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OCCURRENCE OF HUMAN PATHOGENIC MICROSPORIDIA IN IRRIGATION WATER AND ULTRAVIOLET LIGHT AND CHLORINE INACTIVATION OF ENTERIC ADENOVIRUS TYPE 40 AND FELINE CALICIVIRUS

by

Jeanette Ann Thurston

A Dissertation Submitted to the Faculty of the

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In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY

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	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Charles P. Gerba — Dissertation Director

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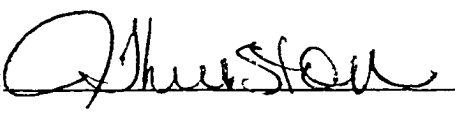
SIGNED: 

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DEDICATION

My family has dedicated their support and love to me throughout my life and although not nearly as important as a contribution, this dissertation is dedicated to them.

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ABSTRACT

The occurrence and disinfectant effectiveness for pathogens which are known or thought to be important in waterborne disease was evaluated. In the first study, the occurrence of human pathogenic microsporidia, *Giardia* cysts and *Cryptosporidium* oocysts in surface waters used for the irrigation of vegetable crops was determined. Twenty-eight percent of the irrigation water samples tested positive for microsporidia, 60 % positive for *Giardia* cysts and 36 % positive for *Cryptosporidium* oocysts. Concentrations of *Giardia* cysts and *Cryptosporidium* oocysts detected in water samples collected in Central American compared to the United States were 559 cysts and 227 oocysts, and 25 cysts and < 19 oocysts per 100 L, respectively. The presence of human pathogenic parasites in irrigation waters used for production of crops traditionally consumed raw suggests that there may be a risk of infection to consumers who come in contact with or consume these products.

In the other investigations, the effectiveness of UV light and free chlorine on the inactivation of feline calicivirus (FCV) and enteric adenovirus type 40 (AD40) was assessed and compared to model viruses, poliovirus type 1 (PV-1) and coliphage MS-2. FCV was used as a model for members of the "Norwalk like virus" (NLV) group. The UV doses required to achieve 99 % inactivation of AD40, coliphage MS-2 and FCV in buffered demand free (BDF) water were 108.6, 58.5 and 16.8 mWs/cm^2, respectively. For chlorine reactions, higher Ct values for high pH and low temperature conditions was observed for FCV and AD40. Both viruses were more resistant to chlorine than the well-studied PV-1. FCV and AD40 were inactivated rapidly by ~0.5 mg/L free chlorine by

≥4.00- and ≥2.54- logs at pH 6 and 5 °C whereas, PV1 was not inactivated by 4.04-logs until 10 min contact time. Experiments conducted with aggregated FCV and PV-1 and experiments conducted in treated groundwater had slower inactivation kinetics than dispersed viral suspensions in BDF water. The high disinfectant decay rate of some experiments was most likely due to the decrease in chlorine concentration throughout the experiment. However, low disinfectant decay rates of the AD40 experiments suggest that aggregation or clumping of the viruses may have occurred. The results of these studies provide information on the effectiveness of two common water treatment disinfectants in waters with different physical and chemical qualities. The results of this study may provide a basis for the establishment of guidelines for proficient application in drinking water treatment.

INTRODUCTION

Problem definition

Waterborne disease

Water can serve as a passive carrier for the transmission of disease by enteric pathogens (Table 1). By definition, waterborne diseases are caused by infectious agents which are excreted in the feces and transmitted by ingestion of contaminated water (34, 55). Excretion in the feces by humans or animals enables enteric pathogens to effectively contaminate the environment, increasing the chances of exposure to other susceptible individuals. Other features that enable them to effectively contaminate, survive and transmit disease through the waterborne route are listed in Table 2. Human populations are exposed to these agents through several routes, including ingestion of water used for drinking and recreational activities. Although considered foodborne, consumption of shellfish and food crops cultivated with contaminated water are other routes by which enteric diseases are transmitted.

In the early 1900's, due to the introduction of filtration and chlorination of water supplies, the waterborne outbreak paradigm in the United States shifted from enteric bacteria, as the primary agents causing waterborne disease, to protozoan parasites and enteric viruses (34). Characteristics of enteric viruses and protozoan parasites, such as increased resistance to drinking water disinfectants, increased environmental stability and low infectious dose may account for their association with more outbreaks of

Table 1 Human pathogens and associated illnesses that are transmitted by ingestion of contaminated water.

	Pathogen	Illness
Bacteria	*Salmonella*	Diarrhea, Typhoid
	Campylobacter jejuni	Diarrhea
	Escherichia coli	Diarrhea
	Shigella	Diarrhea
Viruses	Hepatitis A and E	Hepatitis
	Enteroviruses (polioviruses, coxsckieviruses, echoviruses)	Febrile illness, respiratory illness, meningitis, diarrhea, encephalitis and others
	Rotaviruses (group A and B)	Diarrhea
Protozoa	*Giardia lamblia*	Diarrhea
	Cryptosporidium	Diarrhea
	Entamoeba histolytica	Amebic dysentary
	Cyclospora cayetanensis	Diarrhea
Helminths	*Ascaris lumbricoides*	Ascariasis
	Tricuris trichiura	Trichuriasis-whipworm
Blue-green algae	Cyanobacteria (*Anabaena, Aphanizomenon* and *Microcystis* species)	Toxin poisoning (gastroenteritis, pneumonia)

Adapted from Moe, 1997 and Gerba, 1996

waterborne disease than bacterial pathogens today. The percentage of reported cases of waterborne disease is estimated to be 31 % caused by protozoa (*Giardia* and *Cryptosporidium*), 15 % caused by viral acute gastrointesinal illness (AGI) and 10 % due to bacterial agents (34). However, the etiological agents responsible for approximately half of all reported outbreaks of disease go unrecognized. The agents of AGI have

epidemiologic and clinical characteristics consistent with viral etiology (5).

Control of waterborne transmission of pathogenic microorganisms continues to be a public health concern since it is thought that a large percentage of waterborne outbreaks and their etiological agents go unrecognized (30, 34). In fact, while 12 to 20 waterborne disease outbreaks are documented each year in the United States, it is estimated that the true incidence may be 10 to 100 times higher (34). Even for well-established pathogens, the true incidence is unknown in the United States since reporting waterborne outbreaks and its agents is voluntary, contamination events in water are usually transient and individuals may not seek medical attention since acute gastrointestinal illness (AGI) is usually self-limiting and mild (30, 55). Furthermore, very little, if any information exists on infectious agents that are newly recognized, or "emerging" in water supplies and their impact on waterborne disease outbreaks. For these agents, isolation and identification from the environment or infected individuals may be hampered by a lack of, or inefficient detection methodologies (30, 55).

A number of newly recognized infectious agents that have been implicated in waterborne disease outbreaks and others that have the potential for waterborne transmission, are published in the United States Environmental Protection Agency's (USEPA) Contaminant Candidate List (CCL)(Table 3) (29). Selection of these pathogens was

Table 2 Characteristics that may enhance microbial waterborne potential.

Important Characteristics of Some Waterborne Disease Agents
Excretion in the feces
Human and animal reservoirs
Presence in sewage and surface water
Environmental stability
Low infectious dose
Prolonged asymptomatic shedding
Resistance to water treatment disinfectants
Ability to survive water treatment processes

Table 3 Microorganisms on the USEPA Drinking Water Contaminant Candidate List.

Acanthamoeba
Adenoviruses
Aeromonas hydrophila
Caliciviruses
Coxsackieviruses
Cyanobacteria
Echoviruses
Helicobacter pylori
Microsporidia
Mycobacterium avium intracellulare

based on the need for information regarding the occurrence of these microorganisms in source waters, the effectiveness of current water treatment practices and methodologies for their detection and identification. In effect, the CCL petitions the research community to provide this information, thereby enabling the USEPA to determine if regulatory action, concerning these contaminants, should take place.

Literature Review

Human pathogenic microsporidia

Disease caused by human pathogenic microsporidia is usually gastrointestinal but infections of the respiratory, reproductive, muscle, excretory and nervous systems have been reported (Table 4) (79). Since the onset of the AIDS (acquired immunodeficiency syndrome) epidemic, *E. bienusi* has been the most commonly identified microsporidian followed by *Encephalitozoon intestinalis* as the cause of chronic diarrhea and wasting in AIDS patients (22). Microsporidia have also been implicated in disease affecting immunocompetent individuals (10). *E. bieneusi* and *E. intestinalis* have been implicated in non-HIV-associated, self-limited diarrhea in several immunocompetent hosts (10, 50, 61, 64). Recently, a suspected waterborne outbreak of intestinal microsporidiosis was reported in France where microsporidia were detected in the feces of both immunocompromised and immunocompetent individuals (16). One study has suggested infection by *Encephalitozoon sp.* may be common in the immunocompetent population due to a high seroprevalence against *Encephalitozoon* species (77). In addition,

Table 4 Human pathogenic microsporidia.

Human Pathogenic	Disease	Excretion by Infected	Potential Routes of Infection By Contact
Enterocytozoon bieneusi	Diarrhea, malabsorption, wasting syndrome	Feces	Ingestion of spores
Encephalitozoon cuniculi	Kidney infection and other organs	Urine	Ingestion of spores
E. intestinalis	Diarrhea, renal failure, nose and sinus infections	Feces and urine	Ingestion of spores
E. hellem	Eye, sinus and lung infections	Feces and urine	Direct contact and ingestion
Trachipleistophora hominis, T. anthropophthera Brachiola connori, Pleistophora spp,B.vesicularum	Muscle infections	Feces	Ingestion of spores
Vittaforma corneae, Nosema ocularum, Microsporidium ceylonensis, M. africanum	Eye infections (immunocompetent) Disseminated infection (immunocompromised)	Urine	Direct contact

antibodies to *Encephalitozoon sp.* were detected in 21% of the households in a rural community in Mexico (28). Consequently, in immunocompetent hosts, microsporidiosis may be a common illness that goes unrecognized due to asymptomatic or self-limited infection.

Modes of transmission of human microsporidia include airborne, person-person, waterborne and zoonotic (10). Spores are excreted in the urine, feces and respiratory secretions of infected humans and animals. The potential for zoonotic transmission is evidenced by detection of *Enterocytozoon bieneusi* spores in dogs, rabbits, and pigs (20, 63) and spores of *Encephalitozoan* species in rodents, rabbits, dogs, birds, pigs, monkeys, goats, and cattle (10, 17). Human pathogenic microsporidia have been detected in surface water, tertiary-treated wastewater effluent and ground water (25, 72). Kucerova-Pospisilova (1999) suggested that *Encephalitozoon* spores were resistant to environmental temperatures characteristic of surface waters. Germination and infection of *Encephalitozoon* spores was observed after a week of storage at 4 °C, 22 °C and 33 °C (47). Due to the excretion of microsporidia spores in the feces of humans and animals, survival at environmental temperatures and their detection in water and wastewater, microsporidia have the potential to be waterborne. Table 5 lists key features of human pathogenic microsporidia that may indicate their potential as important waterborne pathogens.

Table 5 Key features of microsporidia that may enhance their potential for waterborne transmission and disease.

Features	Microsporidia	References
Reservoir	**humans and animals**	**Bryan and Schwartz, 1999**
Occurrence in surface water	**yes** **potential outbreak**	**Cotte *et al.*, 1999** **Dowd *et al.*, 1998** **Sparfel *et al.*, 1997**
Occurrence in sewage	**yes**	**Dowd *et al.*, 1998**
Environmental stability	**1-2 week survival at 4, 22 and 33°C (some *Encephalitozoon* species)**	**Kucerova-Pospisilova *et al.*, 1999**
Resistance to water disinfectants	**90 % reduction by 8 min, 2 mg/L chlorine pH 7, 25 °C** **99.9 % reduction by 16 min, , 2 mg/L, pH 7, 25 oC**	**Wolk, *et al.*, 2000**
Other	**many species involved in human disease**	**Bryan and Schwartz, 1999**

Enteric adenovirus

Enteric adenoviruses, serotypes 40 and 41, are important causes of infant and childhood gastroenteritis worldwide (31). Although many of the adenoviruses may produce diarrhea, such as types 31 and members of adenovirus subgroups B and C, adenoviruses type 40 (AD40) and 41 (AD41) are the most important in childhood gastroenteritis (31).

These viruses replicate in the intestinal tract, are excreted in high numbers in feces (up to 10^{11} particles per gram) and are transmitted via the fecal-oral route (1). The symptoms include fever, vomiting, or respiratory symptoms but the prominent symptom is diarrhea (1). While these viruses predominately infect infants and young children, chronic infections have been reported in immunocompromised patients including human immunodeficiency virus (HIV) infected and transplant patients (13, 23, 26).

Adenoviruses are double-stranded (ds), deoxyribonucleic (DNA) icosahedral viruses that are 60-90 nm in size (31). Survival studies have reported that the enteric adenoviruses are more resistant at higher temperatures and in tap water and seawater compared to some members of the enterovirus group (27). Their survival in wastewater, however, was not significantly longer than enteroviruses (27). Occurrence studies involving the detection of enteroviruses and the enteric adenoviruses found higher concentrations of adenoviruses in raw sewage (42). Moreover, their occurrence in the environment may be high since 80 % of all adenoviruses detected in the feces are estimated to be enteric adenoviruses (42).

Very few studies on the effectiveness of water disinfectants for enteric adenovirus inactivation have been reported. However, studies involving the UV inactivation has shown that these viruses are extremely resistant to UV light compared to other enteric viruses (54). While no chlorine inactivation experiments involving enteric adenoviruses have been conducted, it has been reported that respiratory adenoviruses are more sensitive

to chlorine inactivation than poliovirus (15) and differences in chlorine resistance occur amongst adenovirus strains (51).

The increased resistance of adenoviruses to UV light but not to chlorine may indicate different mechanisms of inactivation. It is already well established that UV light damages microbial nucleic acids but since the adenovirus genome consists of dsDNA , it has been suggested that the viral DNA may be repaired by host cell DNA repair systems (7). The principal mechanisms of chlorine inactivation may include destruction of host cell receptors necessary for attachment and infection of the host cell. Although no waterborne outbreaks have been documented for the enteric adenoviruses, information regarding their excretion in high numbers, increased disinfectant resistance, occurrence within the environment and their increased resistance to environmental conditions has instigated their inclusion onto the USEPA's CCL list. Table 6 lists characteristics of these viruses that may contribute to its waterborne disease potential.

Table 6 Key features of enteric adenoviruses that may enhance their potential for waterborne transmission and disease.

Features	Enteric Adenoviruses	References
Reservoir	**humans**	**Foy, 1997**
Concentration excreted in the feces	**10^{11}/gram**	**Albert, 1986**
Occurrence in surface water	**yes**	**Genthe *et al.*, 1995** **Puig *et al.*, 1994** **Tani *et al.*, 1995**
Occurrence in sewage	**yes**	**Hurst *et al.*, 1988** **Puig *et al.*, 1994**
Environmental stability	**longer survival than poliovirus 1 in tap and sea water**	**Enriquez *et al.*, 1995**
Resistance to water disinfectants	**higher UV light doses needed for inactivation compared to any other enteric virus studied to date**	**Meng and Gerba, 1996**
Other	**prolonged shedding (may shed for many months)**	**Horwitz, 1996**
	higher concentration in sewage sludge than enteroviruses	**Hurst *et al.*, 1988**

"Norwalk-like viruses"

Human caliciviruses, specifically the members of the "Norwalk-like virus" (NLV) genus are the most important cause of acute gastroenteritis outbreaks in developing and developed countries (35). NLVs cause a mild, self-limiting gastroenteritis, affecting all age groups and disease is more severe and long-lasting in the immunocompromised (35). Symptoms include nausea, vomiting, diarrhea, abdominal cramps, headache, fever, chills, myalgias and sore throat (44). Characteristics of NLV disease that are important in their transmission are low infectious dose, prolonged asymptomatic shedding in the feces, many genetic and antigenic types and failure to produce lifetime immunity (35).

NLVs are ss ribonucleic acid (RNA) icosahedral viruses ranging in size from 25 to 34 nm (43). They have been identified as the cause of acute gastrointestinal illness in multiple drinking and recreational water outbreaks (49). Further, they have been detected in higher concentrations than rotavirus in sewage in the Netherlands (46) and evidence suggesting possible animal reservoirs has been reported (18, 46, 73). Their survival in ice (12) and at high temperatures (52) also support that these agents are environmentally stable (35).

Unfortunately, no animal or cell culture systems are available that can determine the infectivity of NLVs, thus, research regarding chemical inactivation and survival in water is hampered. Only two studies involving the chlorine inactivation of NLVs, specifically the Norwalk virus, have been reported and conclusions of these studies are contradictory

(45, 68). Methods of detection and virus preparation may be the cause of the conflicting observations. In one study, high concentrations of chlorine, 3.25 mg/L, did not completely inactivate Norwalk virus. In a second study, Norwalk virus was reportedly less resistant to the action of chlorine than poliovirus type 1. In the first and second studies, determination of infectivity was by human infectivity studies and molecular methods (polymerase chain reaction), respectively. In addition, dispersion of the virus suspension was not included in the first study but methods to disperse viral aggregates was performed in the second study. A closely related calicivirus, feline calicivirus, has been suggested as a suitable indicator for NLV inactivation by chlorine and heat due to similar genomic organization and capsid similarities (24, 69). NLVs are important causes of waterborne disease and the characteristics which may contribute to their survival and occurrence in water are listed in Table 7.

Irrigation waters as a source for foodborne disease

Foodborne illness affects approximately 76 million Americans each year and the number of produce-related outbreaks is on the rise (19, 53). The increase in the consumption of fresh fruits, vegetables and organically grown produce, the globalization of produce, and an increase in the immunocompromised population may be causes of the heightened occurrence of produce-related outbreaks (19). Potential sources of these produce-related outbreaks may occur during growth, harvesting, processing and distribution (75).

