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Inactivation of enteric adenovirus and feline calicivirus by ozone

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Abstract

Little information is available regarding the effectiveness of ozone on the inactivation of caliciviruses and enteric adenoviruses. Inactivation experiments were conducted with feline calicivirus (FCV), closely related to the human caliciviruses based on nucleic acid organization and capsid architecture, and adenovirus type 40 (AD40). Experiments were carried out in buffered disinfectant demand free water at pH 7 and 5 °C. *Ct* values; concentration of ozone multiplied by contact time with virus; were determined from application of the efficiency factor hom (EFH) model. *Ct* values for 4-log (99.99%) ozone inactivation at 5 °C and pH 7 ranged from 0.07 to 0.60 mg/l min for AD40 and <0.01 to 0.03 mg/l min for FCV. *Ct* values listed in the US environmental protection agency "Guidance Manual for Compliance with Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources" were higher than *Ct* values generated by this study. Very low ozone residuals (<0.01(mg/l) substantially inactivated FCV and AD40 under the studied conditions.
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1. Introduction

According to the United States Environmental Protection Agency (USEPA) National Primary Drinking Water Standards, enteric viruses must be removed or inactivated by 4-logs (99.99%) from source water by filtration or disinfection, or a combination of these technologies (USEPA, 2001). Viral pathogens can bypass conventional filtration processes due to their small size making disinfection an important treatment barrier between drinking water consumers and viral gastroenteritis. While chlorine is the most common disinfectant in the US for drinking water and wastewater treatment, alternative disinfectants are needed to reduce highly chlorine resistant pathogens, such as *Cryptosporidium parvum*. Ozone is an effective disinfectant for the reduction of protozoan parasites in water (Gyurek et al., 1999; Rennecker et al., 1999; Widmer et al., 2002). There is little information, however, regarding the ability of

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ozone to inactivate adenoviruses and caliciviruses, which are listed on the USEPA's contaminant candidate list (CCL) (Federal Register, 1998).

Based on previous viral disinfection studies, the USEPA published the "Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Sources" (USEPA Guidance Manual) (37). *Ct* values, disinfectant concentration multiplied by contact time between the disinfectant and microorganism, for 2–4 logs viral inactivation by several disinfectants, including ozone, at different pH and temperature conditions are listed in the manual. The Guidance Manual *Ct* values were calculated by multiplying *Ct* values obtained from bench-scale, disinfectant inactivation experiments conducted with dispersed hepatitis A virus in buffered, disinfectant demand-free water by a "safety factor" of three. This safety factor is assumed to assure reduction of not only hepatitis A virus but also other waterborne viruses. The Guidance Manual's *Ct* values guide public water utilities in order to ensure that their disinfection practices meet regulatory viral log inactivation requirements. However, these *Ct* values may not be adequate for emerging viral pathogens who's susceptibility to drinking water disinfectants is largely unknown.

The USEPA, mandated by the Safe Drinking Water Act (SDWA), published the Drinking Water CCL in 1998 (Federal Register, 1998). These contaminants are under regulatory consideration since little to no information regarding health, drinking and wastewater treatment or analytical methodology is currently available. Several enteric viruses, including enteric adenoviruses and caliciviruses, are included on the CCL and were investigated in the current study.

Ozone is a very strong oxidizing agent and an effective alternative to chlorine for pathogen reduction in water. Ozone improves coagulation and is effective at controlling color, taste, and odor in water (AWWA, 1995). Disadvantages of ozone disinfection in water include, (a) short half-life; (b) needs to be generated on-site; (c) corrosive; and (d) ozone gas is toxic (AWWA, 1995). Previous studies have reported that ozone effectively inactivates several viruses in water and sewage (Finch and Fairbairn, 1991; Hall and Sobsey, 1993; Harakeh and Butler, 1985; Herbold et al., 1989; Roy et al., 1982; Shin and Sobsey, 2003; Vaughn et al., 1990), but no information is available concerning the reduction of feline caliciviruses (FCVs) (a surrogate for noroviruses (NV)) and enteric adenoviruses in treated water.

