INACTIVATION OF CRYPTOSPORIDIUM PARVUM OOCYSTS AND
BACILLUS SUBTILIS SPORES BY CHLORINE DIOXIDE IN
LABORATORY REAGENT AND NATURAL WATERS

by

Christopher Zygmunt Radziminski

A thesis submitted in conformity with the requirements
for the degree of Master of Applied Science
Graduate Department of Civil Engineering
University of Toronto

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INACTIVATION OF CRYPTOSPORIDIUM PARVUM OOCYSTS AND BACILLUS SUBTILIS SPORES BY CHLORINE DIOXIDE IN LABORATORY REAGENT AND NATURAL WATERS

Master of Applied Science, A.D. 2000

Christopher Zygmunt Radziminski
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ABSTRACT

The chlorine dioxide inactivation of Cryptosporidium parvum oocysts (Iowa isolate, genotype 2 (C)) was measured at bench scale in various water matrices using in vitro excystation and a most probable number cell culture infectivity assay. In vitro excystation underestimated inactivation compared to the infectivity assay. There was a significant difference ($\alpha=0.05$) in the inactivation of oocysts among the diverse water matrices measured by the infectivity assay but not by in vitro excystation. A $C_l$ of 1 000 mg-min/L was necessary for 2.0 log$_{10}$ inactivation in type I deionised-distilled water adjusted to pH 8 at 22 °C. This degree of resistance to chlorine dioxide by C. parvum oocysts has not been quantified previously in the literature. Bacillus subtilis spores (ATCC 19659) were found to be an unsuitable surrogate for C. parvum oocysts in bench- and pilot-scale chlorine dioxide inactivation studies, but could function as a surrogate in pilot scale filtration removal studies.
ACKNOWLEDGEMENTS

I thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for a postgraduate scholarship, and I thank my two supervisors, Dr. Robert C. Andrews (University of Toronto) and Dr. Christian P. Chauret (Indiana University Kokomo). Thanks to Dr. David M. Bagley (University of Toronto) who was the second reader for this thesis. The pilot scale work was possible through the collaboration of the Regional Municipality of Ottawa-Carleton and the invaluable technical assistance of Mr. John Van Den Oever. For laboratory assistance, I thank Mrs. Robin Creason and Mrs. Nancy Hartman at Indiana University Kokomo and Mr. Quanfang Ye at the University of Toronto.

This work would not have been possible without the excellent undergraduate education I received at the University of British Columbia. I thank the professors and teachers in the Department of Microbiology and Immunology for their instruction and encouragement, especially Dr. William Ramey. Most of all, I thank Dr. David G. Holm: Thanks, Dr. Holm, for starting me on this road.

Many thanks to my parents, my brother Adam, my sister Nicole, and Kiwi of course. Thanks also to all of the amazing people at the Newman Centre at the University of Toronto, including Fr. Robin Koning, S.J., Sr. Anne Lemire, C.S.J., Sr. Winnifred O’Mara, C.S.J. and Fr. Thomas Rosica, C.S.B. (who taught me so much about being concise).
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Portions of this work have also been presented at the Ontario Water Works Association / Ontario Municipal Water Association Joint Annual Conference, Windsor, ON, May 7-10, 2000. and the research has been accepted for presentation at the AWWA Water Quality Technology Conference. Salt Lake City, Utah. November 5-9, 2000.

This work is dedicated to the memories of my grandparents.

"This superdevelopment, which consists in an excessive availability of every kind of material good for the benefit of certain social groups, easily makes people slaves of 'possession' and of immediate gratification, with no other horizon than the multiplication or continual replacement of the things already owned with others still better. This is the so-called civilization of 'consumption' or 'consumerism' which involves so much 'throwing away' and 'waste' ... To 'have' objects and goods does not in itself perfect the human subject, unless it contributes to the maturing and enrichment of that subject's 'being', that is to say, unless it contributes to realization of the human vocation as such."

Sollicitudo Rei Socialis
Pope John Paul II
Radix malorum est Cupiditas

Prologue to the Pardoner's Tale
Geoffrey Chaucer

"... And to you, dead and bloated nation of sleepwalkers, so content to drown in your own rancid apathy that your own minds and the minds of your children are being bought and sold on the auction block by swarthy old hogs – oh, set a place for the auctioneer. He'll be coming to dinner tonight. No need to bother, honey. He'll be coming live via satellite direct, right through our brand new mother f*%&in’ super-mega-screen Home Monitor System ..."

Core
Stone Temple Pilots

Action is consolatory. It is the enemy of thought and the friend of flattering illusions. Only in the conduct of our action can we find the sense of mastery over the Fates.

Nostromo
Joseph Conrad

All our life passes in this way: we seek rest by struggling against certain obstacles, and once they are overcome, rest proves intolerable because of the boredom it produces.

Pensées
Blaise Pascal

Democracy cannot be idolized to the point of making it a substitute for morality or a panacea for immorality ... Its moral value is not automatic, but depends on conformity to the moral law to which it, like every other form of human behavior, must be subject ...

Evangelium Vitae
Pope John Paul II
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<tr>
<td>$A_x$</td>
<td>absorbance at $x$ nm</td>
</tr>
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<td>APHA</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BDCM</td>
<td>bromodichloromethane</td>
</tr>
<tr>
<td>$C$</td>
<td>concentration of disinfectant (mg/L)</td>
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<tr>
<td>CDCP</td>
<td>United States Centers for Disease Control and Prevention</td>
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<td>ClO$_2$</td>
<td>chlorine dioxide</td>
</tr>
<tr>
<td>$Ct$</td>
<td>Concentration (mg/L) x time (min.)</td>
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<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
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<td>DBAA</td>
<td>dibromoacetic acid</td>
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<tr>
<td>DBCM</td>
<td>dibromochloromethane</td>
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<td>DBPs</td>
<td>disinfection byproducts</td>
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<td>DCAA</td>
<td>dichloroacetic acid</td>
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<td>ddH$_2$O</td>
<td>type I distilled deionised water</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EDA</td>
<td>ethylene diamine</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GWUDI</td>
<td>ground water under the direct influence of surface water</td>
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<td>HAA5</td>
<td>haloacetic acids. the sum of the concentrations of mono-. di-. and trichloroacetic acids and mono- and dibromoacetic acids</td>
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<td>human ileocecal adenocarcinoma cells</td>
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<td>HDPE</td>
<td>high density polyethylene</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>ID$_{50}$</td>
<td>Infectious dose 50%</td>
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<td>IESWTR</td>
<td>Interim Enhanced Surface Water Treatment Rule</td>
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<td>Immunoglobulin G</td>
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<td>Immunoglobulin M</td>
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<td>LDPE</td>
<td>low density polyethylene</td>
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<td>lissamine green B</td>
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<td>$\log_{10}$</td>
<td>common (Briggs') logarithm</td>
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<td>monobromoacetic acid</td>
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<td>MCAA</td>
<td>monochloroacetic acid</td>
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<td>MCL</td>
<td>maximum contaminant level</td>
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<td>MCLG</td>
<td>maximum contaminant level goal</td>
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<td>MDCK</td>
<td>Madin Darby canine kidney cells</td>
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<td>MDL</td>
<td>method detection limit</td>
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<td>MPN</td>
<td>most probable number</td>
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<td>MRDL</td>
<td>maximum residual disinfectant level</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NaDCC</td>
<td>sodium dichloroisocyanurate</td>
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<td>NaOCl</td>
<td>sodium hypochlorite</td>
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PBS  phosphate buffered saline
PCR  polymerase chain reaction
PI   propidium iodide
SASP small acid-soluble proteins
Stage 1 DBPR Stage 1 Disinfectants and Disinfection Byproducts Rule
std. dev. standard deviation
$t$  time (min.)
$t = 0$ initial state
$t = eq \text{`}m$ equilibrium state
TCAA  trichloroacetic acid
$\theta$  mean detention time
TOC  total organic carbon
TTHM  total trihalomethanes, the sum of the concentrations of chloroform, bromodichloromethane, dibromochloromethane, and bromofluoride
$U$  unit
USEPA United States Environmental Protection Agency
USOMB United States Office of Management and Budget
$UV_{254}$ ultraviolet absorbance at 254 nm
CHAPTER 1
INTRODUCTION

1.0 CONTEXT

Exposure of immunocompetent humans to pathogenic Cryptosporidium spp. may result in a transient infection ranging in severity from asymptomatic to self-limiting gastroenteritis, with no known specific curative therapy. In immunosuppressed or immunodeficient patients, cryptosporidiosis may lead to a persistent and potentially life-threatening disease. Current and Garcia (1991) estimated that there are 250 to 500 million cases of cryptosporidiosis annually in persons living in Asia, Africa, and Latin America.

Cryptosporidium spp. are intracellular parasites with an environmentally resistant oocyst stage, capable of transmission directly from host-to-host or indirectly by contamination of drinking water, food, or the environment (O’Donoghue, 1995; Meinhardt et al., 1996). Oocysts have been reported in surface water and ground water under the direct influence of surface water (GWUDI), and there are indications that Cryptosporidium spp. oocysts may also be present in plant-treated finished water (Wallis et al., 1996; USEPA, 2000). One significant property of Cryptosporidium in the context of water treatment is the remarkable resistance of oocysts to standard chlorination and chloramination practices. The use of chlorine dioxide as a primary disinfectant may be a strategy to help overcome this difficulty.
There also exist difficulties in measuring *Cryptosporidium* spp. oocyst concentration and viability/infectivity status. Further, technical, economic, and safety considerations associated with *Cryptosporidium* spp. experiments have led to the examination of model organisms (surrogates) in bench-scale disinfection studies.

1.1 PROBLEM STATEMENT & THESIS OVERVIEW

There are scarce published data on the inactivation of *Cryptosporidium parvum* oocysts by chlorine dioxide, especially in non-laboratory reagent waters. Further, the data which do exist are difficult to compare because of differences in *C. parvum* oocyst isolates studied and the viability and infectivity assays used. Here, a most probable number (MPN) – cell culture method with Madin Darby canine kidney cells (MDCK, ATCC CCL-34) is used to measure inactivation as loss of infectivity *in vitro* with respect to time. The assay was a modified version of that used by Slifko *et al.* (1999). Infectious parasitic stages were detected by direct immunofluorescence staining using a fluorescein isothiocyanate (FITC)-labelled polyclonal antibody (Waterborne Inc., New Orleans, LA). An *in vitro* excystation method was used simultaneously to facilitate comparison to published data. Chlorine dioxide inactivation of *C. parvum* oocysts (Iowa isolate, genotype 2 (C), Pleasant Hill Farm, Troy, ID) was examined at bench-scale in laboratory-reagent water as well as post-filtration (pre-disinfection) waters obtained from the Ottawa River (Ontario, Canada), the White River (Indiana, USA), and Lake Michigan (Wisconsin, USA).
The scope of this research was the examination of the inactivation of *C. parvum* oocysts and *Bacillus subtilis* spores (ATCC 19659) by chlorine dioxide in the context of a conventional water treatment plant practicing post-filter disinfection. The major objectives of the research were to:

1. Describe the inactivation of *C. parvum* oocysts by chlorine dioxide in different water matrices.
2. Compare *C. parvum* oocyst inactivation data obtained from *in vitro* excystation and MPN-cell culture methods.
3. Examine *B. subtilis* spores as a possible surrogate for *C. parvum* oocysts in chlorine dioxide inactivation studies by performing bench- and pilot-scale experiments.

To this end, the thesis is split into three sections: Chapter 3, *Bacillus subtilis* spore removal and inactivation at pilot scale; Chapter 4, *Bacillus subtilis* spore inactivation at bench scale; and Chapter 5. *Cryptosporidium parvum* oocyst inactivation at bench scale. The thesis concludes with Chapter 6, which includes a summary, a brief discussion of the research, conclusions, and possible future research directions.

### 1.2 REFERENCES


CHAPTER 2
BACKGROUND

2.0 OVERVIEW OF CRYPTOSPORIDIUM SPP.

2.0.1. INCIDENCE

National reporting for cryptosporidiosis in the United States began in 1995: Between 1995-1998, the annual median of reported cases by the United States Centers for Disease Control and Prevention (CDCP) was 2,900 (range: 2,566 - 3,793; CDCP. 1999). These estimates, reported by the CDCP, are likely underestimates because there is a lack of routine laboratory screening of stool samples for cryptosporidiosis infection. and many with diarrhoeal symptoms simply do not consult a physician; further, cryptosporidiosis was not a reportable disease in six states as recently as 1998 (Berkelman. 1994; Marshall et al., 1997; CDCP. 1999). Indeed, upon introduction of the Interim Enhanced Surface Water Treatment Rule (IESWTR), the United States Environmental Protection Agency (USEPA) estimated that compliance would decrease endemic illness from cryptosporidiosis in the United States by 110,000 to 463,000 cases annually (USEPA. 1998b). Further, to reflect the uncertainty in these figures, the United States Office of Management and Budget (USOMB) estimated that the IESWTR would result in an annual mean reduction of 110,000 to 338,000 cases of cryptosporidiosis, with an estimated benefit of US $0.5 to 1.5 billion (USOMB. 2000).
2.0.2. **Life Cycle**

*Cryptosporidium* spp. are intracellular, extracytoplasmic, protozoan, obligate parasites characterised by a complex, monoxenous life cycle involving an environmentally resistant oocyst stage (O'Donoghue, 1995; Meinhardt et al., 1996). Ultimately, infections are acquired by ingestion or inhalation of pathogenic oocysts excreted by an infected host (O'Donoghue, 1995). The oocysts excyst in the gastrointestinal tract to release four motile sporozoites, which are 5- by 1-μm forms that invade the absorptive epithelial cells in the gastrointestinal tract and locally disrupt the microvilli which cover the host cell (Clark, 1999). Once an intracellular niche is established, eight merozoites are produced by asexual replication and rupture out of the host cell to infect other host cells (Clark, 1999). The sexual stage of the life cycle commences when merozoites differentiate into gamonts, which undergo sexual reproduction to ultimately produce oocysts, which are excreted in the faeces (O'Donoghue, 1995; Clark, 1999). While most of the oocysts produced are thick-walled, about one-in-five are thin-walled and may excyst within the same host to contribute to a persistent infection (O'Donoghue, 1995; Marshall et al., 1997).

2.0.3. **Taxonomy**

Unfortunately, the taxonomy of the genus *Cryptosporidium* is confusing, especially at the species level, and does not appear to be well correlated with phylogeny (O’Donoghue, 1995; Morgan et al., 1999; Xiao et al., 1999). Much of this stems from the use of phenotypic characteristics in taxonomy such as morphological and morphometric parameters, similarities in life cycles, host specificity, and cross-transmission studies, making a distinction between unique isolates and species ambiguous (O’Donoghue, 1995;
Bornay-Llinares et al., 1999; Morgan et al., 1999). Speciation and a clear taxonomy are fundamental to epidemiology because not all oocysts are capable of causing infection in humans (Gasser and O'Donoghue, 1999). Molecular studies indicate that the causative agents of cryptosporidiosis in humans include two distinct genotypes of *C. parvum*, known as genotypes 1 and 2 (or H and C, respectively) (Peng et al., 1997; Xiao et al., 1998; Morgan et al., 1998, 1999; McLauchlin et al., 1999). *C. parvum* genotype 1 (H) is associated with human and possibly non-human primate infection. whereas *C. parvum* genotype 2 (C) is infectious to many mammals, including humans and domestic livestock such as calves (Sulaiman et al., 1998; Clark, 1999; Morgan et al., 1999; Okhuysen et al., 1999). This partitioning into two discrete groups may, however, be an oversimplification of *C. parvum* (Clark, 1999). Further, there is evidence to suggest that the two genotypes are reproductively isolated and may in fact be distinct species (McLauchlin et al., 1999; Morgan et al., 1999). Recent work also implicates *Cryptosporidium felis* as a potential causative agent after molecular typing and morphometric evaluation of oocysts isolated from a cow in the Gdańsk district of Poland revealed that the isolate was identical to isolates found in HIV-infected persons and in cats in Australia (Sargent et al., 1998; Bornay-Llinares et al., 1999; Pieniazek et al., 1999). A few past reports have suggested that other cryptosporidia may be pathogenic to humans, but these could not be established unequivocally because of the unavailability of robust molecular typing methodologies at the time (Bornay-Llinares et al., 1999). In recognition of the ambiguous taxonomy of *Cryptosporidium* spp., the USEPA established a maximum contaminant level goal (MCLG) of 0 for *Cryptosporidium* at the genus level rather than the more specific species level in the IESWTR (USEPA, 1998b).
2.0.4. Caveats

Meinhardt et al. (1996) describe the incidence and severity of cryptosporidiosis as linked to host immunity, environmental factors, and parasite-specific factors. Among the latter are differences in susceptibility to environmental stresses and variability in virulence. Okhuysen et al. (1999) present results suggesting that geographically diverse isolates of C. parvum genotype 2 (C) differ in their virulence as evidenced by three distinct isolates with differing attack rates and infective doses (ID₅₀ range: <10 to 1000 oocysts) in healthy volunteers initially negative for anti-C. parvum status. It has also been suggested that oocyst contact with faecal matter may alter the sensitivity of oocysts to environmental pressures or disinfectants (Robertson et al., 1992; Carpenter et al., 1999; Jenkins et al., 1999). These are important considerations when reviewing inactivation results presented in the literature, and, indeed, in this thesis. Inactivation experiments typically involve a specific isolate of C. parvum recovered from the faeces of a particular host animal and processed through a specific purification protocol. It cannot be ignored that other cryptosporidia may be pathogenic and that different isolates (or, indeed, even different lots of the same isolate) of Cryptosporidium spp. may exhibit different responses when exposed to a given disinfectant.

2.0.5. Environmental surveys

Cryptosporidium spp. oocysts have been reported in surface water and ground water under the direct influence of surface water (GWUDI), in over twenty-five source water surveys across North America (Wallis et al., 1996; USEPA, 2000a). Surveys of water treatment plants across the United States and Canada indicate that Cryptosporidium
spp. oocysts may also be present in finished water (Wallis et al., 1996; USEPA, 2000a). The results of such surveys, however, must be viewed with caution because of inherent sampling difficulties and the detection assays used. Sampling of water bodies tends to be infrequent and involves relatively small volumes of water collected relative to the total volume of the water body. Oocysts, however, are not necessarily distributed homogeneously in terms of time and space in a given body of water (Meinhardt et al., 1996). Further, collection and processing procedures for Cryptosporidium spp. oocyst recovery and identification from environmental samples are wanting, exhibiting poor recoveries and perhaps biased results (Clancy et al., 1994; LeChevallier et al., 1995). The detection assays used for the environmental samples analysed in the surveys neither discriminated between viable (alive) and non-viable (dead) oocysts nor distinguished between the viable isolates present (infective or non-infective). Such distinctions are important because oocysts must be both viable (alive) and infectious to be of concern in the arena of public health (Meinhardt et al., 1996; Gasser and O'Donoghue, 1999). Indeed, as well illustrated by the 1994 cryptosporidiosis outbreak in Las Vegas, Nevada, and the 1998 crisis in Sydney, Australia, measured water contamination by cryptosporidia does not necessarily correlate with disease occurrence (Goldstein et al., 1996; Gasser and O'Donoghue, 1999; Clancy, 2000). Despite the shortcomings, these environmental surveys have established that Cryptosporidium spp. oocysts are present in diverse source waters and may be present in finished waters.
2.0.6. Viability and Infectivity Measurement

Overview. Controlled, laboratory-scale inactivation experiments typically involve high concentrations of Cryptosporidium parvum oocysts (e.g., $10^6$/L, Ruffell et al., 2000) and thus circumvent the sampling difficulties associated with environmental surveys. Further, detection assays capable of determining the viability or infectivity status of oocysts, but not feasible or possible with environmental samples, are available. Such techniques may fall into one or more of the following categories: vital dye staining, in vitro excystation, animal infectivity, tissue culture and genetic analyses (Gasser and O’Donoghue, 1999). Antibody-based detection methods for oocysts in environmental samples or stool specimens do not discriminate between viable and non-viable oocysts and so will not be addressed (Rochelle et al., 1997; Widmer et al., 1999). Fricker and Crabb (1998) point out that oocyst viability and infectivity assessments reflect not only treatment effects, e.g., disinfection, but also the effects of sampling and subsequent sample processing. Any critical assessment of an inactivation experiment must acknowledge this fact.

Viability measurement. Detection methods for Cryptosporidium isolates rely largely upon microscopic examination which limits the application of a method to small-scale operations and necessitates a trained analyst. Vital dye staining is based on the selective inclusion or exclusion of a specific fluorogenic dye by an oocyst. One example is the procedure developed by Campbell et al. (1992), based on the vital dyes 4,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). The method is technically simple and cost-effective, but the consistency and quality of the method may depend upon the history of environmental and/or treatment stresses on the oocysts, as well as on the method used.
for concentrating the sample (Jakubowski et al., 1996). This necessitates the consideration of oocyst permeability when results from environmental and laboratory studies are reviewed (Campbell et al., 1992). The permeability of oocyst walls to DAPI varies between oocysts of a given isolate, and the change in oocyst permeability is itself a time-dependent, progressive process which precludes the simple description of an oocyst as either viable or nonviable (Campbell et al., 1992; Jenkins et al., 1997; Robertson et al., 1998). Other examples of vital dye stains are hexidium. SYTO-9. SYTO-59. and MPR71059. which are purported to differentiate between viable and non-viable oocysts (Belosevic et al., 1997). One drawback of these aforementioned stains is that an oocyst wash step may be required following disinfection to facilitate dye uptake (Belosevic et al., 1997). Further. the dyes have not been widely evaluated under different experimental and field conditions (Belosevic et al., 1997).

