7-1-2003

Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus

Jeanette A. Thurston-Enriquez
University of Nebraska-Lincoln

Charles N. Haas
Drexel University, haas@drexel.edu

Joseph Jacangelo
Montgomery Watson Harza, Lovettsville, Virginia

Charles P. Gerba
University of Arizona, gerba@ag.arizona.edu

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2003, p. 3979–3985 Vol. 69, No. 7
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.7.3979–3985.2003

Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus

Jeanette A. Thurston-Enriquez,[1]* Charles N. Haas,[2] Joseph Jacangelo,[3] and Charles P. Gerba[4]

Agricultural Research Service, U.S. Department of Agriculture, University of Nebraska, Lincoln, Nebraska 68583[1]; School of Environmental Science, Engineering and Policy, Drexel University, Philadelphia, Pennsylvania 19104[2]; Montgomery Watson Harza, Lovettsville, Virginia 20180[3]; and Department of Soil, Water and Environmental Science, University of Arizona, Tucson, Arizona 85721[4]

Received 1 November 2002/Accepted 7 April 2003

***Ct* values, the concentration of free chlorine multiplied by time of contact with virus, were determined for free-chlorine inactivation experiments carried out with chloroform-extracted (dispersed) and non-chloroform-extracted (aggregated) feline calicivirus (FCV), adenovirus type 40 (AD40), and polio virus type 1 (PV-1). Experiments were carried out with high and low pH and temperature conditions. *Ct* values were calculated directly from bench-scale free-chlorine inactivation experiments and from application of the efficiency factor Hom model. For each experimental condition, *Ct* values were higher at pH 8 than at pH 6, higher at 5°C than at 15°C, and higher for dispersed AD40 (dAD40) than for dispersed FCV (dFCV). dFCV and dAD40 were more sensitive to free chlorine than dispersed PV-1 (dPV-1). *Ct*s for 2 log inactivation of aggregated FCV (aFCV) and aggregated PV-1 (aPV-1) were 31.0 and 2.8 orders of magnitude higher than those calculated from experiments carried out with dispersed virus. *Ct*s for 2 log inactivation of dFCV and dAD40 in treated groundwater at 15°C were 1.2 and 13.7 times greater than in buffered-demand-free (BDF) water experiments at 5°C. *Ct* values listed in the U.S. Environmental Protection Agency (EPA) Guidance Manual were close to, or lower than, *Ct* values generated for experiments conducted with dispersed and aggregated viruses suspended in BDF water and for dispersed viruses suspended in treated groundwater. Since the state of viruses in water is most likely to be aggregated and associated with organic or inorganic matter, reevaluation of the EPA Guidance Manual *Ct* values is necessary, since they would not be useful for ensuring inactivation of viruses in these states. Under the tested conditions, dAD40, dFCV, aFCV, dPV-1, and aPV-1 particles would be inactivated by commonly used free chlorine concentrations (1 mg/liter) and contact times (60 to 237 min) applied for drinking water treatment in the United States.**

The Safe Water Drinking Act (SDWA), amended several times since its 1974 enactment, regulates the nation's drinking water supply in order to protect the public's health by creating barriers against water pollution. These barriers include filtration, disinfection, and source water protection. The U.S. Environmental Protection Agency (EPA), mandated by the Safe Water Drinking Act, sets national drinking water standards for protection against biological and chemical contaminants that may threaten drinking water and its sources. The EPA is required to publish a "maximum contaminant level goal," a nonenforceable health goal, and enforceable regulations through the National Primary Drinking Water Regulation. These regulations specify the maximum contaminant level or a treatment technique that has been shown to significantly reduce levels of the contaminant(s) of concern. According to the EPA National Primary Drinking Water Standards, enteric viruses must be removed or inactivated by 4 logs (99.99%) from surface water or groundwater under the direct influence of surface water by filtration and disinfection, or a combination of these technologies (46).

Chlorination of drinking water and wastewater is the most common disinfection practice in the United States (4). Chlorine inactivation studies have demonstrated the susceptibilities of important enteric viruses that have threatened or caused waterborne outbreaks, such as polio virus (5, 38, 40) and hepatitis A virus (HAV) (17, 43). Based on previous viral disinfection studies, the EPA published the Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Sources (EPA Guidance Manual) (45). *Ct* values, disinfectant concentration multiplied by contact time between the disinfectant and microorganism, for 2 to 4 logs viral inactivation by chlorine at different pH and temperature conditions are listed in the manual. Guidance Manual *Ct* values were calculated by multiplying *Ct* values obtained from bench-scale, chlorine inactivation experiments conducted with dispersed HAV in buffered, chlorine-demand-free water by a "safety factor" of 3. The Guidance Manual's *Ct* values guide public water utilities in order to ensure that their disinfection practices meet regulatory microbial log inactivation requirements. However, these *Ct* values may not be adequate for emerging viral pathogens whose susceptibility to drinking water disinfectants is largely unknown.

The EPA, mandated by the SDWA, published the Drinking Water Contaminant Candidate List (CCL) in 1998 (12). Contaminants that are known or anticipated to occur in public water systems are included on the CCL. Contaminants on this list are under regulatory consideration since little to no information regarding health, drinking water 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 present study.