Members of the human calicivirus genus, NV, are a principal cause of nonbacterial acute gastroenteritis (Fankhauser et al., 1998; Kapikian et al., 1996) and have been identified as etiological agents of waterborne outbreaks (Hafliger et al., 2000, Kukkula et al., 1997). Previous outbreaks caused by NV-contaminated ice and cooked shellfish have suggested that these viruses are capable of withstanding harsh environmental conditions (Glass et al., 2000). Their ability to withstand current drinking water disinfection practices is largely unknown since there are no known animal or mammalian cell culture systems that determine NV infectivity. Due to these difficulties, two alternative studies, a human feeding study and a polymerase chain reaction (PCR) based study, have been carried out previously (Keswick et al., 1985; Shin et al., 1998). However, conflicting results between these studies made conclusions regarding NV chlorine resistance difficult. More recently, a NV surrogate, FCV, has been used as a substitute for NV inactivation in several disinfection studies (Nuanualsuwan and Cliver, 2003; Thurston-Enriquez et al., 2003a, b). FCV has similar genome organization (Clarke and Lambden, 2000; Jiang et al., 1993) and capsid architecture (Prasad et al., 2000) compared to NVs and can be easily grown in cell culture. While these characteristics have been used as justifications for employing FCV as a surrogate of NVs, varying susceptibilities to disinfectants between these viruses is likely due to genomic and amino acid composition differences. Chlorine inactivation experiments carried out with FCV, however, resulted in similar conclusions as those reported in the NV PCR-based study (Shin et al., 1998; Thurston-Enriquez et al., 2003a).

Like NV, the enteric adenoviruses, adenovirus 40 (AD40) and 41 (AD41), are also important causes of self-limiting, acute gastroenteritis, especially in children less than 4 years of age (Horowitz, 1996). Enteric adenoviruses have increased environmental stability compared to other enteric viruses (Enriquez et al., 1995), so their presence in sewage and surface water makes them likely contaminants in public water supplies (Hurst et al., 1988; Irving and Smith, 1981). Moreover, enteric adenoviruses and NV were identified as two of the etiological agents causing acute gastroenteritis in a waterborne outbreak in Finland (Kukkula et al., 1997). Enteric adenoviruses are susceptible to chlorine (Thurston-Enriquez et al., 2003a) but are very resistant to UV light (Thurston-Enriquez et al., 2003b).

Based on previous viral disinfection studies, the USEPA published the "Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Sources" (USEPA Guidance Manual) (USEPA, 1989). *Ct* values, (disinfectant concentration (*C*) multiplied by contact time (*t*) between the disinfectant and microorganism), for 2- to 4-logs viral inactivation by ozone and other water disinfectants at different pH and temperature conditions are listed in the manual. Guidance Manual's *Ct* values (mg/l min) direct public water utilities to ensure that disinfection practices meet regulatory microbial log inactivation requirements. However, ozone *Ct* values may not be adequate for caliciviruses and adenoviruses whose susceptibility to this disinfectant is largely unknown.

The objectives of this study were to: (1) compare ozone inactivation of AD40 and FCV; (2) use previously described disinfection models to generate *Ct* values for each virus; and (3) compare predicted *Ct* values to USEPA Guidance Manual *Ct* values.

2. Methods

2.1. Virus propagation and assay

AD40 (strain Dugan), FCV (strain F9), primary liver carcinoma cell line (PLC/PRF/5) and Crandell Feline Kidney (CRFK) cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). AD40 and FCV stocks were propagated, enumerated, concentrated, purified, stored, and titered (most probable number) in the same manner as described by Thurston-Enriquez et al. (2003a, b).

2.2. Ozone production and measurement

Ozone gas was generated using a model #648-G ozone generator manufactured by Bankston Electric Company (Bankston Electric Company, Mansfield, TX, USA). A 500 ml amber bottle containing distilled water was placed into an ice-filled insulated bucket and ozone gas was bubbled into water for at least 2 h to produce a high concentration ozone stock solution. Ozone concentrations were measured according to the Standard Methods for the Examination of Water and Wastewater Indigo Colorimetric Method (Method 4500-O_3 B) (APHA, 1998).

