardia* spp. cysts to UV exposure reported from controlled laboratory studies, and the inoculum size delivered to the experimental gerbils, cyst infectivity should have been essentially eliminated by UV treatment. Several explanations for these weak infections are possible. The inactive trophozoites detected in the intestines may have originated from the original cyst inoculum and may not have been due to *de novo* division of trophozoites in the small intestine. However, Li et al. (2007a) reported that this was not the case and that the detection of even a small number of trophozoites is evidence of some level of infection. It should be noted that gerbils used in the studies described in this thesis were not immunosuppressed prior to experiments. It is also probable that there are genetic and physiological differences between the isolates of indigenous cysts in wastewater and parasites used in the laboratory studies described in this thesis. Furthermore, cysts that have been exposed to environmental stressors present during the wastewater treatment processes may exhibit UV sensitivity that was different from that of the freshly isolated cysts used in the laboratory studies. Taken together, my findings suggest that UV inactivation may not completely eliminate the infectivity of cysts in full-scale field UV reactors, which differs from what has been reported in previous studies. If I disregard my observations of low intensity infections in the gerbils, I would also conclude that the cysts obtained from secondary effluent were not infectious

to gerbils. It is clear from my findings that the infectivity of *G. lamblia* cysts obtained from primary effluent wastewater was substantially reduced by the biological treatment processes. The survivors from the secondary wastewater treatment processes may exhibit different resistance to UV exposure compared to freshly isolated cysts in laboratory. My results, however, clearly indicate that not all of the parasites were inactivated by UV under field conditions.

The cysts detected in primary effluent produced strong or moderate infections in Mongolian gerbils in three of four wastewater treatment plants investigated, which was consistent with the results of previous studies (Garcia et al., 2002). Secondary wastewater treatment processes damaged and reduced the infectivity of cysts since only weak infections were observed in gerbils exposed to cysts isolated from secondary effluent. Garcia *et al.* (2002) found no cysts in the feces of gerbils inoculated with 200 cysts or 1000 cysts recovered from tertiary-treated and chlorine disinfected wastewater. However, they did not examine the presence of trophozoites in the small intestine of gerbils, which may have shown low level infection in exposed gerbils. The absence of cysts in gerbil feces is not an adequate measure of *Giardia* spp. infectivity, as shown by our findings, where no cysts were detected in the feces of exposed gerbils, but the trophozoites were present in the small intestine indicates that they were infectious.

Full-scale UV reactors in the Edmonton and Alberta Capital Region wastewater treatment plants resulted in only a marginal reduction (< 0.5 log) in infectious parasites based on the reduction of the low intensity infections observed upstream and downstream of the reactor. This is less than the reported laboratory results for WB and H3 isolates (Campbell and Wallis, 2002; Linden et al., 2002; Mofidi et al., 2002). Those studies used the presence of cysts in the feces and/or large numbers of active trophozoites in the small intestine as the endpoint of infection. In contrast, in this study, the presence of even a small number of non-active trophozoites was used as the endpoint of infection in the gerbils. A logistic dose-response model (Finch et al., 1993) used to calculate the number of live cysts inoculated to gerbils was generated using freshly isolated *G. lamblia* cysts. Due to differences between freshly isolated parasites and indigenous cysts present in wastewater, it may not be possible to fully compare the logistic dose responses between these two cyst preparations; however, my observations do indicate that the percentages of

infected gerbils was higher than expected based on laboratory studies at UV doses less than 50 mJ/cm^2.

The difference between the field results and the laboratory results may be related to differences in cyst isolates, or to differences in the physical state of the naturally occurring cysts and the purified laboratory cysts, the hydraulic conditions in the UV reactor, the calibration and accuracy of UV irradiation measured on site, and the effect of particles in wastewater matrices. The hydraulic conditions or the dose delivery in the full scale UV reactors were not examined directly in this research. It appears that the UV dose delivered by full scale UV reactors was reasonable based on the coliform reduction. However, the cysts that passed through the UV reactors may not have received the same dose due to the presence of a dose distribution. The UV intensity is not uniformly distributed in multi-lamp UV reactors due to the existence of shadow and reflection effects, which could result in up to a 3-fold spatial variation in the UV intensity within a UV reactor (Bolton, 2000; Jin et al., 2005).

The relationship between the wastewater particles and cysts and the effect of this particle association on the inactivation by UV irradiation was explored using indigenous spores as surrogates in wastewater matrix. There was no significant difference in the UV inactivation of indigenous ASF bacteria present in the wastewater between homogenized samples (spores dispersed) and non-homogenized samples (spores not dispersed) before UV exposure. There was no tailing observed in the UV inactivation curve of indigenous spores at the UV doses up to 40 mJ/cm^2. At this UV dose level, coliform bacteria present in wastewater effluent normally show a tailing effect, due to scattering and incomplete penetration of UV radiation into particles that contain microorganisms (Emerick et al., 2000; Jolis et al., 2001; Loge et al., 1996; Qualls et al., 1983). These researchers observed that at 3 to 4 log inactivation level, the effects of particle association limited the inactivation of coliform bacteria. In my study, the inactivation of indigenous spores was less than 1.5 log at UV 40 mJ/cm^2 for homogenized samples. Mamane-Gravetz and Linden (2004) reported that there was no tailing effect for indigenous spores isolate from surface water and exposed to UV doses up to 100 mJ/cm^2 (2 log inactivation). This suggests that there was no significant effect of particle association on the inactivation of indigenous spores, up to the 2 log inactivation level, in wastewater effluent and surface

water. Perhaps the effects of particles would only become evident at higher log inactivation levels. At low inactivation level, the spores are assumed to be dispersed and the UV radiation will inactivate the spores readily. But at higher doses than those used in this study, the dispersed spores might have been inactivated completely, and the only live spores remaining would be those that were shielded by the particles from UV radiation. Then, the inactivation curve may feature a tailing effect. The interaction between spores and wastewater particles suggests that *G. lamblia* cysts may be more dispersed in wastewater effluent and the inactivation of cysts by UV radiation may not be affected by the particle shielding effect. Particles larger than a critical size, as a function of the size of examined microorganisms, will effectively shield the embedded microorganisms (Emerick et al., 2000; Emerick et al., 1999). A minimum particle size of 10 μm governs the ability of particle to shield coliform bacteria from UV radiation (Emerick et al., 2000), and particles less than 2 μm in diameter are large enough to protect viruses from UV radiation (Templeton et al., 2005), suggesting that a critical particle size is approximately 10 times larger than the microorganisms to shield them. Considering the size of spores (~ 1 μm) and the size of *G. lamblia* cysts (7 to 14 μm), the critical particle sizes to shield cysts should be larger than 80 μm, and study do show that spiked *C. parvum* oocysts readily associated with particulate matter of diameter greater than 80 μm (Tsuchihashi et al., 2003). Only less than 0.001% of secondary effluent particles are larger than 80 μm, comparing with about 30% of particles between 7 and 14 μm measured from Edmonton WWTP. The physichemical characteristics of spores and cysts are similar on the surface charge and hydrophobicity. Both the cysts (Dai et al., 2004) and spores are negatively surface charged as the sludge flocs (Mikkelsen and Keiding, 2002). As reported by Dai et al. (2004), the hydrophobicity of solid surface played a more important role in the adhesion of *G. lamblia* cyst than the surface charge when the contact angle is larger than 90° (hydrophobic). However, the municipal activated sludge flocs have a mean contact angle of 7° to 9° (Sponza, 2003). This also supports the hypothesis that the cysts in wastewater are not likely attached to the wastewater flocs.

The public health significance of the low numbers of infectious cysts present in the wastewater effluents at all four facilities is unclear at this time. However, my results suggest the presence of low numbers of infectious cysts in UV-treated wastewater

effluents which may be of public health concern. Considering the equivalent volumes of wastewater administered to individual gerbils (i.e. 7 to 92 L), it seems unlikely that exposure to the wastewater would produce giardiasis in healthy humans but may cause disease in the highly susceptible immunocompromised individuals such as patients undergoing chemotherapy or HIV infected individuals. Rendtorff and Holt (1954) demonstrated that ingestion of as few as 10 cysts was capable of causing infection in two healthy human volunteers. Assuming that a person swimming one day per week in a river water into which wastewater effluent was discharged for 3 months, the dilution factor of 1:100, and the reduction of cyst infectivity by full scale UV reactors of 0.5 $\log_{10}$, the annual risk of accidental intake of 100 mL of the water is estimated to be 2.5×10^{-4} for Kelowna, 4.5×10^{-4} for Edmonton and 1.1×10^{-3} for Winnipeg, using the model of Ryu et al. (2007). If an annual acceptable level of risk of less than 10^{-4} for a yearly exposure for potable water (Rose et al., 1991) were applied to for the microbial risk assessment, the wastewater effluents studied would pose a public health risk. The actual risk would be lower than this because the cysts in the secondary wastewater were not fully infectious.

It should be noted that different *G. lamblia* parasite isolates produced different levels of infection in gerbils after UV exposure. Also *G. lamblia* could potentially reactivate and divide following exposure to relatively low (i.e. < 10 mJ/cm^2) UV doses as determined by the *in vitro* trophozoite experiment. My results suggest that assessment of UV inactivation of *G. lamblia* cysts in wastewater effluents might not be as straightforward as indicated by different studies. The parasite may reactivate after exposure to low UV doses and small number of cysts present in wastewater may be resistant to UV, resulting in residual low level infections in immunocompetent hosts which may intensify into symptomatic disease in immunocompromised hosts. Although it is difficult to translate low intensity of infections in gerbils to the potential for these parasites to cause disease in humans, the low concentrations of cysts in the finished wastewater effluents and the dilution factor in receiving waters, suggest that these parasites would cause a relatively low public health risk provided that the hosts are immunocompetent. However, these infectious parasites present in finished wastewater may pose a greater public health risk in the cohort of immunodeficient individuals (Neto et al., 2006).

6.2 Conclusions

1. Different isolates of *G. lamblia* cysts were not equally sensitive to UV inactivation, as measured by the Mongolian gerbil model.
2. UV radiation may not completely eliminate the ability of the *G. lamblia* parasites to reproduce at UV dose as high as 40 mJ/cm^2. *G. lamblia* trophozoites were still able to reproduce by binary fission at this dose.
3. The infectivity of *G. lamblia* cysts was reduced by secondary wastewater treatment processes. The cysts obtained from primary effluent strongly or moderately infected the gerbils. The cysts obtained from secondary effluent either upstream or downstream of UV reactor infected the gerbils weakly.
4. The field UV systems provided considerably less inactivation than expected based on the laboratory UV dose response of *Giardia* spp. reported in the literature. The probability of weak infections caused by inoculums of 50 to 1,400 cysts was on average reduced by approximately 10% with 18 to 50 mJ/cm^2 UV doses recorded on-site on the four wastewater UV installations.
5. The cysts present in the wastewater effluent retained residual, low level infectivity following UV treatment. The public health implications of these residual infections are uncertain, but are suspected to be low.
6. The results of the experiments with indigenous aerobic spores indicate that indigenous spores are not significantly associated with wastewater particles in wastewater effluent up to 2 log UV inactivation. This suggests that *G. lamblia cysts,* like inert particles without extracellular polymeric substances, are not likely to associate with particles in wastewater at significant levels.

6.3 Future Studies

Based on the results from this thesis, the following future studies are recommended:

1. The reproduction of trophozoites after UV exposure was measured in *in vitro* culture may lead to a promising infectivity assay to determine the infectivity of

G. lamblia cysts after UV exposure. The infectivity of cysts could be measured by the combination of excystation and in vitro cultivation of trophozoites. It is known from previous studies that the cysts have the ability to excyst after UV exposure and trophozoites are able to reproduce *in vitro* after UV exposure from this study. Research will be focused on the collection and cultivation of excysted trophozoites from the UV exposed cysts to measure the reproduction of trophozoites *in vitro* culture. The results need to be compared and correlated with the animal infectivity assay. The expected results are that the reproduction of trophozoites will be inversely proportional to the UV doses applied to the cysts.

2. Research should be conducted to determine the differential susceptibility of various strains of *G. lamblia* cysts to the UV irradiation from each genotyping group, assemblage A and assemblage B. Differential resistance to UV disinfection was observed between the WB and H3 *G. lamblia* isolates; however, the dataset is limited to conclude statistically that the different genotypes of *G. lamblia* cysts have different survival after UV exposure. With the development and improvement of the molecular methods of genotyping *G. lamblia* cysts, more isolates of *G. lamblia cysts,* belonging to different genotypes, need to be tested at more UV dose range to support this finding.

3. It would be valuable to determine the infectivity of trophozoites after UV exposure in the Mongolian gerbil model at a broad UV dose range, even beyond the practical UV dose range used in water and wastewater industry. The infectivity of UV exposed trophozoites in the gerbils shall be measured simultaneously with the *in vitro* culture method for comparison purpose.