*In vitro* excystation is based on the *in vitro* simulation of conditions of the gut such that sporozoites can emerge from the oocyst stage. Microscopy is conducted subsequently to determine the number of viable oocysts. The technique is easy to use and not costly compared to. *e.g.*, infectivity measurement methods; a further advantage is its resemblance to the first stages of pathogenesis (Jakubowski et al., 1996). However, it requires a high concentration of oocysts to be feasible and is therefore unfit for use with the small number of oocysts found in environmental samples (Fricker and Crabb, 1998). The performance of this method when compared to infectivity assessments will be addressed in the ensuing discussion.
Genetic analyses for *Cryptosporidium* spp. detection are based typically on the polymerase chain reaction (PCR). PCR is sensitive and relatively easy to perform, allows for the simultaneous analysis of large numbers of samples at low cost, does not rely upon microscopic examination, and can be specific enough to distinguish among different species or genotypes (Sluter *et al*., 1997; Morgan and Thompson, 1998). PCR is, however, prone to contamination and may be inhibited by compounds such as humic acids present in samples (Jakubowski *et al*., 1996; Sluter *et al*., 1997; Morgan and Thompson, 1998). The technique is also destructive in that lysis of oocysts is required to release the nucleic acids: this prevents the possibility of subsequent microscopic examination (Fricker and Crabb, 1998).

*Infectivity measurement.* Animal infectivity has been referred to as the benchmark for inactivation assessment in laboratory experiments, but others dispute that a truly acceptable ‘gold standard’ exists (Gasser and O’Donoghue, 1999). Animal tests are expensive, difficult to perform, include several sources of variation inherent to an animal model system, and involve ethical considerations; further, there is currently no standard infectivity model, though neonatal mice of the CD-1 or BALB/c strains appear to be used most often (Finch *et al*., 1993b; Jakubowski *et al*., 1996; Fricker and Crabb, 1998). Indeed, *in vivo* infectivity is now considered by some to be unreliable since *C. parvum* genotype 1 (H) does not regularly infect mice (Peng *et al*., 1997; Gasser and O’Donoghue, 1999). However, when *in vitro* excystation and DAPI/PI vital dye staining are compared to infectivity methods as measures of oocyst inactivation, the former methods have been observed to overestimate infectious oocyst survival (Finch *et al*., 1993a, 1995; Black *et al*.,...
1996; Beiosevic et al., 1997; Bukhari et al., 1999; Slifko et al., 1999). This has led some to believe that animal infectivity is superior to vital dye and in vitro excystation methods in measuring the survival of infectious oocysts following chemical disinfection (Black et al., 1996).

To circumvent some of the difficulties associated with the animal infectivity assay, tissue (cell) culture assays have been developed. The principle of the method is that a monolayer of cells is inoculated with a sample and, after a period of incubation, is observed for evidence of invasion and replication. Various cell lines and incubation conditions have been used successfully for establishing the cell monolayer (Upton et al., 1994). To detect infections of the cell monolayer, different methods have been proposed. PCR techniques targeting specific DNA sequences or mRNA extracts have been described and, from an epidemiological viewpoint, the potential ability of these techniques to speciate may allow the source of an infection to be determined (Rochelle et al., 1997; Morgan and Thompson, 1998; Di Giovanni et al., 1999). Another strategy is the microscopic examination of the cell monolayer to detect the presence/absence of clusters of reproductive stages; antibody staining can be used to enhance visualisation of such foci of infection (Slifko et al., 1999). Such examination provides direct evidence that reproduction has occurred, an advantage when compared to the PCR techniques (Slifko et al., 1999). By using a presence/absence evaluation in combination with the principles of the most probable number (MPN) method of enumerating microbes, infectious oocysts in a sample can be quantified (Slifko et al., 1999). The cell culture approach holds much promise as a sensitive measure of infectivity and appears to be more practical and cost-
effective than animal infectivity; however, the method is still relatively slow, expensive, and labour-intensive (Morgan and Thompson, 1998; Slifko et al., 1999). A further drawback is that some isolates may not infect culture cells even though they may be infective in vivo, and possibly vice versa (Morgan and Thompson, 1998).

Comment. In the absence of an accepted standard measure of *Cryptosporidium* spp. oocyst viability and infectivity, the inactivation data available in the literature become difficult to compare. It is therefore prudent to use more than one detection method simultaneously to not only facilitate comparison to existing data but also to bolster the robustness of newly-gathered data. The methods used in this thesis are *in vitro* excystation, an oocyst viability measuring technique used in various forms by past researchers for inactivation experiments, and an MPN-cell culture assay, an oocyst infectivity method (Chapter 5).

2.1 USE OF A MICROBIAL SURROGATE MEASURE

Besides the difficulties associated with measuring *Cryptosporidium* spp. oocyst concentration in the laboratory, there are several properties of cryptosporidia that make the use of a model organism (surrogate) in bench-scale disinfection studies attractive. The current lack of a continuous *in vitro* cultivation system for *Cryptosporidium* spp. oocysts means that the high concentrations of oocysts typically used in bench-scale experiments must be collected from the faeces of infected animals (Gasser and O'Donoghue, 1999). Coupling this with the technical expertise, expense of materials, potential pathogenicity of cryptosporidia, and time required for *Cryptosporidium* spp. experiments, there are economic and practical limitations to bench-scale work. Desired characteristics of a
microbial surrogate for *Cryptosporidium* spp. inactivation experiments, therefore, include: a resistance to the disinfectant comparable to that of *Cryptosporidium* spp. oocysts, the availability of specific, easy, inexpensive and sensitive enumeration methods, and a non-pathogenic nature. Here, the aerobic spore former *Bacillus subtilis* (ATCC 19659) is investigated as a potential microbial surrogate to *Cryptosporidium parvum* (Iowa isolate, genotype 2 (C). Pleasant Hill Farm, Troy, ID) in bench-scale experiments (Chapters 4 and 5).

At the scale of a water treatment plant, the measurement of *Cryptosporidium* spp. oocysts in raw and finished waters is an inefficient method of gauging plant performance (LeChevallier and Norton, 1995; Lisle and Rose, 1995). LeChevallier and Norton (1995) proposed that utilities develop a raw water database for oocyst occurrence, over a prolonged period of time to reflect potential seasonal variations, and use some other means to determine treatment plant performance. One potential means of achieving this is the seeding of an appropriate microbial surrogate in pilot-scale experiments. Therefore, the inactivation and removal of *B. subtilis* spores (ATCC 19659) is studied at pilot-scale to aid in the analysis of treatment plant performance (Chapter 3).

2.2 THE CHALLENGE OF *CRYPTOSPORIDIUM* SPP. TREATMENT

The recognition that certain isolates of the genus *Cryptosporidium* are causative agents of human morbidity and mortality has presented the North American water treatment industry with a challenge, because conventional treatment strategies (coagulation, flocculation, sedimentation, filtration, chlorine disinfection) may be
inadequate for the removal and/or inactivation of sufficient infective, pathogenic oocysts during a contamination event to safeguard against an outbreak.

One significant property of Cryptosporidium in the context of water treatment is the remarkable resistance of oocysts to standard chlorination and chloramination practices. Venczel et al. (1997) reported that for three replicate experiments at 25 °C in pH 7, oxidant demand free 0.01 M phosphate-buffered water, a 5 mg/L dose of free chlorine for 24 h resulted in essentially no inactivation of C. parvum oocysts (Iowa strain) as measured by infectivity in neonatal BALB/c mice. This is consistent with the animal infectivity and in vitro excystation data of Korich et al. (1990), who reported that at 25 °C in pH 7 water, at least 2 log₁₀ inactivation of C. parvum oocysts required 80 mg/L of free chlorine for 1.5 h of exposure. Additional research demonstrating the resistance of a variety of C. parvum isolates to chlorination under various conditions is present in the literature (Fayer, 1995; Chauret et al., 1998; Moore et al., 1998; Carpenter et al., 1999). Chloramines have not been found to be significantly better in terms of oocyst inactivation (Ransome et al., 1993; Fricker and Crabb, 1998). There are health, economic and practical constraints in achieving such high concentrations of disinfectant and/or contact times in drinking water treatment. Indeed, it is considered unlikely that Cryptosporidium spp. can be controlled effectively by simply increasing Ct values above those commonly employed for chlorine and chloramines (Fricker and Crabb, 1998; USEPA, 2000a).

The USEPA implemented the IESWTR in 1998 in a specific attempt to decrease the level of Cryptosporidium spp. in American finished drinking water supplies by
improving physical removal processes (USEPA, 1998b). The underlying rationale was that more effective and reliable removal of cryptosporidia (and other potentially pathogenic microorganisms) would decrease the reliance on a disinfection barrier (USEPA, 1998b). While endeavouring to ensure the microbiological safety of drinking water, the USEPA addressed simultaneously the potential health risk of disinfectants and disinfection byproducts (DBPs) through the release of the Stage 1 Disinfectants and Disinfection Byproducts Rule (Stage 1 DBPR) which establishes disinfectant and DBP concentration limits (USEPA, 1998a). Revisions to both rules have since been proposed (USEPA, 2000b,c).

The focus of the IESWTR on removal processes highlights the fact that different stages of the water treatment process can be used to control *Cryptosporidium* contamination in drinking water. This has often been referred to as the "multi-barrier approach". Such stages of treatment, including watershed management to minimise oocyst contamination of source water, physical and chemical removal processes, and physical and chemical inactivation, can be each optimised and used in combination by a water utility to reduce the risk of an outbreak of cryptosporidiosis. Indeed, LeChevallier and Norton (1995) suggest that while much more disinfection data are needed under field conditions, effective removal processes coupled with chlorination may already be achieving the goal of a $10^{-4}$ annual risk of cryptosporidiosis. In the context of the "multi-barrier approach", there are many potential strategies possible to surmount the problem posed by pathogenic cryptosporidia in drinking water. Among such strategies is the use of a primary disinfectant other than chlorine.
2.3 CHLORINE DIOXIDE

2.3.1. CHEMICAL PROPERTIES

A potential alternative to chlorine for primary disinfection is chlorine dioxide (ClO₂). At room temperature, chlorine dioxide is a strongly oxidising, yellow to reddish-yellow gas (melting point -59 °C, boiling point +11°C, 67.45 g/mole) with an unpleasant odour similar to that of chlorine (Merck and Co., 1996). At concentrations >10% at atmospheric pressure, it detonates explosively by heat, sunlight, noise, or contact with mercury or carbon monoxide (Merck and Co., 1996). This instability in the gaseous state precludes ClO₂ transport and necessitates its production on-site.

2.3.2. USE AS A DISINFECTANT

Chlorine dioxide has a variety of uses in water treatment, including oxidation of taste and odour-causing organic compounds, colour, reduced iron and manganese, and as a disinfectant (USEPA, 1998a). While there is scarce published data on the inactivation of C. parvum oocysts by chlorine dioxide, especially in non-laboratory reagent waters, there are data to suggest that microbial inactivation is associated with chlorine dioxide and not its disproportionation products such as chlorite and chlorate (Noss and Olivieri, 1985; Harakeh et al., 1988; Liyanage et al., 1997). As mentioned previously, the data which do exist are difficult to compare because of differences in C. parvum oocyst isolates studied and the viability and infectivity assays used. Indeed, there is a range of opinions on the efficacy of chlorine dioxide inactivation of pathogenic Cryptosporidium spp. oocysts. Based on their research, Peeters et al. (1989) deemed treatment of drinking water with chlorine dioxide feasible, while Korich et al. (1990) and Lisle and Rose (1995) felt that the
use of chlorine dioxide, chlorine, or monochloramine alone should not be expected to inactivate *C. parvum* oocysts in drinking water. In review of the chlorine dioxide inactivation data available, Fricker and Crabb (1998) came to the same conclusion as Korich *et al.* (1990). Meinhardt *et al.* (1996) did not disqualify chlorine dioxide entirely, suggesting that the use of chlorine dioxide alone in “high” concentrations – which, they added, may have significant disadvantages – may be efficacious in inactivating *C. parvum* oocysts in drinking water. The opinions of these various researchers are presented to illustrate the need for an expanded data set, which this thesis will help to accomplish. To facilitate comparison and prevent repetition, the inactivation data in the literature are presented adjacent to the data in the following chapters.

While the focus of this thesis is the use of chlorine dioxide as a single, primary disinfectant, it is noted that there may be synergistic effects between chlorine dioxide and other disinfectants. Indeed, Gates (1998) stated that chlorine dioxide is not intended to replace free chlorine or chloramines in drinking water disinfection, but rather to complement standard chlorination. While Ransome *et al.* (1993) found that combinations of ozone and chlorine for disinfection were no more effective than using ozone alone, Liyanage *et al.* (1997) provided evidence of synergy in the inactivation of *C. parvum* oocysts when ozone was followed by chlorine dioxide.

### 2.3.3. Disinfection Byproducts

One concern emerging from the use of disinfectants in drinking water treatment is the potential detrimental health effects of the disinfectants used and their byproducts
(DBPs) to consumers. Several DBPs have been shown to be carcinogenic or to cause adverse reproductive or developmental effects in laboratory animals; epidemiological studies have suggested possible associations between exposure to chlorinated surface water and bladder, rectal, and colon cancers (USEPA, 1998a). However, there is great difficulty in assessing such health risk as there lies uncertainty in the epidemiological and toxicological studies on which the USEPA attempts to set its regulations (USEPA, 1998a). Further, the simultaneous release of the Stage 1 DBPR and the IESWTR highlights the complex question of how to balance risks from pathogenic microbial contaminants and the risks from disinfectants and their byproducts (USEPA 1998a,b). Nevertheless, a maximum residual disinfectant level (MRDL) was set for chlorine dioxide at 0.8 mg ClO₂/L (USEPA, 1998a). Further, the health concerns associated with short-term exposure to chlorine dioxide prompted the USEPA (1998a) to take the additional step of not permitting the use of chlorine dioxide above the MRDL for short periods of time to address specific microbiological contamination problems, unlike the case for chlorine and chloramines.

For the possible DBPs resulting from chlorine dioxide use, maximum contaminant levels (MCLs) were set at 80 μg/L for total trihalomethanes (TTHM, the sum of the concentrations of chloroform, bromodichloromethane, dibromochloromethane, and bromoform), 60 μg/L for haloacetic acids (HAA5, the sum of the concentrations of mono-, di-, and trichloroacetic acids and mono- and dibromoacetic acids), and 1.0 mg/L for chlorite, the major by-product of chlorine dioxide disinfection (Hoigné and Bader, 1994; USEPA, 1998a). Maximum contaminant level goals (MCLGs) were also set at 0 mg/L for bromodichloromethane, bromoform and dichloroacetic acid, 0.06 mg/L for dibromochloro-
methane, 0.3 mg/L for trichloroacetic acid, and 0.8 mg/L for chlorite (USEPA, 1998a). The MCLG of 0 mg chloroform / L has been removed following a court challenge (USEPA, 1998a, 2000c). While MCLGs are non-enforceable, they are established at concentrations at which no known or anticipated undesirable health effects occur (USEPA, 1998a). The scientific uncertainty surrounding the possible health effects of disinfectants and DBPs is well illustrated by the estimated total annual costs and benefits of the Stage 1 DBPR. The USOMB (2000) estimated that this rule will generate between US $3.18 billion in net benefits and 701 million in net costs, based on potential reductions in fatal and non-fatal bladder cancers. It should be noted that possible reductions in colon cancers, rectal cancers, and adverse reproductive and developmental effects were not quantified as part of this analysis (USOMB, 2000).

2.4 REFERENCES


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CHAPTER 3

Bacillus subtilis Spore Removal and Inactivation
At Pilot Scale

3.0 INTRODUCTION

At the scale of a water treatment plant, the measurement of Cryptosporidium spp. oocysts in raw and finished waters is an inefficient method of gauging plant performance (LeChevallier and Norton, 1995; Lisle and Rose, 1995). LeChevallier and Norton (1995) proposed that utilities develop a raw water database for oocyst occurrence over a prolonged period of time to reflect potential seasonal variations, and use some other means to determine treatment plant performance. One potential means of achieving this is the seeding of an appropriate microbial surrogate in pilot-scale experiments. Pilot-scale studies with Bacillus subtilis spores (ATCC 19659) were conducted in July and August 1999 at the Britannia Water Treatment Plant (Ottawa, ON, Canada) to address five main objectives:

1) To describe the inactivation by chlorine dioxide of B. subtilis spores in summer post-filtration (pre-disinfection) Ottawa River water.

2) To examine the effect on inactivation of B. subtilis spores when microbial spiking takes place pre-filtration versus post-filtration.

3) To determine whether B. subtilis spores exposed to a sand-shearing protocol at bench-scale and spiked post-filtration may serve as a surrogate in microbial inactivation studies for untreated spores spiked pre-filtration.
(4) To describe the removal of *B. subtilis* spores via anthracite/sand dual-media filtration.

(5) To describe briefly the disinfection byproducts (DBPs) formed at the chlorine dioxide doses used at pilot-scale.

### 3.1 BACKGROUND

*Cl concept.* The primary disinfection kinetic theory for regulatory use is the Chick-Watson model, which describes disinfection as a chemical reaction (Finch *et al.*, 1993; Lisle and Rose, 1995). The general model is \( \log \left( \frac{N}{N_0} \right) = -kCt \), where: \( N \) is the concentration of organisms at time \( t \), \( N_0 \) is the initial concentration of organisms, \( k \) is the pseudo first-order reaction rate constant, \( C \) is the concentration of disinfectant (mg/L), and \( n \) is the coefficient of dilution, an empirical factor assumed to be 1.0 (Finch *et al.*, 1993; Lisle and Rose, 1995). This assumption is strengthened for chlorine dioxide by the work of Hoigné and Bader (1994), who studied the reaction kinetics of chlorine dioxide with various inorganic and organic compounds and determined that, in all cases tested, the rate law was first order in chlorine dioxide and first order in substrate.

When a desired level of inactivation is required, the equation may be simplified to the more familiar form \( K = Ct \), where \( K \) is a constant for each microorganism to obtain a desired level of inactivation under specific environmental conditions, *e.g.*, temperature, water chemistry (Finch *et al.*, 1993; Lisle and Rose, 1995). This equation implies that the desired level of inactivation will occur for any combination of \( C \) and \( t \) that results in a specific \( K \) value (Finch *et al.*, 1993). Botzenhart *et al.* (1993) studied the chlorine dioxide
inactivation of *Bacillus subtilis* spores at pH 6 and 8 in a continuous-flow bench-scale reactor and reported that, for a given level of inactivation, there was not a decrease in \( Ct \) product with increasing \( C \) as they had observed with other bacteria having a faster rate of inactivation. This report thus strengthens the assumption of Chick-Watson kinetics for *Bacillus subtilis* inactivation by chlorine dioxide.

Indeed, the assumption of Chick-Watson kinetics facilitates straightforward comparisons of disinfection data between researchers using dissimilar initial concentrations of a given disinfectant for different contact times. All \( Ct \) values for chlorine dioxide reported and compared in this thesis involve the underlying assumption of the Chick-Watson model.

*Bench and pilot scale Ct calculations.* Differences in calculating \( Ct \) values arise when laboratory- and pilot-scale experiments are performed, due to differences in the inherent flow regimes of the systems. In bench-scale experiments, the residual disinfectant can be measured over time to allow for a time-integrated \( Ct \) value (Chapters 4, 5). Continuous flow systems such as pilot plants require a tracer study to determine the characteristic contact time \( t \). In the work conducted here, there was no means of measuring chlorine dioxide concentration over time in a specific flow segment, and thus the chlorine dioxide concentration at the clearwell effluent was used as the value for \( C \) (Finch *et al.*, 1993). This approach means that the \( Ct \) values may be underestimated when compared to the integrated approach because the method does not account for a potentially higher concentration of chlorine dioxide at the clearwell influent (Finch *et al.*, 1993).
Bacillus subtilis spore removal. The Interim Enhanced Surface Water Treatment Rule (IESWTR) requires 2 log_{10} removal of Cryptosporidium spp. oocysts for all American public water systems that filter, serve 10,000 or more people, and use surface water or ground water under the direct influence of surface water (GWUDI) (USEPA, 1998b). It is now proposed that this rule be extended to those American public water systems serving fewer than 10,000 (USEPA, 2000). USEPA (2000) reviewed pilot- and full-scale removal studies and concluded that conventional and direct filtration treatment systems with rapid granular filtration and appropriate coagulation, optimised to achieve a turbidity of \(<0.3\) ntu in the filter effluent, should be able to achieve at least 2 log_{10} removal of Cryptosporidium spp. oocysts. The Cryptosporidium spp. oocyst removal rates cited in the various studies reviewed varied widely (1.9 to 5.2 log_{10} removal in optimised conventional treatment pilot plants), which the USEPA (2000) deemed to depend upon water matrix conditions, the filter effluent turbidity, and the stage of the filtration cycle.