Irrigation waters may become contaminated by either sewage introduction or runoff from non-point sources. Rain events may carry fecal contamination from agricultural, domestic and wild animals (including birds) into canals, rivers and wells that serve as sources for irrigation water (19). In a study conducted by Mong and Chinchilla (1995), the presence of *Cryptosporidium* oocysts on fresh vegetables was suspected to be caused by the use of contaminated irrigation waters. The authors also noted a higher number of oocysts on the tested vegetables during the rainy season. Agricultural, urban and industrial runoff most likely contributed to the increased oocyst concentrations observed in their study (56). In addition, irrigation waters used to mix insecticides and fungicides that are sprayed directly onto crops increase the risk of surface contamination by pathogenic microorganisms. The 1996 outbreak of cyclosporiasis may be one example of the transfer of protozoan pathogens from contaminated surface water to produce traditionally eaten raw. The contaminated surface water was mixed with a fungicide and then sprayed onto raspberries before harvest, resulting in more than 1,400 cases of cyclosporiasis (7, 38).

Since pathogens may be present in irrigation waters, it is likely that they may come in contact with, and attach to, the surface of crops. Moreover, human enteric pathogens may be excreted in the feces (microsporidia, *Giardia*, *Cryptosporidium*, enteric viruses and bacteria) or urine (microsporidia) of infected individuals. Consequently, irrigation water sources impacted by agricultural, urban and wildlife pollution may have an

Table 7 Key features of NLVs that may enhance their potential for waterborne transmission and disease.

Features	Norwalk-like viruses	References
Reservoirs	humans	Glass *et al.*, 2000
Concentration excreted in the feces	~10^6/gram lower than other enteric viruses [1]	Monroe, 2000
Occurrence in surface water	yes outbreaks reported	Levy *et al.*, 1998
Occurrence in sewage	yes higher concentration than rotavirus	Koopmans *et al.*, 2000
Environmental stability	survival in ice survival at 60°C	Cannon *et al.*, 1991 McDonnell *et al*, 1997
Resistance to water disinfectants	resistant to a chlorine dose of 3.75 mg/L	Keswick, *et al.*, 1985
Other	lack of longtime immunity; many genetic and antigenic strains; prolonged asymptomatic shedding	Glass *et al.*, 2000

increased occurrence and concentration of enteric pathogens. Since the infectious doses for many enteric pathogens (enteric viruses and protozoan parasites) are low, an increased risk of enteric illness for consumers of crops traditionally eaten raw is likely.

Disinfection in relation to water treatment

Drinking water disinfection has been practiced in the United States since the early 1900's in order to circumvent waterborne disease outbreaks (80). Disinfection does not necessarily destroy all microorganisms but is meant to reduce the number of pathogenic microorganisms to a safe level (33). Examples of chemical and physical disinfectants employed in water treatment are listed in Table 8. In drinking water treatment, disinfection is the final barrier between infectious pathogens and drinking water consumers. In addition, appropriate disinfection of wastewater is also critical since receiving waters may be used for drinking, recreation, irrigating food crops traditionally eaten raw and shellfish harvesting.

Table 8 Disinfectants applied for water treatment purposes.

Chlorine
Chloramines
Ozone
Chlorine dioxide
Potassium permanganate
Ultraviolet light

Disinfection kinetics

Microbial inactivation by chemical and physical disinfectants proceeds at specific reaction rates, are governed by kinetics and affected by a number of factors including concentrations of substrate or disinfectant, temperature, pH and the presence of interfering substances (62). Developed on the basis of experimental data, kinetic models have been used in the formulation of disinfection design criteria for water treatment (36). Specifically, these models attempt to predict the complex interactions of different microorganisms, from bacteria to viruses, with different disinfectants which have different cellular targets and modes of action (36). Consequently, because of these complex interactions, predicting microbial inactivation is difficult.

There are many kinetic models that have been used to predict the interaction of disinfectant with microorganisms but probably the most well known is the Chick-Watson model. This model combines Chick's rate law and the Watson function. According to Chick's rate law, the number of microorganisms destroyed per unit time is proportional

to the number remaining for a given disinfectant concentration. Thus, Chick's law assumes that the reaction between a chemical disinfectant and a microorganism follow first-order kinetics. In this law, however, the effect of varying disinfectant concentrations is not taken into consideration, so Watson derived an empirical logarithmic function called the Watson function. The incorporation of Watson's function into Chick's rate law produces the Chick-Watson pseudo first-order rate law (36) (Table 9). More often however, first-order kinetics are not observed for microbial inactivation by disinfectants, instead various types of inactivation kinetics are observed as demonstrated in Figure 1. Curve 1 illustrates first-order or exponential inactivation kinetics, which usually describes microbial UV light inactivation. Curve 2 represents a shoulder curve which may be caused by one or more of the following, ineffective dispersion of the microorganisms or disinfectants, delays in disinfectant diffusion to critical sites on the microorganism and disinfectant interaction of multiple targets necessary for microbial inactivation (36, 40). Curve 3 displays the effects of clumping or aggregation, presence of different microbial populations having varying disinfectant resistance or increased disinfectant decay (36, 40, 76). While the Chick-Watson can only accurately predict inactivation kinetics represented by Curve 1, other kinetic inactivation models have been mathematically advanced to describe curves which deviate from first-order kinetics.

Hoff (1987) stated that modeling of microbial inactivation behavior as that of a reagent grade chemical reaction with disinfectants in an irreversibly, bi-molecular elementary reaction is overly simplistic. As stated previously, the inactivation kinetics for reactions

involving microorganisms and disinfectants does not usually follow first-order kinetics and the disinfectant concentration does not remain constant throughout the reaction. Other kinetic models which describe linear and nonlinear inactivation kinetics for reactions where disinfectant concentration is constant and for conditions where

Table 9 Examples of kinetic models used for prediction of microbial inactivation by water disinfectants.

Model Name	Kinetic Equation Ln (N_t/N_0) =
Chick's Law	$-kt$
Watson's Function	$C^n t$
Chick-Watson	
Disinfectant demand-free conditions	$-kC^n t$
Disinfectant demand conditions	$-k/k' n(C_0^n - C_f^n)$
Efficiency Facor Hom	
Disinfectant demand	$-k\, C_0^n t'^n [(1-\exp(-nk't/m)/(nk't/m)]^m$

Adapted from Gyurek and Finch, 1998

Figure 1 Typical microbial survival curves.

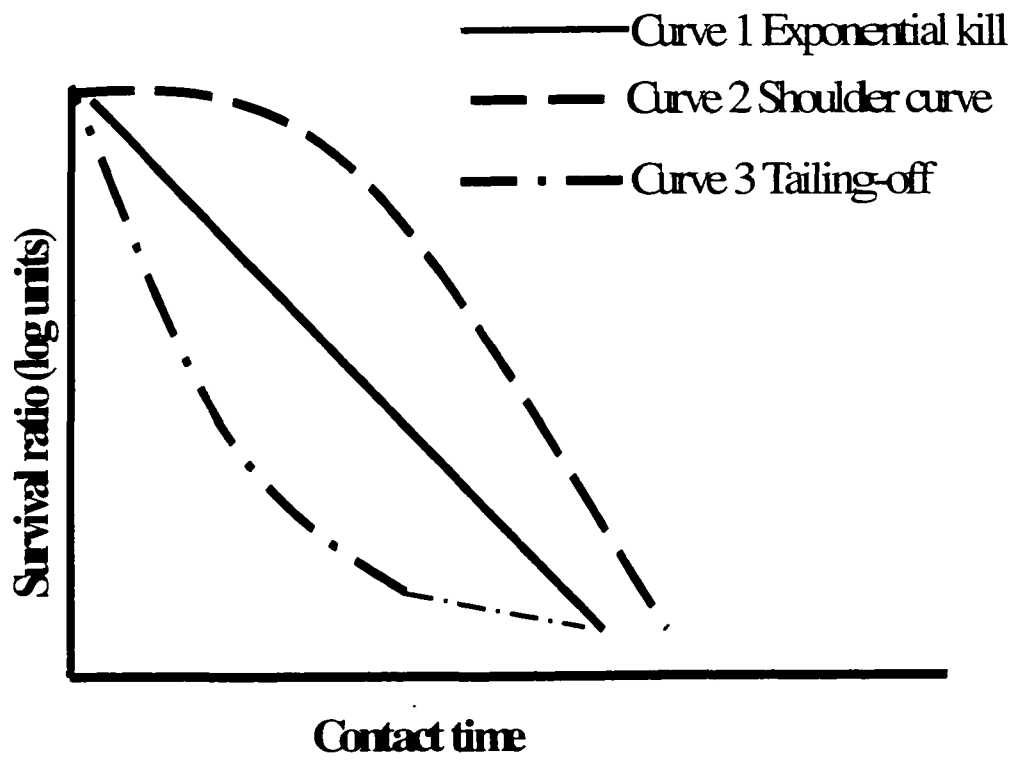


Table 10 Kinetic model parameters and definitions.

Parameter	Definition	Comments
k'	disinfectant decay constant	units usually 1/minutes first-order decay assumed rate assumed to be independent of the microorganisms present
t	time required to achieve a given level of inactivation	units usually in minutes
n	coefficient of dilution	represents the average number of molecules to have combined with the microorganism necessary to cause inactivation
k, m	microbial inactivation constant	specific for the microorganism and set of conditions
C, C_0, C_f	concentration of disinfectant, at time zero and final time of inactivation experiment	units depend on disinfectant

disinfectant decay occurs, have been mathematically derived (36). Examples include the Hom, Hom-Power, Efficiency Factor Hom and the Selleck models (Table 9). Definitions for all kinetic model constants are presented in Table 10. These models have been shown to provide a statistically improved fit to the bench-scale inactivation process over the Chick-Watson model (36, 37).

The Ct concept was derived from the Watson's function where C represents the concentration of the disinfectant and t is the time required to inactivate a predetermined

percentage, usually 99 %, of the population at specific pH and temperature conditions (41). The higher the Ct value, the more resistant the microorganism is to inactivation by the disinfectant whereas a lower value would indicate decreased resistance. By comparing these values, evaluation of the effectiveness of pathogen inactivation by various disinfectants can be determined. In addition, differences between different groups of microorganisms and variability within groups may be ascertained. The Ct method is used by the water treatment industry to help predict the amount of disinfectant needed to inactivate pathogenic microorganisms occurring in the water supply (36). Doses ($\mu Ws/cm^2$) and Ct values ($mg/L_x min$) for UV and chlorine inactivation of enteric viruses are displayed in Table 11 and 12, respectively.

Ultraviolet radiation

Ultraviolet (UV) radiation or ultraviolet light has become a viable disinfectant option for water treatment since no known toxic by-products are produced, no storage or handling of toxic chemicals is required and no taste or odor problems are produced (8). However, disadvantages of UV light over chemical disinfectants, such as chlorine, include; no disinfectant residual is produced, determination of UV dose is difficult and high cost due to lamp fouling and replacement. Since no disinfectant residual is produced, UV treatment of drinking water requires the application of a secondary disinfectant in order to maintain a disinfectant residual within the distribution system (8). However, UV disinfection is practiced more frequently in the wastewater treatment industry due to the

presence of high concentrations of organic matter in wastewater and the increased concern regarding the formation of toxic disinfectant by-products (9, 48).

Low pressure UV disinfection systems expose water to a germicidal wavelength of 254 nm. This wavelength is very close to the absorption maxima of nucleotide bases where the pyrimidines, thymine (T), cytosine (C) and uracil (U), undergo photodecomposition at a much higher rate than the purines (adenine and guanine) (7). The most important class of photoproducts formed by photodecomposition are the 5, 6-cyclobutyl dipyrimidines, more commonly referred to as pyrimidine dimers (7). Three types of dimers may be formed which include T-T, C-C and T-C. Of these, T-T dimers are produced more often since their formation has a greater quantum yield than either C-T or C-C dimers (7). It is the formation of these dimers that inactivates microorganisms by blocking DNA replication (8). Although UV damage also occurs in RNA, it is not as important as damage produced in DNA since RNA is high in copy number and can be replaced if the DNA template is undamaged. However, if the genetic material consists of RNA, as with the enteroviruses and caliciviruses, damage can be lethal (7). Protein damage due to UV absorption at the germicidal wavelength is much lower than nucleic acids, therefore it is not as important in microbial inactivation compared to the formation of pyrimidine dimers (7).

First-order kinetics involving UV inactivation of microorganisms, including viruses, has been reported in the majority of studies. In addition, double stranded (ds) DNA viruses

have been shown to be more resistant than single stranded (ss) RNA viruses and varied resistance amongst closely related viruses has been reported (11, 33, 78). Table 11 lists the UV doses ($\mu Ws/cm^2$) required for the inactivation of selected enteric viruses.

Table 11 UV light inactivation of selected enteric viruses.

Virus	Dosage $\mu W \times s/cm^2$	Reduction (%)	Reference
Poliovirus 1	5,000	90	Wolf, 1990
	7,700	90	Wilson *et al.*, 1992
Rotavirus SA11	25,000	99.9	Chang *et al.*, (1993)
Hepatitis A virus	5,500	90	Wiedenmann *et al.*, 1993
Coxsackievirus B1	15,600	90	Hill *et al.*, 1970
Coxsackievirus B5	15,000	99	Battigelli *et al.*, 1993
Coxsackievirus A9	35,700	99.9	Hill *et al.*, 1970
Echovirus 1	1,250	99	Hill *et al.*, 1970
Echovirus 11	1,410	99	Hill *et al.*, 1970
Adenovirus 40	30,000	90	Meng and Gerba, 1996
Adenovirus 41	23,600	90	Meng and Gerba, 1996
Coliphage MS2	55	99.9	Wiedenmann *et al.*, 1993

Chlorination

For over 100 years, chlorine has been used as a disinfectant for the reduction of disease-causing microorganisms in water (80). In fact, chlorine and related compounds are

applied for the treatment of water more frequently than any other water disinfectant (3). In water treatment, chlorine gas is added to water resulting in two reactions, hydrolysis

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \qquad \text{(EQN. 1)}$$

$$HOCl \rightleftharpoons H^+ + OCl^- \qquad \text{(EQN. 2)}$$

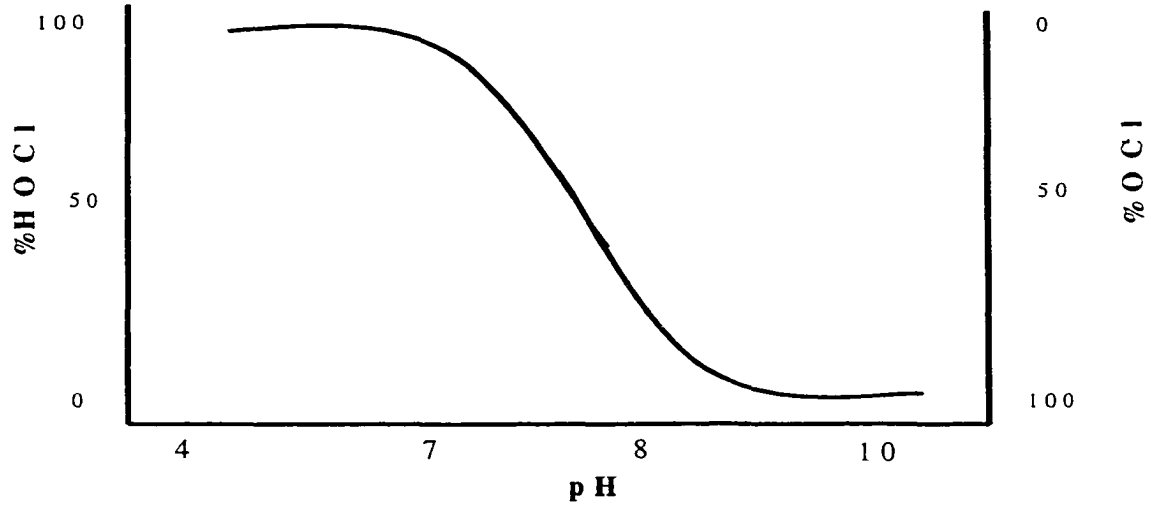
(Equation 1) and ionization (Equation 2), where HOCl is hypochlorous acid and OCl^- is the hypochlorite ion. Free available chlorine refers to the amount of unbound HOCl and OCl^- present in water, whereas total available chlorine refers to both bound and unbound forms. The concentration of HOCl and OCl^- present in a water sample is governed by pH and is described in Figure 2. Furthermore, HOCl is a much stronger oxidant, and therefore better disinfectant, compared to other forms of chlorine (Table 12). Due to chlorine's strong oxidizing properties, it not only reacts with pathogenic microorganisms, but also other easily oxidizable substances present, including metals such as iron and manganese, organic matter, hydrogen sulfide and ammonia. These reactions reduce the chlorine forms to the unreactive chloride ion (80). This effect, where disinfectant is consumed by water constituents, is commonly referred to as disinfectant demand.

Table 12 Estimated effectiveness of residual chlorine forms.

Chlorine form	Estimated Potency Compared to HOCl
Hypochlorous acid (HOCl)	1
Hypochlorite ion (OCl^-)	0.010
Monochloramine (NH_2Cl)	0.007
Dichloramine ($NHCl_2$)	0.013
Trichloramine (NCl_3)	Possibly more effective than $NHCl_2$

Adapted from AWWA, 1995

Figure 2 Distribution of HOCl and OCl^- in water as a function of pH.



Adapted from Bitton, 1994

However, when chlorine combines with ammonia, chloramines are formed in three sequential reactions;

$NH_3 + HOCl \rightarrow NH_2Cl$ (monochloramine) + H_2O (EQN. 3)

$NH_2Cl + HOCl \rightarrow NHCl_2$ (dichloramine) + H_2O (EQN. 4)

$NHCl_2 + HOCl \rightarrow NCl_3$ (nitrogen trichloride or trichloramine) + H_2O (EQN. 5)

in which the di- and trichloramines are eventually oxidized to nitrogen gas. These three competing reactions are affected by pH, temperature, contact time, the ratio of chlorine to nitrogen and most importantly, the initial chlorine and ammonia nitrogen concentrations. Continual application of chlorine will eventually increase the chlorine to nitrogen ratio to 7.6:1 and higher, where chloramines will be oxidized to nitrogen gas, nitrate, and other end products and a free chlorine residual appears. This point in the ammonia-chlorine interaction is termed the breakpoint (80). Ammonia-chlorine reactions are important in wastewater treatment, where naturally-occurring concentrations of ammonia convert applied free chlorine to chloramines. Since chloramines are considerably weaker disinfectants, chlorine is applied until the breakpoint reaction is reached.