4. Future study could be conducted by inoculating more than one strain of *G. lamblia* cysts to the gerbils at the same time to investigate if there is a synergic effect or a competition between strains on the infection of gerbils. As observed in wastewater treatment results, different genotypes of *G. lamblia* cysts were detected in primary effluent and the feces of gerbils inoculated by the same primary effluent. The experiments could be done by changing the combination

of inoculums of assemblage A and assemblage B to the gerbils, and genotyping the cysts collected from the feces of gerbils.

5. The risk of low intensity infection of cysts is not well identified. It is recommended to conduct the cyst infectivity experiments using both untreated gerbils and immunodeficient gerbils in parallel to further investigate the implication of low intensity infections on the public health.

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Appendix A. Raw Data

Table A-1. Dose Response of WB Strain *G. lamblia* in Mongolian gerbils

Trial no.	UV dose (mJ/cm^2)	Inoculum (cysts/gerbil)	Total gerbils inoculated	Infection ratio			No. of gerbils infected
				Strong	Moderate	Weak	
1	0	0	2	0	0	0	0
		100	4	1	0	0	1
		1000	4	0	0	3	3
		10000	4	4	0	0	4
	40	10000	4	0	0	1	1
		100000	4	2	0	1	3
2	0	0	2	0	0	0	0
		10000 (Dynal)	4	2	0	0	2
		10000 (Sucrose)	4	4	0	0	4
		10000 (Percoll)	4	4	0	0	4
	40	10000	4	0	0	2	2
		100000	4	2	0	1	3
3	0	0	2	0	0	0	0
		100	4	0	0	2	2
		1000	4	0	0	1	1
		10000	4	3	0	1	4
	40	10000	4	0	1	1	2
		100000	4	0	0	3	3
4	0	0	2	0	0	0	0
		100	4	0	0	3	3
		1000	4	1	1	2	4
		10000	4	2	1	0	3
	40	10000	4	0	0	3	3
		100000	4	0	0	2	2
5	0	0	2	0	0	0	0
		100	4	0	0	1	1
		1000	4	0	0	3	3
	0.5	2000	5	0	0	1	1
		20000	5	1	1	3	5
6	0	0	2	0	0	0	0
	1	100	4	0	1	2	3
		1000	4	1	0	1	2
		10000	4	4	0	0	4
	0	50000	2	2	0	0	2

Table A-1.2. UV exposure experiment conditions

Trial No. 2 Absorbance = 0.168 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.812 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.039 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 1018 s
Trial No. 3 Absorbance = 0.049 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.924 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.045 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 894 s
Trial No. 4 Absorbance = 0.189 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.786 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.038 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 1052 s
Trial No. 5 Absorbance = 0.946 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.387 Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.018 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 2254 s

Table A-2. Dose Response of H3 Strain *G. lamblia* in Mongolian gerbils

Table A-2.1.

Trial no.	UV dose mJ/cm^2	Inoculum (cysts/gerbil)	Total gerbils inoculated	Infection ratio			No. of gerbils infected
				Strong	Moderate	Weak	
1	0	0	2	0	0	0	0
		10	6	1	0	1	2
		100	4	4	0	0	4
		1000	4	4	0	0	4
		10000	5	5	0	0	5
2	0	0	2	0	0	0	0
		100	4	0	1	1	2
		1000	6	0	0	0	0
	40	10000	6	0	0	0	0
		100000	6	0	0	1	1
3	0	0	2	0	0	0	0
		100	4	4	0	0	4
		1000	4	4	0	0	4
	40	1000	4	0	0	1	1
		10000	8	0	0	3	3
4	0	0	2	0	0	0	0
		10	4	0	0	1	1
		100	4	4	0	0	4
		1000	2	2	0	0	2
	40	10000	4	0	0	0	0
		100000	6	0	0	1	1
5	0	0	2	0	0	0	0
		10	6	0	0	1	1
		100	2	2	0	0	2
	40	10000	6	0	0	1	1
		100000	6	0	0	2	2

Table A-2.2. UV exposure experiment conditions

Trial No. 2 Absorbance = 0.168 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.812 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.039 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 1018 s
Trial No. 3 Absorbance = 0.049 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.924 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.045 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 894 s
Trial No. 4 Absorbance = 0.189 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.786 Radiometer reading at the center of Petri Dish = 0.057 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.038 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 1052 s
Trial No. 5 Absorbance = 0.946 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975 Water factor *Divergence factor = 0.387 Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2 Average Germicidal Irradiance throughout the water volume = 0.018 mW/cm^2 Time for a UV Dose of 40 mJ/cm^2 = 2254 s

Table A-3. Effect of treatment method on the infectivity of cysts in Mongolian gerbils

Trial no.	Treatment method	Inoculum cysts/gerbil	Total gerbils inoculated	Infection ratio			No. of gerbils infected	Precentage of gerbils infected
				Strong	Moderate	Weak		
1	Dynal	10,000	4	2	0	0	2	0.50
	Sucrose	10,000	4	4	0	0	4	1.00
	Percoll	10,000	4	4	0	0	4	1.00
2	NC	0	2	0	0	0	0	0.00
	Sucrose	1000	8	0	2	5	7	0.88
	Dynal	1000	6	2	0	2	4	0.67
3	NC	0	2	0	0	0	0	0.00
	Sucrose	10,000	8	6	0	0	6	0.75
	Dynal	10,000	8	7	0	0	7	0.88
	Heated	10,000	4	0	0	0	0	0.00

Notes: NC = negative control; Heated = Cysts were heated at 65°C for 15 min.

Table A-4. Trophozoites reproduction control experiment

Day 0 13-May-07 0 hr

Experiment condition	Hemocytometer counts								No. of trophozoites in flasks	Geomean	N/N_0
1	2	3	4	5	6	7	8	9	10	11	12
Control	7	1	3	5	1	7	10	0	42,500	58,196	0.73
	7	8	9	13	7	1	5	3	66,250		1.14
	7	8	11	3	5	7	6	9	70,000		1.20
Exposed to Air	0	4	2	7	6	6	9	4	47,500	39,414	1.21
	3	5	7	3	3	0	2	2	31,250		0.79
	4	2	2	10	3	2	8	2	41,250		1.05

Day 1 14-May-07 24 hr

Experiment condition	Hemocytometer counts								No. of trophozoites in flasks	Geomean	N/N_0
1	2	3	4	5	6	7	8	9	10	11	12
Control	15	9	16	27	20	10	16	9	152,500	144,159	2.62
	18	7	15	13	20	10	21	23	158,750		2.73
	13	8	13	15	9	18	14	9	123,750		2.13
Exposed to Air	12	11	5	6	14	8	7	10	91,250	85,864	2.32
	5	11	13	12	7	5	17	4	92,500		2.35
	6	8	1	6	7	15	8	9	75,000		1.90

Day 3 16-May-07 72 hr

Experiment condition	Hemocytometer counts								No. of trophozoites in flasks	Geomean	N/N_0
1	2	3	4	5	6	7	8	9	10	11	12
Control	32	29	43	37	29	26	30	33	323,750	330,591	5.56
	35	33	27	42	33	30	40	39	348,750		5.99
	39	27	28	46	34	27	24	31	320,000		5.50
Exposed to Air	15	9	13	21	12	7	13	13	128,750	159,259	3.27
	29	25	26	21	23	31	27	25	258,750		6.56
	9	12	7	14	8	23	15	9	121,250		3.08

Day 5 18-May-07 120 hr

Experiment condition	Hemocytometer counts								No. of trophozoites in flasks	Geomean	N/N_0
1	2	3	4	5	6	7	8	9	10	11	12
Control	57	63	52	44	71	61	34	57	548,750	362,405	9.43
	31	30	27	37	35	34	28	35	321,250		5.52
	19	25	35	27	39	24	23	24	270,000		4.64
Exposed to Air	33	20	23	28	41	36	25	24	287,500	238,836	7.29
	30	29	43	20	24	25	27	25	278,750		7.07
	17	18	16	20	17	20	14	14	170,000		4.31

Table A-5. Number of cysts collected from feces of gerbils inoculated with trophozoites (UV = 1 mJ/cm^2)

Infection date: Jan 04, 2006

Absorbance = 0.559 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975
Water factor *Divergence factor = 0.552
Radiometer reading at the center of Petri Dish = 0.053 mW/cm^2
Average Germicidal Irradiance throughout the water volume = 0.025 mW/cm^2
Time for a UV Dose of 1 mJ/cm^2 = 40 s

Unit: 10^4 cysts/g feces

Trophozoites	Gerbils	Days after UV exposure					
		5 to 9	12	13	14	15	16
Positive Control	10N	-	0.01	0.56	-	-	0.55
	10R	-	0.33	0.43	1.98	0.97	1.03
	10L	-	-	-	-	-	-
UV exposed	11N	-	0.23	0.79	-	-	-
	11R	-	-	-	-	-	-
	12N	-	0.25	0.51	1.39	0.87	0.29
	12R	-	0.12	0.37	-	-	-
Negative Control	13N	-	-	-	-	-	-
	13R	-	-	-	-	-	-

Table A-6. Small intestine check of gerbils inoculated with WB *G. lamblia* trophozoites (UV=1mJ/cm^2) (Day 16)

Infection date: Jan 04, 2006

Trophozoites	Gerbils	Section A		Section B		Section C	Section D	Note
Positive Control	10N	✓	✓	✓	✓	✓	✓	Strong
	10R	-	-	✓	✓	-	-	Strong
	10L	✓	✓	✓	✓	✓	✓	Moderate
UV exposed	11N	✓	✓	✓	✓	✓	✓	Strong
	11R	-	-	-	-	-	-	None
	12N	✓	✓	✓	✓	✓	✓	Strong
	12R	✓	✓	✓	✓	✓	✓	Strong
Negative Control	13N	-	-	-	-	-	-	None
	13R	-	-	-	-	-	-	None

Table A-7. Trophozoites reproduction after exposure to 1mL/cm^2 UV dose

Date: Jan 04, 2006

Day	counts in 0.1uL								Dilution	Conc.	Geomean
0	4	4	3	7	4	3	3	2	1:1	37500	37500
1	8	12	7	5	9	5	9	3	1:1	72500	72500
2	6	6	6	4	8	4	6	4	1:1	55000	55000
3	4	2	6	4	3	6	2	3	1:1	37500	37500
5	2	9	5	5	5	8	5	8	1:1	58750	58750
6	22	9	11	22	19	16	13	11	1:1	153750	153750
7	37	33	23	34	25	40	30	29	1:1	313750	313750
8	37	36	36	31	31	24	28	25	1:10	3100000	3449094
	43	46	42	42	31	31	24	48	1:10	3837500	
9	55	79	74	58	73	67	48	79	1:10	6662500	6859585
	79	69	65	89	49	89	70	55	1:10	7062500	

Table A-8. Reproduction of WB strain *G. lamblia* trophozoites in Diamond's TYI-S-33 medium

Date: September 9, 2005

UV = 0 mJ/cm^2	Day						
Trial No.	0	1	2	3	4	5	6
1	10000	25900	84000	216300	408100	1155000	1939000
2	10000	39200	63000	307300	399700	1687000	1610000
3	10000	29860	52500	319900	475300	1477000	
Geomean	10000	31181	65252	277041	426406	1422413	1766859
N/N_0	1	3	7	28	43	142	177

Table A-9. Reproduction of WB strain *G. lamblia* trophozoites in Diamond's TYI-S-33 medium

Date: Sept 29, 2005

Absorbance = 0.368 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975
Water factor *Divergence factor = 0.650
Radiometer reading at the center of Petri Dish = 0.058 mW/cm^2
Average Germicidal Irradiance throughout the water volume = 0.032 mW/cm^2
Time for a UV Dose of 1 mJ/cm^2 = 31.274 s
Time for a UV Dose of 5 mJ/cm^2 = 156.4 s
Time for a UV Dose of 10 mJ/cm^2 = 312.7 s

UV dose (mJ/cm^2)	counts in 0.1uL										No. of trophozoites in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8	9	10			
Day 0													
	7	5	0	1	4	3	0	4	3	4	31000	35767	0.87
0	5	1	4	8	6	2	2	5	4	4	41000		1.15
	1	2	5	6	2	3	6	4	1	6	36000		1.01
	1	4	3	2	5	3	4	2	3	0	27000	25718	1.05
1	1	0	1	5	4	2	1	0	4	3	21000		0.82
	1	4	3	3	4	2	4	3	3	3	30000		1.17
	4	5	5	6	6	2	2	4	0	8	42000	37030	1.13
5	6	4	2	4	7	1	2	3	6	4	39000		1.05
	4	3	2	5	3	2	1	0	5	6	31000		0.84
	2	7	6	8	6	3	4	4	5	6	51000	38397	1.33
10	1	7	1	4	2	2	3	5	4	1	30000		0.78
	5	5	5	2	4	6	3	3	2	2	37000		0.96
Day 1													
0	5	3	6	1	1	4	1	6	3	7	37000	39987	1.03
	6	0	8	6	4	7	3	5	7	2	48000		1.34
	5	4	4	10	1	4	2	3	3	0	36000		1.01
1	6	9	5	6	7	4	1	6	2	3	49000	42440	1.91
	4	2	9	4	2	2	4	5	4	4	40000		1.56
	5	6	5	4	1	8	4	4	2	0	39000		1.52
5	5	4	7	9	3	3	2	5	3	4	45000	38869	1.22
	6	1	1	7	1	3	3	2	3	2	29000		0.78
	3	1	4	4	3	6	3	7	6	8	45000		1.22
10	2	5	3	12	4	4	3	5	1	3	42000	39143	1.09
	5	5	0	7	4	4	0	3	2	4	34000		0.89
	2	2	2	5	4	6	9	4	2	6	42000		1.09