The data reviewed by USEPA (2000) involved removal of Cryptosporidium spp. oocysts; however, the seeding of inactivated or viable Cryptosporidium spp. oocysts is not always practical at pilot-scale and under rare circumstances can be done at full-scale (e.g., Nieminski and Ongerth, 1995). Further, directly monitoring naturally occurring Cryptosporidium spp. oocysts in the filter influent and effluent waters of a treatment plant is not an appropriate method to gauge plant performance (LeChevallier and Norton, 1995; Lisle and Rose, 1995; Nieminski and Ongerth, 1995; Rice et al., 1996). The seeding of an appropriate microbial surrogate at pilot-scale may provide a practical alternative.
Payment and Franco (1993) noted that the removal of microorganisms is dependent upon their size and surface charges. Because of the smaller size of \( B. \text{subtilis} \) spores (about 0.5 x 1.0 x 2.0 \( \mu \text{m} \)) compared to \( C. \text{parvum} \) oocysts (about 5.0 \( \mu \text{m} \) diameter). Rice \textit{et al.} (1996) considered spores a conservative indicator of removal efficiency. Rice \textit{et al.} (1996) measured the zeta potentials to describe surface charge characteristics of \( C. \text{parvum} \) oocysts from neonatal bull Holstein calves, as well as \( B. \text{subtilis} \) spores. While the authors noted that zeta potential readings are not a direct measure of removability, they stated that these readings can be used to optimise coagulation. As the pH of spiked pond water increased from 5.0 to 10.5, they measured a zeta potential between \(-16\) and \(-20\) mV for \( B. \text{subtilis} \) spores and between \(-5\) to \(-13\) mV for \( C. \text{parvum} \) oocysts (Rice \textit{et al.}, 1996).

3.2 MATERIALS AND METHODS

3.2.1. PILOT SCALE \textit{Bacillus subtilis} SPORE INACTIVATION EXPERIMENTS

General set-up and water matrix. The Ottawa River (Rivière des Outaouais) serves as the sole water source for the Britannia Water Treatment Plant (Ottawa, ON, Canada). The raw water tends to be high in colour and low in turbidity (Table 3.1). The Britannia Water Treatment Pilot Plant was used to simulate conventional treatment with post-filtration disinfection. Raw water from the Ottawa River was subjected to treatment consisting of coagulation (26-46 mg alum/L and 2.0-3.5 mg activated silicate/L), flocculation (tapered mixing), sedimentation, dual-media filtration, and disinfection in a clearwell which incorporated influent and effluent baffles (Figure 3.1). A flow-rate of 2.5 L/min was
maintained through a dual-media anthracite-sand filter (hydraulic loading rate of 8.22 m/h), with 2.0 L/min being directed to the clearwell and the remainder being directed to a sampling port. When used, chlorine dioxide (ClO₂) was always added at the clearwell influent.

Pilot work was conducted in July and August 1999; physical and chemical characteristics of the water at different stages of the pilot plant are described in Table 3.1. For runs involving microbial spiking, Bacillus subtilis spores (ATCC 19659) were added into the treatment train at the filter influent ("pre-filter spike") or at the filter effluent / clearwell influent ("post-filter spike") (Figure 3.1). The mean detention time (θ) from the filter influent to the clearwell effluent was 66 min., and θ from the clearwell influent to the clearwell effluent was 50 min. (Ballantyne. 1999). After commencing addition of ClO₂ and/or microbial spores (depending on the experiment), the time elapsed before "equilibrium" samples were collected was 140 min. for post-filter spike runs and 180 min. for pre-filter spike runs. Equilibrium conditions at 140 min. for post-filter spike experiments were confirmed in an experiment where chlorine dioxide residuals were monitored over time at the clearwell effluent (Figure A.5). Samples were collected at both t = 0 and t = equilibrium (eq’m) at different points along the pilot plant treatment train for measurement of microbial concentration and chlorine dioxide residual, as shown in Figure 3.1.

Run protocol. The filter was backwashed with air scour using disinfectant-free filter effluent water; 30 min. were allotted for filter ripening before t = 0 samples were
collected. For experiments involving chlorine dioxide addition, the stock chlorine dioxide solution was titrated twice and the flow rate of the diaphragm metering pump (Prominent Fluid Controls Model G/4b, Guelph, ON, Canada) was adjusted to achieve the desired chlorine dioxide concentration in the clearwell by delivery at the clearwell influent. The microbial stock solution was prepared by diluting $10^8$ or $10^9$ spores in 3.5-4.0 L of pH 6 phosphate-buffered water (88.9 mL/L of monopotassium phosphate solution (63.5 g/L KH$_2$PO$_4$) and 11.1 mL/L of disodium phosphate solution (249.2 g/L Na$_2$HPO$_4$·7H$_2$O) added drop-wise to type I distilled deionised water (ddH$_2$O) to pH 6), in a beaker with a continuously-running magnetic stir bar.

3.2.2. MICROBIOLOGY

*Bacillus subtilis* spore stock preparation. Five mL of Tryptone broth (0.1 g/L Tryptone (DIFCO, Sparks, MD), 0.01 g/L yeast extract (DIFCO), 0.01 g/L glucose, 0.08 g/L NaCl, 0.0022 g/L CaCl$_2$) were inoculated with *Bacillus subtilis* spores for 16 h at 37 °C. Purity was verified by a Gram stain. From this stock, 100 µL were added to 100 mL of 1/10 Columbia broth supplemented with MnSO$_4$ (0.035 g/L Columbia dry mix (DIFCO), 10 mL/L of 10 mM MnSO$_4$·4H$_2$O). This was incubated at 160 rpm in a temperature-controlled incubator (Psychroterm™, New Brunswick Scientific, Edison, NJ) set at 37 °C for 4-5 days. A subsequent Malachite Green spore stain verified the presence of spores. The culture was incubated for 10 min. at 70 °C to destroy vegetative cells, and was placed immediately in an ice water bath. Aliquots were centrifuged (Beckman Coulter model GS-6, Fullerton, CA) at 10 000 x g for 11 min. The supernatant was removed, and the pellet was resuspended in 5 mL sterile ddH$_2$O. This suspension was recentrifuged and the
supernatant was again removed. The pellet was resuspended in about 14 mL sterile ddH₂O and stored at 4 °C.

_Sand shearing ("stressing") of Bacillus subtilis spores._ A stock of _Bacillus subtilis_ spores was prepared as described above and enumerated by the spread plate method on nutrient agar plates with 0.015 g/L trypan blue. A volume of this stock was added to sterile ddH₂O with 1 kg/L sterile filter sand (20/40 mesh size, Crystalline Silica, J.T. Products, Kilgore, TX) for a total volume of 10 mL. This was placed in an incubator (Psychroterm™) overnight (14-18 h) set at 200 rpm and 20.0 (±0.5) °C. After 5 min. of settling, the supernatant was removed and concentrated to 1 mL by centrifugation (Fisher Scientific Micro7, Pittsburgh, PA) at 10 000 x g for 5.5 min. This was enumerated as described previously and stored at 4 (±1) °C.

_Microbial sampling._ Duplicate samples of the microbial stock solution, filter effluent, and clearwell effluent were taken for microbial analysis at \( t = 0 \) and \( t = \text{eq}'m \). Settled water samples were also taken during "pre-filter" spike experiments. The microbial stock was sampled using sterile, colourless glass vials with Teflon®-lined caps, while all other samples were collected in sterile, high density polyethylene (HDPE) bottles. All samples were quenched by 1.5% (w/v) Na₂S₂O₃ and stored in the dark at 4 (±1) °C until shipment by overnight courier to Indiana University Kokomo for analysis. Microbial samples were heat-treated in a 75 (±5) °C water bath for 10 min. and enumerated by the spread plate method or by membrane filtration through a 0.45 μm filter (Millipore Corporation, Bedford, MA) on nutrient agar plates with 0.015 g/L trypan blue. All plates were
incubated for 16-20 h at 37.0 (±0.5) °C. Replicate samples were averaged for a particular time point.

*Inactivation and removal calculations.*

"Post-filter" spike experiments. The inactivation of *Bacillus subtilis* spores was calculated as:

\[
\log_{10} \left( \frac{\text{microbial concentration at clearwell influent}}{\text{microbial concentration at clearwell effluent}} \right)
\]  

The microbial concentration at the clearwell effluent was measured as described above. The microbial concentration measured at the filter effluent upstream of spiking was negligible (<0.1%) compared to that calculated as injected at the clearwell influent. The latter was calculated from the equation:

\[
(\text{microbial concentration injected at clearwell influent}) = (\text{microbial stock concentration}) \times (\text{microbial pump rate}) / (\text{clearwell flow rate})
\]  

The concentration of the microbial stock solution was measured as described above. The clearwell flow rate was controlled automatically to 2.0 L/min. The actual flow rate of the microbial pump (Cole-Parmer Instrument Co., peristaltic pump model 7520-25, Chicago, IL) used to deliver the microbial spike was measured at each of *t* = 0 and *t* = eq'm (n≥3 at each time), resulting in two separate calculated values for the microbial concentration injected at the clearwell influent. The mean of these two calculations was used in the computation of spore inactivation for the experiment.

"Pre-filter" spike experiments. The removal of *Bacillus subtilis* spores through the filter was calculated as:
The microbial concentration at the filter effluent was measured as described above. The measured microbial concentration of the settled (pre-filter) water was negligible (<0.1%) compared to that calculated as injected at the filter influent. The latter was calculated from the equation:

\[
\log_{10} \left( \frac{\text{microbial concentration at filter influent}}{\text{microbial concentration at filter effluent}} \right) \quad (3.3)
\]

The concentration of the microbial stock solution was measured as described above. The filter flow rate was held to about 2.5 L/min. The microbial pump rate was measured at each of \( t = 0 \) and \( t = \text{eq'm} \) \( (n \geq 3 \text{ for each time}) \), resulting in two separate calculated values for the microbial concentration injected at the filter influent. The mean of these two calculations was used in the computation of spore removal through the filter for the experiment. The inactivation of spores in the clearwell was calculated according to equation 3.1. The microbial concentration at the clearwell effluent was measured as described above. The microbial concentration measured at the filter effluent was used as the microbial concentration at the clearwell influent.

3.2.3. CHLORINE DIOXIDE

Generation. A 9% hydrochloric acid solution (Sterling Pulp Chemicals, Buckingham, QC, Canada) and a 7.5% sodium chlorite solution (Sterling Pulp Chemicals) were delivered to the reaction chamber of a ProMinent Chlorine Dioxide Generating System (Bello Zon Type CDVa, Heidelberg, Germany) by metering pumps (ProMinent Fluid Controls, Guelph, ON, Canada) to produce chlorine dioxide (ClO₂), which was diluted by
a continuous flow of plant effluent water to fill an enclosed, 100 L high
density polyethylene (HDPE) tank containing round HDPE balls (diameter ≈ 50 mm) to minimise
ClO₂ losses to volatilisation. The ClO₂ stocks used ranged from 250-300 mg/L (purity 96-
99%), as measured by the titration method described below.

**ClO₂ stock titration.** A titration method based on Standard Method 4500-ClO₂ B was
used to measure the stock concentration of ClO₂ (APHA, 1998). To 25 mL of 10% (w/v)
KI solution were added 15 mL of pH 7.0 buffer (25.4 g/L anhydrous KH₂PO₄, 64.4 g/L
Na₂HPO₄). A 20.0 mL sample of the ClO₂ stock was added to this solution and titrated
with 0.1000 N Na₂S₂O₃ until the iodine colour disappeared. The volume of titrant used
was recorded as \( N \) (neutral titration). Next was added 20 mL of 2 N H₂SO₄; the solution
was again titrated with 0.1000 N Na₂S₂O₃ until it became colourless. The volume of
titrant used was recorded as \( A \) (acid titration). The concentration of ClO₂ (g/L) was
calculated from:

\[
[\text{ClO}_2] = 0.27 \times \frac{T}{4} \tag{3.5}
\]

where \( T \) (total volume of titration) = \( A + N \). Purity of the stock solution was determined
from:

\[
\text{Purity of stock solution} (\%) = \left( \frac{5A}{4T} \right) \times 100\% \tag{3.6}
\]

**Residual ClO₂ measurement.** The lissamine green B (LGB) dye assay was used to
measure ClO₂ residual (Hofmann et al., 1998). To 90 mL of sample were added 10 mL of
lissamine green buffer, followed by 1.0 mL lissamine green B dye solution. Two to four
independent samples were collected at the clearwell effluent at each of \( t = 0 \) and \( t = \text{eq' m} \).
The absorbance at 616 nm ($A_{616}$) was recorded on a water-blanked spectrophotometer (LKB Ultrospec II, Biochrom Ltd., Cambridge, UK. or Cary 50 ConcUV-Visible Spectrophotometer, Varian Inc., Palo Alto, CA) using a 1 cm glass cuvette (see Figure A.1 for standard curves). Chlorine dioxide standards were prepared and read regularly (Figure A.2). The method detection limit calculated by Hofmann et al. (1998) in laboratory reagent water (Milli-Q® UV Plus System) at 20 °C was 0.017 mg ClO₂/L.

*Ct calculation.* The mean of the concentration of ClO₂ measurements at $t = \text{eq}'m$ was multiplied by 50 min., the mean detention time of the clearwell, to calculate *Ct.*

### 3.2.4. Physical and Chemical Parameters

*Alkalinity, pH and temperature.* The pH meter (Fisher Scientific Model 15 accumet pH meter, Pittsburgh, PA) was calibrated at two points. Alkalinity was measured using a procedure based on Standard Method 2320B (APHA, 1998). After the pH and temperature of a 100 mL sample were measured, the sample was titrated with 0.005 N H₂SO₄ to pH 4.5. The volume of titrant used (mL) was recorded and multiplied by 2.5 to derive alkalinity (mg CaCO₃/L).

*Chlorate and chlorite.* Amber-glass, 125 mL bottles were half-filled with samples, and residual ClO₂ was purged with nitrogen (N₂) for at least 10 min. using a gas-dispersion tube. A 20 mL colourless glass vial with a Teflon®-lined cap was filled with the purged sample, and 2-3 drops of ethylene diamine stock (EDA; 45 g/L) were added. Samples were shipped to the University of Toronto for analysis in accordance with USEPA method.
300.0 (USEPA, 1993). Ion chromatography was performed on a Dionex DX 500 ion chromatograph equipped with an AS9-SC analytical column (4 x 250 mm), AG9-GC guard column, and CD20 conductivity cell detector (Dionex Corp., Sunnyvale, CA). The sample loop was 50 µL, with pump rates of 2.0 mL/min. for eluent (120 mM H₃BO₃ and 30 mM NaOH) and about 8 mL/min. for regenerant (25 mM H₂SO₄). The detection limit for each of chlorate and chlorite was 0.05 mg/L. Values reported represent the arithmetic mean of duplicate samples. Travel standards containing 0.1 mg/L of each of chlorate and chlorite were prepared from stock solutions, treated with 2-3 drops of EDA, and included in each shipment. Travel blanks containing laboratory-reagent water were treated likewise. Measured mean concentrations (standard deviation) of the travel standards (n=10) were 0.13 (0.04) mg chlorite/L and 0.14 (0.09) mg chlorate/L. Chlorite and chlorate concentrations were less than their detection limits for all travel blanks (n=10).

Colour and UV₂₅₄. Colourless, 100 mL glass bottles were used to collect water which was stored at 4 °C until colour and UV₂₅₄ analyses were conducted. A method developed at the Britannia Water Treatment Plant was used for colour analysis. Filter and clearwell effluent samples were placed into a quartz 4 cm cell, to which was added one drop of pH 11 buffer (1.0 M boric acid and 0.3 M NaOH borate buffer). Raw and settled water samples were first processed through a 0.45 µm syringe-filter. The absorbance at 420 nm (A₄₂₀ units) was recorded on a water-blanked spectrophotometer (LKB Ultrospec II or Cary 50 ConcUV-Visible Spectrophotometer) and the result converted into true colour units (tcu) using a standard curve developed at the Britannia Water Treatment Plant. Standard Method 5910B was used for UV₂₅₄ measurement (APHA, 1998). Filter and
clearwell effluent samples were placed into a quartz 4 cm cell; raw and settled water samples were first filtered through a 0.45 μm syringe-filter. The pH of samples was not adjusted. Absorbance at 254 nm (A_{254} units) was recorded on a distilled-deionised water-blanked spectrophotometer (LKB Ultrospec II or Cary 50 ConcUV-Visible Spectrophotometer). Results are reported as standardised to a 1 cm cell.

*Haloacetic acids (HAA5) and total trihalomethanes (TTHM).* About 30 mg NH₄Cl was added to a 20 mL colourless glass vial with a Teflon®-lined cap. Each vial was filled headspace-free, with separate samples collected for TTHM and HAA5 analysis. TTHM is defined as the sum of the concentrations of chloroform, bromodichloromethane (BDCM), dibromochloromethane (DBCM), and bromoform (USEPA, 1998a). HAA5 is the sum of the concentrations of mono-, di-, and trichloroacetic acids and mono- and dibromoacetic acids (MCAA, DCAA, TCAA, MBAA and DBAA, respectively: USEPA, 1998a). All samples were sent to the University of Toronto for analysis as per USEPA method 551.1 for TTHM and 552.2 for HAA5 (USEPA, 1995). Values reported represent the arithmetic mean of duplicate samples. Analyses for both TTHM and HAA5 were performed using a Hewlett-Packard 5890 Series II Plus gas chromatograph (Mississauga, ON, Canada) with electron capture detection (detector temperature 300 °C). The injector temperature was 200 °C. For HAA5 analysis, a DB-5 capillary column (30 m x 0.25 mm x 1 μm, J&W Scientific Inc., Folsom, CA) was used with 2 μL splitless injection, a carrier gas of helium (1.2 mL/min. at 35 °C), and 1,2,3-trichloropropane as the internal standard. The oven temperature program was 35 °C for 10 min., increased to 100 °C at 2 °C/min., held for 4 min., increased to 120 °C at 10 °C/min., and held for 4 min. Detection limits were
1.6 μg MCAA/L, 1.1 μg DCAA/L, 0.9 μg TCAA/L, 1.9 μg MBAA/L and 1.6 μg DBAA/L. For TTHM analysis, a DB-5 capillary column (30 m x 0.25 mm x 0.25 μm) was used with 1 μL splitless injection, a carrier gas of helium (1.0 mL/min. at 35 °C), and 1,2-dibromopropane as the internal standard. The oven temperature program was 35 °C for 10 min., increased to 80 °C at 5 °C/min., held for 2 min., increased to 100 °C at 10 °C/min., and held for 2 min. Detection limits were 1.4 μg chloroform/L, 1.0 μg BDCM/L, 0.8 μg DBCM/L and 0.9 μg bromoform/L for TTHM analysis. Travel standards were prepared at the University of Toronto, shipped to the Britannia Water Treatment Plant, and then returned with samples for analysis at the University of Toronto. The travel standards for HAA5 included 16.3 μg MCAA/L and 8.3 μg/L of each of DCAA, TCAA, MBAA and DBAA. From HAA5 analyses, the measured mean concentrations (standard deviation) of the travel standards (n=8) were 13.1 (0.8) μg MCAA/L, 7.7 (1.8) μg DCAA/L, 8.2 (2.7) μg TCAA/L, 8.7 (0.5) μg MBAA/L and 8.1 (2.6) μg DBAA/L. The travel standards for TTHM included 4.3 μg/L of each of chloroform, BDCM, DBCM, and bromoform. From TTHM analyses, the measured mean concentrations (standard deviation) of the travel standards (n=8) were 6.1 (0.2) μg chloroform/L, 5.6 (0.3) μg BDCM/L, 6.0 (0.2) μg DBCM/L and 5.6 (0.1) μg bromoform/L.

**Total organic carbon (TOC).** Amber-glass, 40 mL vials with Teflon-lined caps were filled with sample, acidified with three drops of conc. H₂SO₄, and stored at 4 °C until sent to the University of Toronto for analysis (OI Analytical Model 1010 TOC Analyzer with
Model 1051 Autosampler, College Station, TX) in accordance with Standard Method 5310D (APHA, 1998). Travel blanks were prepared in a likewise manner using laboratory reagent water as the sample. Travel standards of 3 mg carbon / L were prepared using anhydrous potassium biphthalate ($\text{C}_8\text{H}_j\text{K}_04$) in laboratory reagent water. The travel standards were measured at 2.72 mg/L (std. dev. 0.09, $n=8$), and the travel blanks were measured at 0.05 mg/L (std. dev. 0.02, $n=8$).

**Turbidity.** Samples for turbidity analysis were collected directly into a glass turbidimeter sample cell and measured immediately (Hach Model 43900 or Model 2100N, Loveland, CO). Results are reported in nephelometric turbidity units (ntu) and in accordance with Standard Method 2130B (APHA, 1998).