The mechanism by which chlorine inactivates viral pathogens is controversial. Viral inactivation may be caused by interaction with the viral capsid proteins and/or the nucleic acid. In some studies, viral RNA had a greater affinity for chlorine than the proteinacious capsid whereas other researchers observed that the virus became noninfectious before damage to the nucleic capsid

Table 13 Chlorine inactivation of selected enteric viruses.

Virus	°C	pH	Chlorine Dose (mg/L)	Estimated Ct (% Reduction)	Reference
Poliovirus 1	5	6	0.5	1.05 (99 %)	Engelbrecht *et al.*, 1980
	5	10	0.5	10.5 (99 %)	Engelbrecht *et al.*, 1980
Hepatitis A	5	6	0.5	2.3 (99.99 %)	Sobsey *et al.*, 1991
cell-associated	5	6	0.5	29 (99.99 %)	Sobsey *et al.*, 1991
Adenovirus 3	4	6	0.10-0.20	0.01-0.08 (99.9 %)	Clarke *et al.*, 1956
Coxsackie B5	5	6	0.5	0.18 (99 %)	Engelbrecht *et al.*, 1980
Echovirus 1	5	6	0.5	0.25 (99 %)	Engelbrecht *et al.*, 1980
Coliphage MS-2	5	6	0.5	0.60 (99.99 %)	Sobsey *et al.*, 1989
Coliphage MS-2	5	8	0.5	8.35 (99.99 %)	Sobsey *et al.*, 1989

occurred (21, 58). Furthermore, depending on the concentration of chlorine, studies have indicated that nucleic acid damage may occur at low (0.8 mg/L) concentrations whereas, high (>0.8 mg/L) chlorine concentrations may result in damage to both the nucleic acid and protein capsid (2).

Like most water disinfectants, the resistance to chlorine is generally higher for protozoan pathogens followed by bacterial spores, viruses and vegetative bacteria. In addition, varied resistance amongst closely related viruses has been reported. Table 13 lists Ct values for chlorine inactivation of viruses.

Factors affecting chlorine and UV disinfection of viruses

The ability of a disinfectant to effectively inactivate viral pathogens is dependent on many physical and chemical characteristics of the disinfectant, the virus itself and the water where the virus and disinfectant are in contact. Water temperature influences the rate of disinfection, where higher temperatures tend to have higher inactivation rates (40). The water pH may affect the ionic species of the disinfectant for chemical disinfectants such as chlorine and chlorine dioxide. The percentage of HOCL is greater than OCl^- at lower compared to higher pH values (Figure 1). In addition, changes in pH above or below the isoelectric point (IEP)of a virus, may alter the conformation of disinfectant target sites on the capsid, reducing or diminishing interaction with the disinfectant (76). Viral aggregation or clumping may also be induced by pH changes, possibly by increasing virion hydrophobicity and therefore, significantly decreasing inactivation rates (76). Concentrations of salt have been reported to significantly increase the viral inactivation rate by disinfectants such as chlorine (66, 67). Finally, like all microorganisms, viruses are more sensitive to some disinfectants compared to others, for example viruses are more susceptible to inactivation by ozone than chlorine.

Dissolved organic matter and particulates (suspended solids; turbidity) present in water are other important factors which may lower the effectiveness of disinfectants such as chlorine and UV light. Dissolved organic matter react (oxidize or adsorb) with chlorine and UV light, reducing the disinfectant concentration available to react with the microorganisms (70). For any disinfectant, particulate matter (organic or inorganic) present in water may consume the disinfectant and protect or shield any embedded or adsorbed microorganisms (70). Particulate matter may also scatter UV light, decreasing the UV intensity applied to the microorganism. However, research conducted with inorganic particulates varies, since some studies conducted with viruses associated with inorganic particles were inactivated similarly to unassociated viruses (70). Nonetheless, removal of particulates is crucial in water treatment so that the potency of applied disinfectants is not compromised (33).

Individual virus properties may affect the efficiency of water disinfection. Viruses have been shown to have varying susceptibilities to disinfectants even within one population or between different viral types (76, 78). The viral particle's state of hydration has also been shown to affect disinfection kinetics, where hydrated viral particles are less resistant than nonhydrated particles (76). Also, laboratory viral strains have been shown to be less resistant to chlorine compared to chlorinated drinking water isolates (59, 65). However, for some viral types, greater resistance of the chlorinated drinking water isolates was not observed (59).

Since the enteric viruses are shed in the feces of infected individuals, they are aggregated and closely associated with cell debris and other fecal solids (82). A few studies have demonstrated the increased resistance of aggregated or debris-associated compared to dispersed viral particles (Table 13) (59, 70, 71). Due to their natural occurrence in source waters, it has been suggested that evaluation of drinking water disinfectant effectiveness should be carried out with these more resistant aggregated and cell-associated forms (71).

Summary and current study

Waterborne disease outbreaks are probably greatly under-reported and the etiological agent is not identified in about 50 % of the reported cases. Emerging enteric pathogens, known or thought to occur in public water supplies, may be important cause of waterborne illness. Information important in assessing if these pathogens are important in waterborne transmission of disease is lacking, such as information regarding occurrence and survival in water, resistance to water treatment processes, presence in sewage, zoonotic transmission and methodologies for detection and determination of viability. Consequently, the USEPA published the CCL list in order to stimulate research in these areas.

In the current study, three investigations were conducted with three of the CCL pathogens; human pathogenic microsporidia, caliciviruses and enteric adenoviruses. In the first study, the detection of human pathogenic microsporidia in surface waters used for the irrigation of vegetable crops was conducted. Since there are no methods which

can determine the infectivity of microsporidia from environmental samples, molecular methods (polymerase chain reaction) were employed for detection of the human pathogenic species. The second and third studies examined the effectiveness of water treatment disinfectants to inactivate feline calicivirus and adenovirus type 40. FCV served as a surrogate for the inactivation of NLVs. Three test waters were used in the disinfection experiments, buffered demand free water and treated ground and surface water. UV disinfection experiments were conducted at pH 7 and room temperature only. However, free chlorine inactivation experiments were conducted at pH 6, pH 8, 5°C and 15°C. A final set of chlorine experiments was conducted involving aggregated (no chloroform extraction performed in viral preparation) viruses. Doses and Ct values for UV light and chlorine inactivation were calculated for each virus using kinetic modeling methods.

Dissertation format

The research reported in the appendices of this dissertation consists of three experiments designed and undertaken by the candidate: 1) Detection of Protozoan Parasites and Microsporidia in Irrigation Waters Used for Crop Production; 2) Inactivation of Feline Caliciviruses and Adenovirus Type 40 by Ultraviolet Radiation; 3) Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus. This format offers the advantage that candidates for the advanced degree in the Department of Soil, Water and Environmental Science are expected to submit their original research to peer review scientific journals for publication. By using this format these papers will essentially be ready for publication.

PRESENT STUDY

The objectives of the current study were to (1) determine the occurrence of human pathogenic microsporidia in irrigation water used for crops eaten raw; (2) the determine the number of *Giardia* cysts and *Cryptosporidium* oocysts in irrigation water used for crops eaten raw; (3) determine the effectiveness of low pressure ultraviolet light to inactivate adenovirus type 40 and feline calicivirus; (4) determine the effectiveness of chlorine to inactivate adenovirus type 40 and feline calicivirus; (5) determine the differences in inactivation kinetics between dispersed and aggregated viruses exposed to chlorine; and (6) determine the effect of treated drinking waters (surface water and groundwater) on the effectiveness of ultraviolet light and chlorine inactivation of adenovirus type 40 and feline calicivirus.

The occurrence and disinfectant effectiveness for pathogens which are known or thought to be important in waterborne disease was evaluated. In the first study, the occurrence of human pathogenic microsporidia, *Giardia* cysts and *Cryptosporidium* oocysts in surface waters used for the irrigation of vegetable crops was determined. Twenty-eight percent of the irrigation water samples tested positive for microsporidia, 60 % positive for *Giardia* cysts and 36 % positive for *Cryptosporidium* oocysts. Concentrations of *Giardia* cysts and *Cryptosporidium* oocysts detected in water samples collected in Central American compared to the United States were 559 cysts and 227 oocysts, and 25 cysts and < 19 oocysts per 100 L, respectively. The presence of human pathogenic parasites in irrigation

waters used for production of crops traditionally consumed raw suggests that there may be a risk of infection to consumers who come in contact with or consume these products.

In the other investigations, the effectiveness of UV light and free chlorine on the inactivation of feline calicivirus (FCV) and enteric adenovirus type 40 (AD40) was assessed and compared to model viruses, poliovirus type 1 (PV-1) and coliphage MS-2. FCV was used as a model for members of the "Norwalk like virus" (NLV) group. The UV doses required to achieve 99 % inactivation of AD40, coliphage MS-2 and FCV in buffered demand free (BDF) water were 108.6, 58.5 and 16.8 mWs/cm^2, respectively. For chlorine reactions, higher Ct values for high pH and low temperature conditions was observed for FCV and AD40. Both viruses were more resistant to chlorine than the well-studied PV-1. FCV and AD40 were inactivated rapidly by ~0.5 mg/L free chlorine by ≥4.00- and ≥2.54- logs at pH 6 and 5 °C whereas, PV1 was not inactivated by 4.04-logs until 10 min contact time. Experiments conducted with aggregated FCV and PV-1 and experiments conducted in treated groundwater had slower inactivation kinetics than dispersed viral suspensions in BDF water. FCV and PV-1 were over 27.7 and 2.7 fold higher inactivation kinetics in the aggregated compared to the dispersed state. The high disinfectant decay rate of some experiments was most likely due to the decrease in chlorine concentration throughout the experiment. However, low disinfectant decay rates observed for experiments conducted in treated groundwater suggest that aggregation or clumping of AD40 may have occurred. The results of these studies provide information on the effectiveness of two common water treatment disinfectants in waters with different

physical and chemical qualities. The results of this study may provide a basis for the establishment of guidelines for proficient application in drinking water treatment.

APPENDIX A:

DETECTION OF PROTOZOAN PARASITES AND MICROSPORIDIA IN IRRIGATION WATERS USED FOR CROP PRODUCTION

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Short title: Detection of parasites in irrigation waters

Key words: *Giardia*, *Cryptosporidium*, *Encephalitozoon intestinalis*, irrigation water, microsporidia

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Abstract

The occurrence of human pathogenic parasites was investigated in irrigation waters used for food crops traditionally eaten raw. Polymerase chain reaction was used to detect human pathogenic microsporidia in irrigation waters from the United States and several Central American countries. In addition, the occurrence of both *Cryptosporidium* oocysts and *Giardia* cysts was determined via immunofluorescent techniques. Twenty-eight percent of the irrigation water samples tested positive for microsporidia, 60% positive for *Giardia* cysts and 36% positive for *Cryptosporidium* oocysts. The average concentrations in samples from Central America containing *Giardia* cysts and *Cryptosporidium* oocysts was 559 cysts and 227 oocysts per 100 L. In samples from the U.S., an average of 25 *Giardia* cysts and < 19 (average detection limit) *Cryptosporidium* oocysts per 100 L were detected. Two of the samples, which were positive for microsporidia, were sequenced and subsequent database homology comparisons allowed the presumptive identification of two human pathogenic species; *Encephalitozoon intestinalis* (94% homology) and *Pleistophora* species (89% homology). The presence of human pathogenic parasites in irrigation waters used for the production of crops traditionally consumed raw suggests that there may be a risk of infection to consumers who come in contact with or consume these products.

Introduction

Foodborne illness affects approximately 76 million Americans each year and the number of produce-related outbreaks is on the rise (5, 16). The increase in the consumption of fresh fruits, vegetables and organically grown produce, the globalization of produce, and an increase in the immunocompromised population may be causes of the heightened occurrence of produce-related outbreaks (5). Potential sources of these produce-related outbreaks may occur during growth, harvesting, processing and distribution (22).

Irrigation waters may become contaminated by either sewage introduction or runoff from non-point sources. Rain events may carry fecal contamination from agricultural, domestic and wild animals (including birds) into canals, river waters and wells that serve as sources for irrigation water (5). Irrigation waters used to mix insecticides and fungicides that are sprayed directly onto crops increase the risk of surface contamination by pathogenic microorganisms. The 1996 outbreak of cyclosporiasis may be one example of the transfer of protozoan pathogens from contaminated surface water to produce traditionally eaten raw. The contaminated surface water was mixed with a fungicide and then sprayed onto raspberries before harvest in Guatemala, resulting in more than 1,400 cases of cyclosporiasis (1, 12).

Two waterborne protozoan pathogens, *Giardia* and *Cryptosporidium*, are transmitted via the fecal-oral route and are important causes of waterborne outbreaks of gastroenteritis. While no foodborne outbreaks due to contaminated irrigation water have been reported,

other foodborne outbreaks have occurred (21). Since these pathogens may be transmitted by animals, are resistant to environmental conditions and only a few cysts and oocysts may cause infection, it is likely that *Giardia* cysts and *Cryptosporidium* oocysts may occur in irrigation waters and survive on irrigated produce (21).

In over half of the reported foodborne outbreaks the etiological agent is unknown (5). Newly recognized agents of waterborne disease are likely causes of foodborne outbreaks due to inappropriate detection methods or the lack of knowledge of these emerging pathogens. One such group of emerging pathogens that has been recently implicated in waterborne disease is the microsporidia (3). Microsporidia are parasites that cause infection in both vertebrates and invertebrates. Potential modes of transmission of human microsporidia include airborne, person-person, waterborne and zoonotic (2). The potential for zoonotic transmission of *Giardia* and *Cryptosporidium* is well documented, whereas evidence for the zoonotic transmission of microsporidia, important in human disease, is increasing (4, 6, 19, 21). *Enterocytozoon bieneusi* has been detected in dogs, rabbits, and pigs (6, 19). Potential reservoirs of *Encephalitozoan* species include rodents, rabbits, dogs, birds, pigs, monkeys, goats, and cattle (2, 4).

Disease caused by microsporidia is usually gastrointestinal but infections of the respiratory, reproductive, muscle, excretory and nervous systems have been reported (26). Since the onset of the AIDS (acquired immunodeficiency syndrome) epidemic, *E. bienusi* has been the most commonly identified microsporidian followed by *Encephalitozoon*

intestinalis as the cause of chronic diarrhea and wasting in AIDS patients (8). Microsporidia have also been implicated in disease affecting immunocompetent individuals (2). *E. bieneusi* and *E. intestinalis* have been implicated in non-HIV-associated, self-limited diarrhea in several immunocompetent hosts (2, 15, 18, 20). Recently, an outbreak of intestinal microsporidiosis was reported in France, where contamination of the drinking water source was suspected as the source of intestinal microsporidia which was detected in the feces of both immunocompromised and immunocompetent individuals (3). One study has suggested infection by *Encephalitozoon sp.* may be common in the immunocompetent population due to a high seroprevalence against *Encephalitozoon* species (25). In addition, antibodies to *Encephalitozoon sp.* were detected in 21% of the households in a rural community in Mexico (11). Consequently, in immunocompetent hosts, microsporidiosis may be a common illness that goes unrecognized due to asymptomatic or self-limited infection.

Due to waterborne transmission of human pathogenic parasites, contamination of waters used for irrigation of produce traditionally eaten raw may increase the risk of infection through the consumption of minimally processed fruits and vegetables. For these reasons, irrigation waters used to irrigate crops traditionally consumed raw, were collected from both Central America and the United States to determine the occurrence of human pathogenic microsporidia, *Giardia* cysts and *Cryptosporidium* oocysts.

Methods

Water sampling

Each water sample was collected from a different surface water source (canal, lake or river) that was used directly (no prior treatment) for irrigation of crops traditionally eaten raw. Table 1.A includes the location, irrigation water source and types of crops impacted by the irrigation waters tested. Water samples were processed according to the United States Environmental Protection Agency (USEPA) Information Collection Rule (ICR) method (24). Water samples (100 – 400 L) were filtered through polypropylene wound filters using the sampling protocols described in the ICR (24). After collection, the filters were placed in separate plastic bags and held in ice-filled coolers until arrival at our laboratory at the University of Arizona.

Purification of parasites

The filters were processed within 96 hours after arrival. Each filter was cut lengthwise and the filter fibers separated into two portions. Each portion was placed into a 4 L beaker containing 1.5 L of eluting solution (24). The fibers were hand washed for a total of 30 min to dislodge any particulates, including microsporidia spores, trapped within the filter matrix. The filters were wrung out by hand after washing and the elution solution was concentrated by centrifugation at 2,000 x g for 10 min in a Beckman GS-6 swinging bucket rotor centrifuge (no brake). The supernatant was aspirated off and the filter sediment resuspended in elution solution. This step was repeated until all of the sample sediment was pelleted into one tube. The supernatant was again discarded and the pellet

was resuspended in 20% formalin. All samples were then stored at 4°C until floatation purification.

Floatation purification

Pelleted water concentrates were further purified by the flotation purification protocol described in the ICR method (24). A portion of the purified sample was then subjected to DNA extraction for detection of microsporidia or immunoflourescent staining and microscopy for the detection of *Giardia* cysts and *Cryptosporidium* oocysts.

Immunoflourescent staining and microscopy

Immunoflourescent staining using Hydrofluor ™ flourescent antibodies (Hydrofluor TM Combo Meridian Diagnostics, Inc., Cincinnati, OH) and detection of *Giardia* cysts and *Cryptosporidium* oocysts was performed according to the ICR methods (24).