UV dose (mJ/cm^2)	counts in 0.1uL										No. of trophozoites in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8	9	10			
Day 2													
0	7	11	5	12	10	3	11	8	5	10	82000	62238	2.29
	6	6	8	4	5	7	9	9	6	10	70000		1.96
	5	5	2	4	10	3	2	1	7	3	42000		1.17
1	9	7	7	7	4	4	1	2	3	0	44000	37516	1.71
	6	10	2	2	3	3	9	2	1	2	40000		1.56
	3	5	4	4	4	1	4	3	1	1	30000		1.17
5	1	1	1	5	3	1	3	0	2	5	22000	24021	0.59
	1	2	4	3	9	3	2	3	1	2	30000		0.81
	1	0	1	0	3	4	1	2	7	2	21000		0.57
10	1	2	1	0	2	1	3	3	3	1	17000	16263	0.44
	2	2	2	2	2	0	0	0	1	0	11000		0.29
	0	4	1	1	3	1	5	2	5	1	23000		0.60
Day 5													
0	2	3	6	6	5	4	4	2	1	5	38000	27070	1.06
	4	2	5	0	4	4	0	4	1	5	29000		0.81
	2	3	3	2	2	0	1	1	2	2	18000		0.50
1	2	2	0	7	2	0	2	2	1	1	19000	22481	0.74
	0	4	1	4	3	4	5	2	1	2	26000		1.01
	1	4	2	5	1	1	0	6	1	2	23000		0.89
5	0	6	1	2	1	8	3	0	5	1	27000	16287	0.73
	0	1	1	0	1	0	1	1	2	3	10000		0.27
	5	2	1	0	1	0	1	3	0	3	16000		0.43
10	0	1	1	1	0	2	0	0	1	0	6000	4243	0.16
	0	0	0	0	1	2	0	0	0	0	3000		0.08
	0	0	0	0	0	0	0	0	0	0	0		0.00

Table A-10. Reproduction of WB strain *G. lamblia* trophozoites in Diamond's TYI-S-33 medium

Date: Jan 12, 2006

Absorbance = 0.391 cm^{-1} Petri factor = 0.870 Reflection factor = 0.975
Water factor *Divergence factor = 0.647
Radiometer reading at the center of Petri Dish = 0.052 mW/cm^2
Average Germicidal Irradiance throughout the water volume = 0.029 mW/cm^2
Time for a UV Dose of 10 mJ/cm^2 = 350 s
Time for a UV Dose of 40 mJ/cm^2 = 1400 s
Time for a UV Dose of 100 mJ/cm^2 = 3500 s

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	No. of trophozoites in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8				
Day 0												
10	29	21	27	22	25	33	30	23	1.0	262500	275625	0.95
	33	27	28	38	19	34	32	20		288750		1.05
40	54	39	49	37	58	62	57	55	1.0	513750	468750	1.86
	51	39	32	42	48	40	54	33		423750		1.54
100	43	59	65	57	86	46	51	69	1.0	595000	630000	2.16
	75	51	47	73	55	91	83	57		665000		2.41
Day 1												
10	27	18	14	31	12	12	19	12	1.0	181250	193125	0.66
	15	22	18	16	22	25	28	18		205000		0.74
40	36	36	26	41	35	30	28	28	1.0	325000	386250	1.18
	44	50	47	34	55	39	41	48		447500		1.62
100	9	8	14	12	15	9	16	13	1.0	120000	124375	0.44
	19	15	13	14	11	8	10	13		128750		0.47
Day 2												
10	7	11	13	8	11	12	9	13	1.0	105000	76250	0.38
	4	9	6	4	2	3	2	8		47500		0.17
40	5	2	2	2	1	2	2	2	0.4	7875	5250	0.03
	1	0	1	0	2	0	1	1		2625		0.01
100	0	0	0	0	0	0	0	0	0.4	0	438	0.00
	1	0	0	0	0	0	1	0		875		0.00
Day 3												
10	14	18	7	11	27	16	10	17	0.4	52500	44406	0.19
	12	7	16	9	4	14	9	12		36313		0.13
40	0	0	2	2	0	2	1	0	0.4	3063	2625	0.01
	0	0	1	2	1	1	0	0		2188		0.01
100	1	0	0	0	1	0	0	0	0.4	875	1094	0.00
	0	0	0	1	1	0	0	1		1313		0.00
Day 4												
10	22	11	11	23	15	5	16	5	0.4	47250	39156	0.17
	5	5	13	13	3	6	14	12		31063		0.11
40	0	0	1	1	0	0	0	1	0.4	1312.5	875	0.00
	0	0	0	0	0	1	0	0		437.5		0.00
100	0	1	0	0	0	0	0	0	0.4	437.5	875	0.00
	1	1	0	0	0	0	1	0		1312.5		0.00

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	No. of trophozoites in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8				
Day 5												
10	4	5	4	7	9	5	12	6	0.4	22750	21219	0.08
	9	7	4	5	3	6	7	4		19687.5		0.07
40	0	1	1	2	0	0	0	1	0.4	2187.5	1969	0.01
	0	1	0	0	0	2	1	0		1750		0.01
100	0	0	0	0	1	0	0	0	0.4	437.5	656	0.00
	1	0	0	0	1	0	0	0		875		0.00
Day 6												
10	4	2	2	5	0	1	2	2	0.4	7875	7875	0.03
	5	2	2	3	7	4	6	4		14437.5		0.05
40	0	0	1	0	0	2	1	0	0.4	1750	1313	0.01
	1	0	0	1	0	0	0	0		875		0.00
100	0	0	0	0	0	1	1	0	0.4	875	438	0.00
	0	0	0	0	0	0	0	0		0		0.00
Day 7												
10	2	1	1	0	2	0	1	1	0.4	3500	3500	0.01
	4	0	0	1	0	0	0	0		2187.5		0.01
40	1	0	0	0	0	0	0	0	0.4	437.5	438	0.00
	0	0	0	0	0	1	0	0		437.5		0.00
100	0	0	0	0	1	0	0	0	0.4	437.5	656	0.00
	0	0	1	0	1	0	0	0		875		0.00
Day 8												
10	0	1	0	0	1	0	0	0	0.4	875	875	0.00
	1	0	0	2	2	1	0	1		3062.5		0.01
40	1	0	0	0	0	0	0	0	0.4	437.5	438	0.00
	0	0	0	0	0	1	0	0		437.5		0.00
100	0	1	0	0	0	0	0	0	0.4	437.5	438	0.00
	0	1	0	0	0	0	0	0		437.5		0.00
Day 11												
10	1	0	4	0	2	2	2	0	0.5	6875	8438	0.02
	2	1	2	2	1	3	4	1		10000		0.04
40	0	0	0	0	0	1	0	0	0.5	625	313	0.00
	0	0	0	0	0	0	0	0		0		0.00
100	0	0	0	0	0	0	0	0	0.5	0	0	0.00
	0	0	0	0	0	0	0	0		0		0.00
Day 12												
10	29	23	23	31	30	41	28	36	1.0	301250	301250	1.09
	-	-	-	-	-	-	-	-				0.00
40	0	0	0	0	0	0	0	0	0.5	0	0	0.00
	0	0	0	0	0	0	0	0		0		0.00
100	0	0	0	0	0	0	0	0	0.5	0	0	0.00
	0	0	0	0	0	0	0	0		0		0.00
Day 13												
10	3	6	14	7	7	14	8	7	1.0	82500	82500	0.30
	0	0	0	0	0	0	0	0		0		0.00
40	0	0	0	0	0	0	0	0	0.5	0	0	0.00
	0	0	0	0	0	0	0	0		0		0.00
100	0	0	0	0	0	0	0	0	0.5	0	0	0.00
	0	0	0	0	0	0	0	0		0		0.00

Table A-11. Reproduction of WB strain *G. lamblia* trophozoites in Diamond's TYI-S-33 medium

Date: May 25, 2006

Absorbance = 0.286 cm^{-1} Petri factor = 0.928 Reflection factor = 0.975
Water factor *Divergence factor = 0.718
Radiometer reading at the center of Petri Dish = 0.055 mW/cm^2
Average Germicidal Irradiance throughout the water volume = 0.036 mW/cm^2
Time for a UV Dose of 10 mJ/cm^2 = 280 s
Time for a UV Dose of 40 mJ/cm^2 = 1120 s
Time for a UV Dose of 100 mJ/cm^2 = 2799 s

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
Day 0													
0	16	21	17	15	21	24	17	17	1.0		185000	187500	0.99
	8	30	22	21	10	24	25	12			190000		1.01
10	13	12	6	19	7	3	4	6	1.0		87500	102500	0.47
	10	12	10	10	11	26	7	8			117500		0.63
40	14	15	12	9	14	7	10	16	1.0		121250	91875	0.65
	2	6	7	8	8	9	8	2			62500		0.33
100	24	18	16	20	22	18	21	30	1.0		211250	230000	1.13
	21	19	20	18	29	25	40	27			248750		1.33
Day 1													
0	68	69	75	76	84	62	64	85	1.0		728750	716875	3.89
	64	71	83	71	76	70	64	65			705000		3.76
10	11	11	9	3	13	14	9	9	1.0		98750	94375	0.53
	12	12	9	13	7	3	8	8			90000		0.48
40	10	8	7	8	5	11	1	15	1.0		81250	75625	0.43
	9	3	9	8	4	11	5	7			70000		0.37
100	11	8	11	8	9	15	12	9	1.0		103750	106250	0.55
	14	12	9	10	10	12	11	9			108750		0.58
Day 2													
0	23	36	30	23	25	37	22	18	1.0		267500	238750	1.43
	24	15	25	27	25	14	19	19			210000		1.12
10	2	3	4	4	1	3	5	2	1.0		30000	35000	0.16
	6	6	3	3	7	1	2	4			40000		0.21
40	1	2	1	1	0	2	0	3	1.0		12500	11875	0.07
	0	1	0	1	1	0	4	2			11250		0.06
100	1	0	0	1	0	2	0	0	1.0		5000	3750	0.03
	0	0	1	0	0	0	0	1			2500		0.01
Day 3													
0	40	40	35	56	47	41	27	30	1.0	1:10	3950000	4162500	21.07
	49	36	32	47	60	34	46	46			4375000		23.33
10	3	5	4	2	1	4	5	5	0.4		12687.5	11156	0.07
	1	2	6	4	2	4	0	3			9625		0.05
40	4	4	2	1	2	3	1	3	0.4		8750	6344	0.05
	3	0	3	0	1	0	1	1			3938		0.02
100	3	2	0	4	3	1	0	1	0.4		6125	5906	0.03
	2	1	1	3	3	0	2	1			5688		0.03

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
Day 4													
0	54	67	47	75	55	44	37	55	1.0	1:10	5425000	6368750	28.93
	78	70	77	85	71	59	77	68			7312500		39.00
10	1	6	1	0	3	1	0	1	0.4		5688	7000	0.03
	3	2	4	2	2	4	1	1			8313		0.04
40	0	0	0	1	0	1	1	1	0.4		1750	1969	0.01
	0	0	2	1	0	0	1	1			2187.5		0.01
100	0	1	1	0	0	0	0	0	0.4		875	1094	0.00
	0	1	0	0	0	0	2	0			1312.5		0.01
Day 5													
0	10	6	5	7	4	8	13	7	1.0	1:100	7500000	8625000	40
	9	9	8	13	10	13	6	10			9750000		52
10	2	1	1	5	1	2	3	1	0.4		7000	7000	0.04
	1	4	0	2	3	0	2	4			7000		0.04
40	0	1	0	0	0	2	0	0	0.4		1312.5	3063	0.01
	1	2	0	2	1	3	1	1			4812.5		0.03
100	1	0	0	0	1	0	0	0	0.4		875	1313	0.00
	1	0	0	1	1	0	1	0			1750		0.01
Day 6													
0	44	32	34	38	46	42	31	26	1.0	1:100	36625000	24062500	195
	15	8	9	10	13	13	6	18			11500000		61
10	3	3	2	1	2	3	1	2	0.4		7437.5	7438	0.04
	1	6	2	3	3	2	4	3			10500		0.06
40	2	0	1	1	0	1	0	2	0.4		3062.5	2188	0.02
	0	1	0	0	0	2	0	0			1312.5		0.01
100	0	0	2	0	0	1	1	0	0.4		1750	2406	0.01
	1	0	1	3	1	0	0	1			3062.5		0.02
Day 7													
0	10	11	14	18	7	13	12	8	1.0	1:500	58125000	48125000	310
	6	6	7	15	5	5	11	6			38125000		203
10	1	0	1	0	2	1	1	3	0.4		3937.5	3938	0.02
	1	2	1	0	0	1	0	1			2625		0.01
40	0	0	0	0	1	0	1	0	0.4		875	1531	0.00
	1	1	0	1	0	1	1	0			2187.5		0.01
100	0	0	0	1	0	0	0	0	0.4		437.5	438	0.00
	1	0	0	0	0	0	0	0			437.5		0.00
Day 8													
0	9	10	15	8	9	14	14	9	1.0	1:500	55000000	61562500	293
	11	15	11	17	18	12	12	13			68125000		363
10	2	0	2	1	0	3	1	2	0.4		4812.5	4813	0.03
	2	0	5	2	2	0	1	0			5250		0.03
40	0	0	1	0	1	1	0	1	0.4		1750	1313	0.01
	0	1	1	0	0	0	0	0			875		0.00
100	0	0	0	0	0	1	1	0	0.4		875	656	0.00
	0	0	1	0	0	0	0	0			437.5		0.00