2.3. Experimental protocol

The experiment was carried out according to protocols described by Thurston-Enriquez et al. (2003a). All glassware was soaked in ozonated water and dried prior to use. Disinfectant demand-free glass beakers containing 50 ml phosphate buffered demand-free (BDF) water at pH 7 were kept at 5 °C in a refrigerated water bath. At least five reaction beakers were analyzed for each experimental condition. The first beaker, containing only BDF water and ozone was measured at 15 s in order to determine the initial ozone dose in the absence of ozone demand from the viral stock. The second and third reaction beakers were inoculated with one of the studied viruses at concentrations that would allow detection of at least 2-logs of viral inactivation. The second and third beakers were also inoculated with ozonated water and immediately stirred. The second beaker was sampled to determine ozone demand of viral stocks and ozone decay. In order to determine viral inactivation, 2 ml samples were taken from the third beaker and immediately inoculated into collection tubes containing 20 µl of sterile 10% sodium thiosulfate solution to quench any residual disinfectant activity. The fourth reaction beaker, or control beaker, contained only virus and BDF water. This control beaker was necessary to (1) determine initial virus concentration for every experiment, and (2) evaluate whether virus inactivation occurred under the tested conditions (in the absence of disinfectants). Two to three replicates (a fifth or sixth reaction beaker identical to the third reaction beaker) were conducted for each virus. Viral samples were kept at 4 °C until assay.

2.4. Kinetic modeling and Ct values

Disinfectant decay and *Ct* values (Tables 1 and 2) were calculated using the efficiency factor hom (EFH) model (Thurston-Enriquez et al., 2003a). Ozone decay constants, k', for each experiment were calculated using the Solver function in Microsoft Excel 2000 (Microsoft Corp.) to regress (using the least-squares method) the first-order kinetic equation:

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

where C and C_0 are ozone residual (mg/l) at time t and time 15 s (closest measurement to time 0), respectively; and k' is the first-order free ozone decay rate constant

Table 1
Summary of parameter estimates for EFH Model and R^2 values for comparison of predicted and observed ozone inactivation curves

O_3 dose[a] (mg/l)	Virus	No. of replicates	k'[b]	k[c]	n[d]	m[e]	R^2
0.49	AD40	2	6.10	75.72	1.53	0.51	0.98
0.30	AD40	2	5.96	8.56	0.03	0.16	0.99
1.00	FCV	3	8.83	9.91	5.01	0.01	0.99
0.06	FCV	2	4.40	98.60	0.69	0.59	0.96

[a]Experiments conducted at pH 7 and 5 °C.
[b]Average disinfectant decay constant for replicate experiments.
[c]Inactivation rate constant.
[d]Coefficient of dilution.
[e]Constant for the inactivation rate law (describes deviation from Chick–Watson kinetics).

Table 2
EFH Model predicted *Ct* values for inactivation of FCV and AD40 under negligible ozone decay ($k' = 0.0001$)

$-\text{Log}_{10}$ inactivation	EFH model values[a]		EPA guidance manual *Ct* value
	AD40	FCV	
	$Ct_{99.0-99.99\%}$ (mg/l min)		
2	0.02[b]	<0.01[e]	0.6
	<0.01[c]	<0.01[f]	
3	0.04[b]	<0.01[e]	0.9
	0.10[c]	0.02[f]	
4	0.07[b,d]	<0.01[e]	1.2
	0.60[c,d]	0.03[f]	

[a] $Ct_{99.0-99.99\%}$ values for experiments conducted at pH 7 and 5 °C.
[b] Ozone dose = 0.49 mg/l.
[c] Ozone dose = 0.30 mg/l.
[d] *Ct* values predicted by EFH Model since 4-log inactivation was not achieved in bench-scale experiments.
[e] Ozone dose = 1.00 mg/l.
[f] Ozone dose = 0.06 mg/l.

(min^{-1}). Viral most-probable-number values for each experiment, grouped by virus type, were fit into the EFH model:

$$\ln N/N_0 = -kC_0t^m[(1 - \exp(-nk't/m)/(nk't/m)], \quad (2)$$

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 (Haas and Joffe, 1994). $\ln N/N_0$ 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 $\ln N/N_0$ for viral disinfection experiments performed with the same virus in order to determine the values for each model's coefficients.