DNA extraction

Total DNA was extracted from the purified samples using a QIAamp Tissue Kit (Quiagen, Inc., Santa Clarita, Calif.) reagents according to slightly modified protocols as described by Dowd et al. (1998). No more than 100 µl of pelleted water concentrate was resuspended in 180 µl of buffer ATL by vortexing. To this, 20 µl of proteinase K (100 mg/ml) was added and the sample was once again vortexed. The sample was then incubated at 55 °C for 4 hr in a shaking water bath. Next, 200 µl of buffer AL was added

and the sample was vortexed thoroughly and incubated in a 70°C water bath for 10 min, followed by a 10 min incubation in a 98°C water bath. Following this, the samples were once again vortexed and then centrifuged at 16,000 x g for 2 min to pellet any solids. The supernatant was transferred to a clean microcentrifuge tube and the pellet was discarded. Next, 210 µl of 100% ethanol was added to the sample, which was then thoroughly vortexed for 1 min. The sample was then added to QIAamp spin columns and centrifuged at 6,000 x g for 1 min. The membrane-captured DNA was subsequently washed twice, according to the manufacturer's instructions, and eluted by adding 100 µl of molecular-grade water, which had been preheated to 70°C, to the column and incubating the columns in a hybridization incubator for 10 min at 70°C. The samples were then centrifuged for 1 min at 16,000 x g. The eluted water was then reapplied to the column and incubated once again to maximize the recovery of DNA from the sample. Finally, the column was centrifuged at 16,000 x g and up to 80 µl of the supernatant was used for the 100 µl polymerase chain reaction (PCR) analysis.

Polymerase chain reaction

The PCR primers used in this analysis have been described in previous research (10). The forward primer (5'- CAC CAG GTT GAT TCT GCC TGA C-3') and the reverse primer (5'-CCT CTC CGG AAC CAA ACC CTG-3') amplify the small subunit ribosomal DNA (SSU-rDNA) of human pathogenic microsporidia. These microsporidia

species include *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Vittaforma cornea* and *Pleistophora sp.*, all of which produce approximately 300 bp amplicon sizes. The PCR conditions were as follows: *Taq* Gold (Perkin-Elmer Corp., Norwalk, Conn.)-induced hot start cycling conditions consisting of 10 min of denaturation at 95°C, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 20 s, and extension at 72°C for 40 s. A final extension step consisting of 5 min at 72°C was included. PCR products were analyzed by agarose gel electrophoresis and observed for bands within the 300 bp region. Bands that were sequenced were cut from this region and purified for sequencing analysis.

Sequencing

PCR products were purified from the gel by using a QIAquick PCR purification kit (Quiagen) and were resuspended in sterile water. The forward PCR primer was then used for dye termination PCR sequencing, which was performed at the University of Arizona's Laboratory of Molecular Systematics and Evolution Sequencing Facility (Tucson, AZ).

SSU-rDNA sequence analysis

Database searching was performed with BLAST 2.0 on the National Center for Biotechnology Information's World Wide Web site (http://www.ncbi.nlm.nih.gov).

Results

Twenty-five different irrigation water samples from Central America (Mexico, Costa Rica and Panama) and the United States were analyzed by immunoflourescent and molecular techniques for the presence of human pathogenic protozoan parasites, *Giardia* cysts, *Cryptosporidium* oocysts and human pathogenic microsporidia. Table 2.A shows the percentage of samples which contained *Giardia* cysts, *Cryptosporidium* oocysts and human pathogenic microsporidia DNA. Twenty-eight percent of all the analyzed samples contained DNA from human pathogenic microsporidia species, 60% had *Giardia* cysts and 36% had *Cryptosporidium* oocysts. For samples collected from Central American countries, 16 %, 59 % and 36 % contained human pathogenic microsporidia DNA, *Giardia* cysts and *Cryptosporidium* oocysts, respectively. Two of the three samples collected from the U.S. contained both human pathogenic microsporidia DNA and *Giardia* cysts. No *Cryptosporidium* oocysts were detected in any of the irrigation water samples collected in the U.S.

The concentration ranges of *Giardia* cysts and *Cryptosporidium* oocysts for all irrigation water samples analyzed are shown in Table 3.A along with the average number of cysts and oocysts detected in positive samples for each country (Costa Rica, Panama, Mexico and U.S.) and the combined averages for samples collected from Central American locations and samples collected from the U.S. The range of *Giardia* cysts for all locations was <5.7 to 17,493 per 100 L and the range of *Cryptosporidium* oocysts was <5.7 to 1,579 per 100 L. The number of cysts and oocysts for all irrigation water samples

that contained these organisms (positive water samples), averaged 369 *Giardia* cysts and 227 *Cryptosporidium* oocysts per 100 L. When comparing samples collected in Central America to those from the U.S., the average concentration of cysts for water samples which were positive for these pathogens was 559 and 25 per 100 L, respectively. There were no *Cryptosporidium* oocysts detected in any of the U.S. irrigation water samples but Central American locations contained an average of 227 oocysts per 100 L. A range of <7.7 to 40 *Giardia* cysts per 100 L was observed in the U.S. samples, compared to a broader range of < 5.7 to 17,493 cysts per 100 L in Central American water samples. *Cryptosporidium* oocysts in Central American irrigation water samples ranged from < 5.7 to 1,579 per 100 L.

Seven out of 25 irrigation water samples from all locations were positive for human pathogenic microsporidia DNA. Two of the seven positive microsporidia PCR products were sequenced and subsequent database homology comparisons allowed the presumptive identification of two human pathogenic microsporidia species. One irrigation water sample collected in Mexico was found to contain DNA from *E. intestinalis*. The percent homology of the National Center for Biotechnology Information's (NCBI) database sequence for *E. intestinalis* compared to the submitted irrigation water sequence was 94 %. For the second PCR product, water sample collected in Costa Rica, the submitted irrigation water sequence had a low homology (89 %) to the *Pleistophora* species sequence located in the NCBI database.

Discussion

Cryptosporidium oocysts, *Giardia* cysts and human pathogenic microsporidia DNA were detected in irrigation waters used for crops which require little processing. Since these pathogens are present in irrigation waters it is likely that they may come in contact with and attach to the surface of crops. In a study conducted by Mong and Chinchilla (1995), the presence of *Cryptosporidium* oocysts on fresh vegetables was suspected to be due to the use of contaminated irrigation waters. The authors also noted a higher number of oocysts on the tested vegetables during the rainy season (17). Agricultural, urban and industrial runoff most likely contributed to the increased oocyst concentrations observed in their study. The irrigation waters in their study and in many of the Central American irrigation waters analyzed in this study are from surface waters that receive industrial, urban and agricultural wastewater. Irrigation water samples were collected from rivers and lakes in Costa Rica and Panama, and from irrigation canals in Mexico and the U.S. Higher concentrations of *Giardia* cysts and *Cryptosporidium* oocysts were detected in water samples collected in the Central American countries as compared to those collected in the U.S. One explanation may be that the locations in Central America appeared to be more heavily impacted by human activity and farm, wild and domestic animal activity than locations in the U.S. Moreover, since human pathogenic microsporidia, *Cryptosporidium* oocysts and *Giardia* cysts are excreted in the feces (microsporidia, *Giardia* and *Cryptosporidium*) or urine (microsporidia) of infected individuals (animals and humans) irrigation water sources impacted by agricultural, urban and wildlife

pollution are more likely to have an increased occurrence and concentration of these pathogens.

Due to the low infectious doses required to induce infection by *Giardia* and *Cryptosporidium*, the concentrations observed in many of the sampled waters may pose a health risk to consumers if these pathogens are associated with irrigated products. The infectious doses are not known for human pathogenic microsporidia species, but 28 % of the sampled waters contained their DNA. Furthermore, two of these positive DNA samples were presumptively identified as the human pathogenic species, *E. intestinalis* and *Pleistophora spp.*, which are known to cause disease in individuals with compromised immune systems (2). *E. intestinalis* has been identified as a cause of cause of traveler's diarrhea and a high seroprevalence of *Encephalitozoon* species has been reported in immunocompetent persons (18, 25).

The methods used for the detection of human pathogenic microsporidia only detect the presence of DNA sequences specific to the human pathogenic microsporidia species and, therefore, do not discriminate between viable and non-viable spores. No methods are currently available to determine the viability of microsporidia spores from environmental samples. The methods used for the detection of *Cryptosporidium* oocysts and *Giardia* cysts can not assess viability either. Although viability was not assessed for any of the irrigation water samples, previous research suggests that these protozoan pathogens are capable of surviving conditions associated with surface waters and the surface of produce.

Kucerova-Pospisilova (1999) suggested that *Encephalitozoon* spores were resistant to environmental conditions characteristic of surface waters. Germination and infection of *Encephalitozoon* spores were not completely killed after a week of storage in water at 4 °C, 22 °C and 33 °C. (14). For *Giardia* cysts and *Cryptosporidium* oocysts, survival studies conducted in surface waters have verified an increased resistance of these pathogens to environmental conditions (7, 13, 21).

The occurrence and concentrations of the studied pathogens may be underestimated due to the methods used in this study. Previously reported efficiencies for the detection of *Giardia* cysts and *Cryptosporidium* oocysts averaged 11% and 26% at the University of Arizona (23). Moreover, recovery of microsporidia is about 4.8 %, lower than the other protozoan parasites analyzed (9). The low recovery of these protozoan parasites may signify that their occurrence is higher than indicated by the results presented.
From the results of this study, microsporidia spores, *Giardia* cysts and *Cryptosporidium* oocysts may come in contact with irrigated crops due to their presence in waters used for irrigation. Moreover, these pathogens may remain viable under conditions characteristic of surface waters and the surface of vegetable crops. Since the infectious doses for *Giardia* cysts and *Cryptosporidium* oocysts are low (no data available for the infectious dose of microsporidia species), the presence of viable organisms in irrigation waters which come in contact with minimally processed crops, may increase the risk of disease to consumers who come in contact or consume these products.

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Table 1.A Location of sampled irrigation waters, irrigation water sources and types of produce affected by these waters.

Location	Irrigation Water Source	Produce Impacted by Sampled Irrigation Waters
Costa Rica	River (all 5 locations)	Cilantro, coffee, tomatoes, celery, lettuce, peppers
Mexico	Agricultural canal (11 locations)	Chili, cilantro, tomatoes, cucumbers, lettuce
Panama	River (5 locations) Lake (1 location)	Tomatoes, bananas, peppers, lettuce, potatoes, carrots, onions
United States	Agricultural canal (all 3 locations)	Lettuce, tomatoes

Table 3.A Concentration ranges and averages for samples which contained cysts and oocysts (immunoflourescent microscopy) in the irrigation waters analyzed in this study.

Protozoan Parasite and Location of Sample	Ranges of Cysts or Oocysts Per 100 L	Average [a, b] number of Cysts or Oocysts Per 100 L
Giardia cysts		
Costa Rica	<52.6 - 17493	6426
Mexico	<5.7 - 8945	227
Panama	<18 - 1800	693
Central America	<5.7 - 17493	559
United States	<7.7 - 40	25
All Locations	<5.7-17493	369
Cryptosporidium oocysts		
Costa Rica	<52.6 – 333	150
Mexico	<5.7 - 1579	612
Panama	<18 - 250	190
Central America	<5.7 - 1579	227
United States	<7.7 - <43	< 19 [c]
All Locations	<5.7-1579	227

[a] Geometric average

[b] Average number of cysts and oocysts in samples containing these pathogens

[c] Average detection limit

Table 2.A Location of sampled irrigation waters, total number of samples at each location and percentage of water samples containing *Giardia* cysts, *Cryptosporidium* oocysts (immunoflourescent staining detection methods) and microsporidia (PCR detection) compared to total number of water samples analyzed.

Location (Total number of samples analyzed)	Percentage of samples positive for microsporidia DNA	Percentage of samples positive for *Giardia* cysts	Percentage of samples positive for *Cryptosporidium* oocysts
Costa Rica (5)	40 %	60 %	60 %
Mexico (11)	27 %	64 %	18 %
Panama (6)	<1 %	50 %	67 %
All Central American Locations (22)	16 %	59 %	36 %
United States (3)	67 %	67 %	<1 %
All Locations (25)	28 %	60 %	36 %

APPENDIX B:

INACTIVATION OF FELINE CALICIVIRUS AND ADENOVIRUS TYPE 40 BY ULTRAVIOLET RADIATION

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Short title: UV inactivation of enteric viruses

Key words: Enteric adenovirus, calicivirus, UV disinfection, feline calicivirus

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Abstract

Little information regarding the effectiveness of ultraviolet (UV) radiation on the inactivation of caliciviruses and enteric adenoviruses is available. Analysis of human calicivirus resistance to disinfectants is hampered by the lack of animal or cell culture methods which can determine their infectivity. The inactivation kinetics of enteric adenovirus type 40 (AD40), coliphage MS-2 and feline calicivirus (FCV), closely related to the human caliciviruses based on nucleic acid organization and capsid architecture, were determined after exposure to low pressure UV radiation in buffered demand free (BDF) water. In addition, UV disinfection experiments were also carried out in treated ground and surface water for FCV and groundwater for AD40. AD40 was significantly more resistant ($p<0.01$) than either FCV or coliphage MS-2 in both BDF and natural waters. The doses of UV required to achieve 99 % inactivation of AD40, coliphage MS-2 and FCV in BDF water were 108.6, 58.5 and 16.8 mWs/cm^2, respectively. The doses of UV required to achieve 99 % inactivation of AD40, coliphage MS-2 and FCV in ground and surface water were slightly less than in BDF water. FCV was inactivated by 99 % by 14.2 and 13.8 mWs/cm^2 in treated ground water and surface water, respectively and a dose of 51.7 mWs/cm^2 was required for 99 % inactivation of AD40 in treated ground water. The results of this study indicate that if FCV is an adequate surrogate for human caliciviruses, then their inactivation by UV radiation is similar to other enteric viruses such as polio virus. In addition, AD40 appears to be more resistant to UV disinfection than previously reported.

Introduction

Disinfection is an important treatment barrier between drinking water consumers and viral gastroenteritis since the small size (~25-80 nm) of enteric viruses may permit their escape from filtration processes (8). Concern for the formation of chlorinated organics and the effectiveness of chemical disinfectants has increased interest by the drinking and waste water industries to consider alternative disinfectants. One option that does not produce any known disinfectant by-products is ultraviolet (UV) radiation (11).

Members of the human calicivirus genus, "Norwalk-like viruses" (NLVs), are an important cause of waterborne outbreaks affecting adults and children in the United States (9). Like NLVs, the enteric adenoviruses, enteric adenovirus 40 (AD40) and 41 (AD41), are also important causes of self-limiting, acute gastroenteritis (9, 14). Following NLVs and rotavirus, enteric adenoviruses are an important cause of childhood (<4 yrs) gastroenteritis (14, 16). These viruses are shed in high numbers in feces and prolonged shedding is typical (9, 14). Although no waterborne outbreaks have been reported for the enteric adenoviruses, increased environmental stability compared to other enteric viruses and their presence in sewage and surface waters has been reported (7, 20, 24). Waterborne outbreaks from swimming have been reported for the nonenteric adenoviruses (6, 17) .

Both caliciviruses and enteric adenoviruses are on the United States Environmental Protection Agency's (USEPA) Drinking Water Contaminant Candidate List (CCL) (25).

These viruses are on the CCL for regulatory consideration since little to no information regarding health, drinking and wastewater treatment or analytical methodologies is currently available. Very few studies have been conducted on the inactivation of these viruses by water disinfectants such as UV light. The objectives of this study were to determine the inactivation kinetics of AD40 and feline calicivirus (FCV) in two water types, buffered-demand-free (BDF) and treated drinking waters (ground and surface water), after exposure to low pressure UV radiation. Since there are currently no known animal or mammalian cell culture systems which can determine infectivity of NLVs, a closely related surrogate, FCV, was used in the current study as a model for NLV inactivation by UV light. FCV has been suggested to be an adequate surrogate for NLV inactivation since it has similar genome organization and capsid architecture (4, 23). The inactivation kinetics of coliphage MS-2, a suggested indicator of viral inactivation by UV light, was also conducted and compared to FCV and AD40 inactivation kinetics (13).

Methods

Virus propagation and assay

AD40 (strain Dugan), FCV (strain F9), primary liver carcinoma cell line (PLC/PRF/5) and Crandell feline kidney (CRFK) cell line were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). AD40 and FCV were propagated and assayed in the PLC/PRF/5 and CRFK cell lines (4, 5, 10). Maintenance media (minimal essential medium containing 10 % fetal bovine serum) was decanted from 162 cm^2 tissue culture flasks containing complete monolayers. The flasks were rinsed with sterile TRIS

buffered saline (Trizma base) (Sigma Chemical Co., St. Louis, MO) and 1 ml of approximately 10^4-10^5 most probable number (MPN)/ml of the viral stock was inoculated onto the monolayer. In order to allow virus attachment the flasks were incubated for 1 hr at room temperature. The flasks were rocked every 15 min throughout the 1 hr incubation. After incubation, 50 ml of maintenance medium (without fetal bovine serum) was inoculated onto the infected cell monolayer and incubated at 37 °C until destruction of at least 90 % of the cell monolayer occurred. The cell culture flasks containing the propagated virus were frozen and thawed, one time for AD40 and three times for FCV. Next, the supernatant was centrifuged at 4°C and 10,000 x g to remove cell debris.

Further purification and concentration of FCV and AD40 was accomplished by performing two successive polyethylene glycol (PEG) precipitation procedures. Briefly, for every 100 mL volume of viral supernatant 9 g of PEG (MW 8,000) and 5.8 g NaCl were added and stirred overnight at 4 °C. Centrifugation at 10,000 x g at 4 °C for 30 min was performed followed by disposal of the supernatant and resuspension of the pellet in BDF water. BDF water was prepared by dissolving 0.54 g Na_2HPO_4 (anhydrous) and 0.88 g KH_2PO_4 (anhydrous) per L of purified water (Nanopure RO purifier, Barnstead, Dubuque, IA) and adjusted to pH 7 with 1 M NaOH and 1 M KH_2PO_4. For dispersion of FCV and AD40 stocks, equal volumes of the viral extracts and chloroform were homogenized by vortexing the mixture for at least 10 min. Once homogenized, the chloroform-viral suspension was centrifuged at 10,000 x g at 4 °C for 15 min. After centrifugation, the upper layer containing the purified viral stock was collected and stored

at 4°C.