### 3.3 RESULTS AND DISCUSSION

#### 3.3.1. *Bacillus subtilis* Spore Removal

In three pilot-scale trials, 1.9, 2.1, and 2.9 log$_{10}$ removal of *B. subtilis* spores was measured through the filter (*Table 3.2*). Rice *et al.* (1996) reported a 0.85 log$_{10}$ removal of aerobic spores between the source water and settled water effluent, and 2.12 log$_{10}$ removal/inactivation between the source water and the chlorinated filter effluent in pilot studies (*Table 3.2*). While the study of Rice *et al.* (1996) is not directly comparable to the present study, the approximately 1.27 log$_{10}$ removal and inactivation of spores during filtration measured by the authors is approximately 1 log$_{10}$ less than measured in the study presented here and may reflect differences in the influent water chemistry, the impact of coagulants, and/or the use of sand versus dual-media anthracite/sand filtration (*Tables 3.1,*
3.2). Similar studies concerning the removal of indigenous, aerobic spore-forming bacteria were conducted by Rice et al. (1996) at four full-scale surface water treatment plants practicing a variety of treatment processes. The removal (calculated from source water to filter effluent) of spores at the water treatment plants ranged from 1.68 to 2.75 log\(_{10}\). 

Nieminski and Ongerth (1995) studied the removal of inactivated *C. parvum* oocysts in a conventional water treatment pilot plant (*Table 3.2*). Because the authors calculated removal from source water to filter effluent, their data are not directly comparable to the pilot-plant *B. subtilis* spore removal data of the present study. However, some useful information can be extracted. First, the average calculated removal reported by Nieminski and Ongerth (1995) is larger than that of the present study. This is consistent with the incorporation of the unit processes of coagulation through filtration in the removal calculation of Nieminski and Ongerth (1995), versus the calculation of removal solely through filtration in the data presented here. Second, the variability in removal of the present study is consistent with the variability of results of Nieminski and Ongerth (1995). Third, the same authors conducted similar experiments in a full scale treatment plant and found inactivated *C. parvum* oocyst removal on the order of 0.5 log\(_{10}\) less than in the corresponding pilot plant tests, but the authors did not attempt to explain this result. In the absence of full scale treatment plant data for the removal of *B. subtilis* spores in the present study, the possible overestimation of removal by the pilot plant relative to the full scale treatment plant is noted. This may be due to differences in optimisation of the treatment trains or effects of scale.
Swerfeger et al. (1999) spiked heat-inactivated \textit{C. parvum} oocysts into filter influent water at a pilot plant treating Ohio River water and measured removal by filtration (\textit{Table 3.2}). The authors subtracted the log$_{10}$ removal through an empty column to obtain the reported values; however, as the authors concede, a full-scale treatment plant would probably achieve some removal by non-media surfaces, \textit{e.g.}, side walls, an effect subtracted out of the reported values (\textit{Table 3.2}; Swerfeger et al., 1999). Nevertheless, three important points emerge from these data. First, the \textit{B. subtilis} spore removal values and associated variation observed in the present study are consistent with the data of Swerger et al. (1999) (\textit{Table 3.2}). Second, spore removal appears conservative compared with oocyst removal, consistent with the hypothesis of Rice et al. (1996). However, it is noted that Swerfeger et al. (1999) only examined a specific isolate of \textit{C. parvum.} Other potentially pathogenic cryptosporidia may vary in size and other morphological characteristics (Bornay-Llinares, 1999), which may affect filter removal performance. Third, while the removal of indigenous, aerobic, spore-forming bacteria was not studied in the present case, the literature lends validity to the use of \textit{B. subtilis} spores (ATCC 19659) as a surrogate measure of \textit{C. parvum} oocyst removal. This helps to support the use of aerobic spores in the evaluation and improvement of treatment plant performance (Nieminski et al., 2000). Indeed, the removal results of the present study may suggest compliance with the \textit{Cryptosporidium} spp. oocyst removal requirement of the IESWTR (USEPA, 1998b).

3.3.2. \textit{Bacillus subtilis} Spore Inactivation

\textit{Unmodified versus sand-sheared "stressed" spores.} Jenkins et al. (1999) studied the
environmental stresses on \textit{C. parvum} oocysts in soils and animal waste piles, and suggested that the abrasive effects of soil particles, possibly resulting from the expansion and contraction of the soil during freeze-thaw cycles, resulted in mechanical disruption of oocysts which was a factor in their inactivation. Parker and Smith (1993) postulated that the abrasive effects between \textit{Cryptosporidium} spp. oocysts and the sand grains of filter media in a water treatment plant may result in increased sensitivity of oocysts to free chlorine. The same authors found a significant increase in the percentage of non-viable \textit{C. parvum} oocysts, as measured by the DAPI/PI assay and compared to controls, after shaking the oocysts for 5 min. at bench-scale with sand (0.33 mm average size, 1.56 uniformity coefficient). When the shaken oocysts were exposed to 1 mg/L free chlorine for a further 5 min., the number of non-viable oocysts increased, though not in a statistically significant way, when compared to those treated with sand only (Parker and Smith, 1993).

In the present study, \textit{B. subtilis} spores were shaken at bench-scale with sand to produce so-called sand-sheared “stressed” spores. These were then spiked post-filtration to measure the inactivation by chlorine dioxide in the clearwell. In comparison to the inactivation of spores without sand-shearing spiked post-filter, the inactivation of “stressed” spores provides no evidence to support the theory that bench-scale sand shearing increases the sensitivity of the \textit{B. subtilis} spores to chlorine dioxide (\textit{Figure 3.2}).

Parker and Smith (1993) suggested that the bench-scale shaking of oocysts with sand is much more severe than the possible abrasive effects of filtration. In the present
study. \textit{B. subtilis} spores were spiked pre-filtration and the inactivation by chlorine dioxide in the clearwell was measured (\textit{Figure 3.2}). Upon comparison of the ClO$_2$ inactivation data of the pre-filtration and post-filtration spiked \textit{B. subtilis} spores, there is no evidence to suggest that that the unit process of filtration increases the sensitivity of the \textit{B. subtilis} spores to chlorine dioxide (\textit{Figure 3.2}). Further, there is no evidence to suggest that bench-scale shaking of oocysts with sand makes \textit{B. subtilis} spores any more sensitive to ClO$_2$ than does filtration of unmodified spores (\textit{Figure 3.2}). Note that these results apply to \textit{B. subtilis} spores; it would be premature to extrapolate these results to \textit{Cryptosporidium} spp. oocysts, because of differences in size and cell physiology.

3.3.3. \textbf{Disinfection Byproducts (DBPs)}

\textit{Chlorite, chlorate and residual chlorine dioxide}. The pilot plant clearwell mean detention time (\(\theta\)) was not varied during the course of the experiments. Thus, within the context of the Chick-Watson model, increased inactivation would necessitate an increase in chlorine dioxide concentration. The increased residual chlorine dioxide measured at the clearwell effluent at \(t = \text{eq}'m\) indeed corresponds to increased inactivation of spores (\textit{Figure 3.3}). The levels of chlorine dioxide in the clearwell effluent for 1.5 and 2.0 log$_{10}$ inactivation were 1.0 and 1.3 mg/L, respectively (\textit{Figure 3.3}). These levels exceed the 0.8 mg ClO$_2$/L maximum residual disinfectant level (MRDL) established by the USEPA (1998a). Chlorite concentrations were below the USEPA (1998a) maximum contaminant level (MCL) of 1.0 mg/L and below the maximum contaminant level goal (MCLG) of 0.8 mg/L for all levels of inactivation studied (\textit{Figure 3.3}). The general trend of increasing concentrations of chlorite and chlorate with increasing chlorine dioxide residual was
expected as the former are the major byproducts associated with chlorine dioxide disinfection.

Total trihalomethanes (TTHM) and haloacetic acids (HAA5). Bromodichloromethane (BDCM) and dibromochloromethane (DBCM) were not detected in the clearwell effluent at \( t = \text{eq'm} \) for the post-filter, unstressed spore spike experiments (Figure 3.4). TTHMs were well within the MCL of 80 \( \mu \text{g/L} \) (Figure 3.4; USEPA, 1998a). Bromoform was not observed until chlorine dioxide was increased sufficiently to effect a 2.0 \( \log_{10} \) inactivation of spores: at this level, a concentration of 1 \( \mu \text{g/L} \) was recorded (Figure 3.4). The MCL of 60 \( \mu \text{g/L} \) for HAA5 and the MCLG of 30 \( \mu \text{g TCAA/L} \) was achieved at all levels of inactivation (Figure 3.5; USEPA, 1998a). The level of DCAA increased as the level of inactivation increased, corresponding to an increased chlorine dioxide residual in the clearwell effluent (Figures 3.3, 3.5; USEPA, 1998a). The general trend of increasing DBPs with increased chlorine dioxide residual are as expected according to chlorine dioxide reaction theory, as more \( \text{ClO}_2 \) would be available to react with precursor materials or to disproportionate.

3.4 SUMMARY

\textit{Bacillus subtilis} spores (ATCC 19659) were observed to be sensitive to chlorine dioxide (\( \text{ClO}_2 \)) inactivation in pH 6 post-filtration (pre-disinfection) Ottawa River water at 23 °C. \textit{e.g.}, a \( C_t \) of 63 mg·min/L being sufficient for 2 \( \log_{10} \) inactivation in a conventional pilot-scale water treatment facility. The pilot scale inactivation data for “stressed” spores provides no evidence to support the hypothesis that sand shearing at bench-scale increases
the sensitivity of the *B. subtilis* spores to chlorine dioxide. For this water matrix, achieving $> 1 \log_{10}$ inactivation of *B. subtilis* spores at pilot-scale exceeded the MRDL of 0.8 mg ClO$_2$/L (USEPA, 1998). The MCL for chlorite was met up to $2 \log_{10}$ inactivation, the highest level of inactivation measured. The MCL for total trihalomethanes of 0.080 mg/L and the MCL for haloacetic acids (HAA5) of 0.060 mg/L were also met up to $2 \log_{10}$ inactivation (USEPA, 1998).

*B. subtilis* spores may be an appropriate, conservative surrogate at pilot-scale to gauge removal of cryptosporidial oocysts. Pilot-scale results may however overestimate full-scale treatment plant removal. There is no evidence in the present study to suggest that anthracite/sand filtration increases the sensitivity of *B. subtilis* spores to chlorine dioxide disinfection.
3.5 FIGURES AND TABLES

Table 3.1. Physical and chemical characteristics of the Ottawa River water after different stages of treatment in the pilot plant.\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Raw water</th>
<th>Pre-filter / settled water</th>
<th>Post-filter effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>23.1</td>
<td>23.3</td>
<td>23.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
<td>5.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3) / L)</td>
<td>17.3</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Turbidity (ntu)</td>
<td>1.1</td>
<td>0.80</td>
<td>0.10</td>
</tr>
<tr>
<td>TOC (mg/L)(^b)</td>
<td>5.95</td>
<td>3.16</td>
<td>2.72</td>
</tr>
<tr>
<td>Colour (tcu)</td>
<td>27.6</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td>UV(<em>{254}) (A(</em>{254}) units)(^c)</td>
<td>0.24</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>ClO(_2) residual (mg/L)</td>
<td>&lt; MDL(^d)</td>
<td>&lt; MDL(^d)</td>
<td>&lt; MDL(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Each datum for "Raw water", "Pre-filter / settled water", and "Post-filter effluent" is the average of 23 independent samples, collected in July and August 1999.

\(^b\) TOC = total organic carbon.

\(^c\) UV\(_{254}\) = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.

\(^d\) MDL = method detection limit, 0.017 mg/L at 20 °C (Hofmann et al., 1998).

Figure 3.1. Schematic of the Britannia pilot plant treatment train. The clearwell included two baffles extending across the width of the clearwell, shown here in profile view. "SP" indicates a sampling point.
Table 3.2. Summary of selected pilot-scale removal data and comparison to the present study.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Water treatment summary</th>
<th>Organism studied</th>
<th>Log(_{10}) removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nieminski and Ongerth (1995)</td>
<td>Coagulation (alum and a cationic polymer), flocculation, sedimentation, and dual-media filtration (anthracite/sand).</td>
<td>Inactivated C. parvum oocysts</td>
<td>2.98 (average removal from source water to filter effluent, range 1.94 to 3.98, std. dev. 0.64, n=8)</td>
</tr>
<tr>
<td>Rice et al. (1996)</td>
<td>Lake water source (pH 8.16, alkalinity 99 mg/L, TOC 4.81 mg/L), subjected to alum coagulation, flocculation, sedimentation, chlorine disinfection, and sand filtration.</td>
<td>Aerobic, spore-forming bacteria indigenous to lake water</td>
<td>0.85 (removal between source water and settled water effluent) and 2.12 (removal/inactivation between source water and chlorinated filter effluent)</td>
</tr>
<tr>
<td>Swerfeger et al. (1999)</td>
<td>Ohio River source water subjected to coagulation (alum and a cationic polymer), flocculation, sedimentation, and dual-media filtration (anthracite/sand). Filter aid ferric sulphate injected upstream of filtration. Settled water: average 23 °C and 1.3 ntu.</td>
<td>Heat-inactivated C. parvum oocysts, spiked at filter influent</td>
<td>1.6, 3.1 and 3.4 (removal by filtration, n=3) and 3.4, 3.9, and 4.2 (removal through a filter with a deeper anthracite layer, n=3)</td>
</tr>
<tr>
<td>Present study</td>
<td>Ottawa River source water (Table 3.1) subjected to coagulation (alum and activated silicate), flocculation, sedimentation and dual-media filtration (anthracite/sand).</td>
<td>B. subtilis spores</td>
<td>1.9, 2.1, and 2.9 (removal by filtration, n=3)</td>
</tr>
</tbody>
</table>

a These removal data are conservative: The log\(_{10}\) removal through an empty column was subtracted to obtain these reported values.

b Most of the heat-inactivated oocysts were observed to retain sporozoites and other morphological characteristics, with < 0.2% reported as empty by the authors.

c Removal of these spores was measured simultaneously to the removal of the heat-inactivated C. parvum oocysts.

d The authors suggested that the removal/inactivation observed during filtration was due largely to physical removal and not chlorine inactivation because the chlorine applied prior to sand filtration (resulting in a chlorine residual in the filter effluent of 2.69 mg/L) had a contact time of < 20 min.

e A laboratory error, as reported by the authors, precluded publication of data for the third trial.

f Same data reported in Figure 3.2.
Figure 3.2. Pilot-scale inactivation of *Bacillus subtilis* spores in Ottawa River water by chlorine dioxide. Three sets of experiments are shown on the graph. *B. subtilis* spores were spiked either pre-filtration ("Prefilter" runs) or post-filtration ("Postfilter" runs). "Postfilter stressed" runs involved the post-filtration spiking of *B. subtilis* spores 'stressed' artificially by sand shearing at bench-scale. The number below each bar represents the \( Ct \) value corresponding to the microbial inactivation data, calculated by multiplying the measured \( \text{ClO}_2 \) residual (mg/L) by the contact time of the clearwell (50 min.). A \( Ct \) value of 0 mg·min/L indicates a run with no addition of \( \text{ClO}_2 \). Where applicable, values for \( \log_{10} \) removal are indicated above the appropriate bar. Sample replicates are as described in the *Materials and Methods*. 
Figure 3.3. Chlorite, chlorate and residual chlorine dioxide concentrations in the clearwell effluent at f=eq'm for pilot-scale Bacillus subtilis spore inactivation studies. The x-axis indicates the log$_{10}$ inactivation of spores by chlorine dioxide corresponding to the data. The method detection limits (MDL) were 0.017 mg chlorine dioxide/L (Hofmann et al., 1998) and 0.05 mg/L for each of chlorite and chlorate. Sample replicates are as described in the Materials and Methods.
Figure 3.4. TTHM concentrations in the clearwell effluent at t<sub>eq'm</sub> for pilot-scale Bacillus subtilis spore inactivation studies. The x-axis indicates the log<sub>10</sub> inactivation of spores by chlorine dioxide corresponding to the data. Each bar represents the mean of two replicate samples. The absence of a bar indicates that the given trihalomethane was below the MDL of 1.4 µg chloroform/L, 1.0 µg BDCM/L, 0.8 µg DBCM/L or 0.9 µg bromoform/L.

Figure 3.5. HAA5 concentrations in the clearwell effluent at t<sub>eq'm</sub> for pilot-scale Bacillus subtilis spore inactivation studies. The x-axis indicates the log<sub>10</sub> inactivation of spores by chlorine dioxide corresponding to the data. The second replicate of HAA5 data corresponding to 1.0 log<sub>10</sub> inactivation did not contain measurable concentrations of MCAA, DCAA, TCAA or MBAA, and so the data plotted at this level of inactivation is of a single observation, rather than the mean of two replicates as for the other data. The absence of a bar indicates that the given haloacetic acid was below the MDL of 1.6 µg MCAA/L, 1.1 µg DCAA/L, 0.9 µg TCAA/L, 1.9 µg MBAA/L or 1.6 µg DBAA/L.
3.6 REFERENCES


CHAPTER 4

BACILLUS SUBTILIS SPORE INACTIVATION AT BENCH SCALE

4.0 INTRODUCTION

There are several properties of cryptosporidia that make the use of a model organism (surrogate) in bench-scale disinfection studies attractive. The current lack of a continuous *in vitro* cultivation system for *Cryptosporidium* spp. oocysts means that the high concentrations of oocysts typically used in bench-scale experiments must be collected from the faeces of infected animals (Gasser and O'Donoghue, 1999). Coupling this with the technical expertise, expense of materials, potential pathogenicity of cryptosporidia, difficulties associated with measuring *Cryptosporidium* spp. oocyst concentration in the laboratory, and time required for *Cryptosporidium* spp. experiments, there are economic and practical limitations to bench-scale work. Desired characteristics of a microbial surrogate for *Cryptosporidium* spp. inactivation experiments, therefore, include: a resistance to the disinfectant comparable to that of *Cryptosporidium* spp. oocysts, the availability of specific, easy, inexpensive and sensitive enumeration methods, and a non-pathogenic nature. Here, the inactivation of *Bacillus subtilis* spores by chlorine dioxide was studied at bench-scale in various water matrices to achieve two main objectives:

1. To examine the effects of pH and water matrix on the inactivation by chlorine dioxide of *B. subtilis* spores.
2. To compare bench- and pilot-scale results for *B. subtilis* spore inactivation.
4.1 BACKGROUND

**pH effects.** Chlorine dioxide disinfection efficiency is not expected to vary with pH. Noss and Olivieri (1985) tested the chlorine dioxide inactivation of f2 bacterial virus (ATCC 15776-B) and presented evidence that chlorine dioxide, and not its disproportionation products such as chlorite and chlorate, was the active disinfecting species. This was confirmed by Harakeh et al. (1988), who tested the inactivation of *Bacillus subtilis* vegetative cells (strain unreported) in phosphate buffered water (pH 7.0) at 23 (±1) °C using a so-called “stabilised” aqueous solution of chlorine dioxide. Liyanage et al. (1997) presented a similar conclusion for “drinking water disinfection conditions” after conducting chlorine dioxide inactivation experiments using *Cryptosporidium parvum* oocysts in oxidant demand-free 0.05 M phosphate buffer (pH 8) at 22 (±1) °C. Further, in contrast to aqueous chlorine (HOCl / ClO⁻), the chemical structure of aqueous chlorine dioxide does not change with pH. As such, the reactivity of chlorine dioxide does not change significantly from pH 2 to 10.5 when the speciation (i.e., the chemical charge) of the substrate does not vary (Hoigné and Bader, 1994). Coupling this with the evidence that free chlorine dioxide is the major disinfecting species, chlorine dioxide inactivation efficiency is not anticipated to vary significantly in the pH range of 2 to 10.5.

The literature, however, contains conflicting reports on the effects of pH on chlorine dioxide efficiency. Botzenhart et al. (1993) reported that ClO₂ disinfection efficiency on *B. subtilis* spores increased when the pH of a continuous-flow, bench-scale reactor was increased from 6 to 8 at temperatures of 5 or 15 °C (water matrix unspecified). Likewise, Berman and Hoff (1984) reported that at 5 °C, simian rotavirus
SA11 was more rapidly inactivated by chlorine dioxide at pH 10 than at pH 6. Chen et al. (1985) showed that the chlorine dioxide inactivation rate of Naegleria gruberi cysts in buffered, chlorine dioxide demand-free ddH₂O water matrices increased when pH increased from 5 to 9. The authors speculated that this may have been the result of changes in the organism surface or an increased effectiveness of the disinfectant with increased pH (Chen et al., 1985). Noss and Olivieri (1985) presented evidence that f2 bacterial virus was more easily inactivated by chlorine dioxide as the pH increased from 5 to 9. Commenting on these results, the authors suggested that there may have been a change in reactivity between chlorine dioxide and the virus, but dismissed the idea of a change in the species of the oxychlorine compound present (Noss and Olivieri, 1985). More specifically, they hypothesised that the results may have been attributable to a change in the chemical structure of the virion and/or the concentration of hydroxyl ions in the solution, if the hydroxyl ions were necessary for the inactivation reaction to occur (Noss and Olivieri, 1985). Similarly, Liyanage et al. (1997), commenting on research that suggested ClO₂ disinfection of Giardia lamblia cysts improved as the pH increased, postulated that the increased efficiency of ClO₂ at a higher pH may be due to possible chemical or physical changes in cyst structure.