Ct value is the disinfectant concentration (mg/l) multiplied by the time (min) when a specific log inactivation, 2, 3 or 4-log (99%, 99.9% and 99.99%) occurred. *Ct* values ($Ct_{99.0\%}$, $Ct_{99.9\%}$ and $Ct_{99.99\%}$) were used to assess viral sensitivity to ozone and compare observed bench-scale inactivation to predicted EFH model $Ct_{99.0-99.99\%}$ values. Generation of predicted *Ct* values for each virus and set of conditions was determined through application of the EFH model parameters. Because of the rapid decay of ozone and tailing of disinfection curves, only viral inactivation values where ozone levels were above 0.001 mg/l (determined through disinfectant decay model) were used with the EFH Model. A value of 0.0001 for k' (conditions of negligible disinfectant decay) was used for EFH model *Ct* values. This value was chosen in order to produce baseline *Ct* values and because k' varied between experiments. As suggested by earlier studies, only disinfectant concentrations similar to amounts applied in the bench-scale experiments were used to calculate *Ct* values (Thurston-Enriquez et al., 2003a, b). R^2 values were calculated using Microsoft Excel 2000 (Microsoft Corp.) to determine the fit of predicted EFH model inactivation curves to observed bench-scale curves.

3. Results

Table 1 lists parameter estimates for EFH model analysis and R^2 values for observed versus predicted inactivation curves. All R^2 values are close to 1.0 indicating that the EFH Model closely predicted observed bench-scale ozone AD40 and FCV inactivation. Table 2 compares EFH Model predicted *Ct* values for minimal disinfectant decay ($k' = 0.0001$) calculated from this study's bench scale experiments to EPA Guidance Manual *Ct* values for 2-, 3-, or 4-log ($Ct_{99.0-99.99\%}$) viral inactivation. Under this study's experimental conditions, EFH Model *Ct* values for AD40 and FCV are far below EPA Guidance Manual *Ct* values for ozone inactivation.

Ozone decayed rapidly during the course of all viral disinfection experiments. For AD40 experiments, ozone decreased from an initial dose of 0.49 mg/l down to an average of 0.11 within 15 s and was not measurable by 2 min. For FCV, a dose of 1.0 mg/l ozone was decreased to a concentration of 0.11 mg/l by 15 s and below detection by 5 min. The practical lower limit of detection for ozone in water by the Indigo Colorimetric Method is between 0.01 and 0.02 mg/l (APHA, 1998). Ozone decayed more slowly in experiments conducted with AD40 at 0.30 mg/l and FCV at 0.06 mg/l ozone dose (Table 1).

For each ozone dose tested, Table 3 lists the $-\log_{10}$ inactivation and associated ozone residual value observed at the contact time listed. Ozone residual values listed in the table were derived from the first-order kinetic disinfectant decay equation. The values in Table 3 demonstrate that ozone was effective at viral inactivation at very low ozone residuals.

4. Discussion

To the best of our knowledge, this is the first report describing the effectiveness of ozone to inactivate FCV and AD40 in water. In comparison to EPA Guidance Manual *Ct* values, $Ct_{99.0-99.99\%}$ values generated by this study demonstrate that EPA Guidance Manual *Ct* values are sufficient for reducing AD40 and FCV by at least 4-logs in treated water under the tested conditions.

Table 3
Average ozone dose, residual, and contact time for low and high inactivation values for AD40 and FCV

AD40			FCV		
$-\text{Log}_{10}$ inactivation	Contact time (min)	Ozone residual (mg/l)[a]	$-\text{Log}_{10}$ inactivation	Contact time (min)	Ozone residual (mg/l)[a]
Ozone dose = 0.49 mg/l			Ozone dose = 1.00 mg/l		
2.63	0.25	0.108	4.28	0.25	0.110
3.28	2.00	<0.001	>4.74	1.20	<0.0001
Ozone dose = 0.30 mg/l			Ozone dose = 0.06 mg/l		
3.04	0.50	0.014	1.85	0.25	0.020
3.55	10	<0.001	2.77	5.00	<0.0001

[a]Ozone residual was determined from disinfectant decay equation for the contact time listed.

Comparison of *Ct* values for the inactivation of the studied viruses suggests that AD40 is more resistant to the effects of ozone compared to FCV. While AD40 ozone disinfection experiments were carried out with higher initial ozone doses, *Ct* values for AD40 were still higher than values calculated for 1- to 4-log inactivation of FCV.