Determination of viral titer before and after UV radiation was accomplished by plating 5 or 10-fold dilutions, in quadruplet, in a 24-well tissue culture tray with the appropriate cells in suspension. The concentration of the viruses was determined by observation of each infected well for cytopathic effect (CPE). Observation for CPE was continued up to 12 and 24 days for FCV and AD40, respectively. The Most Probable Number (MPN) General Purpose Program adapted from Hurley and Roscoe (1983) was used to determine the concentration, MPN/mL, of both AD40 and FCV.

Propagation of MS-2 coliphage was accomplished using the double layer agar technique (1). An 18-24 hr culture of *Escherichia coli* (ATCC 15977) grown in tryptic soy broth (TSB; Difco, Detroit, MI) and 1 ml of this culture was transferred to fresh TSB and grown for 3 hr at 37°C in a shaking water bath. MS-2 stock was serially diluted in BDF water in order to achieve a final concentration of 10^5 plaque forming units (PFU)/ml. A 1 ml suspension of the host cells and 0.1 ml of the phage dilution were mixed in 3 ml of molten overlay agar and poured on to petri dishes containing tryptic soy agar (TSA; 1.5% agar; Difco, Detroit, MI). After 24 hr incubation at 37 °C the bacteriophage was eluted by the addition of approximately 6 ml of BDF water and incubation at room temperature for 1-3 hr. After incubation, the liquid from the plates was collected and centrifuged at 10,000 x g for 10 min to remove bacterial debris. Further purification included chloroform extraction as described for FCV and AD40 processing. Stock phage was

serially diluted in BDF water in order to achieve a concentration that would allow observation of at least 99.99% inactivation for all UV disinfection experiments. Phage samples from the UV radiation experiments were serially diluted in BDF water and plated in duplicate using the double agar technique as described previously. The resulting plaques were enumerated and averaged in order to determine the MS-2 coliphage survival.

UV lamp set-up

The collimated beam apparatus consisted of an 8-watt, low-pressure mercury ultraviolet lamp (Sankyo Denki, G8T5.2N) suspended horizontally in a wood box. For uniform lamp output, the lamp was warmed up for at least 30 min prior to all experiments. Attached to the UV lamp box was the collimating tube, made of black PVC pipe measuring 21 in. long and a diameter of 3 in. Both the UV box and collimating tube were painted with a non-reflective black paint to minimize light diffraction. A stir plate was placed directly under the collimated beam for slow stirring of the viral suspensions. UV intensity of each experiment was determined with a calibrated radiometer (IL2000, photodetector SED 240/NS254/W, International light, Newburyport, MA) by placing the radiometer at approximately the same location and elevation of the water surface of the irradiated samples.

Test waters

For all viruses at least two experiments were performed with BDF water at pH 7 and

room temperature, approximately 22-25 °C. The effects of UV radiation on viral inactivation in treated (coagulation, sedimentation, chlorination and filtration) drinking waters, ground and surface water, was also conducted. Both of the treated waters were dechlorinated by rapid mixing and exposure to UV light until no chlorine was measured by the N, N diethyl-p-phenylenediamine (DPD) method (2). Two experiments were performed with FCV and AD40 suspended in the treated groundwater sample and only one experiment was conducted with FCV suspended in the treated surface water sample. Table 1.B lists the properties of each water type.

Irradiation of samples

Samples were irradiated in sterile Pyrex 60 x 15 mm glass petri dishes containing 10 x 2 mm stir bars stirring at low speed. The viral stock was diluted in either BDF water or one of the two treated drinking waters in order to achieve a total inactivation of 99% and 99.99 % for AD40 and FCV, respectively. The total volume and depth of viral suspension within the petri dish was 14 ml and 1 cm, respectively. Prior to each test, the absorbance of the viral suspension was measured at 254 nm (Spectronic Genesis 5 spectrophotometer, Milton Roy Co., Rochester, NY). Viral suspensions were placed under the collimated beam and irradiated for predetermined times. One to four milliliter samples were withdrawn for viral assay. In addition, control samples, not subjected to UV light, were also collected at the same time. Both the initial and final sampling times were withdrawn from the control petri dishes in order to determine the concentration of viruses in the viral suspension and to determine if viral inactivation occurred in the

absence of UV radiation. Temperature and pH were also determined for each experiment. Each experiment was conducted at least in duplicate with the exception of the FCV inactivation experiment carried out in surface water.

Dose determination

According to Beer's Law the measured intensity was corrected for absorbance of the liquid sample by the following equation:

$I_{ave} = I_o (1-e^{-aeL})/a_eL$

where I_{ave} = intensity average (mW/cm^2), a_e = absorbance of the suspension to the base e, L = depth (cm) of solution irradiated by collimating beam and I_o = average of the measured intensity at time zero (t_0) and at the final time (t_{final}) (mW/cm^2). Dose (mWs/cm^2) was calculated as the product of the average intensity (mW/cm^2) multiplied by the time (s) of UV exposure.

Viral inactivation analysis

Since the inactivation of viruses has been shown to be of a first order type, Chick's Law can be used to describe the kinetics of viral inactivation by UV (21). Viral UV inactivation can be defined by the following equation: $N_t/N_0 = e^{-kit}$; where N_t = the number of viral particles at time t (time of UV exposure), N_0 = number of viral particles at time zero (no UV radiation applied), k = inactivation rate or slope of inactivation curve, i = intensity of UV light energy (mW/cm^2), and t = exposure time (s). $-Log_{10}$ of the survival ratio (Nt/N0) versus dose for each experiment was used to perform regression

analysis for each virus and water type using Quatro Pro (Corel Corporation;Version 9, 1999). Generation of regression curves was accomplished by considering all experiments (observed data) for each virus and water type. The slope of the regression line was used to predict the doses required for 1,2,3 and 4-log inactivation of each virus. The predicted and observed values were plotted on a chart, survival ratio (-log(Nt/NO)) vs. dose, for each set of UV disinfection experiments involving one of the tested viruses suspended in either BDF or treated drinking water.

Results

UV disinfection survival curves for FCV, MS-2 and AD40 for UV disinfection experiments carried out in either BDF or treated natural water are shown in Figures 1.B and 2.B. For each virus studied, the survival ratios, -Log(Nt/N0), were plotted against the UV dose (mWs/cm^2) for examination of UV inactivation kinetics. FCV and MS-2 exhibited typical first order inactivation kinetics. Alternatively, the AD40 inactivation curve demonstrated a slight shoulder at low UV doses followed by linear reduction (Figure 3.B). Neither flattening or tailing of any of the inactivation curves was observed, indicating a lack of clumping or aggregation of the viral suspensions.

Due to difficulties obtaining a high AD40 titer, a minimum of 2-Log reduction was sought for all UV disinfection experiments involving this virus. The predicted doses calculated for 1-4 Logs of inactivation for each of the studied viruses are shown in Table 2.B. In addition, associated R^2 values and inactivation constants are also listed in Table

3.B.

AD40 was the most resistant to UV inactivation for experiments carried out in BDF water followed by coliphage MS-2 and FCV. The doses required for 2-Log inactivation of FCV, MS-2 and AD40, for experiments carried out in BDF water, were 16.8, 58.5 and 108.6 mWs/cm^2, respectively. Furthermore, the doses required to inactivate 4-Logs of FCV, MS-2 and AD40 were 33.6, 117.1 and 217.1 mWs/cm^2, respectively.

Slightly lower doses were required for viral inactivation in experiments carried out in treated drinking waters compared to BDF water. The absorbance of each treated water are listed in Table 1.B. A dose of 8.4 mWs/cm^2 was needed to inactivate FCV in BDF water by 1-Log, whereas 7.1 and 6.9 mWs/cm^2 was required for the same amount of inactivation in treated ground and surface waters, respectively. The treated surface water was more turbid than the ground water, yet very little difference was observed between the doses needed to achieve 1-4-Logs inactivation for experiments conducted in each water type. For example, doses required for a 2-Log inactivation for treated ground and surface water samples were 14.2 and 13.8 mWs/cm^2, respectively. Since no apparent deviation in inactivation kinetics was observed, only one experiment was conducted with FCV suspended in treated surface water. The dose required to achieve 1-Log inactivation of AD40 in treated ground water was 51.7 mWs/cm^2, whereas a dose of 54.3 mWs/cm^2 was required in BDF water. The same trend, higher dose values for reactions carried out in BDF water, was observed for 2,3 and 4-Log inactivation of each virus. MS-2

disinfection experiments were not conducted in any of the treated waters.

Discussion

Currently no laboratory methods are available for the growth and propagation of NLVs in the lab but the closely related FCV has been used as a model system in previous inactivation studies involving physical and chemical disinfectants (4, 23). Results of this study indicate that UV inactivation of FCV is similar to other ssRNA viruses, such as polio virus type 1 (PV-1). Doses required for 1, 3 and 4-Log inactivation of FCV in BDF water were 8.4, 25.2 and 33.6 mWs/cm^2, respectively. Wolf (1990) and Wilson *et al.* (1992) reported doses of 5 and 7.7 mWs/cm^2 for 1-Log inactivation of PV-1, respectively, whereas Hill *et al.* (1970) determined that a dose of 30 mWs/cm^2 to be necessary for 3-Log inactivation. Unlike FCV, doses required to achieve 90-99.99 % inactivation of AD40 were higher than those previously reported for other enteric viruses and adenovirus types. Respiratory and enteric adenoviruses are considerably more resistant to UV radiation than other double stranded (ds) DNA and double and single stranded (ss) RNA viruses. Hara *et al.* (1990) determined that AD19 is 60 times more resistant than EV70 (ssRNA) to low pressure UV radiation and Cameron *et al.* (1979) demonstrated the increased resistance of AD5 over SV40 (dsDNA) and HSV-1 (dsDNA). In addition, Wasserman (1964) reported that adenovirus types 4 and 20 were more resistant to UV light than types 1,19 and 24, demonstrating varied susceptibilities between adenovirus types. In addition, adenovirus type 5 was shown to be more resistant than type 7 in work performed by Cameron *et al.* (1979).

The exceptionally greater UV resistance of AD40 compared to other viruses may be due to physical differences of the viral capsid and nucleic acid. All adenoviruses have dsDNA as their genetic material. It has been suggested that viruses with double-stranded genomes are less susceptible to UV inactivation since only one strand of the nucleic acid may be damaged during disinfection. The undamaged strand may then serve as a template for repair by host enzymes (12, 15). For DNA viruses, host cells may contain the enzymatic machinery to repair damage by excision or recombinational repair. This has been suggested as a reason for the shouldering effect observed in UV disinfection experiments concerning double stranded DNA viruses (12). Repair of RNA viral genomes may not be as likely since excision has not been demonstrated (12). However, multiple infection of host cells, also termed multiplicity of reactivation, may lead to enhanced survival of either DNA or RNA viruses (3, 12, 22). Multiplicity of reactivation has been proposed as the mechanism for enhanced survival after UV disinfection of several viruses, including polio virus and adenovirus (12, 22).

Characteristics of the viral capsid may also influence the effectiveness of UV radiation. Shielding or consumption of UV radiation before reaching the nucleic acid may occur due to the proteinacious capsid or proteins that are directly associated with the nucleic acid within the capsid. The adenovirus capsid is complex, consisting of several capsid proteins and protruding protein fibers, whereas a the calicivirus capsid is composed of only one viral protein and no protein fibers (18, 19). The more complex adenovirus capsid may afford additional protection from UV radiation over simple capsid

architecture as that found with caliciviruses. Furthermore, it may be possible that viral precursors, remaining in the prepared viral stock, may consume or shield UV radiation from infectious adenovirus particles. For adenovirus, only 10-20% of the viral structural polypeptides are assembled into new adenovirus particles (18). Production of defective particles has not been demonstrated for the caliciviruses.

Experiments conducted in the current study with AD40 were shown to be much more resistant to UV radiation than previous work conducted by Meng and Gerba (1996). They reported 30 and 124 mWs/cm^2 required for 1 and 4 Log inactivation of AD40, whereas higher doses of 54.3 and 162.8 mWs/cm^2 were observed in the current study. Differences in viral preparation may be one explanation for these differences. Previous work in our lab has demonstrated that the enteric adenoviruses are sensitive to successive freeze-thawing. Protocols in the previous work by Meng and Gerba (1996) include 5 freeze-thaw steps which may have made the virus more susceptible to UV inactivation whereas only one freeze-thaw step was performed in the current study. Nonetheless, it is evident that the enteric adenoviruses are extremely resistant to UV disinfection when compared to other enteric viruses.

For both FCV and AD40, the doses required for inactivation of 1,2,3- and 4-Log inactivation were very similar for experiments conducted in the treated ground and surface and BDF waters. The physical characteristics, increased turbidity, for the treated drinking waters did not increase the resistance of the tested viruses to UV radiation,

instead the doses were smaller than those observed in BDF water. These differences may have been due to other constituents within the treated water samples that may have enhanced the effects of UV light.

In the current study, 1- and 3- Log inactivation of coliphage MS-2 required a dose of 29.3 and 87.8 mWs/cm^2, respectively. Similarly, Battegelli *et al.* (1993) reported >25 mWs/cm^2 for 1 Log inactivation of coliphage MS-2, although Wilson et al. (1992) and Meng and Gerba (1996) reported lower doses of 18.6 mWs/cm^2 and 14 mWs/cm^2 for 1-Log inactivation. In the current study, the inactivation rate of coliphage MS-2 was approximately 3.5 times slower in comparison to FCV inactivation and the rate was approximately 1.8 times faster than the rate observed for AD40. Although coliphage MS-2 has been suggested as an adequate indicator for enteric virus UV inactivation, these results suggest that it would not be an acceptable indicator for UV inactivation of AD40. However, coliphage MS-2 may serve as an adequate conservative indicator for the UV inactivation of FCV.

The results of this study provide information on the effectiveness of UV radiation of emerging viral enteric pathogens in waters with different physical qualities. The results of this study may provide a basis for the establishment of guidelines for proficient application of UV radiation for drinking water treatment.

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Table 1.B Water quality parameters for BDF, ground and surface waters.

Water Type	Water Source	pH	Absorbance
BDF	**NA[1]**	**7.0**	**<0.001**
Ground water	**Great Miami Aquifer**	**7.9-8.0**	**0.011**
Surface water	**Occoquan Reservoir**	**7.7**	**0.315**

[1]Not applicable

Table 2.B UV doses for 90-99.99 % inactivation of test viruses in BDF, ground and surface water.

Virus	Water Type	90%[1]	99%[1]	99.9%[1]	99.99%[1]
MS-2	**BDF**	**29.3**	**58.5**	**87.8**	**117.1**
FCV	**BDF**	**8.4**	**16.8**	**25.2**	**33.6**
	Ground	**7.1**	**14.2**	**21.2**	**28.3**
	Surface	**6.9**	**13.8**	**20.6**	**27.5**
AD40	**BDF**	**54.3**	**108.6**	**162.8**	**217.1**
	Ground	**51.7**	**103.3**	**155.0**	**206.7**

[1] Inactivation dose (mWs/cm^2)

Table 3.B Inactivation constant (K), standard error and R^2 values obtained by regression analysis for UV disinfection experiments.

Virus	Total number of Experiments	K	Standard error of K	R^2	Water Type
MS-2	3	0.0342	0.0009	0.9526	BDF
FCV	3	0.1190	0.0059	0.8812	BDF
FCV	2	0.1413	0.0065	0.893	Ground
FCV	1	0.1454	0.0110	0.9691	Surface
AD40	4	0.0184	0.0010	0.8488	BDF
AD40	2	0.0194	0.0012	0.8826	Ground

Figure 1.B UV inactivation of viruses in BDF water.

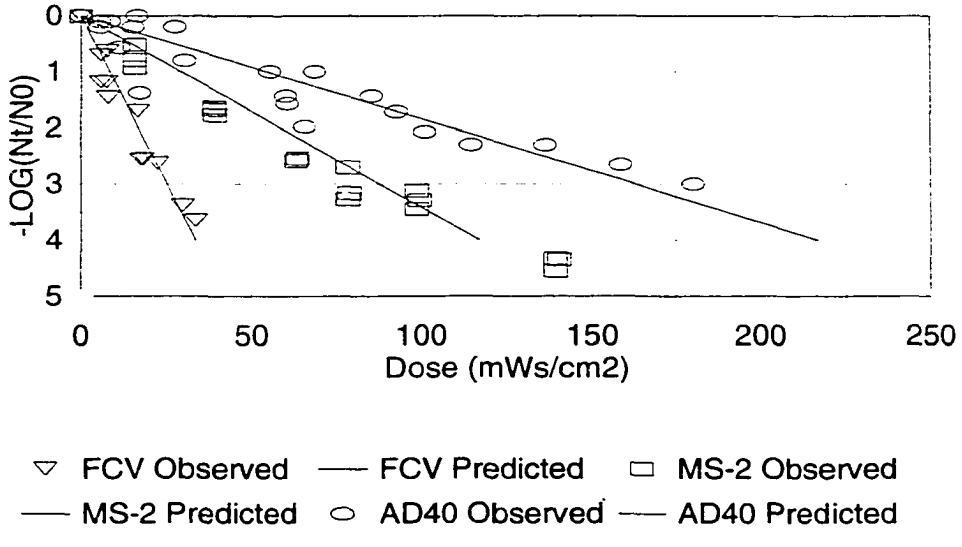


Figure 2.B UV inactivation of FCV and AD40 in treated drinking waters.