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
Day 9													
0	6	0	3	6	6	10	8	8	1.0	1:500	29375000	34687500	157
	6	4	7	11	11	4	11	10			40000000		213
10	0	1	0	1	4	1	0	4	0.7		9625	8313	0.05
	0	2	0	1	3	2	0	0			7000		0.04
40	0	1	0	0	0	1	2	0	0.7		3500	2625	0.02
	0	1	0	0	1	0	0	0			1750		0.01
100	0	1	0	0	1	0	2	0	0.7		3500	3063	0.02
	0	0	0	0	0	1	0	2			2625		0.01
Day 11													
10	6	2	1	5	4	2	3	4	0.7		23625	23625	0.13
	2	3	1	3	4	5	3	5			22750		0.12
40	0	2	1	1	2	1	0	1	0.7		7000	7875	0.04
	2	0	3	1	0	3	0	1			8750		0.05
100	0	1	0	1	0	1	0	0	0.7		2625	4438	0.01
	1	0	0	2	0	1	0	1			6250		0.03
Day 12													
10	4	6	4	4	2	6	5	5	0.7		31500	31500	0.17
	4	2	2	2	6	5	5	2			24500		0.13
40	1	4	1	2	0	1	3	1	0.7		11375	10938	0.06
	0	2	2	2	1	0	2	3			10500		0.06
100	5	5	4	3	3	2	2	2	0.7		22750	17938	0.12
	1	2	2	2	1	4	1	2			13125		0.07
Day 13													
10	4	5	2	3	7	4	5	3	0.7		28875	28875	0.15
	1	2	5	3	1	4	2	4			19250		0.10
40	3	3	4	2	3	3	4	1	0.7		20125	17500	0.11
	4	2	3	3	2	2	1	0			14875		0.08
100	0	2	1	0	1	2	1	2	0.7		7875	9188	0.04
	0	2	1	2	3	2	0	2			10500		0.06
Day 14													
10	5	1	4	5	4	8	8	3	0.7		33250	33250	0.18
	4	3	6	4	2	4	2	3			24500		0.13
40	2	1	4	2	2	0	0	2	0.7		11375	7875	0.06
	0	1	2	0	0	1	1	0			4375		0.02
100	6	2	2	5	6	1	0	5	0.7		23625	17063	0.13
	1	1	1	0	2	3	3	1			10500		0.06

Table A-12. Reproduction of WB strain *G. lamblia* trophozoites in Diamond's TYI-S-33 medium

Date: July 24, 2006

Absorbance = 0.424 cm^{-1} Petri factor = 0.928 Reflection factor = 0.975
Water factor *Divergence factor = 0.622
Radiometer reading at the center of Petri Dish = 0.060 mW/cm^2
Average Germicidal Irradiance throughout the water volume = 0.034 mW/cm^2
Time for a UV Dose of 1 mJ/cm^2 = 29.6 s
Time for a UV Dose of 10 mJ/cm^2 = 296.1 s
Time for a UV Dose of 20 mJ/cm^2 = 592.1 s
Time for a UV Dose of 40 mJ/cm^2 = 1184.2 s
Time for a UV Dose of 100 mJ/cm^2 = 2960.5 s

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
Day 0													
0	52	50	49	60	39	46	52	32	1.0		2.4E+05	2.4E+05	1.0
1	38	36	34	32	41	38	51	37	1.0		1.9E+05	1.9E+05	1.0
10	34	27	34	47	45	41	36	34	1.0		1.9E+05	1.9E+05	1.0
20	33	29	50	40	37	31	36	43	1.0		1.9E+05	1.9E+05	1.0
40	41	44	48	42	47	41	26	51	1.0		2.1E+05	2.1E+05	1.0
100	44	36	35	48	48	44	63	40	1.0		2.2E+05	2.2E+05	1.0
Day 1													
0	47	47	33	41	45	40	45	45	0.5		4.3E+05	5.0E+05	1.8
	61	36	51	80	57	61	65	45	0.5		5.7E+05		2.4
1	94	45	50	57	54	67	44	58	0.5		5.9E+05	4.6E+05	3.1
	38	33	45	23	28	35	39	30	0.5		3.4E+05		1.8
10	27	28	27	22	42	30	24	39	0.5		3.0E+05	3.7E+05	1.6
	34	52	42	33	56	40	28	68	0.5		4.4E+05		2.4
20	42	28	24	26	31	31	34	26	0.5		3.0E+05	2.4E+05	1.6
	22	17	20	20	22	18	13	16	0.5		1.9E+05		1.0
40	32	31	25	22	27	27	25	31	0.5		2.8E+05	2.8E+05	1.3
	27	31	38	23	25	25	26	33	0.5		2.9E+05		1.3
100	28	17	15	14	8	16	16	14	0.5		1.6E+05	1.2E+05	0.7
	6	9	9	4	9	7	6	14	0.5		8.0E+04		0.4
Day 2													
0	11	13	12	11	20	18	14	15	0.5	1:10	1.4E+06	1.3E+06	6.0
	17	10	10	10	13	9	12	13	0.5		1.2E+06		4.9
1	6	2	3	2	6	9	2	1	0.5	1:10	3.9E+05	6.3E+05	2.0
	6	8	16	7	4	20	3	5	0.5		8.6E+05		4.5
10	22	30	27	21	45	18	26	26	0.5		2.7E+05	2.8E+05	1.4
	22	45	21	29	29	21	25	34	0.5		2.8E+05		1.5
20	1	2	0	3	0	0	2	0	0.5		1.0E+04	1.9E+04	0.1

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
	2	4	2	1	4	4	3	2	0.5		2.8E+04		0.1
40	0	0	1	0	1	0	0	0	0.5		2.5E+03	1.9E+03	0.0
	0	1	0	0	0	0	0	0	0.5		1.3E+03		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 3													
0	11	9	13	10	10	3	13	9	0.5	1:100	9.8E+06	8.3E+06	41.1
	9	3	10	6	8	2	7	9	0.5		6.8E+06		28.4
1	15	9	10	9	15	9	7	12	0.5	1:10	1.1E+06	1.1E+06	5.6
	10	8	16	11	7	12	16	6	0.5		1.1E+06		5.6
10	16	20	14	17	17	20	18	22	0.5		1.8E+05	1.6E+05	1.0
	20	20	18	13	7	10	15	16	0.5		1.5E+05		0.8
20	1	0	0	2	3	0	1	0	0.5		8.8E+03	6.9E+03	0.0
	0	2	0	0	1	0	1	0	0.5		5.0E+03		0.0
40	1	0	0	0	0	0	0	0	0.5		1.3E+03	6.3E+02	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 4													
0	13	12	10	11	19	11	18	10	0.5	1:100	1.3E+07	1.7E+07	54.7
	15	23	21	22	27	21	25	21	0.5		2.2E+07		92.1
1	13	19	20	12	13	12	11	12	0.5	1:10	1.4E+06	1.2E+06	7.3
	10	9	9	10	14	10	7	9	0.5		9.8E+05		5.1
10	9	13	7	9	11	14	13	12	0.5		1.1E+05	1.1E+05	0.6
	14	14	12	7	6	14	11	12	0.5		1.1E+05		0.6
20	1	0	1	2	1	2	0	0	0.5		8.8E+03	5.6E+03	0.0
	0	0	1	0	0	0	1	0	0.5		2.5E+03		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 5													
0	88	70	71	72	64	90	74	81	0.5	1:100	7.6E+07	5.6E+07	321.1
	23	45	36	41	41	37	20	38	0.5		3.5E+07		147.9
1	9	12	10	14	12	12	14	9	0.5	1:10	1.2E+06	1.4E+06	6.0
	12	22	21	13	15	13	11	17	0.5		1.6E+06		8.1
10	1	1	0	1	3	3	0	0	0.5		1.1E+04	1.9E+04	0.1
	5	0	2	3	1	4	1	5	0.5		2.6E+04		0.1
20	0	0	0	0	0	1	0	0	0.5		1.3E+03	6.3E+02	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 6													
0	8	3	7	8	9	4	7	2	0.5	1:10 00	6.0E+04	6.2E+07	0.3
	7	7	3	6	4	11	9	4	0.5		6.4E+04		0.3
1	48	63	39	33	58	43	29	66	0.5	1:10	4.7E+05	4.7E+06	2.5
	51	29	37	24	47	28	24	35	0.5		3.4E+05		1.8
10	0	0	0	0	0	0	0	0	0.5		0.0E+00	6.3E+02	0.0
	0	0	0	0	1	0	0	0	0.5		1.3E+03		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 7													
0	11	9	9	9	10	8	4	7	0.5	1:10 00	8.4E+07	7.4E+07	352.6
	6	5	5	5	8	9	7	7	0.5		6.5E+07		273.7
1	36	61	68	37	67	51	49	61	0.5	1:10	5.4E+06	5.4E+06	28.0
	-	-	-	-	-	-	-	-					
10	0	0	0	1	0	0	0	0	0.5		1.3E+03	1.3E+03	0.0
	1	0	0	0	0	0	0	0	0.5		1.3E+03		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 8													
0	5	8	6	1	8	5	10	5	0.5	1:10 00	6.0E+07	7.7E+07	252.6
	3	16	13	8	10	10	5	10	0.5		9.4E+07		394.7
1	42	53	46	29	58	38	43	43	0.5	1:10 0	4.4E+07	4.4E+07	229.3
	37	54	43	51	42	33	31	43	0.5		4.2E+07		217.6
10	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 9													
0	6	6	3	4	5	4	1	1	0.5	1:1000	3.8E+07	4.4E+07	157.9
	8	5	4	4	5	3	7	4	0.5		5.0E+07		210.5
1	44	29	34	44	39	27	43	45	0.5	1:100	3.8E+07	4.5E+07	198.7
	48	49	49	40	69	48	48	64	0.5		5.2E+07		270.4
10	0	0	0	1	0	0	0	0	0.5		1.3E+03	6.3E+02	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 10													
0	2	6	3	9	7	13	5	4	0.5	1:1000	6.1E+07	5.2E+07	257.9
	5	4	3	2	5	5	2	8	0.5		4.3E+07		178.9
1	126	99	124	102	90	90	102	124	0.5	1:100	1.1E+08	1.2E+08	558.3
	181	105	124	146	120	135	127	113	0.5		1.3E+08		684.7
10	1	0	2	0	2	0	0	0	0.5		6.3E+03	3.8E+03	0.0
	0	0	0	0	1	0	0	0	0.5		1.3E+03		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 11													
0	17	10	17	15	11	9	9	10	0.5	1:100	1.2E+07	9.1E+06	51.6
	6	6	7	11	3	3	6	5	0.5		5.9E+06		24.7
1	4	5	6	4	3	5	5	6	0.5	1:1000	4.8E+07	4.8E+07	247.6
	5	4	9	6	4	6	3	4	0.5		5.1E+07		267.1
10	0	0	0	0	0	0	0	0	0.5		0.0E+00	2.5E+03	0.0
	0	2	1	0	1	0	0	0	0.5		5.0E+03		0.0
20	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0

UV dose (mJ/cm^2)	counts in 0.1uL								Final pellet volume (mL)	Dilution	No. of trophs in flasks	Geomean	N/N_0
	1	2	3	4	5	6	7	8					
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
Day 15													
0	10	8	8	11	2	10	5	11	0.5	1:100	8.1E+06	7.7E+06	34.2
	8	7	10	6	12	3	5	7	0.5		7.3E+06		30.5
1	19	15	11	16	18	18	12	14	0.5	1:100	1.5E+07	1.5E+07	80.1
	21	17	12	11	15	18	20	16	0.5		1.6E+07		84.7
10	13	9	4	6	7	10	9	9	0.5	1:10	8.4E+05	8.3E+05	4.5
	10	8	7	12	10	6	4	8	0.5		8.1E+05		4.4
20	0	0	0	0	1	0	0	0	0.5		1.3E+03	1.3E+03	0.0
	0	1	0	0	0	0	0	0	0.5		1.3E+03		0.0
40	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0
100	0	0	0	0	0	0	0	0	0.5		0.0E+00	0.0E+00	0.0
	0	0	0	0	0	0	0	0	0.5		0.0E+00		0.0