Members of the human calicivirus genus, Norwalk-like vi-

* Corresponding author. Mailing address: USDA-ARS, 120 Keim Hall, University of Nebraska East Campus, Lincoln, NE 68583-0934. Phone: (402) 472-8935. Fax: (402) 472-0516. E-mail: jthurston2@unl.edu.

ruses (NLVs), are a principal cause of nonbacterial acute gastroenteritis (11, 27) and have been identified as the etiological agents of waterborne outbreaks (20, 28, 30, 31). Caliciviruses range in diameter from 27 to 40 nm and have a single-stranded RNA genome and an icosahedral capsid structure. Commonly reported symptoms include diarrhea and vomiting. Previous outbreaks caused by NLV-contaminated ice and cooked shellfish have suggested that these viruses are capable of withstanding harsh environmental conditions (16). 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 NLV infectivity. Due to these difficulties, two studies, a human feeding study and a PCR-based study, have been carried out previously (29, 40). Conflicting results, however, between these studies make conclusions regarding NLV chlorine resistance difficult. More recently, an NLV surrogate, feline calicivirus (FCV), has been used as a surrogate for NLV inactivation (8, 34, 41) since FCV has similar genome organization (6, 26) and capsid architecture (36) compared to NLVs and it can be easily grown in cell culture.

Like NLVs, the enteric adenoviruses, enteric adenoviruses 40 (AD40) and 41 are also important causes of self-limiting, acute gastroenteritis, especially in children less than 4 years old (22). These viruses are considerably larger than NLVs, 70 to 90 nm in size, and the capsid structure is complex. The adenovirus icosahedron contains 240 hexons, 12 pentons, and 12 fibers that extend from each penton base, and its genome consists of linear, double-stranded DNA. Enteric adenoviruses are shed in high numbers in the feces (1) and, like NLVs, are typically shed in the feces for long periods, and infection can be caused by low numbers of viral particles (16, 22). Enteric adenoviruses have greater environmental stability than other enteric viruses (10), and their presence in sewage and surface water makes them likely contaminants in public water supplies (24, 25). Moreover, enteric adenoviruses and NLVs were identified as two of the etiological agents causing acute gastroenteritis in a waterborne outbreak in Finland (30), and waterborne outbreaks of pharyngoconjuctivitis from swimming have been reported for nonenteric adenoviruses (15, 35).

In the present study, *Ct* values were calculated for free-chlorine inactivation experiments conducted with dispersed AD40 (dAD40) and dispersed FCV (dFCV) (NLV surrogate) in two water types, buffered-demand-free (BDF) and treated groundwater at high and low pH and temperature conditions. Experiments were also conducted with dispersed poliovirus type 1 (dPV-1) in BDF water at pH 6. Inactivation of PV-1 by chlorine was carried out in order to compare *Ct* values generated by this study to previously reported PV-1 inactivation experiments. Extensive information regarding poliovirus inactivation by chlorine is available in the literature (5, 9, 14, 21, 38–40); and, therefore, it has been used as a model virus for investigations involving disinfection (2, 17, 33, 40). In order to determine *Ct* values for each virus and experimental condition, the efficiency factor Hom (EFH) model was applied to the observed bench-scale results in order to determine *Ct* values for 2-, 3- and 4-log inactivation. *Ct* values for dFCV and dAD40 were developed to evaluate the current chlorination practice's ability to inactivate these viruses in two water types and under different pH and temperature conditions.

While the goal of this project was to determine *Ct* values for free-chlorine inactivation of dispersed viruses, additional experiments were conducted with aggregated FCV (aFCV) and aggregated PV-1 (aPV-1,) since most viruses in surface water and wastewater are likely aggregated or associated with organic matter (42). The choice of viruses for these additional experiments was due to ease of propagation and assay of both viruses and since early studies, using nondispersed Norwalk virus preparations, reported that it survived >3 mg of free chlorine/liter (29). Freon, an organic solvent previously used for virus purification, has been reported to yield a virus preparation with a very high proportion (95%) of single particles (13). In the present study, chloroform, another organic solvent, was used in place of Freon because it is commercially available. Virus preparations that were chloroform extracted are referred to as dispersed, and those that were not chloroform extracted are termed aggregated.

The objectives of this study were the following: (i) to compare viral inactivation by free chlorine for three viruses under various pH, temperature, and water quality conditions; (ii) to analyze viral inactivation using previously described disinfection models that predict *Ct* values; and (iii) to compare predicted *Ct* values to observed viral inactivation of bench scale experiments, EPA Guidance Manual *Ct* values, and commonly applied U.S. chlorine disinfection practices.

MATERIALS AND METHODS

Virus propagation and assay. AD40 (strain Dugan), FCV (strain F9), the primary liver carcinoma cell line (PLC/PRF/5), and the Crandell feline kidney (CRFK) cell line were obtained from the American Type Culture Collection (Rockville, Md.). PV-1 (LSc-2ab) and the buffalo green kidney monkey (BGM) cell line were acquired from available stocks of the Environmental Microbiology Laboratory 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 the PLC/PRF/5, CRFK, and BGM cell lines, respectively (8, 10, 18). Maintenance media (Eagle's minimum essential medium containing 10% fetal bovine serum) was decanted from 162-cm^2 tissue culture flasks containing complete cell monolayers. The monolayers were rinsed with Tris-buffered saline (Trizma base; Sigma Chemical Co., St. Louis, Mo.), and 1 ml of approximately 10^3 to 10^5 most probable number (MPN) of viral stock/ml was inoculated onto the monolayer. Flasks were rocked every 15 min for 1 h, after which 50 ml of maintenance medium (without fetal bovine serum) was inoculated onto the infected cell monolayer. Flasks were incubated at 37°C until ≥90% cell monolayer destruction. One freeze-thaw step for AD40 and three freeze-thaw steps for FCV and PV-1 were performed to release virus particles from host cells. The supernatant was centrifuged at 4°C and 10,000 × *g* to remove cell debris. Further purification and concentration was accomplished by polyethylene glycol precipitation. For every 100-ml volume of viral supernatant, 9 g of polyethylene glycol (Molecular weight, 8,000) and 5.8 g of NaCl was added, and the amended supernatant was stirred overnight at 4°C. Centrifugation at 10,000 × *g* at 4°C for 30 min was followed by disposal of the supernatant and resuspension of the pellet in BDF water, pH 7. Dispersion of the viral stocks was accomplished by combining equal volumes of the viral extracts and chloroform followed by vortexing for at least 10 min. Once homogenized, the chloroform-viral suspension was centrifuged at 10,000 × *g* at 4°C for 15 min, and the upper layer, containing purified viral stock, was collected and termed dispersed. For aggregated viral stocks, no chloroform extraction was performed. All viral stocks were stored at 4°C until use.