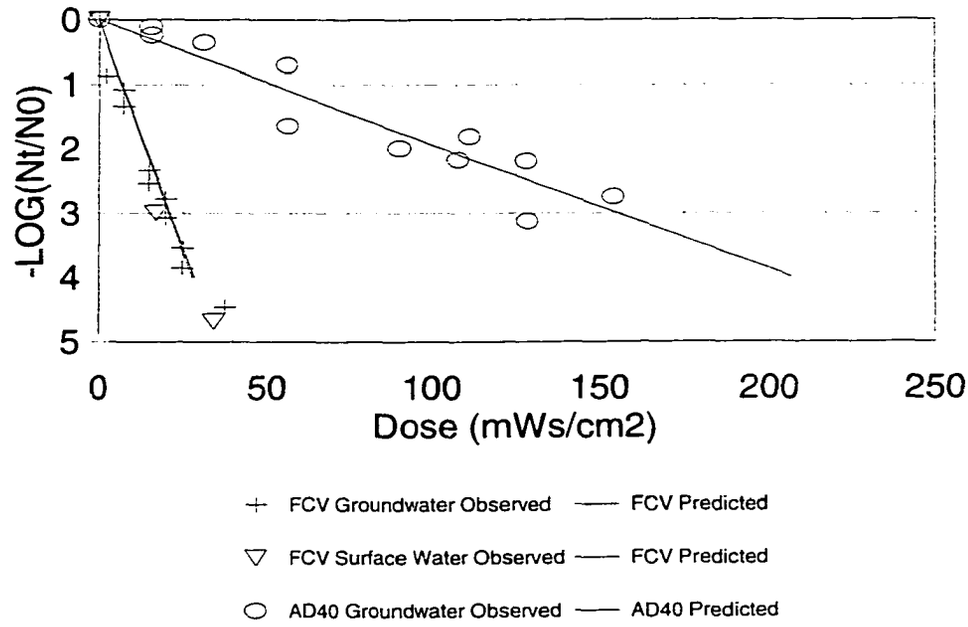
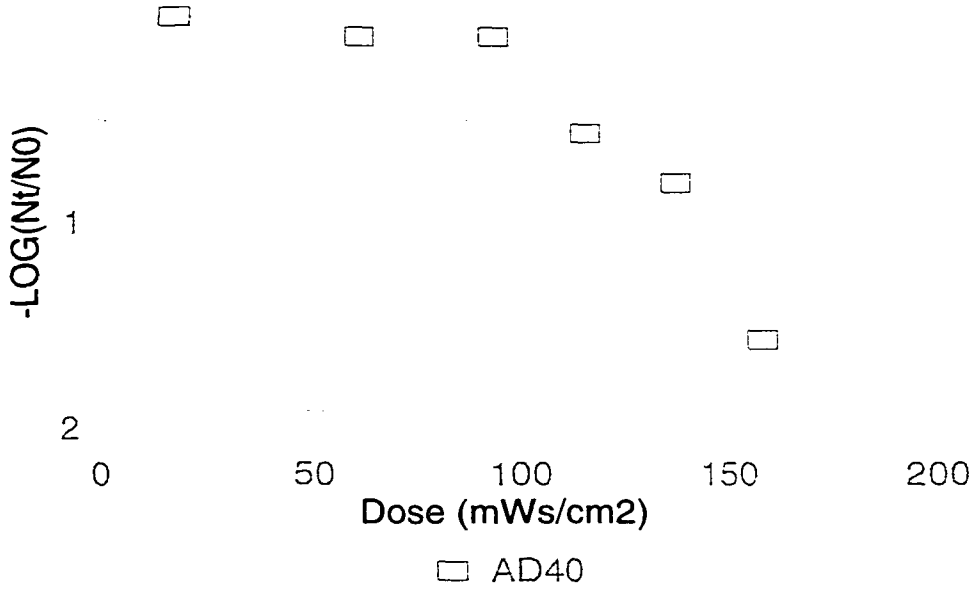


Figure 3.B Shouldering effect of AD40 at low doses.



APPENDIX C:

CHLORINE INACTIVATION OF DISPERSED ADENOVIRUS TYPE 40 AND FELINE

CALICIVIRUS

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Short title: Chlorine inactivation of adenovirus type 40 and feline calicivirus

Key words: Enteric adenovirus, calicivirus, chlorine, disinfection, feline calicivirus

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Abstract

The free chlorine inactivation kinetics of chloroform extracted (dispersed) and non-chloroform extracted (aggregated) feline calicivirus (FCV), adenovirus type 40 (AD40) and poliovirus type 1 (PV-1) in two water types at high and low pH and temperature conditions, were determined. Ct values for each experimental condition were generally higher at pH 8 than pH 6 and for AD40 compared to FCV, although both viruses were very sensitive to free chlorine compared to PV-1. FCV and AD40 were inactivated to ≥4.34 and ≥2.54- Logs at a free chlorine dose of 0.5 mg/L in buffered demand-free (BDF) water at pH 6 and 5°C by 15 s, whereas 4.04- Logs of PV-1 was inactivated by 10 min. At pH 8, FCV was inactivated by >4-Log by 3.5 min and ≥2.5-Logs of AD40 was inactivated by 15 s. FCV and PV-1 were much more resistant to free chlorine inactivation in the aggregated state compared to the dispersed state. The Ct for a 3-Log inactivation at pH 7, 5°C and approximately 1.0 mg/L free chlorine for aggregated FCV and PV-1 were 6.37 and 3.81 $mg/L_x min.$, respectively. The Ct for 2- Log inactivation of FCV and AD40 in treated ground water at 15 °C were 1.2 and 15.1 times greater than Ct values for experiments conducted in BDF water at 5°C. Tailing and low disinfectant decay rates for AD40 disinfected in treated ground water suggest that additional aggregation or clumping may have occurred. If this is the case, aggregated AD40 may be more resistant to free chlorine than the other two viruses studied. The results of this study suggest that conventional drinking water disinfection is sufficient for the inactivation of the studied viruses. However, if treatment fails or if these aggregated forms are able to enter the distribution system post-treatment, where contact time or

disinfectant concentration may not be adequate, there may be an increased risk of exposure of infectious viral pathogens.

Introduction

Disinfection is an important treatment barrier between drinking water consumers and viral gastroenteritis since the small size (~25-80 nm) of enteric viruses may permit their escape from filtration processes (7). Chlorination is the most widely applied disinfectant in the United States for drinking and wastewater disinfection (1). The effectiveness of chlorine for the inactivation of enteric viruses such as poliovirus has been well-studied (15). However, considerably less information is available on the effectiveness of chlorine for inactivation of increasingly important enteric viruses such as adenovirus and Norwalk virus.

Members of the human calicivirus genus, "Norwalk-like viruses" (NLVs), are an important cause of waterborne outbreaks affecting adults and children in the United States (8). Like NLVs, the enteric adenoviruses, enteric adenovirus 40 (AD40) and 41 (AD41), are also important causes of self-limiting, acute gastroenteritis (8, 11). Following NLVs and rotavirus, enteric adenoviruses are the next most important cause of childhood (<4 yrs) gastroenteritis (11, 13) . Both the enteric adenoviruses and NLVs are shed in high numbers in the feces and prolonged shedding is typical (8, 11). Although no drinking waterborne outbreaks have been reported for the enteric adenoviruses, increased environmental stability compared to other enteric viruses and their presence in sewage

and surface waters has been reported (6, 18, 23). Waterborne outbreaks of pharyngoconjuctivitis from swimming have been reported for non-enteric adenoviruses (5, 17).

Both caliciviruses and enteric adenoviruses are on the United States Environmental Protection Agency's (USEPA) Drinking Water Contaminant Candidate List (CCL) (25). These viruses are on the CCL for regulatory consideration since little to no information regarding health, drinking and wastewater treatment or analytical methodologies is currently available. The objectives of this study were to determine the inactivation kinetics of dispersed AD40 and feline calicivirus (FCV) in two water types, buffered-demand-free (BDF) and treated ground water after exposure to free chlorine. Also, since it has been reported that most viruses are aggregated or associated with organic matter in surface and waste waters (22), chlorine disinfection experiments were also conducted with virus stocks of FCV and PV-1 that did not undergo chloroform extraction. Chloroform and other organic solvents used for virus purification have been shown to yield a virus preparation with a very high proportion (95 %) of single particles (Floyd and Sharp, 1975; Sobsey, personal communication). Therefore, the virus preparations which were chloroform extracted are referred to as "dispersed" and those that were not chloroform extracted are termed "aggregated". Since there are currently no known animal or mammalian cell culture systems which can determine infectivity of NLVs, a closely related surrogate, FCV, was used in the current study as a model for NLV inactivation by free chlorine. FCV has been suggested to be an adequate surrogate for

NLV inactivation since it has similar genome organization and capsid architecture (3, 21). In order to determine Ct values for each virus and experimental condition, the Efficiency Factor Hom (EFA) model was applied to the observed bench-scale results in order to determine the Ct for 2, 3- and 4- Log inactivation.

Methods

Virus propagation and assay

AD40 (strain Dugan), FCV (strain F9), primary liver carcinoma cell line (PLC/PRF/5) and Crandell feline kidney (CRFK) cell line were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). PV-1 (LSc-2ab) and the Buffalo Green kidney Monkey (BGM) cell line was acquired from available stocks of the Environmental Microbiology Laboratory of Dr. Charles P. Gerba at the University of Arizona. AD40, FCV and PV-1 were propagated and assayed in the same manner. Cell lines used for propagation of AD40, FCV and PV-1 were PLC/PRF/5, CRFK and BGM cell lines, respectively (3, 4, 9). Maintenance media (Eagle's minimal essential medium containing 10 % fetal bovine serum) was decanted from 162 cm^2 tissue culture flasks containing complete cell monolayers. The flasks were rinsed with TRIS buffered saline (Trizma base) (Sigma Chemical Co., St. Louis, MO) and 1 ml of approximately 10^4-10^5 most probable number (MPN)/ml of the viral stock was inoculated onto the monolayer. In order to allow virus attachment, the flasks were incubated for 1 hr at room temperature. The flasks were rocked every 15 min throughout the 1 hr incubation. After incubation, 50 ml of maintenance medium (without fetal bovine serum) was inoculated onto the infected cell monolayer and incubated at 37 °C until destruction of at least 90 % of the cell

monolayer occurred. The cell culture flasks containing the propagated virus were frozen and thawed. One freeze-thaw step for AD40 and three freeze-thaw steps for FCV and PV-1 were conducted. Following freeze-thawing of the supernatant, centrifugation at 4°C and 10,000 x g was performed in order to remove cell debris.

Further purification and concentration of the studied viruses was accomplished by performing two successive polyethylene glycol (PEG) precipitation procedures. Briefly, for every 100 mL volume of viral supernatant 9 g of PEG (MW 8,000) and 5.8 g NaCl were added and stirred overnight at 4 °C. Centrifugation at 10,000 x g at 4 °C for 30 min was performed followed by disposal of the supernatant and resuspension of the pellet in BDF water. BDF water was prepared by dissolving 0.54 g Na_2HPO_4 (anhydrous) and 0.88 g KH_2PO_4 (anhydrous) per L of deionized, disinfectant demand-free water (Nanopure RO purifier, Barnstead, Dubuque, IA). The pH was adjusted with 1 M NaOH or 1 M KH_2PO_4. BDF water was stored in disinfectant demand-free bottles at 4 °C until use. For dispersion of the viral stocks, equal volumes of the viral extracts and chloroform were homogenized by vortexing the mixture for at least 10 min. Once homogenized, the chloroform-viral suspension was centrifuged at 10,000 x g at 4 °C for 15 min. After centrifugation, the upper layer containing the purified viral stock was collected. For the aggregated viral stocks, no chloroform extraction was performed. The viral stocks were stored at 4°C.

Determination of viral titer before and after chlorine disinfection was accomplished by

plating 5- or 10-fold dilutions, in quadruplet, in a 24-well tissue culture tray with the appropriate cells in suspension. The concentration of the viruses was determined by observation of each infected well for cytopathic effect (CPE). Observation for CPE was continued for up to 12 days for FCV and PV-1 and 24 days for AD40. Second passage was performed for several FCV assays. The Most Probable Number (MPN) General Purpose Program adapted from Hurley and Roscoe (1983) was used to determine the concentration (MPN/mL) of AD40, FCV, and PV-1.

Reagents and glassware

Initially, all glassware was soaked overnight in approximately 50 mg/L chlorine solution followed by rinsing with demand-free water and baking in an oven for at least 2 hr at 200°C. After this initial treatment, only soaking in chlorine and rinsing in demand free water was performed for all glassware. Reagent grade chlorine (5 % sodium hypochlorite; J.T. Baker Co., Phillipsburg, NJ) was diluted in chlorine demand-free water to prepare a stock solution of approximately 150 mg/L.

Test waters

All disinfection experiments were conducted in either buffered demand free (BDF) or treated ground water. Treated ground water was obtained from Cincinnati Water Works where conventional drinking water treatment (coagulation, filtration and chlorination) was applied. The pH of the treated ground waters, between 8.0 and 8.2, was not adjusted. Furthermore, turbidity (Nephelomentric Turbidity Units) and total organic carbon (mg/L)

are 0.05-0.30 and 0.7-1.0, respectively. Dechlorination of these waters was accomplished by rapid mixing and exposure to ultraviolet light until no chlorine was detected by application of the N, N diethyl-p-phenylenediamine (DPD) method using a Hach DR2000 direct reading spectrophotometer (Hach, Loveland, CO).

Experimental protocol

Bench-scale disinfection experiments were performed using chlorine-demand free glass beakers containing 50 mL of virus diluted to the appropriate concentration in BDF or treated ground water at the appropriate temperature (5°C or 15 °C). All beakers were placed in a refrigerated water bath in order to maintain the test temperature throughout the disinfection experiment. Immediately prior to each experiment, the free chlorine concentration of the stock solution was measured by the DPD method. Once the free chlorine stock concentration was known, the volume necessary to achieve the desired final free chlorine concentration in 50 mL of test water was calculated. The exact concentration of the free chlorine dose was determined for each experiment. One reaction beaker containing only BDF water was inoculated with free chlorine and measurements of the free chlorine residual at 15s were determined using the DPD method. Two other reaction beakers, containing the appropriate volume of virus stock in order to achieve 2- and 4- Log inactivation of AD40 and FCV in 50 mL of test water, were inoculated with the appropriate volume of the free chlorine stock solution and immediately stirred. One beaker was sampled in order to determine the free chlorine concentrations at the beginning and end of each disinfection reaction. In order to

determine viral inactivation by free chlorine, 2 mL samples were taken from the second beaker at predetermined times throughout the reaction. Residual chlorine was immediately quenched by placing the 2 mL samples into collection tubes containing 20 μL of a sterile 10 % sodium thiosulfate solution. In addition, a control beaker containing only virus and test water was sampled in order to determine the virus concentration and if virus inactivation occurred under the tested pH and temperature conditions. All viral samples were kept on ice during the experiment and then stored at 4 °C until viral assay.

Kinetic modeling

Chlorine decay constants, k', for each experiment were calculated using the Solver function in Microsoft Excel 2000 (Microsoft Corp.) to regress the first-order kinetic equation (Equation 1) using the least-squares method:

$$C = C_0 \exp(-k't) \qquad (1)$$

where C and C_0 are the disinfectant residual (mg/L) at time t and time zero, respectively; and k' is the first-order disinfectant decay rate constant (min^{-1}). The MPN values for each experiment, grouped by virus type and pH and temperature conditions, were fit into both the Chick-Watson and the Efficiency Factor Hom (EFH) model, Equations 2 and 3, respectively;

$$\text{Ln } N/N_0 = -k/k'n \times (C_0^n - C_f^n) \qquad (2)$$

$$\text{Ln } N/N_0 = -kC_0t^m \times [(1-\exp(-nk't/m)/ (nk't/m)] \qquad (3)$$

where k is the inactivation rate constant, n is the coefficient of dilution and m is the constant for the inactivation rate law which describes deviation from ideal Chick-Watson

kinetics (10). Ln N/N_0 is the natural Log of the survival ratio (number of viruses remaining at time t divided by the number at time zero). Microsoft Excel Solver (Microsoft Excel 2000, Microsoft Corp.) was used to minimize the sum of squares of the difference between the observed and predicted Ln N/N0 for viral disinfection experiments performed with the same virus and conditions, in order to determine the values for each model's coefficients. Inactivation curves, -Log (Nt/N0) (Log inactivation) versus time (min) were created using Microsoft Excel in order to compare observed and predicted Log inactivation values. The observed curve's plotted values consist of the average Log inactivation value of replicate bench-scale experiments versus sampling time.

Ct values

Generation of Ct values, for each virus and set of conditions, were accomplished by application of the EFH model coefficients and the k' equal to 0.0001 (conditions of negligible disinfectant decay). The EFH model was chosen over the Chick-Watson model since fit the bench-scale values more closely (lowest SSQ values) for all free chlorine experiments performed. The Ct value is the concentration (mg/L) of the disinfectant multiplied by the time (min) when a specific Log inactivation (2, 3 or 4-Logs) occurred. Only free chlorine concentrations similar to what was applied in the bench-scale experiments were used in determination of the Ct values. Free chlorine concentrations used which were considerably higher or lower than what was applied in the bench scale experiments produced unreasonable Ct values.

Results

Thirty one free chlorine disinfection reactions, involving AD40, FCV and PV-1, were conducted with different free chlorine concentrations, temperature, pH and water quality conditions. In addition, free chlorine disinfection reactions were conducted for comparison of the inactivation rates of aggregated and dispersed FCV and PV-1 particles. Four Log inactivation was attempted for all experiments involving FCV and PV-1. However, due to difficulties in propagation and purification of AD40, only 2-Log inactivation was attempted for experiments conducted in BDF and ground water and no 15°C or aggregated experiments were performed.