Difficulties in comparisons to literature. One of the primary difficulties when comparing the B. subtilis inactivation data presented in this study to literature values is the use of different strains and preparations of microorganisms, and different experimental conditions. These factors may influence the observed inactivation kinetics with a particular disinfectant. For example, Herbold et al. (1989) noted that the literature
contains conflicting reports on the resistance of enteroviruses to ozone, and noted the significant role of differences in laboratory conditions. Sommer and Cabaj (1993) used three different methods to prepare *B. subtilis* spores (ATCC 6633) and studied their disinfection by ultraviolet light; they reported that the inactivation curves depended on the method used to prepare the spores. Jeng and Woodworth (1990) studied the chlorine dioxide gas sterilisation of a variety of microbial spores, including several environmental isolates of *B. subtilis*. To facilitate comparison of the chlorine dioxide resistance of the different isolates, the authors arbitrarily set the resistance to chlorine dioxide gas of *B. subtilis* subsp. *niger* spores (ATCC 9372) to 100% (Jeng and Woodworth, 1990). The authors reported 73.51% and 0.00% resistance for the spores of two environmental isolates of *B. subtilis* subsp. *niger* and 67.71%, 68.83% and 34.88% resistance for the spores of three different isolates of *B. subtilis* (Jeng and Woodworth, 1990). Taylor et al. (2000) studied the inactivation of five different strains of *Mycobacterium avium* at 23 °C and found that the *Ct* values for 3 log_{10} inactivation ranged from 51 to 204 mg·min/L using chlorine and from 2 to 11 mg·min/L using chlorine dioxide.

*Spores versus vegetative cells.* *B. subtilis* spores and not vegetative cells were used in the disinfection experiments presented here because of the greater environmental resistance of spores. Knott et al. (1995) studied the development of resistance of *B. subtilis* 168 to the chlorine releasing agents sodium dichloroisocyanurate (NaDCC) and sodium hypochlorite (NaOCl) during the differentiation from vegetative cell to spore. The authors presented clear evidence of an increased resistance of spores versus vegetative cells to an initial 10 ppm dose of each disinfectant for an exposure time of 10 min. at 20 °C (Knott et al.,
1995). Sabli et al. (1996) studied the hypochlorite inactivation of B. subtilis 168 strain PS346 spores, wild type for \(\alpha/\beta\)-type small acid-soluble proteins (SASP) and B. subtilis 168 strain PS361 spores, which lack the ability to produce \(\alpha\) and \(\beta\) SASP. The authors presented evidence that SASP contributes to hypochlorite resistance by protecting spore DNA (Sabli et al., 1996).

4.2 MATERIALS AND METHODS

4.2.1. BENCH SCALE BACILLUS SUBTILIS SPORE INACTIVATION EXPERIMENTS

General set-up. The reaction vessels used in experiments were low density polyethylene (LDPE) bottles (Fisher Scientific, Pittsburgh, PA) of approximately 565 mL capacity. A select few experiments involved 60 mL capacity glass bottles (VWR Canlab, Mississauga, ON, Canada) with Teflon®-lined aluminum crimp seals. The bottles were placed in a dark, temperature-controlled incubator (Psychroterm™, New Brunswick Scientific, Edison, NJ) and shaken at 150 rpm. The average temperature of the incubator over the course of the experiments was 21.4 °C (range 20.5-22.5 °C). The water matrix under study was brought to temperature in the incubator overnight.

Water matrices. The laboratory-reagent waters studied included type I deionised-distilled water (ddH₂O)-phosphate buffered to pH 6 and ddH₂O adjusted to pH 8 with 0.5 M NaOH (no buffering) (Table 4.1). The former was prepared by adding a phosphate buffer (88.9 mL/L of monopotassium phosphate solution (63.5 g/L KH₂PO₄) and 11.1 mL/L of disodium phosphate solution (249.2 g/L Na₂HPO₄·7H₂O)) drop-wise to ddH₂O to pH 6. Post-filtration, pre-disinfection Ottawa River water (Ontario, Canada) was collected
September 21, 1999, and used by January 18, 2000. Depending on the experiment, the Ottawa River water was used unmodified, adjusted to pH 8 with 0.5 M NaOH, or adjusted to pH 4.5 with 2 N H₂SO₄ (Table 4.2).

*Bacillus subtilis inactivation experiments.* Each bottle received 565 mL of the water matrix under study. *Bacillus subtilis* spores were prepared as described in Section 3.2.2. The spores were added to a concentration of approximately 10⁶ colony forming units / mL and chlorine dioxide (ClO₂) was added to a target concentration of 2 mg/L. At various time intervals, 12 or 22 mL samples were taken by syringe (BD Brand 30 cc polypropylene syringe, Fisher Scientific) with 10 mL of sample being used for the lissamine green B (LGB) assay to measure for ClO₂ residual (Section 4.2.2) and with the remainder being quenched by 1.5% (w/v) Na₂S₂O₃ for spore enumeration. Microbial samples were heat-treated in a 75 (±5) °C water bath for 10 min. and enumerated by the spread plate method on nutrient agar plates with 0.015 g/L trypan blue or by membrane filtration through a 0.45 μm filter (Millipore Corporation, Bedford, MA). All plates were incubated for 16-20 h at 37.0 (±0.5) °C. Only those spread plates with a colony count in the range of 30-300 and filters with a colony count in the range of 20-200 were used in data processing. Replicate samples were averaged for a particular time point. The inactivation of *Bacillus subtilis* spores at some time *t* was calculated as:

\[
\log_{10} \left( \frac{\text{microbial concentration at time } t}{\text{initial microbial concentration}} \right)
\] (4.1)
4.2.2. Chlorine Dioxide and Statistical Analyses

Generation and titration of ClO₂ stock. A modified version of Standard Method 4500-CIO₂ was used in the generation of chlorine dioxide (ClO₂) for bench-scale work (APHA, 1998). A 25% (w/v) NaClO₂ reagent solution was pumped into a gas-generating bottle containing 12 N H₂SO₄. The Cl₂ scrubber bottle contained a 10% (w/v) NaClO₂ solution and the ClO₂ trap was filled with ddH₂O and kept cold using ice. An additional ClO₂-trap bottle with 10% (w/v) KI was added to the end of the series. The ClO₂ stock solutions had an average purity of 99% (range: 97-100%. n=9). The stock solution of ClO₂ was titrated as described for the pilot-scale Bacillus subtilis spore inactivation experiments (Section 3.2.3).

Residual ClO₂ measurement. The lissamine green B (LGB) dye assay was used to measure ClO₂ residual as described for the pilot-scale Bacillus subtilis spore inactivation experiments (Section 3.2.3), except that one-ninth of each of the components was used. The absorbance at 616 nm (A₆₁₆) was recorded on a water-blanked spectrophotometer (Spectronic 21D, Milton Roy, Rochester, NY) using a 1 cm glass cell. At least three independently prepared blank ClO₂ samples were read daily, and ClO₂ standards were prepared and run weekly (Figure A.2).

Ct calculation. Ct was calculated by simple integration of the ClO₂ residual concentration up to the given sample time. For a given time point, the Ct was calculated by first multiplying the measured ClO₂ concentration by the amount of time elapsed since
the last ClO₂ measurement. This value was then added to the Ct value calculated at the previous time point, to give the overall Ct value for a desired sampling time.

Statistical analyses. General Linear Models (GLMs) were constructed with the common (Briggs') logarithm of inactivation as the dependent measure and with Ct as the independent variable using SAS (version 8, SAS Institute. Cary, NC). Model adequacy checks were performed for each of these models and included residual plots, q-q plots, normality tests and boxplots. Pairwise comparisons were performed using the Bonferroni (Dunn) t-Test (α=0.05) when significant effects were found to be present.

4.2.3. Physical and chemical parameters

Alkalinity, pH and TOC. The pH of the water matrix was measured at the start of an experiment (Jenco Instruments Inc. Model 6071 or Model 671P, San Diego, CA). Alkalinity was measured as described in Section 3.2.4. TOC was measured as described in Section 3.2.4, except that no travel blanks or travel standards were prepared. Independently prepared TOC standards of 4 mg/L were measured to 3.52 mg/L (standard deviation 0.34 mg/L, n=3).

UV₂₅₄. Colourless 20 mL glass vials were used to collect water which was stored at 4 (±1) °C until sent to the University of Toronto for analysis in accordance with Standard Method 5910B (APHA, 1998). The pH of samples was not adjusted. Absorbance at 254 nm (A₂₅₄) was recorded on a water-blanked spectrophotometer (Hewlett-Packard
8452A diode array spectrophotometer, Mississauga, ON, Canada) using a 5 cm quartz cell. Results are reported as standardised to a 1 cm cell.

**4.3 RESULTS AND DISCUSSION**

**4.3.1. pH EFFECTS**

There was a higher rate of inactivation (α=0.05) of *Bacillus subtilis* spores (ATCC 19659) by chlorine dioxide at 21.5 °C in ddH$_2$O adjusted to pH 8 versus ddH$_2$O-buffered to pH 6 (*Figure 4.1; Table 4.3*). Conversely, there is no evidence (α=0.05) of a differing sensitivity to chlorine dioxide for spores at 21.5 °C in post-filtration Ottawa River water at pH 6 or 8, but there was a significant difference (α=0.05) when each of these matrices were compared to Ottawa River water at pH 4.5 (*Figure 4.2; Table 4.3*). While there is the possible confounding factor of water matrix effects, if spore structure were affected by pH or if an increase in the concentration of hydroxyl ions increased the chlorine dioxide inactivation rate, one might expect such effects to have been manifest consistently in the post-filtration Ottawa River water experiments. The difference in the inactivation kinetics of the ddH$_2$O matrices may lie with the different chemistries of the water matrices used: The pH 6 ddH$_2$O water included a phosphate buffer, while the pH 8 ddH$_2$O matrix was simply pH adjusted with NaOH base. The presence of the phosphate buffer may have afforded some indirect or direct protection to the spores. Ruffell *et al.* (2000) noted qualitatively that the chlorine dioxide inactivation curves for *Cryptosporidium parvum* oocysts (Iowa strain, genotype 2 (C)) were essentially the same at 20 °C in 0.01 M phosphate buffered solutions at pH 6 and 8. However, in a borate and carbonate buffered solution at pH 10, the authors stated that there was a faster inactivation rate, though no
statistical evidence of significance was presented (Ruffell et al., 2000). Whether this is
the result of the increased pH or a change in the buffer solution is equivocal; further, the
use of an entirely different microorganism by Ruffell et al. (2000) limits extrapolation of
their results to the *B. subtilis* inactivation experiments of the present study. No hypothesis
is offered to explain the post-filtration Ottawa River water results.

4.3.2. Water matrix effects

Laboratory-scale inactivation experiments reported in the literature tend to be
conducted in buffered "pure" water matrices, which aids in the comparison of data
between researchers. However, the extrapolation of such results to natural waters may be
invalid. No significant difference (α=0.05) in the ClO₂ inactivation of *B. subtilis* spores
was observed when comparing post-filtration Ottawa River water at pH 8 to ddH₂O at pH
8; however, a significant difference (α=0.05) did exist when comparing the latter to post-
filtration Ottawa River water at pH 4.5 or 6 (*Figures 4.2, 4.3; Table 4.3*). Likewise, there
was a significant difference (α=0.05) in the inactivation of spores in post-filtration Ottawa
River water at pH 4.5, 6 or 8 when compared to ddH₂O phosphate buffered to pH 6
(*Figure 4.3; Table 4.3*). It therefore appears that spore inactivation data in ddH₂O
phosphate buffered to pH 6 were more conservative than the unmodified post-filtration
Ottawa River water at pH 6, whereas ddH₂O adjusted to pH 8 provided comparable
inactivation data to post-filtration Ottawa River water adjusted to the same pH.
4.3.3. **Bench scale and pilot scale results**

To assess the applicability of the bench-scale *B. subtilis* inactivation results to represent inactivation at a water treatment facility, results were compared to pilot-scale results presented previously in Chapter 3 (*Figure 4.2; Table 4.4*). A significant difference ($\alpha=0.05$) was observed between the pilot-scale results and the bench-scale inactivation experiments performed using post-filtration Ottawa River in its unmodified (pH 6) and pH 8 adjusted forms. No significant difference ($\alpha=0.05$) was observed when the pilot-scale results were compared to the bench-scale results using post-filtration Ottawa River water acidified to pH 4.5 (*Table 4.4*). This may be a result of the fact that, for the pilot runs corresponding to the data in *Figure 4.2*, the pH of the clearwell effluent at $t=0$ averaged 6.0 ($n=4$), while the pH at $t=eq'm$ averaged 4.6 ($n=4$). However, it is noted that the method used to calculate $Ct$ values for the pilot plant data was necessarily different from the bench-scale data because of the spatial variation in chlorine dioxide concentration in a continuous flow-through system as compared to the assumption of perfect mixing in the bench-scale enclosed reactor system (see Section 3.1). This facilitated the use of an integration technique for the calculation of $Ct$ values for the bench scale data, whereas the $Ct$ values used for the pilot plant results may be conservative as only the chlorine dioxide residual in the clearwell effluent was measured. Therefore the slope of the pilot-scale data may be biased with respect to the slope of the bench-scale inactivation data.

Linear regressions of the bench and pilot-scale inactivation data, as plotted in *Figures 4.1 and 4.2*, were carried out to gauge the Chick-Watson model as an approximation for the observed data (*Table 4.5*). Four of the six data sets had $r^2$ values
greater than 0.9 (Table 4.5). The Chick-Watson model appears to be a reasonable approximation of the chlorine dioxide inactivation of *Bacillus subtilis* spores under the experimental conditions of the present study.

4.3.4. **Comparison to literature data**

*Spores versus vegetative cells.* Harakeh et al. (1988) reported that for an initial ClO₂ dose of 0.75 mg/L, a 5 min. contact time was sufficient to result in 4.52 log₁₀ kill of *B. subtilis* vegetative cells (unreported strain) at 23 (±1) °C in phosphate buffered water (pH 7.0). This datum corresponds to a theoretical maximum *Ct* value of 3.75 mg·min/L. While the strain of *B. subtilis* was unreported, the data are consistent with the expectation that vegetative cells would be more sensitive than spores to disinfection by ClO₂ (Figure 4.1). The hypochlorite kill curves published by Sabli et al. (1996) display a lag before viability reduction of *B. subtilis* spores begins; no lag was observed with the chlorine dioxide inactivation of spores in the present study (Figures 4.1-4.4).

**Comparison to free chlorine inactivation data.** Bloomfield and Arthur (1989) studied the inactivation of spores of *B. subtilis* (NCTC 10073) in phosphate buffered solutions (pH 7.4, unknown temperature) by NaOCl. For comparison to the *B. subtilis* inactivation data presented here, it is interesting to note that an NaOCl dose equivalent of 100 mg/L available free chlorine for a 5 min. contact period resulted in approximately 3 log₁₀ inactivation of *B. subtilis* spores (Bloomfield and Arthur, 1989). Without information on disinfectant decay, this datum corresponds to a theoretical maximum *Ct* value of 500 mg·min/L. Rice et al. (1996) studied the inactivation by dilute NaOCl of aerobic
spores indigenous to local river water. The authors diluted the water 1:10 using 0.05 M chlorine demand-free phosphate buffer (final pH 6.86, 23 °C) and reported that a $Ct$ of 315 mg·min/L (based on total available chlorine) was required for 3 log$_{10}$ inactivation, and that a $Ct$ of 114 mg·min/L was required for 2 log$_{10}$ inactivation. For comparison, 3 log$_{10}$ inactivation of *Bacillus subtilis* spores in ddH$_2$O buffered to pH 6 was achieved at a $Ct$ of about 150 mg·min/L in the present study (Figure 4.1). While the use of a different spore-forming bacteria under different environmental conditions cannot be ignored, it appears that NaOCl may be less effective than ClO$_2$ for inactivating *B. subtilis* spores.

Williams and Russell (1991) studied the inactivation by NaOCl of *B. subtilis* 8236 spores in phosphate buffered water (pH 7.4) at 21 °C. The authors reported that 100 mg/L available chlorine for a contact time of 5 min. was sufficient to produce a 3 log$_{10}$ kill (Williams and Russell, 1991). This corresponds to a theoretical maximum $Ct$ value of 500 mg·min/L. Interestingly, 50 mg/L available chlorine for a contact time of 10 min., resulting in the same theoretical maximum $Ct$ value, only produced 1 log$_{10}$ kill (Williams and Russell, 1991). Indeed, other results of Williams and Russell (1991) also seem discordant with the Chick-Watson disinfection model, a model which implies that a desired level of inactivation will occur for any combination of $C$ and $t$ that results in the same value (Section 3.1; Finch *et al.*, 1993). However, all of the data here cited from the different studies are consistent with the statement that NaOCl appears less effective than ClO$_2$ for inactivating *B. subtilis* spores.
Comparison to chlorine dioxide inactivation data. Botzenhart et al. (1993) reported that a Ct of 25 mg·min/L resulted in 4 log_{10} inactivation of *B. subtilis* spores in a continuous-flow, bench-scale reactor at 5 °C in a pH 8 water matrix. The spores of the *B. subtilis* isolate used by Botzenhart et al. (1993) appeared to be more sensitive to ClO₂ than the spores of the isolate used in the present study, but this is not anomalous, as presented in the previous discussion.

Ballantyne (1999) conducted *B. subtilis* spore inactivation experiments with chlorine dioxide in Milli-Q® water phosphate buffered to pH 6 and in unmodified post-filter (pre-disinfection) Ottawa River water at 20 °C using the same isolate as the present study. The reaction vessels used by Ballantyne (1999) were 60 mL glass bottles (described in Section 4.2.1), unlike the 565 mL LDPE bottles used for most of the experiments in the present study. There was no statistical difference (α=0.05) among the *B. subtilis* inactivation results of the present study in ddH₂O-buffered to pH 6 (21.5 °C) in both glass and LDPE bottles and the Ct values proposed by Ballantyne (1999) for spores in Milli-Q® water phosphate buffered to pH 6 (20 °C) (*Figure 4.4*). Similarly, the proposed Ct values of Ballantyne (1999) for inactivation in post-filtration Ottawa River water (pH 6, 20 °C) were not significantly different (α=0.05) from the inactivation results of the present study in unmodified, post-filtration Ottawa River water at 21.5 °C in LDPE bottles (*Figure 4.2*). Each of the two aforementioned data sets were significantly different (α=0.05) when compared pair-wise to the spore inactivation data of the present study in ddH₂O-buffered to pH 6.
4.4 SUMMARY

For *Bacillus subtilis* bench scale experiments, there was no significant difference ($\alpha=0.05$) in the sensitivity to chlorine dioxide for *B. subtilis* spores at 21.5 °C in post-filtration Ottawa River water at pH 6 or 8. There was, however, a significant difference ($\alpha=0.05$) in the chlorine dioxide inactivation rate of *B. subtilis* spores at 21.5 °C in ddH$_2$O phosphate buffered to pH 6 and in ddH$_2$O adjusted to pH 8. There was no significant difference ($\alpha=0.05$) in the chlorine dioxide inactivation of *B. subtilis* spores at 21.5 °C in ddH$_2$O adjusted to pH 8 and in post-filtration Ottawa River water adjusted to pH 8, but there was a significant difference ($\alpha=0.05$) in the inactivation of spores in ddH$_2$O-buffered to pH 6 and unmodified post-filtration Ottawa River water at the same pH. Under certain conditions, there was no significant difference ($\alpha=0.05$) in the bench- and pilot-scale inactivation of *B. subtilis* spores, strengthening the extrapolation of bench scale results to pilot scale. The inactivation results were consistent with literature values.

4.5 FIGURES AND TABLES

Table 4.1. Physical and chemical characteristics of the ddH$_2$O used in the bench-scale inactivation studies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ddH$_2$O buffered to pH 6</th>
<th>ddH$_2$O adjusted to pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.9 (n=6)</td>
<td>8.2 (n=5)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO$_3$/L)</td>
<td>17.8 (n=6)</td>
<td>7.0 (n=3)</td>
</tr>
<tr>
<td>TOC (mg/L)$^a$</td>
<td>0.27 (n=6)</td>
<td>0.28 (n=3)</td>
</tr>
<tr>
<td>UV$<em>{254}$ (A$</em>{254}$ units)$^b$</td>
<td>0.01 (n=6)</td>
<td>0.01 (n=3)</td>
</tr>
</tbody>
</table>

$^a$ TOC = total organic carbon.

$^b$ UV$_{254}$ = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.
Table 4.2. Physical and chemical characteristics of the post-filtration (pre-disinfection) Ottawa River water used in the bench-scale inactivation studies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unmodified Ottawa River water</th>
<th>Ottawa River water adjusted to pH 8</th>
<th>Ottawa River water adjusted to pH 4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.1 (n=7)</td>
<td>8.1 (n=4)</td>
<td>4.3 (n=1)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO₃ / L)</td>
<td>7.0 (n=5)</td>
<td>21.3 (n=4)</td>
<td>0 c (n=1)</td>
</tr>
<tr>
<td>TOC (mg/L) a</td>
<td>2.59 (n=4)</td>
<td>3.03 (n=3)</td>
<td>2.60 (n=1)</td>
</tr>
<tr>
<td>UV₃₅₄ (A₃₅₄ units) b</td>
<td>0.06 (n=2)</td>
<td>0.07 (n=3)</td>
<td>0.06 (n=1)</td>
</tr>
</tbody>
</table>

a TOC = total organic carbon.
b UV₃₅₄ = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.
c The sample pH was < 4.5 and therefore no alkalinity could be measured by the titration method.