Determination of viral titer before and after chlorine disinfection was accomplished by assaying 5- or 10-fold dilutions, in quadruplet, in 24-well tissue culture trays with the appropriate cells in suspension. Viral concentration was determined by observation for cytopathic effect. Observation for cytopathic effect was continued for up to 12 days for FCV and PV-1 and 24 days for AD40. An MPN general-purpose program adapted from the method of Hurley and Roscoe was used to determine the concentration (MPN/ml) of virus (23).

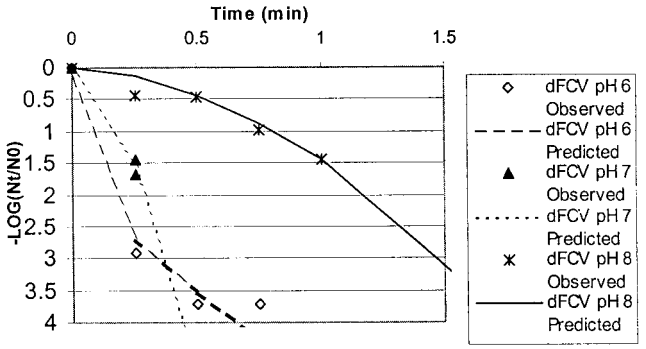

FIG. 1. dFCV BDF water experiments (5°C, 0.17-mg/liter free chlorine dose at pH 6; 0.16-mg/liter free chlorine dose at pH 7 and 8).

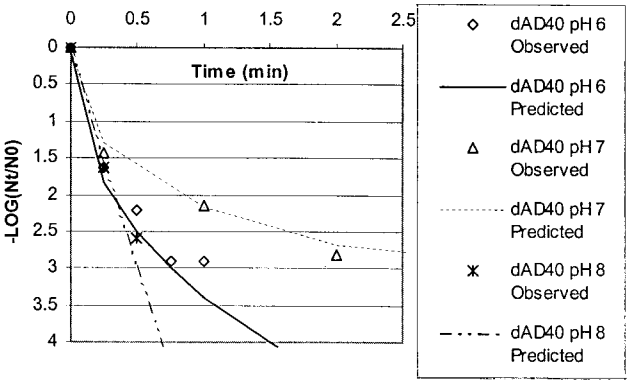

FIG. 2. Dispersed AD40 BDF water experiments (5°C, 0.17-mg/liter free chlorine dose at pH 6 and 7; 0.31-mg/liter free chlorine dose at pH 8).

Reagents and glassware. Glassware was made chlorine demand free by overnight soaking in a solution of at least 100 mg of free chlorine/liter. Beakers were then rinsed with chlorine-demand-free water and baked for at least 2 h at 200°C. After this initial treatment, only soaking in free chlorine and rinsing in demand-free water was performed for all glassware. Reagent-grade chlorine (5.0% sodium hypochlorite; J.T. Baker Co., Phillipsburg, N.J.) was diluted in chlorine-demand-free water to prepare a free chlorine stock solution of approximately 150 mg/liter. Dilution of this stock solution, in chlorine-demand-free water, was carried out to achieve free chlorine concentrations used in disinfection experiments.

Test waters. All disinfection experiments were conducted in either BDF or treated groundwater. BDF water was prepared by dissolving 0.54 g of Na_2HPO_4 (anhydrous) and 0.88 g of KH_2PO_4 (anhydrous) per liter of deionized, chlorine-demand-free water (Nanopure RO purifier; Barnstead, Dubuque, Iowa). The pH was adjusted with 1 M NaOH or 1 M KH_2PO_4. BDF water was stored in chlorine-demand-free bottles at 4°C until use. Treated groundwater was obtained from the Cincinnati Water Works, where conventional drinking water treatment (coagulation, filtration, and chlorination) was applied. Measured treated groundwater water quality parameters were pH (8.0 to 8.2), turbidity (0.04 nephelometric turbidity unit, and total organic carbon (0.7 to 1.0 mg/liter). Prior to all disinfection experiments, treated groundwater was dechlorinated by rapid mixing and exposure to UV light until no chlorine was detected by the *N*,*N*-diethyl-p-phenylenediamine (DPD) method (3), using a Hach (Loveland, Colo.) DR2000 spectrophotometer.

Experimental protocol. Chlorine-demand-free glass beakers containing 50 ml of BDF water or treated groundwater were placed in a refrigerated water bath in order to maintain the tested temperature (5 or 15°C) throughout the disinfection experiment. Immediately prior to each experiment, the free chlorine concentration of the stock solution was measured by the DPD method, and the volume necessary to achieve the initial free-chlorine dose in each experimental beaker was calculated.