The detection limit was observed at the initial sampling time of 15 s for experiments in which applied free chlorine doses ranged from 0.51- 0.54 mg/L at pH 6 (Table 1.C). Disinfection experiments conducted in BDF water at pH 6 and 5°C resulted in ≥4.34 and ≥2.54- Logs reduction for dispersed FCV and AD40, respectively. At pH 8, FCV was reduced by > 4-Logs between 15s and 3.5 min and AD40 by ≥2.54-Log by 15 s. Furthermore, experiments conducted with dispersed FCV suspended in treated ground water resulted in ≥4.85-Logs reduction by 15 s.

Both the Chick-Watson (not shown here) and the EFH models were used to predict chlorine inactivation kinetics for every experimental condition applied to each virus. In all cases, the EFH model produced the lowest sum of squares (SSQ) value between predicted and observed values compared to the Chick-Watson model, therefore, the EFH

model was used for the analysis of all disinfection experiments. Table 2.C lists the parameter estimates for the EFH model analysis and the number total of experiments conducted for each condition. The SSQ values are an indication of how well the EFH model's predicted curves fit the curves generated using the observed values. A high SSQ value indicates that the EFH model's predicted curves do not coincide well with the curves created with values from the bench-scale experiments. A high SSQ value may be caused by variation in the inactivation kinetics between experimental replicates, resulting in the predicted curves lying in-between the observed replicate curves. The highest sum of squares values include experiments conducted with dispersed AD40 at pH 8 and 5°C, aggregated PV-1 at pH 6 and 7, and dispersed FCV suspended in treated ground water. The lowest sum of squares values were calculated for dispersed FCV reactions conducted at 5 °C at pH 6 and 8. The conditions with the highest disinfectant decay rates include dispersed AD40 and FCV experiments conducted at pH 6 and all experiments conducted in treated ground water. High k' values indicate that the disinfectant concentration decreased throughout the duration of the experiment, whereas a low k' indicates minimal disinfectant decay occurred. For all experimental conditions, the predicted and observed inactivation curves are shown in Figures 1.C-8.C.

The EFH model was also used to calculate Ct values for disinfectant decay-free conditions (k' = 0.0001) where only free chlorine concentrations similar to the concentrations applied in the bench-scale experiments were used (Tables 3.C-4.C). Ct values calculated with free chlorine concentrations, other than what was applied in the

actual experiments, created ambiguous values. For example, using a free chlorine concentration of 1 mg/L for AD40 inactivation at pH 6 and 5 °C generates a Ct value of 0.23 mg/L×min, however, ≥2.54-Logs was reduced by 15 s by a free chlorine dose of 0.5 mg/L.

Due to the rapid inactivation rate of dispersed FCV at 15 °C, no Ct values could be determined for experiments conducted at pH 6. However, Ct for experiments conducted at pH 8 and 15 °C were determined. Ct values for 3- and 4-Log inactivation for dispersed FCV at 15 °C, pH 8 and a free chlorine dose of 0.15 mg/L were calculated to be 0.08 and 0.10 mg/L×min., respectively. For experiments conducted at 5 °C, dispersed AD40 appears to be more resistant to inactivation by chlorine than dispersed FCV at pH 6 and 8 (Table 3.C). Differences in Ct values between the two viruses are greatest for 3- and 4-Log inactivation . For example, Ct values calculated for 3-Log inactivation at pH 6 are 0.05 and 0.14 mg/L×min for FCV and AD40, respectively. Table 4.C describes the differences in Ct values between dispersed and aggregated PV-1. Aggregated PV-1 has higher Ct values than those calculated for dispersed PV-1. On average, aggregated PV-1 had about 2.7-fold slower inactivation kinetics than dispersed PV-1. Similarly, experiments conducted with aggregated FCV (pH 7, 5°C and a dose of 1.0 mg/L free chlorine) produced a Ct value that is approximately 27.7 times higher for 3-Log inactivation than the Ct value calculated for dispersed FCV experiments conducted at pH 8, 5°C and a free chlorine dose of 0.15 mg/L (Table 5). In comparison to aggregated PV-1, aggregated FCV had about 1.7-fold slower inactivation kinetics, where the Ct values

for 3-Log reduction of PV-1 and FCV were 3.81 and 6.37 mg/L×min, respectively. Ct values for disinfection experiments conducted with dispersed FCV and AD40 suspended in treated ground water were 0.21 and 1.51 mg/L×min, respectively (Table 6.C). Compared to experiments conducted in BDF water, the Ct values were 1.17 and 15.1 times higher for FCV (similar conditions) and AD40 (free chlorine dose of 0.5 mg/L) in treated groundwater, respectively.

Inactivation curves, Log inactivation versus time, for all experiments which were analyzed by the EFH model are represented in Figures 1.C-8.C. These figures include the average Log inactivation (if multiple reactions conducted) calculated for every sampling time throughout each experiment (observed inactivation curves). Inactivation curves predicted by application of the EFH model (predicted inactivation curves) are also represented. Figures 1.C and 2.C represent the inactivation kinetics of the chlorine disinfection reactions conducted with dispersed FCV suspended in BDF water at 5 and 15 °C and pH 6 and 8. For experiments conducted at 5 °C, dispersed FCV was inactivated by an average of 3.53-Logs by 45 s and 1.33-Logs by 1 min at pH 6 and 8, respectively. Experiments conducted at pH 8 produced a shoulder curve whereas the pH 6 curve appears to tail-off before complete inactivation at 1 min. Free chlorine inactivation experiments conducted with FCV at 15 °C had higher inactivation rates at pH 6 compared to pH 8 (Figure 2.C). Inactivation rates were much higher for experiments conducted at 15 °C compared to 5°C, since ≥3.81-Logs was inactivated by 45 s for both pH conditions. Due to this high inactivation rate, conclusions regarding the shape of the inactivation

curves are difficult, however they appear to be linear in nature.

Figure 3.C represents predicted and observed chlorine inactivation curves of dispersed AD40 suspended in BDF water at pH 6 and 8, free chlorine dose of 0.17 mg/L and 5 °C. Experiments conducted at pH 6 had greater inactivation rates than those carried out at pH 8. For example, AD40 was inactivated by and average of 2.48- and 1.86-Logs at 30 s for experiments conducted at pH 6 and 8. Both inactivation curves displayed tailing although this may be explained by the high disinfectant decay rates observed for all experiments.

Figures 4.C and 5.C describe the inactivation kinetics of chlorine inactivation of dispersed FCV and AD40 suspended in treated ground water at pH 8-8.2 and 15 °C. For both viruses, the inactivation rates are lower for the experiments conducted in treated ground water at 15 °C compared to similar experiments conducted in BDF water at 5 °C. This is especially true for the experiments conducted with AD40, since a free chlorine dose of 0.48 mg/L was applied. Additionally, tailing of the inactivation curves is pronounced, especially for the experiment conducted with FCV. FCV experiments had a high average disinfectant decay rate compared to all experiments conducted in BDF water. In addition, the average disinfectant decay rate for AD40 was lower than those calculated for BDF water experiments.

Chlorine disinfection reactions were also conducted with aggregated and dispersed PV-1 suspended in BDF water at a free chlorine dose of 0.50 mg/L, pH 6 and 5 °C (Figure 6.C).

As expected, PV-1 was more resistant in the aggregated state. At 10 min, an average of 4.07-Logs and 2.54-Logs inactivation was observed for dispersed and aggregated PV-1, respectively. Under these conditions, it takes over 4.5 times the time to inactivate 4-Logs aggregated PV-1 compared to dispersed PV-1 particles. One additional aggregated PV-1 experiment was conducted for comparison to experiments conducted with aggregated FCV (Figure 7.C). All experiments were carried out in BDF water at pH 7 and 5 °C. Free chlorine doses for FCV and PV-1 averaged 1.03 mg/L and 0.93 mg/L, respectively. Comparison of aggregated FCV and PV-1 inactivation curves indicates that FCV may be more resistant than PV-1 in an aggregated state. Tailing of the inactivation curves is not likely caused by disinfectant decay rates since the k' values for all reactions conducted with aggregated FCV and PV-1 were low (Table 2.C).

A comparison of the predicted chlorine inactivation rates for experiments conducted with dispersed PV1, AD40 and FCV particles suspended in BDF water at pH 6 and 5 °C are illustrated in Figure 8.C. PV-1 is the most resistant to chlorine inactivation, even at the higher free chlorine dose of 0.5 mg/L, followed by AD40 and FCV at lower free chlorine doses of 0.17 mg/L and 0.15 mg/L, respectively.

Second passage of FCV cell assays did not result in lower inactivation rates, i.e., the titer did not increase, therefore only one cell passage was conducted. However, second passages and allowing AD40 cell assays to incubate for 24 days did produce an increase in viral titer. In addition, since allowing the AD40 assays to incubate for 24 days

appeared to be more sensitive than second passage, it was conducted for the majority of assays.

Discussion

The results of this study indicate that both dispersed AD40 and FCV are considerably less resistant to free chlorine inactivation than dispersed PV-1. FCV and AD40 were inactivated in BDF water by >4.0- and ≥2.5-Logs within 15 s at pH 6 after doses ranging from 0.51-0.54 mg/L of free chlorine were applied. In addition, at pH 8 > 4-Logs of FCV and ≥2.54-Logs of AD40 were inactivated by 3.5 min and 15 s, respectively. In the United States, the reported average free chlorine residual is approximately 1.0 mg/L and the average and median contact times are 237 and 60 min, respectively (1, 26). Under these disinfection practices, both dispersed FCV and AD40 would be rapidly inactivated to > 4.0- and > 2.5-Logs, respectively.

Early work reported for chlorine disinfection at 4 and 25 °C of the closely related adenovirus, adenovirus type 3 (AD3), also demonstrated a high susceptibility to free chlorine (2). For experiments conducted at 4 °C, 3-Logs were inactivated by 0.13 to 0.42 min. and 1.3 to 3 min. at pH 6 and 8; respectively, with a free chlorine dose ranging from 0.1 to 0.20 mg/L. Similar to the current study, Clarke *et al.* (1956) concluded that inactivation times for AD3 were higher for high pH and low temperature conditions. Another study conducted by Liu *et al.*, (1971) demonstrated that differences in chlorine resistance exist amongst adenovirus strains, where 4 Log inactivation for adenovirus

types 3, 7a and 12 was observed at 4.8, 12.5 and 13.5 min., respectively (15, 16). All experiments were carried out in potable water at a free chlorine dose of 0.5 mg/L, 2°C, and pH 7.8. Differences in virus preparation between the two studies may have caused the reported differences in AD3 resistance to chlorine inactivation. Information on the viral preparation for the Liu *et al.*, (1971) was not provided (15).

Shin *et al.*, (1998) reported the decreased chlorine resistance of Norwalk virus compared to PV-1. The ability of reverse transcriptase polymerase chain reaction (RT-PCR) to amplify viral nucleic acids was used by these authors as a measure of viral infectivity. Norwalk virus units per ml were inactivated by 30 min at an initial dose of 1.0 mg/L at pH 6 and 5 °C. However, the loss of infectivity may have been sooner than indicated since comparable cell culture infectivity studies demonstrated that the RT-PCR method over-estimated the time for viral inactivation. For example, 4-Logs inactivation of PV-1 at a chlorine dose of 1.0 mg/L at pH 6 and 5 °C occurred at 10 and 30 min for the cell culture infectivity and RT-PCR methods, respectively (20). If FCV is an adequate model for chlorine inactivation of Norwalk virus, the two studies provide evidence that dispersed Norwalk virus is not more resistant than PV-1 and current drinking water disinfection practices are adequate for their inactivation. However, a study conducted by Keswick *et al.*, (1985) suggested that Norwalk virus is extremely resistant to chlorine inactivation. In this study, a dose of 3.75 mg/L failed to inactivate this virus, causing 5 of 8 volunteers to seroconvert to Norwalk virus and 4 exhibited illness (12).

Sobsey *et al.*, (1991) suggested that Ct values should be based on inactivation studies conducted with aggregated or cell-associated viruses. Since viral clumps are formed within the host cell and are not dispersed upon release in the feces, viruses which occur in water are most likely associated with organic debris, are aggregated or clumped (24). In the work performed by Sobsey *et al.*, (1991) hepatitis A virus (HAV) was more resistant to free chlorine when no dispersion or purification of the viral stock was performed in comparison to purified and dispersed viral preparations. In the current study, inactivation curves of PV-1 and FCV that lacked the chloroform extraction procedure in their preparation, displayed tailing and a low disinfectant decay values which indicates aggregation of the viral suspensions. The aggregated viral preparations were more resistant to free chlorine inactivation than stocks which where dispersed by chloroform extraction. Surprisingly, FCV appears to be more resistant to inactivation by free chlorine than PV-1 in the aggregated state. Aggregated FCV and PV-1 subjected to a free chlorine dose of 1 mg/L at pH 7 and 5 °C had Ct values for 3-Log inactivation of 6.37 and 3.81, respectively. Furthermore, a low Ct value of 0.23 was calculated for dispersed FCV experiments in BDF water at pH 8 and a lower chlorine dose of 0.15 mg/L, resulting in an inactivation rate over 27.7 times slower for aggregated compared to dispersed FCV. If FCV is an adequate model for Norwalk virus, the results reported by Keswick *et al.*, (1985) may not be surprising. No viral purification or dispersion of the Norwalk virus stock (derived from feces) was performed in that work, therefore the viral inoculant was most likely aggregated and associated with cell debris or other organic matter. Aggregation and association with organic matter may serve to shield the virus from

disinfectant exposure (22, 24). In another study performed with an FCV stock that lacked purification procedures, only 1.55-Logs was inactivated after a 10 min. contact time and a chlorine dose of 0.22 mg/L (19). Due to the effects of aggregation and association with organic debris, viruses are more likely to survive drinking water disinfection than dispersed viral particles. Moreover, viruses are usually aggregated when released from infected cells and the size range (0.5-1.0 μm) of some of these aggregates are not effectively removed by filtration processes (14).

Several experiments were conducted with dispersed FCV and AD40 suspended in treated ground water samples. Like experiments conducted with aggregated viral suspensions, the inactivation curves exhibited tailing. The tailing effect may be produced due to viral aggregation, decrease of disinfectant concentration or conformational change of the viral capsid (24). Since the disinfectant decay constants for experiments conducted with FCV in treated ground water are higher than those calculated for experiments conducted in BDF water, the tailing effect may be due to a decrease in disinfectant concentration throughout the experiment. However, for experiments conducted with AD40, the k' value calculated for experiments conducted in treated ground water were lower than the BDF water values, therefore disinfection decay may not be the primary cause of the tailing effect. Furthermore, the AD40 experiment performed in treated ground water displayed much slower inactivation kinetics at a higher free chlorine dose compared to those experiments performed in BDF water. Groundwater constituents may have afforded AD40 increased free chlorine resistance since shifts in pH or salt concentration

can cause aggregation and conformational changes in the viral capsid (24). If aggregation is the cause for the increased chlorine resistance, AD40 may be more resistant in this state over either PV-1 or FCV.

EFH model constants, k, *n*, and *m*, were determined by applying the model to results acquired through bench-scale disinfection experiments. The constants are assumed to be characteristic for the inactivation kinetics of a particular virus under specific pH and temperature conditions. These constants may then be used for calculating Ct values for individual viruses and specific conditions. The predicted curves fit the observed curves well for the first 2- to 3-Logs of inactivation for each studied virus. Tailing of the predicted curves generated very high Ct values, especially for experiments conducted with aggregated viral preparations and treated ground water. Due to model parameters generated, determination of Ct values with free chlorine concentrations other than what was applied in the actual experiments created questionable values. Due to the complicated inactivation kinetics of the tested viruses, the constants determined by the EFH model may not accurately predict Ct values for free chlorine concentrations other than what was used in the actual experiments.

Conclusions

In the current study, information on the free chlorine resistance of dispersed AD40, FCV and PV-1 and aggregated FCV and PV-1 subjected to varying pH and temperature conditions was obtained. The results of this study indicate that dispersed FCV, AD40 and

PV-1 particles would be inactivated by commonly used concentrations of free chlorine and contact times applied for drinking water treatment. In comparison, however, experiments conducted with aggregated virus had approximately 3- and >28-fold slower inactivation kinetics for dispersed PV-1 and FCV, respectively. Consequently, if treatment fails or if these aggregated forms are able to enter the distribution system post-treatment, where contact time or disinfectant concentration may not be adequate, there may be an increased risk of exposure of infectious viral pathogens.

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Table 1.C Free chlorine inactivation experiments in which the detection limit was observed.

Experimental Conditions	AD40 Free Chlorine Dose (mg/L)	AD40 $-Log_{10}$ (Nt/N0) at 15 s	FCV Free Chlorine Dose (mg/L)	FCV $-Log_{10}$ (Nt/N0)/ at time (s)
pH 6 BDF Water 5 °C	0.52	≥2.54	0.51	≥4.74/15 s
pH 8 BDF Water 5°C	0.52 0.52	≥2.54 ≥2.54	0.50	>4.00/ between 15 s and 3.5 min
pH 8-8.2 Treated Ground Water 15 °C	NA[1]	NA	0.54 0.54	≥4.85/ 15 s ≥4.85/ 15 s

[1] NA = not applicable

Table 2.C Summary of parameter estimates for EFH model.

Experimental conditions	Virus	# of Experiments	k'[1] (min^{-1})	k	n	m	SSQ[2]
Dispersed virus pH 6 5°C BDF water	AD40	3	2.86	7.28	0.01	0.31	4.29
	FCV	2	0.18	11.18	0.08	0.28	2.76
	PV-1	2	0.04	3.37	-0.12	0.36	8.20
Dispersed virus pH 8 5°C BDF water	AD40	3	0.42	20.27	0.71	0.38	11.93
	FCV	2	0.03	6.43	0.36	1.74	0.86
Dispersed virus pH 8 15 °C BDF water	FCV	2	0.01	117.85	1.10	1.27	1.27
Aggregated virus pH 7 5°C BDF water	FCV	2	0.05	4.26	-0.21	0.26	5.51
	PV-1	1	0.01	4.96	0.51	0.23	28.20
Aggregated virus pH 6 5°C BDF water	PV1	2	0.02	3.52	0.50	0.38	25.13
Dispersed virus pH 8-8.2 15 °C Treated ground water	AD40	2	0.15	3.09	-0.23	0.22	2.96
	FCV	2	0.24	16.60	0.76	0.43	18.05

[1] Average k' value
[2] Sum of squares

Table 3.C Ct values for dispersed FCV and AD40 in BDF water at 5°C.