Table 4.3. Pair-wise comparisons of bench-scale inactivation by chlorine dioxide of Bacillus subtilis spores in various water matrices at 21.5 °C.

<table>
<thead>
<tr>
<th>Water matrix</th>
<th>Significant difference in inactivation rate (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-filtration, unmodified (pH 6) Ottawa River water</td>
</tr>
<tr>
<td>Post-filtration, unmodified (pH 6) Ottawa River water</td>
<td>-</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water adjusted to pH 8</td>
<td>No</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water adjusted to pH 4.5</td>
<td>Yes</td>
</tr>
<tr>
<td>ddH₂O-buffered to pH 6</td>
<td>Yes</td>
</tr>
<tr>
<td>ddH₂O adjusted to pH 8</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 4.4. Comparison of bench- to pilot-scale *Bacillus subtilis* inactivation results.

<table>
<thead>
<tr>
<th>Bench-scale, post-filtration Ottawa River water matrix</th>
<th>Significant difference compared to pilot-scale results (α=0.05)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified (pH 6)</td>
<td>Yes</td>
<td>0.0086</td>
</tr>
<tr>
<td>Adjusted to pH 8</td>
<td>Yes</td>
<td>0.0082</td>
</tr>
<tr>
<td>Adjusted to pH 4.5</td>
<td>No</td>
<td>0.8104</td>
</tr>
<tr>
<td>Ct values proposed by Ballantyne (1999)*, pH 6</td>
<td>Yes</td>
<td>0.0154</td>
</tr>
</tbody>
</table>

* Ct values proposed for *B. subtilis* spores in post-filtration Ottawa River water at 20 °C.

Table 4.5. Linear regressions of *Bacillus subtilis* inactivation results.

<table>
<thead>
<tr>
<th>Bench-scale water matrix</th>
<th>$r^2$</th>
<th>$k$ (L/mg·min)*</th>
<th>y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O-buffered to pH 6</td>
<td>0.962</td>
<td>-0.0183</td>
<td>-0.157</td>
</tr>
<tr>
<td>ddH₂O adjusted to pH 8</td>
<td>0.820</td>
<td>-0.0431</td>
<td>-0.721</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water adjusted to pH 4.5</td>
<td>0.959</td>
<td>-0.0450</td>
<td>0.476</td>
</tr>
<tr>
<td>Post-filtration, unmodified (pH 6) Ottawa River water</td>
<td>0.909</td>
<td>-0.0582</td>
<td>0.301</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water adjusted to pH 8</td>
<td>0.734</td>
<td>-0.0407</td>
<td>-0.474</td>
</tr>
<tr>
<td>Ottawa River pilot plant data*</td>
<td>0.957</td>
<td>-0.0281</td>
<td>-0.117</td>
</tr>
</tbody>
</table>

* $k$ is the pseudo first-order reaction rate constant, taken as the slope of the linear regression of the data as plotted in *Figures 4.1 and 4.2* (Section 3.1).

* The Ottawa River pilot plant inactivation data plotted in *Figure 4.2* are used here.
Figure 4.1. Effect of pH on ClO$_2$ inactivation of *Bacillus subtilis* spores in ddH$_2$O at 21.5 ºC.

Figure 4.2. Effect of pH on ClO$_2$ inactivation of *Bacillus subtilis* spores in post-filtration, pre-disinfection Ottawa River water at 21.5 ºC. Pilot plant inactivation data (see Chapter 3) for *B. subtilis* spores by ClO$_2$ are shown for comparison. The Ct values proposed by Ballantyne (1999) for *B. subtilis* spores in post-filtration, pre-disinfection Ottawa River water at 20 ºC are also shown.
Figure 4.3. Effect of water matrix on ClO₂ inactivation of Bacillus subtilis spores at 21.5 °C. Data are from Figures 4.1 and 4.2.

Figure 4.4. Chlorine dioxide inactivation of Bacillus subtilis spores in ddH₂O-buffered to pH 6 at 21.5 °C. The reaction vessel was either a low-density polyethylene (LDPE) bottle or a glass bottle. Data for the former are from Figure 4.1. The Ct values proposed by Ballantyne (1999) for B. subtilis spores in pH 6 phosphate buffered Milli-Q® water at 20 °C are shown for comparison.
4.6 REFERENCES


CHAPTER 5

CRIPTOSPORIDIUM PARVUM OOCYST INACTIVATION

AT BENCH SCALE

5.0 INTRODUCTION

There is scarce published data on the inactivation of Cryptosporidium parvum oocysts by chlorine dioxide, especially in non-laboratory reagent waters. Further, the data which do exist are difficult to compare because of differences in C. parvum oocyst isolates studied and the viability and infectivity assays used. Here, a most probable number (MPN) – cell culture method with Madin Darby canine kidney cells (MDCK, ATCC CCL-34) was used to measure inactivation as loss of infectivity in vitro with respect to time. An in vitro excystation method was used simultaneously to facilitate comparison to published data. Chlorine dioxide inactivation of C. parvum oocysts (Iowa isolate, genotype 2 (C), Pleasant Hill Farm, Troy, ID) was examined at bench-scale in laboratory-reagent water as well as post-filtration (pre-disinfection) waters obtained from the Ottawa River (Ontario, Canada), the White River (Indiana, USA), and Lake Michigan (Wisconsin, USA) to achieve three main objectives:

1) To examine the effect of water matrix on the inactivation at 22 °C of C. parvum oocysts by chlorine dioxide.

2) To compare C. parvum oocyst inactivation data obtained from in vitro excystation and most probable number (MPN)-cell culture methods.
(3) To examine *B. subtilis* spores as a possible surrogate for *C. parvum* oocysts in disinfection studies by examining data from bench- and pilot-scale studies using chlorine dioxide.

5.1 BACKGROUND

*Water matrix effects.* Inactivation efficiency tends to be negatively influenced by increasing turbidity, TOC and colour, as during storm run-off; particulates, for example, may protect microbes from the disinfectant (Sobsey, 1989; Lisle and Rose, 1995). Medema *et al.* (1998) examined the sedimentation kinetics of *C. parvum* oocysts and noted that a significant proportion of the oocysts attached readily to organic biological particles in the secondary effluent studied. The authors noted that attachment likely influences the efficacy of physical removal and disinfection processes (Medema *et al.*, 1998). Ransome *et al.* (1993) studied the chlorine inactivation of *C. parvum* oocysts in a 0.001 M sodium hydrogen carbonate buffered borehole water matrix adjusted to pH 7.0 with NaOH, and added settled sewage in some experiments to provide about 50 thermotolerant coliforms / mL. The authors suggested qualitatively that the addition of the settled sewage did not appear to have an effect on the chlorine inactivation kinetics, though no statistical results were presented (Ransome *et al.*, 1993). Carpenter *et al.* (1999) found that, under "recreational water conditions", faecal material had a protective effect on chlorine inactivation of *C. parvum* oocysts. Other studies have also suggested that oocyst contact with faecal matter may alter the sensitivity of oocysts to environmental pressures or disinfectants (Robertson *et al.*, 1992; Jenkins *et al.*, 1999). Here, inactivation
of oocysts was tested in various laboratory reagent and natural waters to determine if such parameters affected chlorine dioxide disinfection.

5.2 MATERIALS AND METHODS

5.2.1. Bench scale Cryptosporidium parvum inactivation experiments

General set-up. The general set-up was as described for the bench scale Bacillus subtilis spore inactivation experiments, except that only the 565 mL capacity low density polyethylene bottles were used as the reaction vessels (Section 4.2.1). The average temperature of the incubator during the course of the Cryptosporidium parvum experiments was 21.9 °C (range 21.3-22.4 °C).

Water matrices. The laboratory-reagent waters studied included type I deionised-distilled water (ddH$_2$O)-phosphate buffered to pH 6 and ddH$_2$O adjusted to pH 8 with NaOH (no buffering) (Table 5.1). Both matrices were prepared as described for the bench-scale Bacillus subtilis spore inactivation experiments (Section 4.2.1). The other water matrices examined included the Ottawa River (Ontario, Canada), the White River (Indiana, USA), and Lake Michigan (Wisconsin, USA). All were collected from water treatment plants, post-filtration and prior to the addition of disinfectant, with the exception of the Lake Michigan water which was pretreated with ozone. Upon receipt, all waters were stored at 4 (±1) °C. Background information on the characteristics of the Ottawa River water as received, and following pH adjustment can be found in Table 5.2 and Sections 3.2.1 and 4.2.1.
The White River water was collected January 24, 2000. and used February 15, 2000 (Table 5.3). The water was collected at a conventional water treatment plant (Muncie, IN) with a water supply amalgamating the White River, Prairie Creek Reservoir, and well sources. The water used in the experiments was subjected to pretreatment (KMnO₄, Cl₂, powdered activated carbon, lime, and/or caustic soda), coagulation (ferric chloride and a cationic polymer), flocculation, sedimentation and dual-media filtration (sand with either granular activated carbon or garnet).

The Lake Michigan water was collected on February 17, 2000, at a conventional water treatment plant (Milwaukee, WI), and used February 22, 2000 (Table 5.3). The source water received an unquantified amount of ozone and quenched by peroxide, prior to coagulation by alum. The water was collected after settling and filtration, but prior to the addition of free chlorine at the clearwell influent.

Experimental protocol. Each bottle received 560 mL of the water matrix under study. The Cryptosporidium parvum strain used was the Iowa isolate, genotype 2 (C), originally from Iowa and maintained at Pleasant Hill Farm (Troy, ID) with calves as the host animal. Two lots of oocysts were used (lot #99-23, shed November 11, 1999, and lot #00-3, shed January 18, 2000), and were each shipped in 50 mL PBS with 1000 U penicillin and 1000 µg streptomycin. For each reaction vessel, 5.0 mL of C. parvum stock was centrifuged for 10 min. at 1700 x g (Beckman Coulter model GS-6, Fullerton, CA). The supernatant was removed, the oocysts were resuspended in 5.0 mL of the water matrix under study and recentrifuged, the supernatant was removed, and a fresh 5.0 mL of the
water matrix under study was added. This was added to a reaction vessel to achieve a target concentration of approximately $10^5$ oocysts/mL. Chlorine dioxide was added to obtain a target residual of either 2 mg/L or 10 mg/L (measured within two minutes after addition). At various time intervals (up to 120 min. from the addition of ClO$_2$), 22 mL samples were collected by syringe (BD Brand 30 cc polypropylene syringe, Fisher Scientific), with 10 mL of sample being used for the LGB assay to measure for ClO$_2$ residual, as detailed below, and with the remainder being quenched by the addition of 1.5% (w/v) Na$_2$S$_2$O$_3$ for oocyst analysis. The microbial samples were concentrated from 10 mL to 600 μL by centrifugation (1700 x g on Beckman Coulter model GS-6 and 7200 x g on Fisher Scientific Micro7, Pittsburgh, PA). To control for sample processing, duplicate haemocytometer counts were performed on an aliquot of each sample which was stained with Crypt-a-Glo™, an FITC-labelled monoclonal IgM antibody genus-specific to Cryptosporidium (Waterborne Inc., New Orleans, LA). Samples were viewed using an Olympus BX-60 epifluorescence microscope (Olympus Corporation, Tokyo, Japan) with excitation at 450-480 nm (Plate 5.1). The average of the relative standard deviations amongst the haemocytometer counts for each run was 21% for C. parvum oocyst lot #99-23 (range: 12-40%) and 23% for C. parvum oocyst lot #00-3 (range: 15-28%) (Figure A.3). The samples were then used for the MPN-cell culture assay and in vitro excystation procedure as described in the following sections.

5.2.2. MPN-CELL CULTURE ASSAY

*Slide preparation.* The MPN-cell culture assay used was modified from Slifko *et al.*, 1999. Madin Darby canine kidney (MDCK) cells (ATCC CCL-34) were cultured in
RPMI 1640 medium with 25 mM HEPES and 300 mg/L L-glutamine (Cellgro®, Fisher Scientific, Pittsburgh, PA) and supplemented with 10% (v/v) foetal bovine serum (Cellct®, ICN Biomedicals, Aurora, OH) and passaged every 3-4 days. To each well of an eight well slide (Lab-Tek®, Nalge Nunc International, Naperville, IL) was added 150 μL of RPMI 1640 medium, followed by 150 μL of MDCK cell suspension. The slides were sealed in a bag (Bio-Bag™ Environmental Chamber Type C. Becton Dickinson and Company, Cockeysville, MD) with a CO₂ capsule (5-10% CO₂ atmosphere in the bag) and incubated at 37 °C for 24 h to a confluency of 70-80%. The media was then removed and each well was washed with PBS and received 150 μL of fresh media.

*Slide inoculation.* Serial, ten-fold dilutions of treated and control oocysts were prepared up to a dilution of 10⁻⁵. From a selected range of at least three sequential dilutions, five wells were inoculated with 50 μL each of a given dilution, for a total of at least 15 wells for a given sample. No sample was added to negative control wells. Dilutions prepared from the oocyst stock were used in the positive control wells. The slides were then sealed in a bag with a CO₂ capsule as above and incubated at 37 °C for 40 h.

*Fixing and staining slides.* The slides were removed from their bags, and each well was washed four times with 0.03% (v/v) Tween-20 in PBS. The cells were fixed by adding 100 μL methanol to each well for 10 min. at room temperature. Following a wash with PBS, about 125 μL PBS was added to each well for 30 min. The PBS was removed and 50 μL of a 1% (v/v) horse serum solution in water (ICN Biomedicals, Aurora, OH) was added to each well for a 1 h incubation at 37 °C. The wells were then washed once with
PBS, and received 50 µL of a 1-in-20 dilution of FITC-labelled Sporo-Glo™ polyclonal rat IgG antibody (Waterborne Inc., New Orleans, LA) directed against the intracellular stages of *C. parvum*. Slides were incubated in the dark at room temperature for 30 min. with gentle rocking. The wells were then rinsed once with PBS, and the chamber walls were pulled off the slide. To each well was added 8 µL of a DABCO-glycerol solution (2% (w/v) 1,4-diazabicyclo(2.2.2)-octane in glycerol), and a glass cover-slip was sealed over the wells using clear nail polish. Slides were stored in the dark at 4 °C and read within four weeks.

*Slide reading.* Slides were viewed at 400x using an Olympus BX-60 epifluorescence microscope with excitation at 450-480 nm. A "positive" score was marked if both invasion and replication were evident, detected as three or more fluorescing units corresponding to the size expected for infectious stages of *C. parvum* in close proximity in the same field of view for a given well; otherwise a "negative" score was assigned (*Plate 5.2*). MPN values with the Salama correction for bias and 95% Loyer and Hamilton confidence intervals were derived from the MPN Calculator software program version 4.04 available from the USEPA web site (http://www.epa.gov/nerlcwww/other.htm). MPN results reported as improbable by the software were excluded. All negative control wells were read as negative (*n=24*). The results of the positive control wells are shown in *Figure A.4*.

5.2.3. *IN VITRO EXCYSTATION*

Samples and a positive control consisting of a 10⁻¹ dilution of the oocyst stock
were also assessed by in vitro excystation as described previously (Chauret et al., 1998), with the exception that the incubation time for excystation was 2 h rather than 4 h. Before and after excystation, the number of fully excysted oocysts ("shells"), partially excysted oocysts ("partials"), and intact oocysts ("fulls") were counted such that the total number of oocysts examined was at least 100. The viability (%) was then calculated as:

\[
\text{viability} = \left(\frac{\text{shells} + \text{partials in excysted fraction}}{\text{total number of oocysts examined in excysted fraction}}\right) \times 100 - \left(\frac{\text{shells in original fraction}}{\text{total number of oocysts examined in original fraction}}\right) \times 100.
\]  

(5.1)

Positive controls using the oocyst stock were run in each experiment. For oocyst lot #99-23, the average viability was 91.7% (standard deviation 4.5%, n=5), and for oocyst lot #00-3, the average viability was 94.3% (standard deviation 4.2%, n=4).

5.2.4. CHLORINE DIOXIDE AND STATISTICAL ANALYSES

The generation and titration of ClO₂, the measurement of residual ClO₂ and the calculation of \(Ct\) values were described in Section 4.2.2. For experiments involving an initial concentration of ClO₂ greater than 2.5 mg/L, two independently prepared 1-in-5 (v/v) dilutions of a given sample were measured by the lissamine green B (LGB) dye assay, with the water matrix under study serving as diluent. The stock solution of ClO₂ was titrated as described in Section 3.2.3. Statistical analyses were performed as described in Section 4.2.2.

5.2.5. PHYSICAL AND CHEMICAL PARAMETERS

The measurements of alkalinity, pH, TOC, and \(\text{UV}_{254}\) were done as described for the bench-scale Bacillus subtilis spore inactivation experiments (Section 4.2.3). The DPD
ferrous titrimetric method (Section 4500-Cl. F., APHA, 1998) was used to measure residual free chlorine and monochloramine.

5.3 RESULTS AND DISCUSSION

5.3.1. IN VITRO EXCYSTATION AND MPN-CELL CULTURE RESULTS

The inactivation of Cryptosporidium parvum oocysts (Iowa isolate, genotype 2 (C)) by chlorine dioxide was investigated at bench-scale using various water matrices at 22 °C. Two methods were used to measure the inactivation of oocysts: in vitro excystation and an MPN-cell culture assay. The in vitro excystation and MPN-cell culture data (Figure 5.1) both suggest a strong resistance of the oocysts to chlorine dioxide. Especially evident at Ct values greater than 400 mg·min/L, the MPN-cell culture method measured greater kill than the in vitro excystation assay for the experimental conditions examined. In ddH₂O adjusted to pH 8, a Ct of 1 000 mg·min/L resulted in approximately 0.5 log₁₀ kill as measured by in vitro excystation, versus approximately 2.0 log₁₀ kill as measured by MPN-cell culture (Figure 5.1). Indeed, the inactivation rate as measured by the MPN-cell culture was statistically greater (α=0.05) when compared to in vitro excystation for all of the water matrices, except for ddH₂O-buffered to pH 6 and post-filtration Ottawa River water adjusted to pH 8 (Table 5.4). Given the scatter in the data and the limited range of kill measured in the latter two water matrices (Ct greater than 200 mg·min/L was not tested, nor did one datum exceed 0.5 log₁₀ inactivation), the statistical results from comparing the measurement assays in these two water matrices is not surprising (Figure 5.1).
The discrepancy between *in vitro* excystation and infectivity methodologies for a given set of conditions has been reported elsewhere (Finch *et al.*, 1993, 1995; Black *et al.*, 1996; Belosevic *et al.*, 1997; Bukhari *et al.*, 1999; Slifko *et al.*, 1999). Indeed, Fayer *et al.* (1991), in studying cryopreservation protocols for *C. parvum* oocysts, observed that while sporozoites released by excystation from frozen oocysts appeared normal under phase-contrast microscopy, they were uninfectious to mice. The authors therefore suggested that excystation and morphologic criteria may not be reliable indicators of infectivity. Despite this, *in vitro* excystation continues to be used as a viability measure and as a conservative surrogate measure for infectivity, even though the distinction between viable, infective and viable, non-infective oocysts has important implications for public health.

*In vitro* excystation attempts to simulate conditions of the digestive tract to stimulate the release of sporozoites from oocysts as in an *in vivo* infection. Therefore, infectivity is not measured, but rather the viability of the oocysts in terms of the ability for the excystation process to occur. The MPN-cell culture method attempts to simulate conditions of *in vivo* infection by providing host cells to allow for the first stages of an infection. The MPN-cell culture method is therefore a measure of infectivity. The distinction between viability and infectivity is important because it is possible that an oocyst may be able to excyst, and would thus be measured as viable by the *in vitro* excystation assay, but may in some way be damaged to arrest the infection process. On the other hand, an oocyst unable to excyst and therefore measured as non-viable by *in vitro* excystation, is almost certainly incapable of causing an infection. Thus, the MPN-cell culture assay may measure greater kill than *in vitro* excystation.
Based on linear regressions of the inactivation data as plotted in Figure 5.1, the Chick-Watson model appears to be a poor approximation for the chlorine dioxide inactivation of Cryptosporidium parvum oocysts, as measured by both in vitro excystation and MPN-cell culture methods, under the experimental conditions of the present study (Table 5.5). All of the data sets measured by in vitro excystation and four of the six data sets measured by the MPN-cell culture assay had $r^2$ values less than 0.9 (Table 5.5). While the MPN-cell culture inactivation data corresponding to post-filtration White River and Lake Michigan waters had $r^2$ values greater than 0.9, the substantial $y$-intercept values for these two matrices (greater than 1) suggest that the pure Chick-Watson model may not be a good approximation (Table 5.5).