Four experimental reaction beakers were analyzed for every experimental condition. The first reaction beaker, containing only BDF water, was measured at 15 s in order to determine the initial (at 15 s) free-chlorine dose (in the absence of any chlorine demand that may occur with groundwater or viral addition). The second and third reaction beakers were inoculated with the appropriate virus, FCV, AD40, or PV-1, at a concentration that would allow detection of 2- to 4-log inactivation in either BDF or treated groundwater. Both of these beakers were then inoculated with the free-chlorine stock solution and immediately stirred. The second beaker was sampled to determine the free chlorine concentration at the beginning (15 s) and end of each disinfection reaction to determine free-chlorine decay during the experiment. In order to determine viral inactivation by free chlorine, 2-ml samples were taken from the third (reaction) beaker at predetermined times throughout the reaction. Residual free chlorine was immediately quenched by placing the 2-ml samples into collection tubes containing 20 μl of sterile 10% sodium thiosulphate solution. The fourth reaction beaker contained only virus and test water and was considered to be representative of viral concentrations in beakers two and three. This viral control beaker was necessary to determine the initial virus concentration in the reaction beaker and to evaluate whether virus inactivation occurred under the tested pH and temperature conditions (in the absence of chlorine). Viral samples were kept on ice during the experiment and then stored at 4°C until assay.

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

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

where C and C_0 are free-chlorine residual (mg/liter) at time t and time 15 s (closest measurement to time zero), respectively; and k' is the first-order free chlorine decay rate constant (min^{-1}). The MPN values for each experiment, grouped by virus type, pH, and temperature conditions, were fit into both the Chick-Watson and the EFH models, equations 2 and 3, respectively:

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

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

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 (19). 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). Excel Solver (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 and conditions, in order to determine the values for each model's coefficients. Inactivation curves, $-\log (Nt/N_0)$ (log inactivation) versus time (min) were created using Microsoft Excel in order to compare observed and predicted log inactivation values (Fig. 1 to 5).

***Ct* values and ranges.** The *Ct* value is the concentration (milligrams per liter) of free chlorine multiplied by the time (min) when a specific log inactivation, 2, 3, or 4 log (99, 99.9, and 99.99%) occurred. *Ct* values were used to assess viral sensitivity to chlorine and compare observed inactivation values to predicted EFH model *Ct* values. *Ct* ranges, lowest to highest values, were calculated using observed bench-scale log inactivation values from replicate experiments. *Ct* ranges were generated by multiplying the initial free-chlorine dose of each experiment by the time in which approximately 2-, 3-, or 4-log inactivation occurred. Generation of *Ct* values for each virus and set of conditions was determined through application of the EFH model parameters (Table 1). A

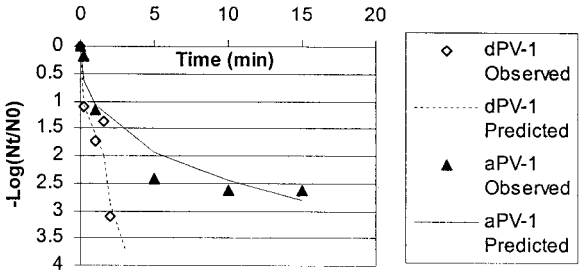

FIG. 3. Dispersed and aggregated PV-1 5°C BDF water experiments (0.5-mg/liter free chlorine dose, pH 6).

TABLE 1. Summary of parameter estimates for EFH model and R^2 values for comparison of predicted and observed free-chlorine inactivation curves

Experimental conditions	Virus	No. of replicates	k'^a (min^{-1})	k	n	m	R^2
pH 6, 5°C, BDF water	dAD40	2	2.86	8.34	0.04	0.45	0.95
	dFCV	2	0.18	0.07	−2.54	0.28	0.95
	dPV-1	2	0.04	1.64	−1.17	0.28	0.93
pH 7, 5°C, BDF water	dAD40	2	0.19	55.55	1.31	0.43	0.99
	dFCV	2	0.03	232.59	1.04	1.64	1.00
pH 8, 5°C, BDF water	dAD40	3	0.42	38.92	1.08	0.50	0.96
	dFCV	2	0.03	6.43	0.36	1.74	1.00
pH 8, 15 °C, BDF water	dFCV	2	0.01	117.85	1.10	1.27	0.99
pH 7, 5°C, BDF water	aFCV	2	0.05	4.26	−0.21	0.26	0.94
pH 6, 5°C, BDF water	aPV-1	2	0.05	3.52	0.50	0.38	0.84
pH 8–8.2, 15°C, treated groundwater	dAD40	2	0.15	3.09	−0.23	0.22	0.96
	dFCV	2	0.24	16.60	0.76	0.43	0.84

[a] Average k' value for replicate experiments.

value of 0.0001 for k' (conditions of negligible disinfectant decay) was used for model prediction of *Ct* values. This value was chosen in order to produce baseline *Ct* values and since k' varied between experiments. Only free chlorine concentrations similar to amounts applied in the bench-scale experiments were used to calculate *Ct* values.

Statistical analysis. R^2 values were calculated to determine the fit of predicted EFH model inactivation curves to observed bench-scale curves (Table 1). F tests were carried out to determine whether differences in viral inactivation were significant ($P < 0.05$) between different pH and temperature conditions, viral type, viral state (aggregated and dispersed), and water type (BDF and treated groundwater). Microsoft Excel was used for all statistical calculations.