	-Log $_{10}$ Inactivation **Free chlorine = 0.15 mg/L**	**Ct (mg/L x min)** **FCV[1]**	**Ct (mg/L x min)** **AD40[1]**
pH 6	2	0.01	0.04
	3	0.05	0.14
	4	0.13	0.34[2]
pH 8	2	0.18	0.10
	3	0.23	0.31[2]
	4	0.27[2]	0.65[2]

[1] Ct predicted values based on no chlorine decay conditions
[2] Amount of inactivation not achieved in actual experiments

Table 4.C Ct values for aggregated and dispersed PV-1 at pH 6 and 5°C.

-Log$_{10}$ Inactivation **Free chlorine = 0.5 mg/L**	**Ct (mg/L x min)** **PV-1[1] Dispersed**	**Ct (mg/L x min)** **PV-1[1]Aggregated**
2	0.93	2.58
3	2.86	7.60
4	6.35	16.36[2]

[1]Ct predicted values based on no chlorine decay conditions
[2] Amount of inactivation not achieved in actual experiments

Table 5.C Ct values for aggregated FCV and PV-1 at 5°C, pH 7.

-Log_{10} Inactivation **Free chlorine = 1.0 mg/L**	**Ct (mg/L x min)** **PV-1[1]**	**Ct (mg/L x min)** **FCV[1]**
2	1.04	NA
3	3.81	6.37
4	9.57	19.12[2]

[1]Ct predicted values based on no chlorine decay conditions
[2] Amount of inactivation not achieved in actual experiments

Table 6.C Ct values for FCV and AD40 in treated ground water at 5°C and pH 8-8.2.

-Log_{10} Inactivation	**Ct (mg/L x min)** **FCV[1]** **Free chlorine = 0.15 mg/L**	**Ct (mg/L x min)** **AD40[1]** **Free chlorine = 0.50 mg/L**
2	0.21	1.51
3	0.56	9.69[2]
4	1.10[2]	36.09[2]

[1]Ct predicted values based on no chlorine decay conditions
[2] Amount of inactivation not achieved in actual experiments

Figure 1.C Predicted and observed inactivation curves of dispersed FCV in BDF water; 0.15 mg/L free chlorine and 5 °C (arrow indicates detection limit for assay).

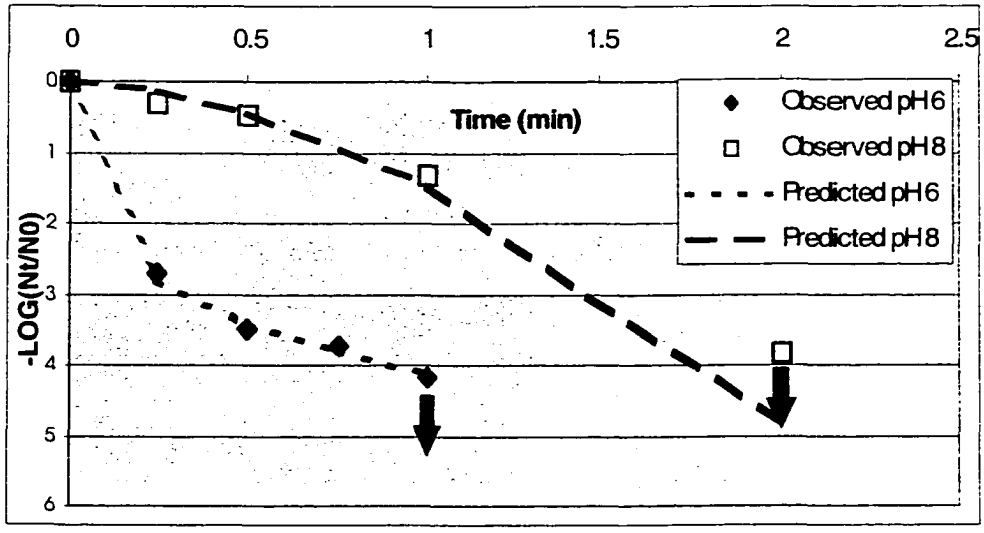


Figure 2.C Predicted and observed inactivation curves for dispersed FCV in BDF water; 0.16 mg/L free chlorine and 15 °C (arrow indicates detection limit of assay).

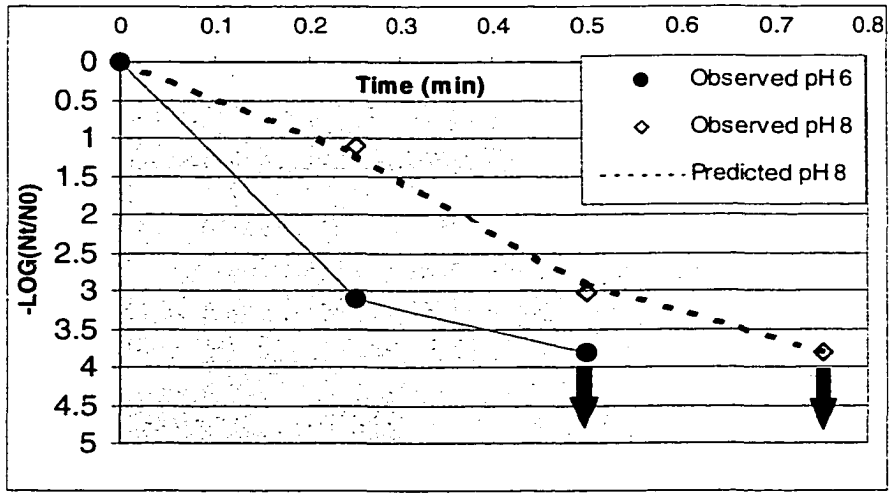


Figure 3.C Predicted and observed inactivation curves of dispersed AD40 in BDF water; 0.17 mg/L free chlorine and 5 °C (arrow indicates detection limit reached).

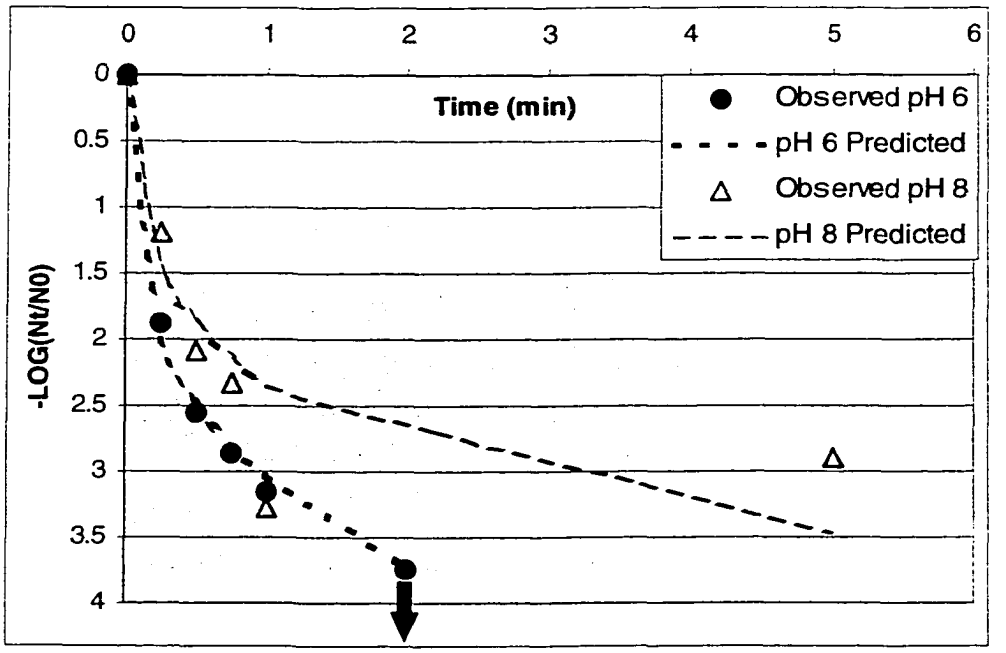


Figure 4.C Predicted and observed inactivation curves of dispersed FCV in treated groundwater; 0.18 mg/L free chlorine, 15 °C and pH 8-8.2.

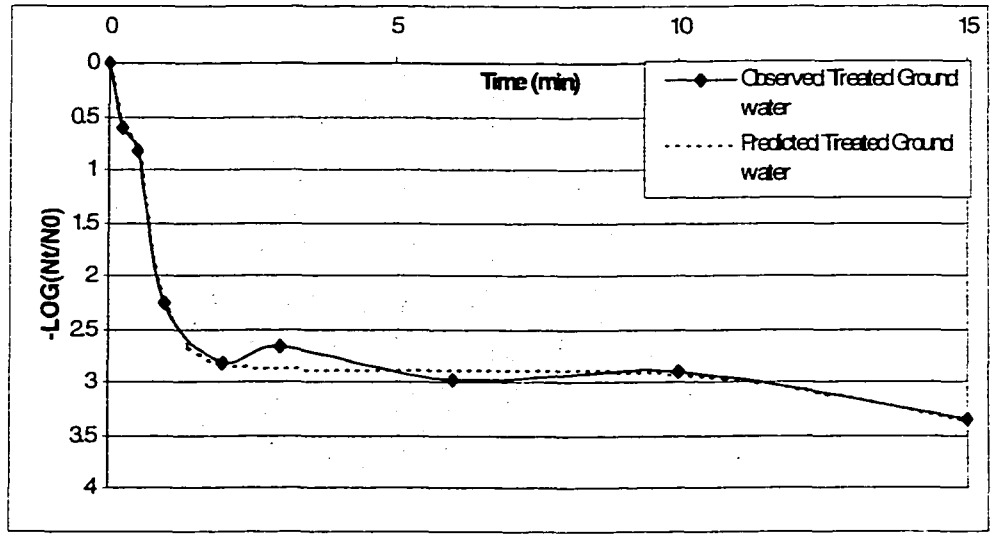


Figure 5.C Comparison of predicted and observed inactivation curves of dispersed AD40 in treated ground water; 0.48 mg/L free chlorine, 15 °C and pH 8-8.2.

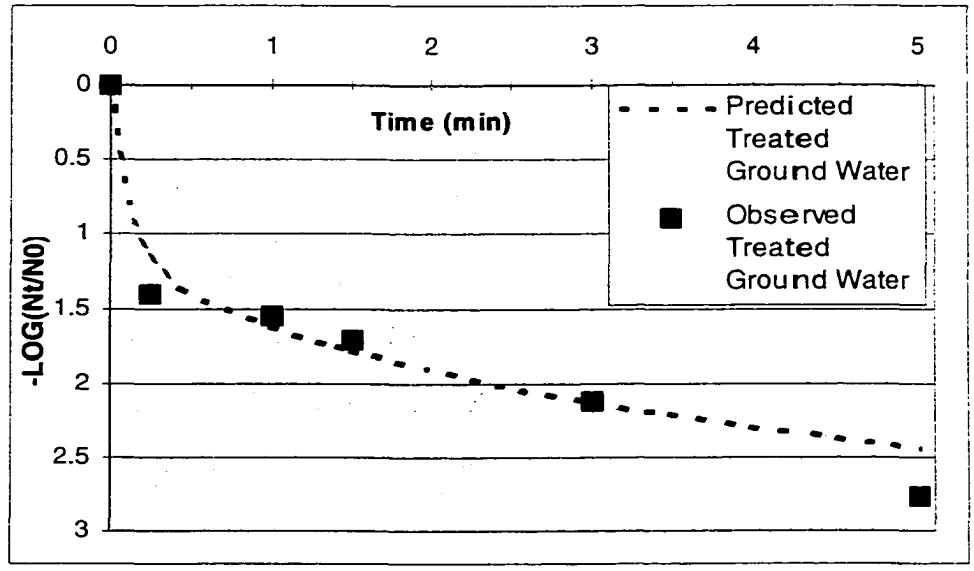


Figure 6.C Comparison of observed and predicted inactivation curves of aggregated and dispersed PV1 particles in BDF water; 0.5 mg/L free chlorine, pH 6 and 5 °C.

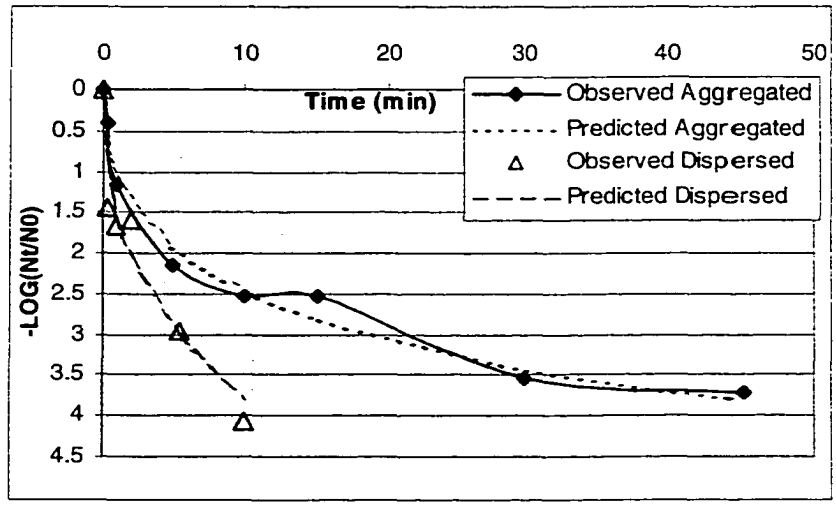


Figure 7.C Comparison of observed and predicted inactivation curves of aggregated FCV and PV1 particles suspended in BDF water; ~1 mg/L, pH 7 and 5 °C.

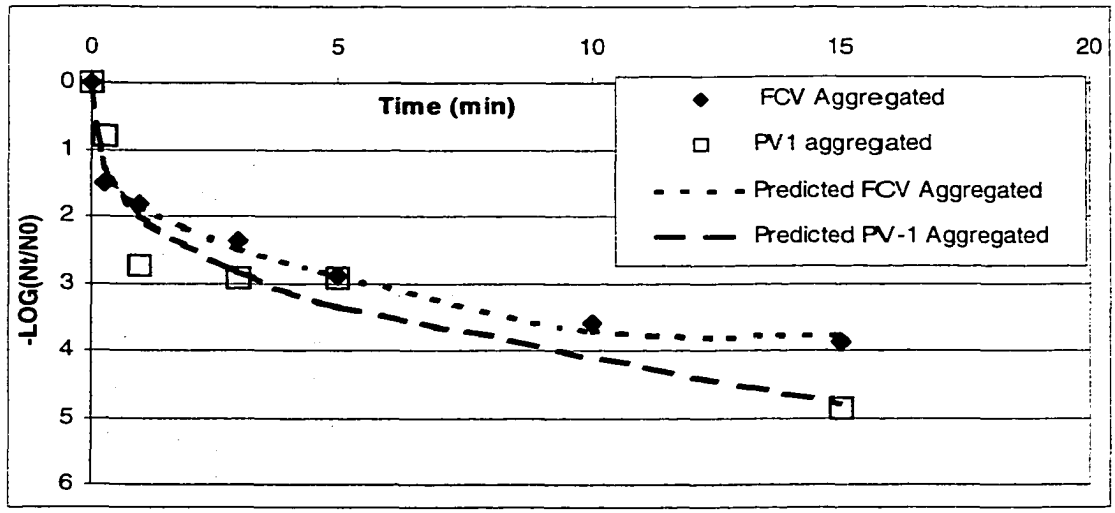
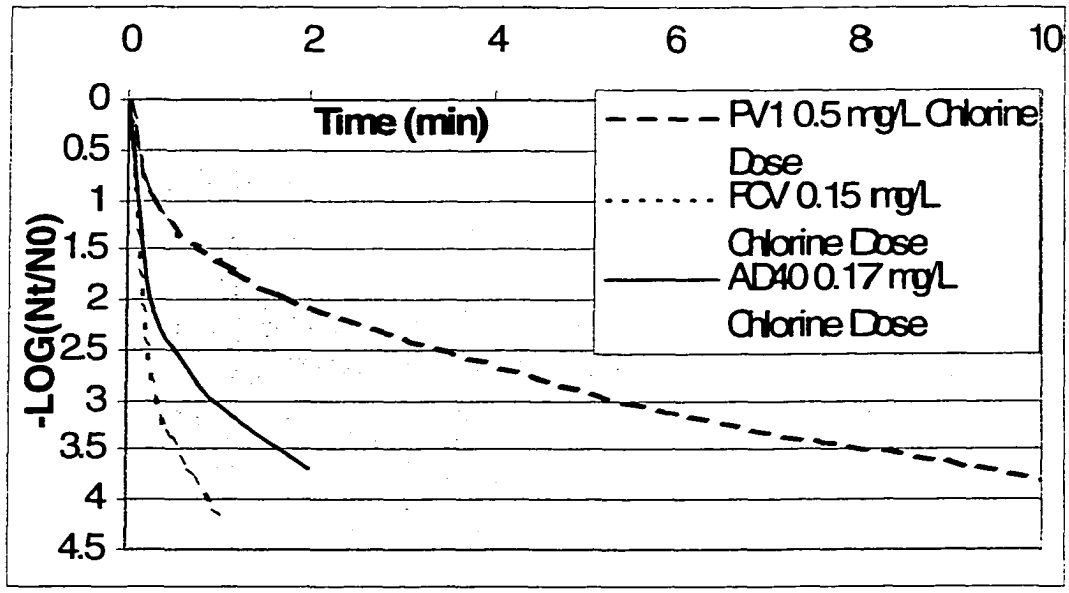


Figure 8.C Comparison of predicted inactivation curves of dispersed PV1, AD40 and FCV suspended in BDF water; pH 6 and 5 °C.



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