Table A-13. Details of Wastewater Quality Parameters during Sampling Periods

Table A-13.1. Edmonton Wastewater Treatment Plant

Sampling date	TSS (mg/L)	UVT%	Fecal coliform		Total coliform	
			U/S UV	D/S UV	U/S UV	D/S UV
May-04	4	58	6,800	38	110,000	1,770
Jun-04	4	63 to 64	7,700	52	110,000	450
Jul-04	4	61	5,900	12	80,000	190
Aug-04	5	63	20,000	46	300,000	510
Dec-04	4	62	7,800	15	12,000	380

Table A-13.2. Kelowna Wastewater Treatment Plant

Sampling date	TSS (mg/L)	UVT%	Fecal coliform		Total coliform	
			U/S UV	D/S UV	U/S UV	D/S UV
Jun-04	0.9 to 1.2	64.5	1 to 2,000	0	24,900 to 35,000	0 to 5.4
Jul-04	0.2 to 0.3	63 to 65	0 to 3,000	0	13,200 to 30,900	0 to 1
Mar-05	0.9 to 1.1	62 to 65	8,500	0 to 1	86,500 to 98,800	1 to 3.1
Apr-05	0.9 to 1.0	62 to 64	1,000 to 2,000	0 to 1	8,600 to 12,200	1 to 3.1
May-05	1.0	60 to 62	18,900 to 20,100	0	110,600 to 133,400	0 to 2
Jun-05	0.2 to 0.4	63 to 64	1,000 to 2,000	0	10,800 to 10,900	1 to 3

Table A-13.3. Winnipeg Wastewater Treatment Plant

Sampling date	TSS (mg/L)	UVT%	Fecal coliform		Total coliform	
			U/S UV	D/S UV	U/S UV	D/S UV
Aug-04	8 to 12	65*	-	-	-	-
Jun-05	13 to 18	65*	230,000	4,300	1,500,000	9300[&]

* Online data. UVT% of grab samples are between 48% and 55% measured by filtered samples in the lab.

[&] There was a laboratory error with the total coliform test and the results for this parameter are considered presumptive.

Table A-13.4. Alberta Capital Region Wastewater Treatment Plant

Sampling date	TSS (mg/L)	UVT%	Fecal coliform		Total coliform	
			U/S UV	D/S UV	U/S UV	D/S UV
May-05	4	51 to 55	56,000	320	310,000	2,300
Jun-05	3	58	16,000	210	120,000	20

Table A-14. ***Giardia lamblia*** **Cyst recovery test**

Table A-14.1. Recovery test on Microscope counting

Date: May 11, 2004

	Count 1	Count 2	Average	Total average	Recovery (%)
Slide 1	83	81	81.7	84.6	73.1
Slide 2	98	*80*	89		
Slide 3	125	*116*	120.5	115.75	STDEV
Slide 4	121	*101*	111		7.6

Note: Slide 1 and 2 labelled with A100FLR-1x FL; Slide 3 and 4 without Antibody as reference.

Date: May 12, 2004

	Count 1	count 2	Average	Total average	Recovery (%)
Slide 1	98	*80*	89	73	83.4
Slide 2	82	*32*	57		
Slide 3	88	*77*	82.5	87.5	STDEV
Slide 4	84	*101*	92.5		28.5
Slide 5	0	*0*			

Note: Slide 1 and 2 labelled with A100FLR-1x FL; Slide 3 and 4 without Antibody as reference; Slide 5 is negative control.

Date: May 25, 2004

	Count 1	Count 2	Average	Total average	Recovery (%)
Cy3	98	95	97	85	70.8
FITC	73	*74*	73.5		STDEV
Calculated	120	120			13.3

Note: Using Cy3 labeled spiking suspension as sample.

Table A-14.2. Initial recovery (50 L DI water)

Date	Number of cysts				Mean recovery%	
	Calculated	Count 1	Count 2	Count 3	Average	STDEV
5/17/2004	400	213	157	181	39.5%	9.0%
5/20/2004	400	150	116			

Table A-14.3. Spiked matrix recovery

Date: 2004-06-17

Trial no.	Count 1	Count 2	Mean Recovery%		
			Average	Total average	STDEV
1	46	46	38%	23%	22%
2	9	9	8%		

Using GB wastewater spiked with Texas Red labeled *G.lamblia*.
Number of cysts spiked = 120

Table A-15. Detailed UV reactor sampling information

Wastewater Treatment Plant	Sampling date	Sampling site	Sample Volume (L)	Flow rate (ML/d)	UV dose (mJ/cm^2)	Cysts enumerated (no./100L)	Cysts calculated (no./100L)
Edmonton	5/27/2004	U/S	200		23.8 to 32.4	860	3739
		D/S	140		23.8 to 32.4	901	3917
	6/14/2004	U/S	200		23.6 to 26.9	1239	5387
		D/S	120		23.6 to 26.9	1200	5217
	7/14/2004	U/S	200		23.6	838	3643
		D/S	200		25.3 to 30.1	900	3913
	8/5/2004	U/S	190		23.5	2175	9457
		D/S	190		23.6 to 32.9	1988	8643
	12/1/2004	U/S	175		23.1	1371	5961
		D/S	165		32.7	1636	7113
Kelowna	6/21-22/2004	U/S	459		50	539	2343
		D/S	826		50	689	2996
	7/19-20/2004	U/S	1135		50	295	1283
		D/S	1151		50	126	548
	3/14/2005	U/S	365		50	4918	21383
		D/S	645		50	2736	11896
	4/25/2005	U/S	550		50.6	314	1365
		D/S	510		50.6	441	1917
	5/16/2005	U/S	630		50.6	215	935
		D/S	790		50.6	234	1017
	6/6/2005	U/S	836		50.6	198	861
		D/S	897		50.6	142	617
Winnipeg	8/9/2004	U/S	190		38.8 to 39.1	2275	9891
		D/S	160		48.4 to 32.8	3138	13643
	6/13/2005	U/S	110		18.6	3068	13339
		D/S	110		18.6	3114	13539
Alberta Capital Region	5/25/2005	U/S	90		35	917	3987
		D/S	90		39	1000	4348
	6/01/2005	U/S	120		57	1500	6522
		D/S	120		43	1604	6974

Table A-16. Alberta Capital Region WWTP ASF bacteria inactivation by UV reactor

Table A-16.1

Sample time: 08/02/2006 AM — UV dose: 40 mJ/cm^2

Volume (mL)	Trial No.	Sample volume					conc.	Geomean
		0.1	1	2.5	10	25		
With Homogenization								
Before UV	1	11	77		TNTC		9350	10695
	2	14	108		TNTC		12400	
	3	12	91		TNTC		10550	
After UV	1	0		3		53	166	200
	2	0		7		53	246	
	3	0		3		68	196	
Without Homogenization								
Before UV	1	5	105		TNTC		7750	8298
	2	4	106		TNTC		7300	
	3	13	72		TNTC		10100	
After UV	1	0		4		51	182	205
	2	0		7		49	238	
	3	0		5		49	198	

Table A-16.2

Sample time: 08/09/2006 PM — UV dose: 16 mJ/cm^2

Volume (mL)	Trial No.	Sample volume					conc.	Geomean
		0.1	1	2.5	10	25		
With Homogenization								
Before UV	1	7	65		TNTC		6750	7336
	2	9	82		TNTC		8600	
	3	7	66		TNTC		6800	
After UV	1			3		53	166	200
	2			7		53	246	
	3			3		68	196	
Without Homogenization								
Before UV	1	6	47		TNTC		5350	5584
	2	10	55		TNTC		7750	
	3	2	64		TNTC		4200	
After UV	1			20		181	762	928
	2			21		239	898	
	3			25		214	928	

Table A-16.3

Sample time: 08/15/2006 AM UV dose: 20 mJ/cm^2

Volume (mL)	Trial No.	Sample volume					conc.	Geomean
		0.1	1	2.5	10	25		
With Homogenization								
Before UV	1	8	61		TNTC		7050	7570
	2	16	70		TNTC		11500	
	3	3	77		TNTC		5350	
After UV	1	0		25		TNTC	1000	944
	2	0		21		TNTC	840	
	3	0		25		TNTC	1000	
Without Homogenization								
Before UV	1	10	68		TNTC		8400	8365
	2	8	83		TNTC		8150	
	3	9	81		TNTC		8550	
After UV	1	0		23		TNTC	920	944
	2	0		22		TNTC	880	
	3	0		26		TNTC	1040	

Table A-17. Alberta Capital Region WWTP ASF bacteria inactivation by collimated beam UV apparatus

Date: 08/03/2006

Absorbance	W/ Homogenization	w/o Homogenization
with standard sampler	0.259	0.242
with integrating sphere attachment	0.228	0.2160

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.691

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.035 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 279.5 s

Time for a UV Dose of 20 mJ/cm^2 = 559.1 s

Time for a UV Dose of 40 mJ/cm^2 = 1118.2 s

Table A-17.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	12	92				10600	10006
	2	17	100				13500	
	3	7					7000	
10	1		60		TNTC		6000	6032
	2		53		TNTC		5300	
	3		69		TNTC		6900	
20	1			57		TNTC	2280	2380
	2			56		TNTC	2240	
	3			66		TNTC	2640	
40	1			10		138	476	477
	2			11		126	472	
	3			10		141	482	

Table A-17.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	12	92				10600	10006
	2	17	100				13500	
	3	7					7000	
10	1		63		TNTC		6300	6132
	2		60		TNTC		6000	
	3		61		TNTC		6100	
20	1			66		TNTC	2640	2477
	2			58		TNTC	2320	
	3			62		TNTC	2480	
40	1			10		148	496	545
	2			17		152	644	
	3			12		134	508	

Table A-17.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	12	92				10600	10006
	2	17	100				13500	
	3	7	-				7000	
10	1		48		TNTC		4800	4440
	2		38		TNTC		3800	
	3		48		TNTC		4800	
20	1			61		TNTC	2440	2249
	2			55		TNTC	2200	
	3			53		TNTC	2120	
40	1			14		106	492	437
	2			10		112	424	
	3			10		100	400	

Table A-18. Alberta Capital Region WWTP ASF bacteria inactivation by collimated beam UV apparatus

Date: 08/08/2006

Absorbance	W/ Homogenization	w/o Homogenization
with standard sampler	0.262	0.247
with integrating sphere attachment	0.233	0.225

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.707

Radiometer reading at the center of Petri Dish = 0.052 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.034 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 293.8 s

Time for a UV Dose of 20 mJ/cm^2 = 587.6 s

Time for a UV Dose of 40 mJ/cm^2 = 1175.1 s

Table A-18.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	11	93				10150	9154
	2	8	87				8350	
	3	9	91				9050	
10	1		38		TNTC		3800	3442
	2		37		TNTC		3700	
	3		29		TNTC		2900	
20	1			TNTC		TNTC		
	2			TNTC		TNTC		
	3			TNTC		TNTC		
40	1			33		140	940	819
	2			20		128	656	
	3			33		116	892	

Table A-18.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	11	93				10150	9154
	2	8	87				8350	
	3	9	91				9050	
10	1		43		TNTC		4300	4591
	2		50		TNTC		5000	
	3		45		TNTC		4500	
20	1			54		TNTC	2160	2184
	2			52		TNTC	2080	
	3			58		TNTC	2320	
40	1			15		88	476	400
	2			7		109	358	
	3			9		98	376	

Table A-18.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	10	80				9000	8454
	2	7	80				7500	
	3	10	79				8950	
10	1		22		TNTC		2200	2361
	2		26		TNTC		2600	
	3		23		TNTC		2300	
20	1			57		TNTC	2280	2061
	2			50		TNTC	2000	
	3			48		TNTC	1920	
40	1			-		89	356	360
	2			-		102	408	
	3			-		80	320	

Table A-19. Alberta Capital Region WWTP ASF bacteria inactivation by collimated beam UV apparatus

Date: 08/10/2006

Absorbance	W/ Homogenization	w/o Homogenization
with standard sampler	0.239	0.229
with integrating sphere attachment	0.216	0.205

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.726

Radiometer reading at the center of Petri Dish = 0.048 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.032 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 320.1 s

Time for a UV Dose of 20 mJ/cm^2 = 639.9 s

Time for a UV Dose of 40 mJ/cm^2 = 1279.9 s

Table A-19.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	7	65				6750	7336
	2	9	82				8600	
	3	7	66				6800	
10	1		31		TNTC		3100	3165
	2		31		TNTC		3100	
	3		33		TNTC		3300	
20	1			25		TNTC	1000	1052
	2			26		TNTC	1040	
	3			28		TNTC	1120	
40	1			11		116	452	485
	2			13		113	486	
	3			13		-	520	