RESULTS

Free-chlorine disinfection reactions, carried out in duplicate, involving AD40, FCV, and PV-1, were conducted with two different water types and high and low temperature and pH conditions. Free-chlorine disinfection experiments were also conducted to compare aggregated and dispersed FCV and PV-1 particles. Four-log inactivation was attempted for all experiments involving FCV and PV-1; however, only 2-log inactivation was achieved in some AD40 experiments due to low AD40 viral stock concentrations.

The EFH model could not be applied to dFCV and dAD40 experiments conducted with 0.50- to 0.54-mg/liter free chlorine doses due to rapid viral inactivation. At pH 6 and 5°C, dFCV and dAD40 were reduced by ≥4.3 and ≥2.5 logs, respectively, by 15 s. At pH 8 and 5°C, dFCV was reduced by >4 logs between 15 s and 3.5 min and dAD40 was reduced by ≥2.5 log by 15 s. Experiments were also carried out with dFCV suspended in treated groundwater, and ≥4.9-log inactivation by 15 s was observed with a 0.50-mg/liter free chlorine dose. Also, no *Ct* values were calculated for dFCV experiments conducted in BDF water and at 15°C (free-chlorine dose, 0.15 mg/liter). At pH 6, 4 logs of dFCV was inactivated by 15 s. *Ct* values for dFCV (15°C, pH 8, and free-chlorine dose of 0.15 mg/liter) for 3- and 4-log inactivation were 0.08 and 0.10 mg/liter × min. No BDF water experiments were carried out for dAD40 at 15°C and 0.15-mg/liter chlorine dose.

Both the Chick-Watson (not shown here) and EFH models were used to predict free-chlorine inactivation kinetics for every experimental condition applied to each virus. In all cases, the EFH model produced the best fit to the observed bench-scale inactivation curves. Thus, the EFH model was used for analysis of all disinfection experiments. Table 1 lists parameter estimates for EFH model analysis and R^2 values for observed versus predicted inactivation curves. The conditions with the highest free-chlorine decay rates (k') include dAD40 experiments conducted at pH 6. High k' values indicate that the free chlorine concentration significantly decreased throughout the duration of the experiment, whereas a low k' value indicates that minimal disinfectant decay occurred.

Only free chlorine concentrations similar to the concentrations applied in the bench-scale experiments were used to generate EFH-model *Ct* values. Free chlorine concentrations, other than what was applied in the actual experiments, created ambiguous *Ct* values. For example, the EFH-model predicted *Ct* value for 1 mg of free chlorine/liter at pH 6 and 5°C is 1.37

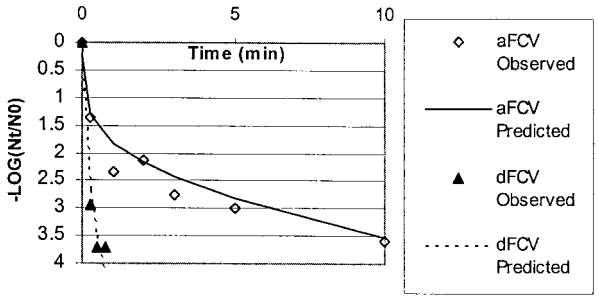

FIG. 4. Dispersed (0.15-mg/liter free chlorine dose) versus aggregated (1.0-mg/liter free chlorine dose) FCV inactivation in BDF water (5°C, pH 6).

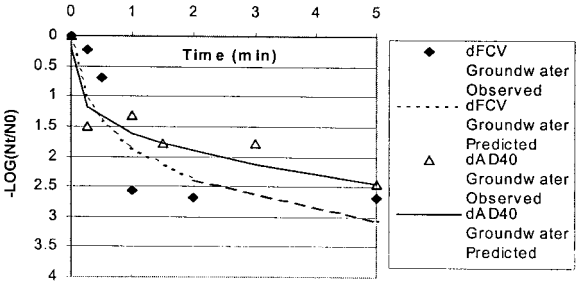

FIG. 5. Treated-groundwater experiments conducted with free chlorine doses of 0.5 and 0.18 mg/liter for AD40 and FCV, respectively (15°C, pH 8).

TABLE 2. Predicted *Ct* and EPA Guidance Manual values and ranges for dFCV, dAD40, and dPV-1 chlorine-inactivation experiments in BDF water (5°C, pHs 6, 7, and 8)

pH	$-\text{Log}_{10}$ inactivation	*Ct* value/range (mg/liter × min)		dPV-1	EPA Guidance Manual *Ct* value
		dFCV	dAD40		
6	2	0.02/<0.04	0.05/0.04–0.13	0.93/1.0–2.75	4
	3	0.07/0.04–0.08	0.11/0.09–0.17	2.87/1.0–5.0	6
	4	0.19/0.11–0.15	0.22/0.17–0.34	6.36/<10	8
7	2	0.05/<0.08	0.15/0.04–0.17	NA[a]	4
	3	0.06/<0.08	0.38/0.34–0.85	NA	6
	4	0.07/<0.08	0.75/NO[b]	NA	8
8	2	0.18/<0.32	0.11/<0.08–0.16	NA	4
	3	0.23/<0.32	0.17/0.16–0.23	NA	6
	4	0.27/<0.32	0.24/0.16–0.23	NA	8

[a] Not applicable (experiments not conducted).
[b] Amount of inactivation not observed in actual experiments.

mg/liter × min for 2 logs dAD40 inactivation. In actual experiments, however, a *Ct* value equivalent to 0.13 mg/liter/min for ≥2.54-log inactivation was observed for replicate experiments. Tables 2 to 4 list EFH model-predicted *Ct* values, *Ct* ranges that were observed in bench-scale experiments, and EPA Guidance Manual *Ct* values for 2-, 3-, or 4-log viral inactivation. *Ct* values and ranges for all 5°C BDF water experiments were lower than EPA Guidance Manual values.