5.3.2. COMPARISON TO FREE CHLORINE INACTIVATION DATA

Although obtained using a different isolate and under different experimental conditions than the literature cited herein, the results of the present study suggest that chlorine dioxide is more effective than free chlorine for the inactivation of Cryptosporidium spp. oocysts (Table 5.6). Moore et al. (1998) used in vitro excystation and found that the percentage viability of C. parvum oocysts (from cervine faeces) remained constant at 84-97% during a 15 day exposure to an NaOCl dose of 1 mg/L (theoretical maximum $Ct$ of 21 600 mg·min/L). Venczel et al. (1997) reported that for three replicate experiments at 25 °C in pH 7, oxidant demand free 0.01 M phosphate-buffered water, a 5 mg/L dose of free chlorine for 24 h (theoretical maximum $Ct$ of 7 200 mg·min/L) resulted in essentially no inactivation of C. parvum oocysts (Iowa strain) as measured by infectivity in neonatal BALB/c mice. In the present study, a
Ct of 1,000 mg-min/L with chlorine dioxide as the disinfectant resulted in approximately 0.5 log₁₀ kill as measured by in vitro excystation and approximately 2.0 log₁₀ kill as measured by MPN-cell culture for C. parvum oocysts in ddH₂O adjusted to pH 8 (Figure 5.1). Additional studies report the resistance of a variety of C. parvum isolates to chlorination under various conditions (Fayer, 1995; Chauret et al., 1998). Chloramines have not been found to be significantly better than chlorination in terms of oocyst inactivation (Ransome et al., 1993; Fricker and Crabb, 1998).

5.3.3. EFFECT OF WATER MATRIX ON INACTIVATION

No significant difference was observed in the inactivation of oocysts among the various water matrices (p=0.0667) as measured by in vitro excystation (Figure 5.1A). This was not the case with the MPN-cell culture assay (Figure 5.1B). The inactivation in post-filtration Lake Michigan water was significantly different (α=0.05) from all of the other water matrices tested; similarly, inactivation in post-filtration White River water was significantly different (α=0.05) from all of the water matrices tested except for ddH₂O adjusted to pH 8. No specific characteristic of the post-filtration Lake Michigan water could be identified readily to explain why the chlorine dioxide inactivation rate (as measured by MPN-cell culture) of C. parvum oocysts was greatest (α=0.05) in this water matrix when compared to the others studied (Figure 5.1B; Tables 5.1-5.3). The inactivation in ddH₂O-buffered to pH 6 was significantly different (α=0.05) from inactivation in ddH₂O adjusted to pH 8; however, it is again noted that the data for the former water matrix is available up to a Ct of about 200 mg-min/L, whereas the latter water matrix has inactivation data for Ct values past 1,100 mg-min/L (Figure 5.1B).
There were no other significant differences (α=0.05) evident upon pair-wise comparison of the water matrices.

5.3.4. COMPARISON TO CHLORINE DIOXIDE INACTIVATION DATA

In comparison to the literature data, the results from the chlorine dioxide inactivation of *C. parvum* oocysts in the present study are disparate and suggest a level of resistance to chlorine dioxide not quantified previously (Table 5.7). For example, Ruffell *et al.* (2000) studied the chlorine dioxide inactivation of *C. parvum* oocysts (Iowa isolate, genotype 2 (C), obtained from a calf at the University of Arizona) at 20 °C in 0.01 M phosphate buffered solutions in ddH₂O (Corona-Vasquez, 2000). To gauge inactivation, the authors used a “modified” *in vitro* excystation method involving sporozoites which reportedly measured more kill than the unmodified method under some conditions (Ruffell *et al.*, 2000). They reported approximately $2.0 \log_{10}$ kill of oocysts in pH 8 buffered ddH₂O at a $Ct$ of 150 mg-min/L (20 °C) (Ruffell *et al.*, 2000). Under similar conditions in the present study, an equivalent level of kill, as measured by the MPN-cell culture, required a $Ct$ of about 1000 mg-min/L (22 °C) (*Figure 5.1*).

Finch *et al.* (1995) provided chlorine dioxide inactivation results for a 0.05 M phosphate buffer, pH 8 water matrix at 25 °C using animal infectivity as the measure for *C. parvum* oocyst concentration. After applying a safety factor of two, Finch *et al.* (1995) proposed that for $1 \log_{10}$ inactivation of *C. parvum* oocysts by chlorine dioxide, a $Ct$ of 60 mg-min/L would be required; for $2 \log_{10}$, 80 mg-min/L; and for $3 \log_{10}$, 140 mg-min/L. Ruffell *et al.* (2000) also proposed $Ct$ values for 25 °C, but did not apply a safety factor.
Taking their data and applying a safety factor of two, the proposed $Ct$ values of Ruffell et al. (2000) are 80 mg-min/L for $1 \log_{10}$ inactivation, 140 for $2 \log_{10}$ inactivation, and 190 for $3 \log_{10}$ inactivation. Given the variability in the *C. parvum* oocyst inactivation data in the literature, these proposed $Ct$ values are in good agreement with the literature cited, but are discordant with the results of the present study (*Table 5.7*). Indeed, the inactivation data of the present study are the anomaly rather than the rule when compared to the literature and suggest a substantially higher resistance level of *C. parvum* oocysts to chlorine dioxide.

5.3.5. **Reconciliation of Disparate Chlorine Dioxide Inactivation Results**

*Cryptosporidium parvum isolates.* With no significant difference ($\alpha=0.05$) among the diverse water matrices for the *in vitro* excystation-measured chlorine dioxide inactivation results presented in *Figure 5.1*, and based on the MPN-cell culture measured inactivation among the laboratory-reagent water matrices, the use of different water matrices in the literature cited (*Table 5.7*) is, on its own, an unlikely explanation for the disparate results. It is unlikely that the resistance of the oocysts to chlorine dioxide was an experimental artefact resulting from a problem with the chlorine dioxide stock solution. The inactivation of *B. subtilis* spores by chlorine dioxide was studied at bench- and pilot-scales, and results were not found to be statistically different ($\alpha=0.05$) under certain conditions (*Chapter 4*). Two different chlorine dioxide generation methods had been used at bench- and pilot-scales, producing ClO$_2$ stocks with an average purity of 99% ($n=9$) at bench scale and 98% ($n=36$) at pilot scale (*Chapters 3, 4*). Further, the *B. subtilis* inactivation results were consistent with the literature (*Chapter 4*). The quality assurance /
quality control data for chlorine dioxide validate the measured chlorine dioxide values (Figure A.2). The disagreement between the literature cited and the present study could therefore be due to inter- and/or intra-oocyst stock differences resulting from genetic variation, oocyst purification methods, experimental protocols, or a combination therein.

Clinical results have been published suggesting that geographically diverse isolates of *C. parvum* genotype 2 (C) differ in their infectivity for humans (Okhuysen et al., 1999). Further, the spores of different isolates of *Bacillus subtilis* have been shown to exhibit different sensitivities to chlorine dioxide gas (Jeng and Woodworth, 1990; see Chapter 4). Taylor et al. (2000) examined the inactivation of five different strains of *Mycobacterium avium* at 23 °C and found that the Ct values for 3 log10 inactivation ranged from 51 to 204 mg·min/L using chlorine, and from 2 to 11 mg·min/L using chlorine dioxide. Rennecker et al. (1999) reported one isolate of *C. parvum* (Louisiana strain) to be more sensitive to ozone inactivation than another isolate (Iowa strain) treated under identical conditions. It therefore appears reasonable to suggest that different isolates of *C. parvum* may differ in their sensitivity to chlorine dioxide.

Comparison of inactivation data from different researchers is difficult because, without a practical, continuous *in vitro* cultivation system capable of producing oocysts in the concentrations used in bench scale studies, no standard reference strains exist (Gasser and O'Donoghue, 1999). Experimenters therefore tend to rely on oocysts passaged in animal hosts, which are subsequently cleaned and purified from the faeces *via* differing protocols and stored in different matrices. Interestingly, there is a close relationship
between the isolates used in the present study and those used in the research of Ruffell et al (2000). Ruffell et al. (2000) used the Iowa isolate, genotype 2 (C), maintained at the University of Arizona; the oocyst stock used in this research was the Iowa isolate, genotype 2 (C), maintained at Pleasant Hill Farm, ID. Both isolates trace their origins to oocysts isolated by Dr. Harley Moon (Iowa State University, Ames) from the faeces of a calf near Ames, Iowa in the mid-1980’s (Corona-Vasquez, 2000). But yet there is a marked difference in chlorine dioxide sensitivity of the oocysts in the present study and Ruffell et al. (2000) (Table 5.7). The inoculation of different animals by a specific oocyst isolate does not guarantee a pure culture of oocysts, e.g., there is the potential for oocyst contamination from a naturally occurring infection. Further, the possibility of evolutionary divergence of the isolates cannot be excluded.

Indeed, in addition to variation in chlorine dioxide sensitivity between unique isolates, there is evidence for an inconsistent response to disinfectants of different batches of a particular “isolate”. Ruffell et al. (2000) used two different shipments of oocysts for chlorine dioxide inactivation experiments at pH 10 and 20 °C and noted qualitatively that one lot of oocysts appeared more resistant than the other. Rennecker et al. (1999), however, noted qualitatively that two different lots of C. parvum oocysts (Iowa strain) used in experiments six months apart showed similar responses to ozone treatment. Slifko et al. (1999) described significant lot-to-lot variability in their measurements of viability and infectivity of C. parvum oocysts obtained from Pleasant Hill Farm; e.g., infectivity, as measured by a cell culture assay, ranged from 3.1 to 63.5% in eight oocyst lots tested. This variation in C. parvum oocyst viability from different batches has been observed by
others (Belosevic et al., 1997). Conversely, in the present study, the infectivity of the oocysts in the positive control, as measured by the MPN-cell culture assay, did not differ significantly between experiments using a particular batch of oocysts or between experiments using different batches (Figure A.4). Further, two C. parvum oocyst stocks were used for inactivation experiments in ddH₂O adjusted to pH 8 for the present study. There was no statistical difference in the inactivation rate between the two studies when using the MPN-cell culture assay or in vitro excystation (p=0.7208 and p=0.6656, respectively). However, if there are indeed significant differences in oocyst response to treatments among oocyst lots from a given supplier, then the potential for such differences between suppliers and the possible ramifications in terms of inter-laboratory comparison of disinfection studies cannot be ignored.

Oocyst purification protocol. Ruffell et al. (2000) proposed that lot-to-lot variability may result from pathological differences upon infection of the host or differences in subsequent oocyst processing. In the context of environmental sampling for C. parvum oocysts. Fricker and Crabb (1998) cautioned that measurements of viability reflected not only the effects of the environment and/or treatment, but also the effects of sample collection and processing, e.g., shear forces and changes in osmotic potential. This rationale may be extended to disinfection studies on C. parvum oocysts collected and purified from animal faeces: The sensitivity of the oocysts used may be affected by the oocyst preparation method. Robertson et al. (1993) used three different isolates of C. parvum oocysts in studies to maximise the efficiency of in vitro excystation and found that the isolates not only had different excystation efficiencies, but also responded
differently to pre-incubation treatments. The authors hypothesised that, while this could have been the result of strain difference, it was more likely a result of different oocyst purification procedures used (Robertson et al., 1993). Slifko et al. (1999) hypothesised that the lot-to-lot variability they observed in viability and infectivity of the oocysts may have resulted from oocyst processing, such as the use of ethyl ether in the defatting of faeces, and proposed to evaluate different purification assays and their effects on oocysts in the future.

It has been reported that contact of C. parvum oocysts with faecal matter may alter the sensitivity of oocysts to environmental pressures, including chlorine. Different recovery protocols of oocysts from faeces may therefore have been a contributing factor to the differences in chlorine dioxide inactivation kinetics. The cleaning process itself, including the use of preservatives and/or bacteriocidal compounds such as potassium dichromate, formalin, sodium hypochlorite, and sodium meta-periodate, may alter oocyst structure, permeability, and sensitivity to disinfectants; these factors may also vary with oocyst age (Reduker et al., 1985; Reduker and Speer, 1985; Campbell et al., 1993; Moore et al., 1998; Gasser and O'Donoghue, 1999). The use of these chemicals may produce oocysts that are not representative of those found in environmental water samples (Belosevic et al., 1997).

Specialised, density centrifugation techniques are often used in Cryptosporidium spp. oocyst recovery procedures to separate oocysts from debris and may influence disinfection studies by damaging oocysts or selectively concentrating a particular subset
of oocysts (Table 5.8). The C. parvum oocysts used by Ruffell et al. (2000) were purified using discontinuous sucrose and caesium chloride centrifugation gradients and shipped in approximately 1 mL volumes containing 0.01% (w/v) Tween 20 solution, 100 U penicillin/mL, 100 μg gentamicin/mL, and 100 μg streptomycin/mL (Corona-Vasquez. 2000). In the present study, ethyl ether extraction was used to remove fat and faecal debris and was followed by centrifugation to remove residual ether and bacteria during the purification of oocysts from faeces (Mason. 2000). The remaining faecal material and bacteria were removed by a one-step sucrose gradient and repeating washing; the oocysts were then shipped in 50 mL PBS containing 20 U penicillin/mL and 20 μg streptomycin/mL (Mason. 2000). While there are obvious gaps in the information available to compare the purification protocols, it seems reasonable to suggest that the difference in inactivation kinetics observed could be at least partly explained by different oocyst preparation protocols, which may have selectively concentrated oocysts and/or altered the sensitivities of the oocysts to chlorine dioxide. For example, the use of a sucrose gradient for centrifugation in the present study, versus discontinuous sucrose and caesium chloride centrifugation gradients by Ruffell et al. (2000), may have resulted in a stock consisting of a higher percentage of fully intact, viable oocysts in the present study. Presumably, with a higher percentage of structurally intact oocysts in the population, inactivation of the oocysts would appear to be less efficient. Other elements in the sample processing procedure, including contact of the oocysts with faecal matter and use of oxidants, may have affected the stocks of oocysts used in these studies such that oocyst resistance to chlorine dioxide was altered.
Experimental sampling protocol. The particular sampling procedure used may also have affected the inactivation results of this and other studies. The present study used a different protocol from that of Ruffell et al. (2000). First, less than a quarter of the total reactor volume was withdrawn over the course of the experiment, whereas the sampling procedure as described in the experiments of Ruffell et al. (2000) appeared to exhaust the entire volume of the reactor vessel over the course of an experiment. This may have affected oocyst recovery. Second, the concentration of oocysts in the reactor vessel of Ruffell et al. (2000) was approximately $10^3$ oocysts/mL, necessitating a filtration step during sample collection. versus approximately $10^5$ oocysts/mL in the reactor vessel of the experiments here. This extra step could potentially have altered the sensitivity or the viability status of the oocysts, perhaps through mechanical disruption of the oocysts via interaction with the filter membrane, which would be manifest by more rapid inactivation kinetics. Further, while a seventeen-fold concentration step was carried out on each sample via centrifugation in the present study, oocyst recovery was checked by carrying out duplicate haemocytometer counts on all samples after processing (Figure A.3); no mention of such a procedure was made by Ruffell et al. (2000).

5.3.6. USE OF *BACILLUS SUBTILIS* SPORES AS A SURROGATE

The sensitivity of *B. subtilis* spores to chlorine dioxide when compared with *C. parvum* oocysts under similar environmental conditions precludes the use of the spores as a direct surrogate for chlorine dioxide inactivation of the studied *C. parvum* isolate (Figure 5.2). Among the fundamental characteristics of a microbial surrogate for *Cryptosporidium* spp. inactivation experiments is a resistance to the disinfectant
comparable to that of Cryptosporidium spp. oocysts. Using the data from ddH₂O adjusted to pH 8 as an example, a Ct of 1000 mg·min/L resulted in approximately 2.0 log₁₀ kill of C. parvum oocysts as measured by MPN-cell culture assay (Figure 5.2). This level of inactivation was achieved at a Ct 20 times less with B. subtilis spores (Figure 5.2). Pair-wise comparisons of the chlorine dioxide inactivation of B. subtilis spores to C. parvum oocysts (as measured by in vitro excystation or the MPN-cell culture assay) when controlling for water matrix confirmed that the inactivation of spores was significantly different from that of the oocysts: All p values from such comparisons were < 0.0001. The aerobic spore former B. subtilis (ATCC 19659) is therefore an inappropriate direct microbial surrogate for the C. parvum isolate used here in chlorine dioxide disinfection studies.

5.4 SUMMARY

The inactivation of Cryptosporidium parvum oocysts at bench-scale, as measured by the most probable number (MPN) cell-culture assay, suggests that the oocysts employed in this study were very resistant to chlorine dioxide in various water matrices at 22 °C. e.g., in ddH₂O adjusted to pH 8, a Ct of 1 000 mg·min/L was necessary to achieve approximately 2.0 log₁₀ kill. The results presented here are anomalous when compared to the literature, in that the high level of oocyst resistance to chlorine dioxide has not been previously quantified. The in vitro excystation method underestimated inactivation when compared to the MPN cell culture assay, a result consistent with the literature. Ct values based on in vitro excystation data may therefore be unnecessarily conservative.
There was no significant difference (α=0.05) in the in vitro excystation measured chlorine dioxide inactivation rate of *C. parvum* oocysts at 22 °C in post-filtration Ottawa River water in its unmodified (pH 6) or base-adjusted forms (pH 8), post-filtration Lake Michigan water (pH 7.3), or post-filtration White River water (pH 7.6). There was a significant difference (α=0.05) in oocyst inactivation among some of the water matrices as measured by the MPN-cell culture assay. *B. subtilis* spores (ATCC 19659) are an inappropriate surrogate for *C. parvum* oocyst inactivation studies using chlorine dioxide, with a sensitivity to the disinfectant far exceeding that of *C. parvum* oocysts.

### 5.5 FIGURES AND TABLES

**Table 5.1.** Physical and chemical characteristics of the ddH2O used in the bench-scale inactivation studies.\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ddH2O buffered to pH 6</th>
<th>ddH2O adjusted to pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.9 (n=6)</td>
<td>8.2 (n=5)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3)/L)</td>
<td>17.8 (n=6)</td>
<td>7.0 (n=3)</td>
</tr>
<tr>
<td>TOC (mg/L)(^b)</td>
<td>0.27 (n=6)</td>
<td>0.28 (n=3)</td>
</tr>
<tr>
<td>UV(<em>{254}) (A(</em>{254}) units)(^c)</td>
<td>0.01 (n=6)</td>
<td>0.01 (n=3)</td>
</tr>
</tbody>
</table>

\(^a\) This table is a duplicate of Table 4.1. 
\(^b\) TOC = total organic carbon. 
\(^c\) UV\(_{254}\) = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.
Table 5.2. Physical and chemical characteristics of the post-filtration (pre-disinfection) Ottawa River water used in the bench-scale inactivation studies.\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unmodified Ottawa River water</th>
<th>Ottawa River water adjusted to pH 8</th>
<th>Ottawa River water adjusted to pH 4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.1 (n=7)</td>
<td>8.1 (n=4)</td>
<td>4.3 (n=1)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3)/L)</td>
<td>7.0 (n=5)</td>
<td>21.3 (n=4)</td>
<td>0 (d) (n=1)</td>
</tr>
<tr>
<td>TOC (mg/L)(^b)</td>
<td>2.59 (n=4)</td>
<td>3.03 (n=3)</td>
<td>2.60 (n=1)</td>
</tr>
<tr>
<td>UV(<em>{254}) (A(</em>{254}) units)(^c)</td>
<td>0.06 (n=2)</td>
<td>0.07 (n=3)</td>
<td>0.06 (n=1)</td>
</tr>
</tbody>
</table>

\(a\) This table is a duplicate of Table 4.2.

\(b\) TOC = total organic carbon.

\(c\) UV\(_{254}\) = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.

\(d\) The sample pH was < 4.5 and therefore no alkalinity could be measured by the titration method.

Table 5.3. Physical and chemical characteristics of the post-filtration (pre-disinfection) White River and Lake Michigan waters used in the bench-scale inactivation studies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>White River</th>
<th>Lake Michigan</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.6 (n=1)</td>
<td>7.3 (n=1)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3)/L)</td>
<td>(&gt; 64.3 (c) (n=1)</td>
<td>NR(^e)</td>
</tr>
<tr>
<td>Turbidity (ntu)</td>
<td>&lt; 0.05 (d) (n=1)</td>
<td>0.05 (n=1)</td>
</tr>
<tr>
<td>TOC (mg/L)(^b)</td>
<td>1.99 (n=2)</td>
<td>1.49 (n=3)</td>
</tr>
<tr>
<td>UV(<em>{254}) (A(</em>{254}) units)(^c)</td>
<td>NR(^e)</td>
<td>0.02 (n=2)</td>
</tr>
<tr>
<td>Cl(_2) residual (mg Cl(_2)/L)</td>
<td>NR(^e)</td>
<td>&lt; MDL(^f) (n=1)</td>
</tr>
</tbody>
</table>

\(a\) TOC = total organic carbon.

\(b\) UV\(_{254}\) = ultraviolet absorbance at 254 nm. The pH of samples was not adjusted.

\(c\) The alkalinity in the raw and final plant-treated waters was 340 and 334 mg/L, respectively, as measured by the utility.

\(d\) Measured prior to shipment to laboratory.

\(e\) NR = not recorded.