Table A-19.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	7	65				6750	7336
	2	9	82				8600	
	3	7	66				6800	
10	1		37		TNTC		3700	3562
	2		33		TNTC		3300	
	3		37		TNTC		3700	
20	1			25		TNTC	1000	1091
	2			28		TNTC	1120	
	3			29		TNTC	1160	
40	1			4		52	184	176
	2			5		44	188	
	3			3		49	158	

Table A-19.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	6	47				5350	5584
	2	10	55				7750	
	3	2	64				4200	
10	1		36		TNTC		3600	3833
	2		34		TNTC		3400	
	3		46		TNTC		4600	
20	1			27		TNTC	1080	990
	2			17		TNTC	680	
	3			33		TNTC	1320	
40	1			-		94	376	412
	2			-		102	408	
	3			-		114	456	

Table A-20. Alberta Capital Region WWTP ASF bacteria inactivation by collimated beam UV apparatus

Date: 08/18/2006

Absorbance	W/ Homogenization	w/o Homogenization
with standard sampler	0.246	0.235
with integrating sphere attachment	0.223	0.218

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.713

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.036 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 285.9 s

Time for a UV Dose of 20 mJ/cm^2 = 574.0 s

Time for a UV Dose of 40 mJ/cm^2 = 1146.1 s

Table A-20.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	8	61				7050	8220
	2	16	70				11500	
	3	6	77				6850	
10	1		28		TNTC		2800	2809
	2		22		TNTC		2200	
	3		36		TNTC		3600	
20	1			39		TNTC	1560	1753
	2			48		TNTC	1920	
	3			45		TNTC	1800	
40	1			12		93	426	344
	2			5		82	264	
	3			7		111	362	

Table A-20.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	8	61				7050	8220
	2	16	70				11500	
	3	6	77				6850	
10	1		12		TNTC		1200	1957
	2		25		TNTC		2500	
	3		25		TNTC		2500	
20	1			34		TNTC	1360	1565
	2			44		TNTC	1760	
	3			40		TNTC	1600	
40	1			7		58	256	271
	2			5		66	232	
	3			10		67	334	

Table A-20.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	10	68				8400	8363
	2	9	83				8650	
	3	8	81				8050	
10	1		32		TNTC		3200	3129
	2		33		TNTC		3300	
	3		29		TNTC		2900	
20	1			38		TNTC	1520	1389
	2			29		TNTC	1160	
	3			38		TNTC	1520	
40	1			9		81	324	350
	2			10		89	356	
	3			6		93	372	

Table A-21. Alberta Capital Region WWTP Secondary effluent spiked with Bacillus ATCC 6633 inactivation by collimated beam UV apparatus

Date: 08/19/2006

Wastewater: upstream UV reactor wastewater spiked with ATCC 6633

Absorbance	W/ Homogenization	w/o Homogenization
with integrating sphere attachment	0.214	0.207

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.724

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.036 mW/cm^2

Time for a UV Dose of 20 mJ/cm^2 = 552.4 s

Time for a UV Dose of 40 mJ/cm^2 = 1104.8 s

Table A-21.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			29	5	3.8E+05	4.2E+05	
	2			49	5	5.0E+05		
	3			38	4	3.9E+05		
20	1			6	0	3.0E+04	4.7E+04	0.95
	2			2	0	1.0E+04		
	3			6	0	3.0E+04		
40	1		1	5		2.6E+04	1.7E+04	1.4
	2		0	0		0.0E+00		
	3		0	0		0.0E+00		

Table A-21.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			29	5	3.8E+05	4.2E+05	
	2			49	5	5.0E+05		
	3			38	4	3.9E+05		
20	1			0	1	5.0E+04	3.3E+04	1.10
	2			0	0	0.0E+00		
	3			0	0	0.0E+00		
40	1		54	8		6.7E+04	5.1E+04	0.91
	2		50	0		2.5E+00		
	3		50	5		5.0E+04		

Table A-21.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			35	6	3.7E+05	3.5E+05	
	2			43	5	4.4E+05		
	3			24	5	2.6E+05		
20	1			5	2	6.4E+04	4.0E+04	0.94
	2			2	0	1.8E+04		
	3			6	0	5.5E+04		
40	1			1	0	9.1E+03	9.1E+03	1.95
	2			0	0	0.0E+00		
	3			1	0	9.1E+03		

Table 22. Alberta Capital Region WWTP Secondary effluent spiked with Bacillus ATCC 6633 inactivation by collimated beam UV apparatus

Date: 08/24/2006

Wastewater: upstream UV reactor wastewater spiked with ATCC 6633

Absorbance	W/ Homogenization	w/o Homogenization
with integrating sphere attachment	0.241	0.233

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.699

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.035 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 285.9 s

Time for a UV Dose of 20 mJ/cm^2 = 571.8 s

Time for a UV Dose of 40 mJ/cm^2 = 1143.6 s

Table A-22.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			219	19	2.2E+06	2.2E+06	
	2			223	25	2.3E+06		
	3			224	22	2.2E+06		
10	1		TNTC	117		1.2E+06	1.1E+06	0.29
	2		TNTC	112		1.1E+06		
	3		TNTC	-				
20	1		TNTC	123		1.2E+06	1.1E+06	0.29
	2		TNTC	90		9.0E+05		
	3		TNTC	134		1.3E+06		
40	1			64	9	6.6E+03	7.5E+03	2.47
	2			68	5	6.6E+03		
	3			100	7	9.7E+03		

Table A-22.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			219	19	2.2E+06	2.2E+06	
	2			223	25	2.3E+06		
	3			224	22	2.2E+06		
10	1		TNTC	138		1.4E+06	1.6E+06	0.14
	2		TNTC	157		1.6E+06		
	3		TNTC	189		1.9E+06		
20	1		TNTC	38		3.8E+05	4.0E+05	0.75
	2		TNTC	44		4.4E+05		
	3		TNTC	37		3.7E+05		

Table A-22.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			178	21	1.8E+06	1.9E+06	
	2			204	26	2.1E+06		
	3			193	17	1.9E+06		
10	1			121	12	1.2E+06	1.2E+06	0.22
	2			105	12	1.1E+06		
	3			121	13	1.2E+06		
20	1		192	25		2.0E+05	2.1E+05	0.96
	2		211	20		2.1E+05		
	3		232	27		2.4E+05		
40	1	15	2			1.5E+03	2.1E+03	2.96
	2	18	5			2.1E+03		
	3	31	1			2.9E+03		

Table A-23. Alberta Capital Region WWTP Secondary effluent spiked with Bacillus ATCC 6633 inactivation by collimated beam UV apparatus

Date: 08/24/2006

Wastewater: upstream UV reactor wastewater spiked with ATCC 6633

Absorbance	W/ Homogenization	w/o Homogenization
with integrating sphere attachment	0.212	0.207

Petri factor = 0.950 Reflection factor = 0.975 Water factor *Divergence factor = 0.724

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Average Germicidal Irradiance throughout the water volume = 0.036 mW/cm^2

Time for a UV Dose of 10 mJ/cm^2 = 276.2 s

Time for a UV Dose of 20 mJ/cm^2 = 552.4 s

Time for a UV Dose of 40 mJ/cm^2 = 1104.8 s

Table A-32.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			214	17	2.1E+06	1.7E+06	
	2			146	19	1.5E+06		
	3				17	1.7E+06		
10	1		TNTC	110		1.1E+06	1.0E+06	0.24
	2		TNTC	101		1.0E+06		
	3		TNTC	92		9.2E+05		
20	1		220	18		2.2E+05	2.2E+05	0.90
	2		227	35		2.4E+05		
	3		202	25		2.1E+05		
40	1	34	4			3.5E+03	3.5E+03	2.70
	2	30	2			2.9E+03		
	3	34	2			4.4E+03		

Table A-23.2. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)				Concentration (cfu/100mL)	Geomean	Log N/N_0
		10^{-2}	10^{-3}	10^{-4}	10^{-5}			
0	1			142	14	1.4E+06	1.8E+06	
	2			185	12	1.8E+06		
	3			164	23	2.3E+06		
10	1		TNTC	78		7.8E+05	8.1E+05	0.35
	2		TNTC	84		8.4E+05		
	3		TNTC	81		8.1E+05		
20	1		161	13		1.6E+05	1.7E+05	1.03
	2		172	21		1.8E+05		
	3		171	18		1.7E+05		
40	1	22	5			2.5E+03	2.8E+03	2.80
	2	29	1			2.7E+03		
	3	34	4			3.5E+03		

Table A-24. Edmonton Gold Bar WWTP ASF bacteria inactivation by UV reactor

Table A-24.1

Sample time: 06/07/2006 AM — UV dose: 24 mJ/cm^2

Volume (mL)	Trial No.	Sample volume				conc. (cfu/100mL)	Geomean
		0.1	1	10	100		
With Homogenization							
Before UV	1	3	62	TNTC		6200	5759
	2	11	56	TNTC		5600	
	3	6	55	TNTC		5500	
After UV	1		2	25	TNTC	250	263
	2		2	28	TNTC	280	
	3		1	26	TNTC	260	
Without Homogenization							
Before UV	1	14	76	TNTC		7600	5819
	2	3	54	TNTC		5400	
	3	7	48	TNTC		4800	
After UV	1		5	23	TNTC	230	248
	2		1	30	TNTC	300	
	3		5	22	TNTC	220	

Table A-24.2

Sample time: 06/19/2006 PM — UV dose: 24 mJ/cm^2

Volume (mL)	Trial No.	Sample volume				conc. (cfu/100mL)	Geomean
		0.1	1	10	100		
With Homogenization							
Before UV	1	5	56		TNTC	5600	5533
	2	7	63		TNTC	6300	
	3	6	48		TNTC	4800	
After UV	1				166	166	235
	2			26	183	222	
	3			52	187	354	
Without Homogenization							
Before UV	1	4	45		TNTC	4500	4752
	2	7	53		TNTC	5300	
	3	4	45		TNTC	4500	
After UV	1			22	189	205	198
	2			16	187	174	
	3			23	209	220	

Table A-24.3

Sample time: 06/28/2006 AM UV dose: 24 mJ/cm^2

Volume (mL)	Trial No.	Sample volume					conc.	Geomean
		0.1	1	2.5	10	25		
With Homogenization								
Before UV	1	3	42		TNTC		3600	4017
	2	3	45		TNTC		3750	
	3	5	46		TNTC		4800	
After UV	1			4		36	152	108
	2			3		34	128	
	3			0		32	64	
Without Homogenization								
Before UV	1	3	36		TNTC		3600	3393
	2	1	31		TNTC		3100	
	3	2	35		TNTC		3500	
After UV	1			8		51	204	224
	2			6		63	252	
	3			5		55	220	

Table A-25. Edmonton WWTP ASF bacteria inactivation by collimated beam UV apparatus

Date: 06/28/2006

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Absorbance	w/ Homogenization	w/o Homogenization
with standard sampler	0.334	0.275
Petri factor	0.950	
Reflection factor	0.975	
Water factor × Divergence factor	0.691	0.721
Average Germicidal Irradiance (mW/cm^2)	0.035	0.036
Time for a UV Dose of 10 mJ/cm^2	277.0	277.3
Time for a UV Dose of 20 mJ/cm^2	555.0	554.6
Time for a UV Dose of 40 mJ/cm^2	1109.0	1109.2

Table A-25.1 Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	3	42				3600	5061
	2	3	45				3750	
	3	5	46				9600	
10	1		20		TNTC		2000	1966
	2		19		TNTC		1900	
	3		20		TNTC		2000	
20	1			44		TNTC	1760	1539
	2			35		TNTC	1400	
	3			37		TNTC	1480	
40	1			TFTC		63	252	242
	2			TFTC		62	248	
	3			TFTC		57	228	

Table A-25.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	3	42				3600	5061
	2	3	45				3750	
	3	5	46				9600	
10	1		16		TNTC		1600	1664
	2		16		TNTC		1600	
	3		18		TNTC		1800	
20	1			31		TNTC	1240	895
	2			19		TNTC	760	
	3			19		TNTC	760	
40	1			8		88	336	323
	2			9		82	344	
	3			5		96	292	

Table A-25.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	3	36				3300	3338
	2	1	31				2050	
	3	2	35				5500	
10	1		21		TNTC		2100	2074
	2		25		TNTC		2500	
	3		17		TNTC		1700	
20	1			13		TNTC	520	822
	2			23		TNTC	920	
	3			29		TNTC	1160	
40	1			5		81	262	257
	2			7		78	296	
	3			5		59	218	

Table A-26. Edmonton WWTP ASF bacteria inactivation by UV reactor

Date: 07/06/2006

Radiometer reading at the center of Petri Dish = 0.054 mW/cm^2

Absorbance	w/ Homogenization	w/o Homogenization
with standard sampler	0.413	0.352
with integrating sphere attachment	0.305	0.295
Petri factor	0.950	
Reflection factor	0.975	
Water factor × Divergence factor	0.639	0.667
Average Germicidal Irradiance (mW/cm^2)	0.032	0.033
Time for a UV Dose of 10 mJ/cm^2	285.0	282.9
Time for a UV Dose of 20 mJ/cm^2	570.1	565.8
Time for a UV Dose of 40 mJ/cm^2	1140.1	1131.6