Tables 2 and 3 list *Ct* values predicted by the EFH model and observed *Ct* ranges for dPV-1 and aPV-1. For 2-log inactivation at 5°C and pH 7, aPV-1 *Ct* values are 2.8 orders of magnitude higher than those for dPV-1. *Ct* values and ranges for aPV-1 2-log inactivation are very close to the EPA Guidance Manual *Ct* value of 4 mg/liter × min and exceed values listed for 3- and 4-log inactivation. Experiments conducted with aFCV, dosed with 1 mg of free chlorine/liter, have a 31.0 times larger 2-log *Ct* value than dFCV exposed to a 0.15-mg/liter free chlorine dose. Furthermore, *Ct* values and ranges for 3- and 4-log inactivation of aFCV exceed EPA Guidance Manual *Ct* values.

Ct values for disinfection experiments conducted with dFCV and dAD40 suspended in treated groundwater at 15°C are shown in Table 4. For 2-log inactivation, dAD40 and dFCV *Ct* values were 13.7 and 1.2 orders of magnitude higher than *Ct*s generated from BDF experiments. dFCV *Ct*s are lower than

TABLE 3. Predicted *Ct* values and observed ranges for aFCV and aPV-1 chlorine-inactivation experiments in BDF water (pH 6 or 7 and 5°C)

pH	$-\text{Log}_{10}$ inactivation	*Ct* value/range[a] (mg/liter/min)		EPA Guidance Manual *Ct* value
		aFCV	aPV-1	
6	2	NA[b]	2.58/2.5–5.0	4
	3	NA	7.60/7.5–22.5	6
	4	NA	16.36/7.5–22.5	8
7	2	1.55/0.25–3	NA	4
	3	8.74/5–10	NA	6
	4	29.60[c]/NO[c]	NA	8

[a] Range includes *Ct* values for both replicates.
[b] NA, not applicable (experiments not conducted).
[c] Amount of inactivation not observed in actual experiments.

TABLE 4. *Ct* values and ranges for dFCV and dAD40 chlorine-inactivation experiments conducted in treated groundwater (15°C, pH 8 to 8.2)

$-\text{Log}_{10}$ inactivation	*Ct* value/range (mg/liter × min)		EPA Guidance Manual *Ct* values
	dFCV	dAD40	
2	0.21/0.09–0.36	1.51/0.72–2.4	4
3	0.56/1.08–2.7	9.69[a]/NO[a]	6
4	1.10[a]/NO[a]	36.09[a]/NO[a]	8

[a] NO, amount of inactivation not observed in actual experiments

EPA Guidance Manual *Ct* values; however, higher values were predicted for 3- and 4-log dAD40 inactivation.

A comparison of observed inactivation curves with EFH model predicted curves are shown in Fig. 1 to 5 for dispersed and aggregated viruses. The data points termed "observed" in the charts include values obtained from bench-scale experiments, and "predicted" lines were generated by application of the EFH model.

Highly significant ($P < 0.001$) differences in viral inactivation were observed for temperature, pH, water type, and viral state (aggregated or dispersed). Viral inactivation rates were higher at pH 6 than at pH 8 (dFCV and dAD40), higher at 15°C than at 5°C (dFCV), higher for dispersed than for aggregated (FCV and PV-1), and higher in BDF than in treated groundwater (dFCV and dAD40) experiments. The sensitivity of the studied viruses, in the dispersed state, to chlorine inactivation was generally greatest for dFCV, followed by dAD40, followed by dPV-1 for experiments conducted in BDF water.

DISCUSSION

In the United States, the reported average free chlorine residual used in drinking water treatment is approximately 1.0 mg/liter, and the average and median contact times are 237 and 60 min, respectively (4, 47). Considering these common chlorination practices, the results of this study suggest that 4-log inactivation of dFCV, dAD40, aFCV, dPV-1, and aPV-1 would occur under the tested pH, water, and temperature conditions. Low residual chlorine concentrations that can occur in some sections of the distribution system, however, may not be high enough for inactivation of viruses that bypass treatment, i.e., viruses that are introduced post-drinking water treatment processes.

Adenovirus type 3 is reported to have a greater susceptibility to free chlorine than PV-1 at 4 and 25°C (7). In this study, the *Ct* values (4 and 25°C, 3-log inactivation) ranged from 0.013 to 0.084, with higher *Ct* values at higher pH values and lower temperatures. Minimal chlorine demand was reported for the viral stock and bench scale viral inactivation experiments.

Shin et al. (40) reported that PV-1 was more resistant to free-chlorine inactivation than Norwalk virus. The ability of reverse transcriptase PCR (RT-PCR) to amplify viral nucleic acids was used by these authors as a measure of free-chlorine inactivation. Norwalk virus PCR units per milliliter were undetectable by 30 min (initial dose of 1.0 mg/liter, pH 6, and 5°C). Viral inactivation may have been sooner than indicated, however, since comparable cell culture infectivity studies demonstrated that the RT-PCR method overestimated the time for PV-1 inactivation. For example, 4 logs of PV-1 inactivation,

determined by cell culture infectivity assays and RT-PCR, occurred by 10 and 30 min, respectively (40). These authors included steps to purify and disperse their viral stocks and measure residual disinfectant at the end of inactivation experiments. Evidence from this and the present study's results suggest that the studied caliciviruses are more sensitive to chlorine disinfection than PV-1 at pH 6 and 5°C.