Results presented by this study further demonstrate that ozone is a very effective water disinfectant for viruses in water. AD40 and FCV were both readily inactivated by low ozone doses of 0.30 and 0.06 mg/l, respectively. $Ct_{99.99\%}$ values corresponding with these low ozone doses were 0.60 and 0.03 for AD40 and FCV, respectively. At higher initial ozone concentrations, FCV was reduced by 4.28-logs within 15 s by 1.00 mg/l ozone dose. Previous studies have also demonstrated the effectiveness of ozone for viral inactivation in buffered disinfectant demand-free water. For example, poliovirus type 1 was inactivated by 2-logs within 30 s at 0.15 mg/l ozone dose and a non-purified poliovirus type 1 viral stock was also readily inactivated (Ellis, 1991). Hepatitis A virus was reduced by 5-logs within 1 min at 1 mg/l ozone (Vaughn et al., 1990) and Norwalk virus was reduced by over 3-logs by 10 s at 0.37 mg/l ozone dose, pH 7, and 5 °C (Shin and Sobsey, 2003). Adenovirus type 2 $Ct_{99.99\%}$ values of 0.17 and >2.0 mg/l min were calculated from graphs describing adenovirus type 2 inactivation by 0.2 mg/l ozone dose at pH 7 (Riley et al., 2002). Ozone residuals at these two contact times, however, were not described (Riley et al., 2002). Differences in ozone residual between these two disinfection experiments may have been the cause for the large differences in *Ct* values. Specific information regarding experimental methods used in many ozone disinfection studies is lacking. The manner in which disinfection experiments are designed can have a great impact on the outcome of viral inactivation. Disparities between viral disinfection studies make comparison and conclusions regarding disinfectant effectiveness difficult (Thurston-Enriquez et al., 2003a, b).

The idea of ozone threshold levels in water has been described by several researchers (Ellis, 1991). It has been suggested that there is an ozone concentration that must be achieved for adequate disinfection. Ozone threshold levels of 1.0, 0.7, and 0.1 mg/l have been suggested by various researchers (Ellis, 1991). Disinfection effectiveness of ozone below these levels has been suggested to be non-existent or extremely poor (Ellis, 1991). Results of this study, however, demonstrate that even very low ozone residuals can inactivate AD40 and FCV particles in water. Batch reactors used in this study resulted in ozone decay throughout each experiment, unlike continuous ozone flow reactors used by previous research groups (Emerson et al., 1982; Roy et al., 1982; Shin and Sobsey, 2003). Using previously described equations for disinfectant decay (Thurston-Enriquez et al., 2003a, b), ozone residual was calculated for different contact times for each FCV and AD40 batch experiment. For AD40 experiments dosed at 0.30 mg/l ozone, residuals averaged 0.015 mg/l and less than 0.0001 mg/l by 30 s and 10 min, respectively. Even at these low ozone residuals, the concentration of AD40 observed at 30 s was further reduced by 67% by 10 min. Similar reduction was observed for FCV, where FCV concentrations at 15 s were reduced by an average of 76% by 10 min contact time. Ozone residuals for these FCV experiments were 0.02 mg/l at 15 s compared to <0.001 mg/l at 45 s. Coliphage MS-2 was inactivated by 4-logs within 20 s at ozone concentrations less than 0.04 mg/l (Finch and Fairbairn, 1991) and a nonpurified poliovirus type 1 viral suspension was completely inactivated in 2 min by an ozone concentration of 0.05 mg/l (Ellis, 1991). While not as effective as higher ozone concentrations, these results suggest that very low ozone concentrations are capable of lethal damage to viral particles.

All disinfection experiments were carried out with BDF water that was inoculated with purified (removal of cell debris and cell culture media) and relatively dispersed (chloroform extraction) AD40 and FCV viral stocks. Removal of cell debris and cell culture media significantly reduces ozone demand in viral stocks. Results obtained by these controlled disinfection studies provide baseline information necessary for understanding ozone efficacy against CCL viral pathogens in treated water. Moreover, this information is important for evaluation of ozone as a primary or secondary disinfectant for inactivation of the studied viruses under the tested water conditions. Previous chlorine disinfection studies have demonstrated that these viruses are inactivated at different rates depending on viral and water quality conditions (Thurston-Enriquez et al., 2003a). Thus, further studies are needed to determine whether EPA Guidance Manual ozone *Ct* values are adequate for reducing AD40 and FCV in an aggregated state and in natural waters.

5. Conclusions

- Under the conditions of this study, ozone is very effective in inactivating FCV and AD40 in treated water and at low ozone concentrations.
- *Ct* values listed in the US EPA's Guidance Manual are adequate for 4-log inactivation of FCV and AD40 under the conditions of this study.
- Low ozone residuals (<0.01 mg/l) observed after 10 min contact time, continued to reduce FCV and AD40 by 67% and 76%, respectively.

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