\(f\) MDL = method detection limit, 0.1 mg/L.
Table 5.4. Statistical comparison of MPN-cell culture and in vitro excystation as measures of inactivation of Cryptosporidium parvum oocyst inactivation by chlorine dioxide.\(^a\)

<table>
<thead>
<tr>
<th>Water matrix</th>
<th>Significant difference in MPN-cell culture and in vitro excystation measured inactivation ((\alpha=0.05))</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH_2O-buffered to pH 6</td>
<td>Yes(^b)</td>
<td>0.0080</td>
</tr>
<tr>
<td>ddH_2O adjusted to pH 8</td>
<td>Yes</td>
<td>0.0019</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water, unmodified (pH 6)</td>
<td>Yes</td>
<td>0.0331</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water, adjusted to pH 8</td>
<td>No</td>
<td>0.1941</td>
</tr>
<tr>
<td>Post-filtration White River water</td>
<td>Yes</td>
<td>0.0174</td>
</tr>
<tr>
<td>Post-filtration Lake Michigan water</td>
<td>Yes</td>
<td>0.0026</td>
</tr>
</tbody>
</table>

\(^a\) Both assays were used simultaneously on the same set of samples in a given experiment.

\(^b\) The inactivation rate was greater as measured by in vitro excystation compared to MPN-cell culture in this water matrix only. It is noted that the slope of the MPN-measured inactivation was positive (see Figure 5.1B, Table 5.5).

Table 5.5. Linear regressions of Cryptosporidium parvum inactivation results.

<table>
<thead>
<tr>
<th>Water matrix</th>
<th>In vitro excystation</th>
<th>MPN-cell culture assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(r^2) (L/mg-min)(^a)</td>
<td>(k) (L/mg-min)(^a)</td>
</tr>
<tr>
<td>ddH_2O-buffered to pH 6</td>
<td>0.511</td>
<td>0.000084</td>
</tr>
<tr>
<td>ddH_2O adjusted to pH 8</td>
<td>0.867</td>
<td>-0.00056</td>
</tr>
<tr>
<td>Post-filtration, unmodified (pH 6) Ottawa River water</td>
<td>0.333</td>
<td>-0.00139</td>
</tr>
<tr>
<td>Post-filtration Ottawa River water adjusted to pH 8</td>
<td>0.317</td>
<td>-0.00111</td>
</tr>
<tr>
<td>Post-filtration White River water, pH 8</td>
<td>0.436</td>
<td>-0.00032</td>
</tr>
<tr>
<td>Post-filtration Lake Michigan water, pH 7</td>
<td>0.830</td>
<td>-0.00078</td>
</tr>
</tbody>
</table>

\(^a\) \(k\) is the pseudo first-order reaction rate constant, taken as the slope of the linear regression of the data as plotted in Figure 5.1 (Section 3.1).
Table 5.6. Summary of selected data for the inactivation of Cryptosporidium parvum oocysts by free chlorine and comparison to chlorine dioxide inactivation data of the present study.

<table>
<thead>
<tr>
<th>Reference*</th>
<th>Experimental summary</th>
<th>$Ct$ (mg·min/L)$^b$</th>
<th>$\log_{10}$ inactivation</th>
<th>Viability or infectivity measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korich et al. (1990)</td>
<td>C. parvum, pH 7 water, 25 °C, 80 mg free chlorine/L for 1.5 h</td>
<td>7 200</td>
<td>at least 2.0</td>
<td>mouse infectivity</td>
</tr>
<tr>
<td>Ransome et al. (1993)</td>
<td>C. parvum, 0.001 M sodium hydrogen carbonate buffered borehole water adjusted to pH 7.0 with NaOH, 5 118 mg chlorine/L for 24 h</td>
<td>7 369 920</td>
<td>0.92</td>
<td>in vitro excystation</td>
</tr>
<tr>
<td>Venczel et al. (1997)</td>
<td>C. parvum (Iowa strain), oxidant demand free 0.01 M phosphate-buffered pH 7 water, 25 °C, 3 replicates, 5 mg free chlorine/L for 24 h</td>
<td>7 200</td>
<td>essentially no inactivation</td>
<td>neonatal BALB/c mice</td>
</tr>
<tr>
<td>Moore et al. (1998)</td>
<td>C. parvum (cervine faeces), 1 mg NaOCl/L for 15 days</td>
<td>21 600</td>
<td>no change in % viability</td>
<td>in vitro excystation</td>
</tr>
<tr>
<td>Carpenter et al. (1999)</td>
<td>(1) C. parvum (AUCP-1 isolate), chlorine demand free deionised water, 20 °C, 2 or 10 mg HOCl/L dose</td>
<td>&gt; 8 640</td>
<td>$Ct$ required for loss of infectivity</td>
<td>neonatal BALB/c mice</td>
</tr>
<tr>
<td></td>
<td>(2) Same as (1), but with water “balanced” between pH 7.2-7.8 and 200-400 ppm CaCl$_2$ added to simulate “recreational water conditions”</td>
<td>&gt; 5 760</td>
<td>$Ct$ required for loss of infectivity</td>
<td>neonatal BALB/c mice</td>
</tr>
<tr>
<td></td>
<td>(3) Identical water and chlorine conditions as (1), but in the presence of faeces</td>
<td>28 800</td>
<td>oocysts remained infectious</td>
<td>neonatal BALB/c mice</td>
</tr>
<tr>
<td>Present study</td>
<td>C. parvum (Iowa strain), ddH$_2$O adjusted to pH 8, 22 °C, chlorine dioxide disinfectant (for comparison); data from Figure 5.1</td>
<td>1 000 2.0$^c$</td>
<td>MPN-cell culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 000 0.5$^c$</td>
<td>$in vitro$ excystation</td>
<td></td>
</tr>
</tbody>
</table>

* Only selected data from each reference are here presented.

$^b$ With the exception of the present study, all $Ct$ values listed are the theoretical maximum $Ct$, calculated by multiplying the initial disinfectant dose by the exposure time.

$^c$ Approximate value.
Table 5.7. Summary of selected data for the inactivation of *Cryptosporidium parvum* oocysts by chlorine dioxide and comparison to the present study.

<table>
<thead>
<tr>
<th>Reference*</th>
<th>Experimental summary</th>
<th>Ct (mg·min/L)</th>
<th>Log₁₀ inactivation</th>
<th>Viability or infectivity measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peeters <em>et al.</em> (1989)</td>
<td><em>C. parvum</em>, room temperature, 0.43 mg ClO₂/L at start, 0.22 mg ClO₂/L at end, contact time of 30 min.</td>
<td>12.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.24 (1.0&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>mouse infectivity</td>
</tr>
<tr>
<td>Korich <em>et al.</em> (1990)</td>
<td><em>C. parvum</em>, 0.01 M phosphate-buffered pH 7 water, 25 °C, 1.3 mg ClO₂/L at start, as low as 0.4 mg ClO₂/L at end, contact time of 60 min.</td>
<td>78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0 (2.0&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>mouse infectivity</td>
</tr>
<tr>
<td>Liyanage <em>et al.</em> (1997a)</td>
<td>(1) <em>C. parvum</em>, oxidant demand-free 0.05 M phosphate-buffered pH 8 water, 22 (±1) °C, 2.0 mg ClO₂/L at start, contact time of 30 min.</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99</td>
<td>neonatal CD-1 mice</td>
</tr>
<tr>
<td></td>
<td>(2) Same as (1) but for a contact time of 61 min.</td>
<td>122&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.57</td>
<td>neonatal CD-1 mice</td>
</tr>
<tr>
<td></td>
<td>(3) Same as (1) but with 3.3 mg ClO₂/L at start, contact time of 116 min.</td>
<td>383&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt; 3.22</td>
<td>neonatal CD-1 mice</td>
</tr>
<tr>
<td>Finch and Li (1999)</td>
<td><em>C. parvum</em>, 0.05 M phosphate-buffered pH 6 water, 22 °C, 2 trials</td>
<td>120</td>
<td>1.8 and 2.1</td>
<td>mouse infectivity</td>
</tr>
<tr>
<td>Ruffell <em>et al.</em> (2000); Corona-Vasquez (2000)</td>
<td>(1) <em>C. parvum</em> (Iowa isolate, genotype 2 (C), from a calf at the University of Arizona), 0.01 M phosphate-buffered pH 8 ddH₂O, 20 °C</td>
<td>150</td>
<td>2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>modified <em>in vitro</em> excystation</td>
</tr>
<tr>
<td></td>
<td>(2) Same as (1) but at 4 °C</td>
<td>900</td>
<td>2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>modified <em>in vitro</em> excystation</td>
</tr>
<tr>
<td>Present study</td>
<td><em>C. parvum</em> (Iowa strain), ddH₂O adjusted to pH 8, 22 °C; data from Figure 5.1</td>
<td>1 000</td>
<td>2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>MPN-cell culture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 000</td>
<td>0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td><em>in vitro</em> excystation</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only selected data from each reference are here presented.
<sup>b</sup> Ct value is the theoretical maximum Ct, calculated by multiplying the initial disinfectant dose by the exposure time. Note that Liyanage *et al.* (1997b) provided data for the final and initial ClO₂ residuals in their *C. parvum* oocyst inactivation experiments, and in all cases the final residual was 60% or less of the initial value.
<sup>c</sup> Recalculation by Finch *et al.* (1995).
<sup>d</sup> Approximate value.
Table 5.8. Possible effects of specialised, density centrifugation techniques on Cryptosporidium spp. oocyst stocks.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Density gradient centrifugation technique</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilani and Sekla (1987)</td>
<td>Percoll</td>
<td>&quot;Clean&quot; oocysts not recovered; pre-treatment of oocysts with NaOCl resolved this, but authors cautioned that possible damage to the oocyst wall may preclude the use of such oocysts from immunological studies.</td>
</tr>
<tr>
<td></td>
<td>Caesium chloride</td>
<td>Suggested this technique would be appropriate for biochemical and immunological studies on Cryptosporidium spp. oocysts.</td>
</tr>
<tr>
<td>Bukhari and Smith (1995)</td>
<td>Sucrose</td>
<td>Viable, intact oocysts concentrated selectively, while nonviable oocysts were lost &quot;frequently&quot;. Fricke and Crabb (1998) speculated that oocysts with a fault in their wall would fill with sucrose and thus sediment.</td>
</tr>
</tbody>
</table>
Plate 5.1. Portion of a haemocytometer slide with antibody-labelled Cryptosporidium parvum oocysts. The slide was viewed under an epifluorescence microscope with excitation at 450-480 nm. The image in this plate was inverted to enhance viewing.

Plate 5.2. Portion of an MPN-cell culture well slide displaying evidence of infection and replication by Cryptosporidium parvum on an MDCK cell monolayer. The slide was viewed under an epifluorescence microscope with excitation at 450-480 nm. The image in this plate was inverted to enhance viewing.
Figure 5.1. Effect of water matrix on inactivation of Cryptosporidium parvum oocysts by ClO₂ at 22 °C, as measured simultaneously by panel A: in vitro excystation and panel B: MPN-cell culture. Note the different scales used on the y axes.
Figure 5.2. Inactivation by ClO₂ of Cryptosporidium parvum oocysts at 22 °C, as measured by the MPN-cell culture assay, and of Bacillus subtilis spores at 21.5 °C. The former data set is from Figure 5.1B and the latter from Figures 4.1 and 4.2.

5.6 REFERENCES


Corona-Vasquez, B. (2000). University of Illinois at Urbana-Champaign. Personal communication.


6.0 SUMMARY & DISCUSSION

_Bacillus subtilis_ spores (ATCC 19659) were sensitive to chlorine dioxide inactivation in post-filtration (pre-disinfection), unmodified (pH 6) Ottawa River water at 23 °C. e.g., a $Ct$ of 63 mg-min/L being sufficient for 2.0 $\log_{10}$ inactivation in a conventional (coagulation, flocculation, sedimentation) pilot-scale water treatment facility. The pilot scale inactivation data for "stressed" spores provided no evidence to support the hypothesis that bench scale sand shearing increases the sensitivity of _B. subtilis_ spores to chlorine dioxide. Further, there was no evidence in the present study to suggest that anthracite/sand filtration conducted at pilot-scale increases the sensitivity of _B. subtilis_ spores to chlorine dioxide disinfection.

_B. subtilis_ spores may serve as a conservative surrogate at pilot-scale to gauge removal of cryptosporidial oocysts. Pilot-scale results may, however, overestimate full-scale treatment plant removal on the order of 0.5 $\log_{10}$.

Achieving $> 1 \log_{10}$ inactivation of _B. subtilis_ spores in Ottawa River water at pilot scale resulted in a violation of the maximum residual disinfectant level (MRDL) of 0.8 mg ClO₂/L (USEPA, 1998). The maximum contaminant level (MCL) of 1.0 mg chlorite/L and the maximum contaminant level goal (MCLG) of 0.8 mg chlorite/L were met up to 2 $\log_{10}$ inactivation, the highest level of inactivation measured (USEPA, 1998). The MCL
for total trihalomethanes (TTHM) of 0.080 mg/L and the MCL for haloacetic acids (HAA5) of 0.060 mg/L were also met up to 2 log₁₀ inactivation (USEPA. 1998).

The results from the chlorine dioxide inactivation of *B. subtilis* spores at bench- and pilot-scales are consistent with literature values. Under certain conditions, there was no significant difference (α=0.05) in the bench- and pilot-scale inactivation results of chlorine dioxide on *B. subtilis* spores, strengthening the extrapolation of bench scale results to pilot scale. A pair-wise comparison of the bench-scale *B. subtilis* inactivation results suggests that water matrix may influence chlorine dioxide efficacy.

The inactivation of *Cryptosporidium parvum* oocysts at bench scale, as measured by the most probable number (MPN) cell culture assay, suggests that the oocysts are very resistant to chlorine dioxide in various water matrices at 22 °C. e.g., in ddH₂O adjusted to pH 8. a Ct of 1 000 mg-min/L was necessary to achieve approximately 2.0 log₁₀ kill. Although obtained using a different isolate and under different experimental conditions, the results of the present study in comparison to the literature suggest that chlorine dioxide is more effective than free chlorine for the inactivation of *Cryptosporidium* spp. oocysts.

As measured by *in vitro* excystation, there was no significant difference (α=0.05) in the chlorine dioxide inactivation rate of *C. parvum* oocysts at 22 °C in post-filtration Ottawa River water in its unmodified (pH 6) or base-adjusted forms (pH 8), post-filtration Lake Michigan water (pH 7.3), or post-filtration White River water (pH 7.6). However,
there was a significant difference (α=0.05) in oocyst inactivation among some of the water matrices as measured by the MPN-cell culture assay.

The use of chlorine dioxide as a primary disinfectant in a water treatment facility may not be a compelling strategy for reducing the concentration of infective *Cryptosporidium* spp. oocysts between raw and plant-treated finished waters. To achieve just 0.5 log\(_{10}\) inactivation of oocysts in pH 8 ddH\(_2\)O at 22 °C, a \(Ct\) of 350 mg·min/L was required. Even by maintaining a chlorine dioxide residual at the maximum level of 0.8 mg/L established by the USEPA (1998), over 7 h of contact time would be necessary to achieve this \(Ct\). There are potential impracticalities in achieving such high \(Ct\) values in a water treatment plant.

The multi-barrier approach therefore remains an important concept in water treatment for limiting the concentration of infective oocysts in plant-treated finished waters. Disinfection is but one unit process that can be implemented to help reduce infective oocyst contamination in finished water; other stages of treatment, including watershed management to minimise oocyst contamination of source water, physical and chemical removal processes, and other physical and chemical inactivation processes, can be each optimised to help achieve this same goal and decrease the reliance on the disinfection barrier.

Indeed, more disinfection data for *Cryptosporidium* spp. oocysts are needed under field conditions. It has been suggested that effective turbidity and particle count
reductions along with chlorination may already be achieving the goal of a 10⁻⁴ annual risk of infection (LeChevallier and Norton, 1995). Further, elimination of pre-disinfection practices for purposes of controlling disinfection byproducts may actually be injurious to the goal of providing a microbiologically safe drinking water, since the application of disinfectant at the start of the water treatment train may provide the longer contact times necessary for sufficient Ct values to effect a certain degree of inactivation (LeChevallier and Norton, 1995).

6.1 CONCLUSIONS

1. The inactivation of Cryptosporidium parvum oocysts at bench scale, as measured by the most probable number (MPN) cell culture assay and in vitro excystation, suggests that the oocysts are very resistant to chlorine dioxide in various water matrices at 22 °C. The results presented here are anomalous when compared to the literature, in that the high level of oocyst resistance to chlorine dioxide has not been previously quantified.

2. The in vitro excystation method underestimated inactivation when compared to the MPN-cell culture assay, a result consistent with the literature. Ct values based on in vitro excystation data may therefore be conservative. Further, differences in oocyst inactivation among various water matrices measured to be significant (α=0.05) by the MPN-cell culture assay were not necessarily measured to be significant by in vitro excystation.
3. *B. subtilis* spores (ATCC 19659) are an inappropriate surrogate for *C. parvum* oocyst inactivation studies using chlorine dioxide, with a sensitivity to the disinfectant far exceeding that of *C. parvum* oocysts.

6.2 RECOMMENDATIONS

1. Research into the chlorine dioxide sensitivity of different pathogenic isolates of cryptosporidia would help to reconcile the disparity between the results of the present study and the literature. Further, an investigation of the effects on the chlorine dioxide sensitivity of oocysts collected and purified *via* different protocols would be valuable.

2. A comparative study of the MPN-cell culture and animal infectivity assays would help to determine the utility of MPN-cell culture as a surrogate for *in vivo* infection.

3. Epidemiological and experimental studies would be aided greatly by continued work on a taxonomical system for cryptosporidia that more closely reflects phylogeny.

4. Continued research into the development of a continuous, *in vitro* cultivation system capable of producing high concentrations of oocysts and the development of an infectivity assay less labour-intensive and less time-consuming than the MPN cell culture assay would also be beneficial.
5. Further research should continue on possible synergistic effects between chlorine dioxide and other disinfectants. Possible disinfection byproducts from the use of multiple disinfectants in this manner must not be overlooked.

6. The search for a surrogate measure for chlorine dioxide inactivation of *C. parvum* oocysts should focus on other environmentally resistant microbial life forms, such as the spores of *Clostridium perfringens*. However, the results of this study cannot be extrapolated to disqualify *B. subtilis* spores from different environmental isolates or laboratory preparations of a given isolate for use as a surrogate measure.

6.3 REFERENCES


APPENDIX A

QUALITY ASSURANCE / QUALITY CONTROL DATA

This appendix contains quality assurance / quality control (QA/QC) data and the standard curves used for the measurement of chlorine dioxide in the bench- and pilot-scale experiments performed. *Figures A.1a and A.1b* are the standard curves for the lissamine green B assay, and *Figure A.2* contains the QA/QC charts of chlorine dioxide standards analysed during the course of the experiments. The haemocytometer data and the MPN-cell culture positive control data for the *Cryptosporidium parvum* oocyst inactivation experiments are shown in *Figures A.3 and A.4* respectively. The experimental determination of the time corresponding to an “equilibrium” condition in the pilot plant clearwell was gauged by measurement of chlorine dioxide residual over time in the clearwell effluent; these data are displayed in *Figure A.5*. 
Figure A.1a. Standard curve for the lissamine green B (LGB) assay in bench-scale experiments. Each data point represents a standard prepared independently of others and measured on a Spectronic 21D spectrophotometer. The solid line is a linear regression through the data ($R^2 = 0.99, n=33$).
Figure A.1b. Standard curves for the lissamine green B (LGB) assay in pilot-scale experiments. Each data point represents a standard prepared and measured independently of others. The solid line in each graph is a linear regression through the data. Panel A: Cary 50 ConcUV-Visible spectrophotometer (R² = 0.99, n=24). Panel B: LKB Ultrospec II spectrophotometer (R² = 0.96, n=43).
Figure A.2. QA/QC charts for ClO₂ standards analysed by the lissamine green B (LGB) assay in bench- and pilot-scale experiments. Each data point represents a standard prepared and measured independently of others. The solid horizontal line on each graph represents the mean of the data set. The dashed lines above and below this represent the mean ± 1 standard deviation (s.d.) and ± 2 s.d. Panel A: 1.0 mg/L standards in pilot-scale runs; average = 0.96 mg/L (n=14). Panel B: 2.0 mg/L standards in pilot-scale runs; average = 2.02 mg/L (n=8). Panel C: 2.0 mg/L standards in bench-scale runs; average = 2.18 mg/L (n=59).
Figure A.3. Haemocytometer counts from bench-scale *Cryptosporidium parvum* inactivation experiments. Duplicate haemocytometer counts were performed on all of the samples collected (n=5) from a given reaction vessel in a given experiment. Each data point represents the average of the counts from a particular vessel, with the error bars representing one standard deviation about the mean. The data are subdivided on the basis of oocyst lot used. The average of the relative standard deviations amongst the haemocytometer counts for each run was 21% for lot #99-23 (range: 12-40%) and 23% for lot #00-3 (range: 15-28%).
Figure A.4. MPN-cell culture positive control data from bench-scale *Cryptosporidium parvum* inactivation experiments. The oocyst stock used for a particular experiment served as the positive control. The error bars represent the 95% Loyer and Hamilton confidence intervals. Panel A: Oocyst lot #99-23, shed November 11, 1999. Panel B: Oocyst lot #00-3, shed January 18, 2000.
Figure A.5. Experimental determination of equilibrium conditions in the clearwell, gauged by measurement of chlorine dioxide residual over time in the clearwell effluent. Chlorine dioxide addition commenced at $t = 0$ min. All data points recorded as having a concentration $> 2.0$ mg/L are based on extrapolations of the lissamine green B standard curve displayed in Figure A.1b(A).