Disparities between viral disinfection studies make conclusions regarding the effectiveness of chlorine difficult. Differences in viral preparation and disinfectant decay rate can have large effects on viral inactivation. In this study, the manner in which viral stocks were prepared, chloroform extracted versus non-chloroform extracted, made large differences in chlorine inactivation rates. Viral preparations that are cell associated or aggregated decrease viral inactivation rates and therefore increases *Ct* values. Cell-associated or aggregated viruses probably result in a "protective effect," as reported by Sobsey et al. (42) for chlorine inactivation of cell-associated HAV. If *Ct* values are calculated based only on the initial free chlorine dose, and if the chlorine decay constant, k', is large, the *Ct* values will be higher than those in experiments that have negligible k'. Disinfection studies should, therefore, report the initial disinfectant dose and the disinfectant decay rate throughout the inactivation experiment. Earlier studies have shown that the type of buffer, potassium chloride (5) and sodium chloride (39), used for suspending viruses during chlorine disinfection experiments can increase viral inactivation. Properties of cell lines, such as their ability to repair damaged nucleic acid or their sensitivity to viral infection, can also affect observations regarding viral inactivation. Consequently, the manner in which disinfection experiments are designed can have a great impact on the outcome of viral inactivation. Researchers should strive to conduct these types of experiments in a similar manner and report all pertinent information regarding viral preparation, chlorine dose and residuals, and overall experimental design.

The EPA Guidance Manual For Compliance with the Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources advises water utility operators of "design, operating and performance criteria for specific surface water quality conditions to provide optimum protection of public health through multiple barrier treatment." Utility operators can monitor chlorine concentrations at various points within the treatment train and calculate travel time of treated water. *Ct* values of the system can be compared to guidance manual *Ct* values to determine if adequate viral inactivation is being achieved. The manual's *Ct*s are based on HAV inactivation studies performed by Sobsey et al. (43). These experiments were conducted with dispersed viruses suspended in BDF water. A safety factor of 3 was multiplied by the highest *Ct* value determined from HAV chlorine disinfection experiments at pHs 6 through 9 and chlorine concentrations of 0.5 to 0.2 mg/liter. The manual's *Ct* values are considerably higher than those determined for dFCV and dAD40 in BDF water. However, *Ct* values for aggregated viral particles exceeded those listed in the EPA Guidance Manual for 3- and 4 log-reduction. Consequently, Guidance Manual *Ct* values need reassessment in order to guide water utilities for achieving sufficient inactivation of viruses in the aggregated state.

Sobsey et al. (42) suggested that *Ct* values should be based on inactivation studies conducted with aggregated or cell-associated viruses, since viruses that occur in water are probably associated with organic debris, are aggregated, or are clumped (42). Furthermore, the size range (0.5 to 1.0 μm) of some of these aggregates means that they are not effectively removed by filtration processes (32, 42). In the work performed by Sobsey et al. (42), HAV was more resistant to free chlorine when no dispersion or purification of the viral stock was performed. In the present study, inactivation curves of aPV-1 and aFCV that lacked a chloroform extraction procedure in their preparation displayed tailing and increased inactivation times, indicating aggregation or clumping of the viral suspensions. The aggregated viral preparations were more resistant to free-chlorine inactivation than stocks which were dispersed by chloroform extraction. Thus, the results reported by Keswick et al. (29) may not be surprising. In this study, a dose of 3.75 mg of free chlorine/liter, 25°C, pH 7.4, and contact time of 30 min (Ct = 112.5 mg/liter × min) failed to inactivate Norwalk virus, causing five of eight volunteers to seroconvert, and four became ill (29). 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 (42, 44). In another study performed with an FCV stock that lacked purification procedures, only 1.55 logs was inactivated by a chlorine dose of 0.22 mg/liter after a 10-min contact time (37). Due to the effects of aggregation and association with organic debris, viruses are more likely to survive drinking water disinfection than dispersed viral particles.

In experiments conducted with dFCV and dAD40 suspended in treated groundwater at 5°C, *Ct*s were higher than in experiments conducted in BDF water at pH 8. The higher *Ct* values for viruses in groundwater were unexpected, since the rate of microbial inactivation generally increases by a factor of 2 or 3 as temperature increases by 10°C (21). Groundwater disinfection inactivation curves for both viruses exhibited tailing. Tailing of inactivation curves can be caused by viral aggregation, decrease of disinfectant concentration, or adsorption to particulates allowing viral protection from disinfectant action (44). Viral aggregation can occur because of viral capsid conformational changes caused by pH condition or salts (44). The groundwater's pH probably did not stimulate viral aggregation, since similar *Ct*s were not observed for experiments carried out at pH 8 in BDF water. Disinfectant decay constants for dFCV groundwater experiments are higher than those calculated for BDF water, suggesting that the tailing effect is due to a decrease in disinfectant concentration throughout the experiment. However, for experiments conducted with dAD40, the k' value was lower than that with BDF water; thus, low chlorine residuals may not be the tailing effect's primary cause. Moreover, *Ct* values for dAD40 experiments performed in treated groundwater were much larger, even at the higher free chlorine dose of 0.5 mg/liter, than for BDF water experiments (0.15 mg/liter). Groundwater constituents may have afforded dAD40, and possibly dFCV, increased resistance to free chlorine by enhancing viral aggregation or protection via adsorption to particles. If aggregation is the cause of the increased chlorine resistance, dAD40 may be more resistant in this state

than either dPV-1 or dFCV. Chlorine-inactivation studies are needed to make conclusions regarding the chlorine resistance of aggregated AD40 particles.