Table A-26.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	9	54				7200	6032
	2	7	45				5750	
	3	6	46				5300	
10	1		37		TNTC		3700	3393
	2		33		TNTC		3300	
	3		32		TNTC		3200	
20	1			27		TNTC	1080	1012
	2			24		TNTC	960	
	3			25		TNTC	1000	
40	1			11		103	426	260
	2			2		94	228	
	3			9			180	

Table A-26.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	9	54				7200	6032
	2	7	45				5750	
	3	6	46				5300	
10	1		28		TNTC		2800	2595
	2		26		TNTC		2600	
	3		24		TNTC		2400	
20	1			30		TNTC	1200	1160
	2			29		TNTC	1160	
	3			28		TNTC	1120	
40	1			9		63	306	232
	2			2		61	162	
	3			5		76	252	

Table A-26.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	1	49				2950	3533
	2	2	41				3050	
	3	4	58				4900	
10	1		30		TNTC		3000	2363
	2		20		TNTC		2000	
	3		22		TNTC		2200	
20	1			26		TNTC	1040	1081
	2			23		TNTC	920	
	3			33		TNTC	1320	
40	1			4		37	154	156
	2			3		39	138	
	3			5		39	178	

Table A-27. Edmonton WWTP ASF bacteria inactivation by UV reactor

Date: 07/11/2006

Radiometer reading at the center of Petri Dish = 0.061 mW/cm^2

Absorbance	w/ Homogenization	w/o Homogenization
with standard sampler	0.309	0.297
with integrating sphere attachment	0.300	0.287
Petri factor	0.950	
Reflection factor	0.975	
Water factor × Divergence factor	0.699	0.705
Average Germicidal Irradiance (mW/cm^2)	0.039	0.040
Time for a UV Dose of 10 mJ/cm^2	253.2	251.1
Time for a UV Dose of 20 mJ/cm^2	506.4	502.2
Time for a UV Dose of 40 mJ/cm^2	1012.9	1004.4

Table A-27.1. Without Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	2	44				3200	3643
	2	5	56				5300	
	3	1	47				2850	
10	1		37		TNTC		3700	3557
	2		38		TNTC		3800	
	3		32		TNTC		3200	
20	1			23		TNTC	920	959
	2			20		TNTC	800	
	3			30		TNTC	1200	
40	1			5		129	358	325
	2			7		83	306	
	3			7		86	312	

Table A-27.2. UV followed by Homogenizer

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	2	44				3200	3643
	2	5	56				5300	
	3	1	47				2850	
10	1		28		TNTC		2800	3109
	2		29		TNTC		2900	
	3		37		TNTC		3700	
20	1			30		TNTC	1200	913
	2			22		TNTC	880	
	3			18		TNTC	720	
40	1			6		95	310	323
	2			6		80	280	
	3			12		75	390	

Table A-27.3. Homogenizer followed by UV

UV doses (mJ/cm^2)	Trial No.	Sample volume (mL)					Concentration (/100mL)	Geomean
		0.1	1	2.5	10	25		
0	1	2	43				3150	3783
	2	8	45				6250	
	3	1	45				2750	
10	1		25		TNTC		2500	2976
	2		31		TNTC		3100	
	3		34		TNTC		3400	
20	1			22		TNTC	880	995
	2			25		TNTC	1000	
	3			28		TNTC	1120	
40	1			5		70	240	271
	2			9		70	320	
	3			5		79	258	

Appendix B. Statistics Analysis

Table B-1. Logistic model using pooled WB strain data set

cysts	no. of gerbils inoculated	no. of gerbils infected	proportion of gerbils infected	log d	p/(1-p)	lnP/(1-p)	b0+b1*Col 5	(Col 7 - Col 8)^2
1	2	3	4	5	6	7	8	9
100	16	7	0.44	2.00	0.78	-0.25	-0.398	0.022
1,000	16	11	0.69	3.00	2.20	0.79	1.082	0.086
10,000	16	15	0.94	4.00	15.00	2.71	2.561	0.022
							SUM =	0.129

Use least square analysis,

b0 = -3.36

b1= 1.48 ID_{50} = 186

Table B-2. Logistic model using pooled H3 strain data set

cysts	no. of gerbils inoculated	no. of gerbils infected	proportion of gerbils infected	log d	p/(1-p)	lnP/(1-p)	b0+b1*Col 5	(Col 7 - Col 8)^2
1	2	3	4	5	6	7	8	9
10	16	4	0.25	1.00	0.33	-1.10	-1.099	0.000
100	18	16	0.89	2.00	8.00	2.08	2.079	0.000
							SUM =	0.000

Use least square analysis,

b0 = -4.28

b1= 3.18 ID_{50} = 22

Table B-3. Student's *t*-test on the sucrose and IMS method

Treatment method	Percentage of gerbils infected		
	1	2	3
Sucrose method	0.88	1.00	0.75
Dynal IMS method	0.67	0.50	0.88

Student's *t*-test results

	Sucrose	*DYNAL*
Mean	0.88	0.69
Variance	0.03	0.07
df	2	2

t Stat= 0.83 P value = 0.49

$t_{0.025,2}$ = 4.30

$t < t_{0.025,2}$, therefore, null Hypothesis is true and there is no significant difference betweeen the infectivity of *Giardia* spp. cysts treated by the sucrose method and Dynal IMS method.

Table B-4. Statistics analysis of log inactivation of WB and H3 strain cysts at UV dose of 40 mJ/cm^2

Isolate	Trial no.	Inoculated cysts per gerbil, d_0	Total no. of gerbils inoculated	Total no. of gerbils infected	Proportion infected, P	Infectious cysts, d	Inactivation, -log d/d_0	Mean Inactivation
WB		10000	4	1	0.25	34	2.5	
	1	100000	4	3	0.75	1027	2.0	2.2
		10000	4	2	0.5	186	1.7	
	2	100000	4	3	0.75	1027	2.0	1.9
		10000	4	2	0.5	186	1.7	
	3	100000	4	3	0.75	1027	2.0	1.9
		10000	4	3	0.75	1027	1.0	
	4	100000	4	2	0.5	186	2.7	1.9
H3		1000	6	0	0	< 7	> 2.2	
		10000	6	0	0	< 7	> 3.2	
	2	100000	6	1	0.17	7	4.2	4.2
		1000	4	1	0.25	10	2.0	
	3	10000	8	3	0.38	15	2.8	2.4
		10000	4	0	0.00	<10	> 3.0	
	4	100000	6	1	0.17	7	4.2	4.2
		10000	6	1	0.17	7	3.2	
	5	100000	6	2	0.33	13	3.9	3.5

Student's *t*-Test: Results

$t = -3.707$ $t_{0.025,3} = 3.182$

$t > t_{0.025,3}$, therefore, there is significant difference betweeen the infectivity of WB strain and H3 *Giardia lamblia* cysts at 40 mJ/cm^2 UV exposure.

Table B-5. Modeling of reproduction of WB strain G. lamblia trophozoites

Equation: $N_T = K_a N_a t + K_d N_d t$
Parameters: Ka = 0.356 d^{-1} Kd = 1.388 d^{-1}

Table B-5.1.
Assume 10 cysts alive after UV exposure

Days	N_T	N_a	N_d	logN/N_0
0	100,000	10	99,990	0.00
1	4,118	23	4,095	-1.39
2	219	52	168	-2.66
3	124	117	7	-2.91
4	266	265	0	-2.58
5	603	603	0	-2.22
6	1,368	1,368	0	-1.86
7	3,105	3,105	0	-1.51
8	7,047	7,047	0	-1.15
9	15,996	15,996	0	-0.80
10	36,308	36,308	0	-0.44
11	82,414	82,414	0	-0.08
12	187,068	187,068	0	0.27
13	424,620	424,620	0	0.63
14	963,829	963,829	0	0.98
15	2,187,762	2,187,762	0	1.34

Table B-5.2.
Assume 1000 cysts alive after UV exposure

Days	N_T	N_a	N_d	logN/N_0
0	100,000	1,000	99,000	0.00
1	6,324	2,270	4,054	-1.20
2	5,318	5,152	166	-1.27
3	11,702	11,695	7	-0.93
4	26,546	26,546	0	-0.58
5	60,256	60,256	0	-0.22
6	136,773	136,773	0	0.14
7	310,456	310,456	0	0.49
8	704,693	704,693	0	0.85
9	1,599,558	1,599,558	0	1.20
10	3,630,781	3,630,781	0	1.56
11	8,241,381	8,241,381	0	1.92
12	18,706,821	18,706,821	0	2.27
13	42,461,956	42,461,956	0	2.63
14	96,382,902	96,382,902	0	2.98
15	218,776,162	218,776,162	0	3.34

Table B-5.3.
Assume 10,000 cysts alive after UV exposure

Days	N_T	N_a	N_d	logN/N_0
0	100,000	10,000	90,000	0.00
1	26,385	22,699	3,686	-0.58
2	51,674	51,523	151	-0.29
3	116,956	116,950	6	0.07
4	265,461	265,461	0	0.42
5	602,560	602,560	0	0.78
6	1,367,729	1,367,729	0	1.14
7	3,104,560	3,104,560	0	1.49
8	7,046,931	7,046,931	0	1.85
9	15,995,580	15,995,580	0	2.20
10	36,307,805	36,307,805	0	2.56
11	82,413,812	82,413,812	0	2.92
12	187,068,214	187,068,214	0	3.27
13	424,619,564	424,619,564	0	3.63
14	963,829,024	963,829,024	0	3.98
15	2,187,761,624	2,187,761,624	0	4.34

Table B-5.4.
Assume 100,000 cysts alive after UV exposure

Days	N_T	N_a	N_d	logN/N_0
0	100,000	100,000	0	0.00
1	275,994	275,994	0	0.44
2	626,470	626,470	0	0.80
3	1,422,001	1,422,001	0	1.15
4	3,227,751	3,227,751	0	1.51
5	7,326,558	7,326,558	0	1.86
6	16,630,297	16,630,297	0	2.22
7	37,748,526	37,748,526	0	2.58
8	85,684,053	85,684,053	0	2.93
9	194,491,220	194,491,220	0	3.29
10	441,468,784	441,468,784	0	3.64
11	1,002,074,475	1,002,074,475	0	4.00
12	2,274,573,631	2,274,573,631	0	4.36
13	5,162,974,737	5,162,974,737	0	4.71
14	11,719,254,887	11,719,254,887	0	5.07
15	26,601,124,757	26,601,124,757	0	5.42

Table B-6. Paired t-test of *G. lamblia* cysts concentrations between upstream and downstream of UV reactors

Sampling site	Sampling date	Upstream		Downstream		*dj*	dj^2
		Total sampling volume (L)	Cysts conc. (cysts/100L)	Total sampling volume (L)	Cysts conc. (cysts/100L)		
Edmonton	May-04	200	860	140	901	-41	1,681
	Jun-04	200	1239	120	1200	39	1,521
	Jul-04	200	838	200	900	-62	3,844
	Aug-04	200	2175	200	1988	187	34,969
	Dec-04	175	1371	165	1636	-265	70,225
Kelowna	Jun-04	1135	539	1151	689	-150	22,500
	Jul-04	365	295	645	126	169	28,561
	Mar-05	550	4918	510	2736	2,182	4,761,124
	Apr-05	630	314	790	441	-127	16,129
	May-05	836	215	897	234	-19	361
	Jun-05	459	198	826	142	56	3,136
Winnipeg	Aug-04	190	2,275	160	3,138	-863	744,769
	Jun-05	110	3,068	110	3,114	-46	2,116
Alberta Capital Region	May-05	90	917	90	1,000	-83	6,889
	Jun-05	120	1,500	120	1,604	-104	10,816
Total						873	5,708,641
Mean			1,381		1,323	58	
n			15		15	15	
STDEV,s			1,293		1,028	636	

From equation $t_0 = \dfrac{\bar{d}}{S_d / \sqrt{n}}$, $t_0 = 0.3545$

At $\alpha = 0.05$ and degree of freedon of 14, $t_{0.025,14} = 2.5095 > t_0$

Therefore, the null hypothesis Ho is accepted. There is no significant difference in the concentration of cysts between upstream and downstream of the UV reactors.

Table B-7. Paired t-test of inactivation of ASF bacteria by field UV reactor with and without homogenization

	UV dose (mJ/cm^2)	Without Homogenization	With Homogenization	dj	dj^2
Alberta Capital Region	40	1.73	1.61	0.12	0.0134
	16	0.96	0.83	0.14	0.0187
	20	0.93	0.95	-0.02	0.0005
Edmonton	24	1.42	1.46	-0.05	0.0022
	24	1.29	1.39	-0.10	0.0093
Total				0.088	0.0441
Mean		1.26	1.25	0.02	
n		5	5	6	
STDEV,s		0.33	0.34	0.10	

From equation $t_0 = \dfrac{\bar{d}}{S_d / \sqrt{n}}$, $t_0 = 0.382$

At $\alpha = 0.05$ and degree of freedon of 4, $t_{0.025,4} = 2.776 > t_0$

Therefore, the null hypothesis Ho is accepted. There is no significant difference in the inactivation of ASF bacteria between homogenization and without homogenization of upstream wastewater of the UV reactors.

Appendix C. Analytical Methods

C-1. One step procedure for preparation of solution of Percoll

Percoll (from bottle) may be diluted directly to make a final working solution of known density by the following procedure.

In a measuring cylinder, add 1/10 desired final volume, 1.5M NaCl or 2.5M sucrose (2.g. 100 mL for 100 mL working solution). To this, add the required volume of Percoll.

Calculate using the formula shown below:

$$v_0 = v\left(\frac{\rho - 0.1\rho_{10} - 0.9}{\rho_0}\right)$$

Where:

V0 = volume of Percoll (from bottle), mL

V = volume of the final working solution, mL

ρ = Desired density of the solution, g/mL

ρ0 = Density of Percoll (from bottle), g/mL

ρ10 = Density of 1.5M NaCl (1.058 g/mL) or density of 2.5M sucrose (1.316 g/mL)

Sample densities:

Percoll (from bottle) = 1.130 g/mL

1.5 M NaCl = 1.058 g/mL

2.5 M sucrose = 1.316 g/mL

Preparation of working solution of sodium chloride

Volume of working solution = 100 mL

Molarity, M = 1.5

Molecular weight of NaCl, MW = 58.44 g

Required mass of NaCl = Molarity (M) × Volume (L) × Molarity weight (WM)

= 1.5 × 0.1 × 58.44 g / 100 mL = 8.766 g / 100 mL

Preparation of working solutions of Percoll:

Volume of working solutions = 100 mL

1. Percoll density 1.05 g/mL

   Working solution of percoll of density 1.05 g/mL in 1.5 M NaCl

   Volume of Percoll required, $V = 100\left(\frac{1.05 - 0.1\times1.058 - 0.9}{1.130 - 1}\right) = 33.85$ mL

   Final solution:

   10 mL 1.5 M NaCl , 33.85 mL Percoll, and 56.15 mL distilled water

2. Percoll density 1.09 g/mL

   Working solution of percoll of density 1.09 g/mL in 1.5 M NaCl

   Volume of Percoll required, $V = 100\left(\frac{1.09 - 0.1\times1.058 - 0.9}{1.130 - 1}\right) = 64.62$ mL

   Final solution:

   10 mL 1.5 M NaCl , 64.62 mL Percoll, and 25.38 mL distilled water

C-2. 1M Sucrose solution preparation

1. On a stir plate, dissolve the molar mass of sucrose 342.3 g into a 1000 mL flask
2. Slightly heat the flask
3. Top with Milli-Q water to a total volume of 1000 mL

C-3. Texas Red stained *G. lamblia* cysts preparation

Giardia lamblia cysts used was bought from Waterborn™ Inc. and was H3 isolate suspended in 5% Formalin /PBS/0.01%Tween 20.

- Transfer 100 µL of Giardia solution into a microcentrifuge tube and adding 400 µL Milli-Q water.
- Adjust pH larger than 8.5 using approximately 10 µL of 1.0 N NaCl.

- Add Texas Red power into the tube, vortex 5 sec to mix.
- Incubate the tube at 4°C for 4 hours.
- Centrifuge the tube at 10,000 rpm for 10 min.
- Remove all the supernatant and re-suspend the pellet in 750 μL Milli-Q water.
- Count under 200 × magnificent microscope.

The above procedure for preparation of Texas Red stained *G. lamblia* cysts referred the following paper:

Titus, J. A., Haugland, R., Sharrow, S. O., and Segal, D. M. (1982) Texas red, a hydrophilic, red-emitting flourophore for use with flourescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *Journal of Immunological Methods*, **50**, 193-204.

G. lamblia cysts	Counts								Average
Before staining	10	11	11	12	10	15	8	12	11
After staining	8	13	13	16	9	15	14	20	12

Appendix D. *Giardia muris* acid treatment experiment measured by the mouse model

D-1. Protocol for *G. lamblia* cysts detection from laboratory gerbils

1. Place the Mongolian gerbils on false-bottom cages with a small amount of water in the cages to soften the feces.
2. Ddispense 1.5 mL Milli-Q water into 14 mL centrifuge tubes and weigh the tubes.
3. After 2 hours, collect feces in corresponding tubes and re-weigh the tubes.
4. Emulsify the content of each tube and gently pipette onto 3 mL of 1 M sucrose solution in glass culture tubes.
5. Centrifuge the culture tubes for 15 mins at 285 × g at 4° C.
6. Following centrifugation, the Giardia layer was directly above the sucrose layer. Remove and discard the top layer by pipette. The subsequent milky layer was pipetted onto 40 μm filters resting on 15 mL centrifuge tubes. Top the tubes with Milli-Q water and centrifuge for 10 min at 388 × g at 4° C.
7. The suspension was concentrated into 0.5 mL and the pellet was resuspended by gently pipetting the solution up and down.
8. Enumerate and count the number of cysts using hemocytometer under 200 × magnification by bright microscope.

D-2. Method of *G. muris* collection and concentration

G. muris strain used in this experiment was originally isolated by Roberts- Thomson from a golden hamster and maintained in the laboratory since 1981 using bi-weekly passage in CD-1 mice or C3H/HeN mice.

Procedure:

1. Place the mice into false-bottom cages for 2 hours each day post infection.
2. Ddispense 1.5 mL Milli-Q water into 14 mL centrifuge tubes and weigh the tubes.
3. After 2 hours, collect feces in corresponding tubes and re-weigh the tubes.
4. Emulsify the content of each tube and gently pipette onto 3 mL of 1 M sucrose solution in glass culture tubes.

5. Centrifuge the culture tubes for 15 mins at 400 × g at 4° C.
6. Following centrifugation, the cyst rich layer at the water-sucrose interface was carefully collected and pipetted onto 40 μm filters resting on 15 mL centrifuge tubes.
7. Top the tubes with Milli-Q water and centrifuge at 600 × g for 10 min at 4° C.
8. The suspension was concentrated into 0.5 mL and the pellet was resuspended by gently pipetting the solution up and down.
9. Enumerate and count the number of cysts using hemocytometer under 200 × magnification by bright microscope.

D-3. *G. muris* pH effect experiment

Freshly collected *G. muris* cysts were split into two parts, one part for control and the other part for the acid treatment.

Acid treated cysts:

- Centrifuge G. muris sample after sucrose flotation separation, remove all the supernatant.
- Re-suspend the pellet by adding 1 mL of 0.1 N HCl and incubate at room temperature for 10 min.
- Add 100 μL of 1.0 N NaCl and incubate at 4 degree for about 4 hours.
- Inoculate both control and acid treated cysts to 5 mice each at a concentration of 10,000 to 100,000 cyst per mouse (Adjust according to the hemocytometer count).
- Check feces of mice from Day 1 to Day 10 post-inoculation, or the cysts produced by mice stable in three continuous days. The procedure to
- Compare the cysts per gram feces produced by control mice group and mice inoculated by acid treated cysts.

Control cysts:

- Let the control cysts incubate at room temperature for 10 min and store at 4 degree as above test. Then inoculate to mice at the same time with acid treated cysts.

Table D-1. Mice feces check results for *G. muris* - acid treated vs. control (Unit: 10^4cysts/g feces)

Infection date: 09/21/2004 Day 1

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.88	-	-	-	-	-	-	two mice
	1-2	0.615	-	-	-	-	-	-	two mice
	1-3	0.204	-	-	-	-	-	-	one mice
	1-4								
	1-5								
2 Acid treated	2-1	0.406	-	-	-	-	-	-	
	2-2	0.376	-	-	-	-	-	-	
	2-3	0.38	-	-	-	-	-	-	
	2-4	0.228	-	-	-	-	-	-	
	2-5	0.357	-	-	-	-	-	-	

Day 2

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.725	-	-	-	-	-	-	two mice
	1-2	0.684	-	-	-	-	-	-	two mice
	1-3	0.252	-	-	-	-	-	-	one mice
	1-4								
	1-5								
2 Acid treated	2-1	0.41	-	-	-	-	-	-	
	2-2	0.382	-	-	-	-	-	-	
	2-3	0.256	-	-	-	-	-	-	
	2-4	0.347	-	-	-	-	-	-	
	2-5	0.324	-	-	-	-	-	-	

Day 3

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.877	-	-	-	-	-	-	two mice
	1-2	0.961	-	-	-	-	-	-	two mice
	1-3	0.319	-	-	-	-	-	-	one mice
	1-4								
	1-5								
2 Acid treated	2-1	0.467	-	-	-	-	-	-	
	2-2	0.616	-	-	-	-	-	-	
	2-3	0.521	-	-	-	-	-	-	
	2-4	0.310	-	-	-	-	-	-	
	2-5	0.656	-	-	-	-	-	-	

Day 4

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.447	1	1	0	0	1	1	
	1-2	0.162	7	4	5	5	5	32	
	1-3	0.737	0	0	0	0	0	0	
	1-4	0.885	2	4	3	2	3	3	
	1-5	0.482	112	120	110	69	103	213	
2 Acid treated	2-1	0.465	3	0	0	1	1	2	
	2-2	0.567	0	4	1	1	2	3	
	2-3	0.496	124	134	103	125	122	245	
	2-4	0.632	5	2	6	5	5	7	
	2-5	0.360	80	70	70	72	73	203	

Day 5

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.745	118	144	128	126	129	173	
	1-2	0.593	10	12	13	11	12	19	
	1-3	0.386	137	285	301	299	256	662	
	1-4	0.458	189	165	222	179	189	412	
	1-5	0.258	243	261	310	252	267	1033	
2 Acid treated	2-1	0.239	53	59	55	57	56	234	
	2-2	0.265	245	261	185	257	237	894	
	2-3	0.197	249	243	299	233	256	1299	
	2-4	0.424	114	120	113	192	135	318	
	2-5	0.329	173	107	136	134	138	418	

Day 6

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.25	304	254	254	216	257	1028	
	1-2	0.503	362	480	342	320	376	748	
	1-3	0.826	604	608	816	704	683	827	
	1-4	0.753	500	558			529	703	
	1-5	0.263	222	212			217	825	
2 Acid treated	2-1	0.123	140	162	114	128	136	1106	
	2-2	0.856	176	140	114	150	145	169	
	2-3	0.527	364	462	386	488	425	806	
	2-4	0.434	192	240	232	184	212	488	
	2-5	0.635	748	732			740	1165	

Day 7

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.562	107	145	108	84	111	198	
	1-2	0.387	84	77	72	70	76	196	
	1-3	0.393	428	356	442	448	419	1065	
	1-4	0.75	426	382	418	359	404	539	
	1-5	0.479	574	606	598	632	590	1232	
2 Acid treated	2-1	0.327	179	173	159	142	163	499	
	2-2	0.285	147	138	119	153	139	489	
	2-3	0.569	264	236	213	247	240	422	
	2-4	0.562	444	379	346	429	400	711	
	2-5	0.634	687	492	616	527	590	930	

Day 9

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.421	192	160	248	322	231	548	
	1-2	0.25	184	146	104	148	146	582	
	1-3	0.402	184	322	352	322	295	734	
	1-4	0.389	470	440	306	350	455	1170	
	1-5	0.406	104	118	180	162	111	273	
2 Acid treated	2-1	0.411	148	106	110	126	123	298	
	2-2	0.274	96	114	62	64	84	307	
	2-3	0.617	330	354	310	340	334	541	
	2-4	0.402	172	164	188	108	158	393	
	2-5	0.208	136	138	140	100	137	659	

Day 10

Cage No.	mouse labeled	Δ weight (g)	Count 1	Count 2	Count 3	Count 4	Average	Cysts/g feces	Note
1 Control	1-1	0.755	212	186	200	198	199	264	
	1-2	0.81	606	520	312	382	455	562	
	1-3	1.044	140	136	154	110	135	129	
	1-4	0.388	56	88	80	94	72	186	
	1-5	0.307	192	208	166	154	200	651	
2 Acid treated	2-1	0.526	160	176	194	194	181	344	
	2-2	0.366	76	82	106	104	92	251	
	2-3	0.581	92	58	52	92	74	127	
	2-4	0.610	136	80	100	120	109	179	
	2-5	0.580	126	52	94	100	89	153	

Table D-2. ANOVA analysis

Source of Variation	SS	*df*	MS	F	P-value	F crit
Control vs. Acid treatment	20124	1	20124	0.344	0.560	3.974
Duration	7871906	8	983988	16.799	9.037×10^{-14}	2.070
Interaction	255252	8	31906	0.545	0.819	2.070
Within	4217371	72	58575			
Total	12364652	89				

Since F < F crit, there is no significant difference between the number of cysts produced from mice inoculated with control *G. muris* cysts and from mice inoculated with acid treated *G. muris* cysts.

This indicates that the acid treatment during the IMS procedure did not affect the infectivity of cysts in the animal assay.