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 and can be used to calculate *Ct* values at various chlorine levels. EFH model predicted curves fit the curves generated from the observed bench-scale experiments well for all BDF experiments. For groundwater and aggregated virus experiments, however, very high *Ct* values were predicted for 4-log inactivation (bench scale inactivation values lacking). Additional studies will determine if these values are accurate. Prediction of *Ct* values with free chlorine concentrations that were not used in the bench-scale 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 bench-scale experiments.

ACKNOWLEDGMENT

We thank the American Water Works Association Research Foundation for funding this project (project 442).

REFERENCES

1. **Albert, M. J.** 1986. Enteric adenoviruses. Arch. Virol. **88:**1–17.
2. **Alvarez, M. E., M. Aguilar, A. Fountain, N. Gonzalez, O. Rascon, and D. Saenz.** 2000. Inactivation of MS-2 phage and poliovirus in groundwater. Can. J. Microbiol. **46:**159–165.
3. **American Public Health Association.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
4. **American Water Works Association.** 2000. Committee report: disinfection at large and medium-sized systems. J. Am. Water Works Assoc. **92:**32–43.
5. **Berg, G., and H. Sanjaghsaz.** 1995. KCl potentiated inactivation of poliovirus-1 by free chlorine at pH 4.5. J. Virol. Methods **53:**113–119.
6. **Clarke, I. N., and P. R. Lambden.** 2000. Organization and expression of calicivirus genes. J. Infect. Dis. **181:**S309–S316.
7. **Clarke, N. A., R. E. Stevenson, and P. W. Kabler.** 1956. The inactivation of purified type 3 adenovirus in water by chlorine. Am. J. Hygiene **64:**314–319.
8. **Doultree, J. C., J. D. Druce, C. J. Birch, D. S. Bowden, and J. A. Marshall.** 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. J. Hosp. Infect. **41:**51–57.
9. **Engelbrecht, R. S., M. J. Weber, B. L. Salter, and C. A. Schmidt.** 1980. Comparative inactivation of viruses by chlorine. Appl. Environ. Microbiol. **40:**249–256.
10. **Enriquez, C. E., C. J. Hurst, and C. P. Gerba.** 1995. Survival of the enteric adenovirus-40 and adenovirus-41 in tap, sea, and waste-water. Water Res. **29:**2548–2553.
11. **Fankhauser, R. L., J. S. Noel, S. S. Monroe, T. Ando, and R. I. Glass.** 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J. Infect. Dis. **178:**1571–1578.
12. **Federal Register.** 1998. Announcement of the drinking water contaminant candidate list. Fed. Regist. **63:**10273–10287.
13. **Floyd, R., J. D. Johnson, and D. G. Sharp.** 1976. Inactivation by bromine of single poliovirus particles in water. Appl. Environ. Microbiol. **31:**298–303.
14. **Floyd, R., and D. G. Sharp.** 1978. Viral aggregation: effects of salts on the aggregation of poliovirus and reovirus at low pH. Appl. Environ. Microbiol. **35:**1084–1094.
15. **Foy, H. M., M. K. Cooney, and J. B. Hatlen.** 1968. Adenovirus type 3 epidemic associated with intermittent chlorination of a swimming pool. Arch. Environ. Health **17:**795–802.
16. **Glass, R. I., J. Noel, T. Ando, R. Fankhauser, G. Belliot, A. Mounts, U. D. Parashar, J. S. Bresee, and S. S. Monroe.** 2000. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. J. Infect. Dis. **181:**S254–S261.
17. **Grabow, W. O. K., P. Coubrough, C. Hilner, and B. W. Bateman.** 1984. Inactivation of hepatitis A virus, other enteric viruses and indicator organisms in water by chlorination. Water Sci. Technol. **17:**657–664.
18. **Grabow, W. O. K., D. L. Puttergill, and A. Bosch.** 1992. Propagation of adenovirus type-40 and type-41 in the PLC/PRF/5 primary liver-carcinoma cell-line. J. Virol. Methods **37:**201–208.
19. **Gyurek, L. L., and G. R. Finch.** 1998. Modeling water treatment chemical disinfection kinetics. J. Environ. Eng.-ASCE **124:**783–793.
20. **Hafliger, D., P. Hubner, and J. Luthy.** 2000. Outbreak of viral gastroenteritis due to sewage-contaminated drinking water. Int. J. Food Microbiol. **54:**123–126.
21. **Hoff, J. C.** 1986. Inactivation of microbial agents by chemical disinfectants EPA 600 S2–86 067. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
22. **Horowitz, M. S.** 1996. Adenoviruses, p. 2149–2171. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven, Philadelphia, Pa.
23. **Hurley, M. A., and M. E. Roscoe.** 1983. Automated statistical analysis of microbial enumeration by dilution series. J. Appl. Microbiol. **55:**159–164.
24. **Hurst, C. J., K. A. McClellan, and W. H. Benton.** 1988. Comparison of cytopathogenicity, immunofluorescence and in situ DNA hybridization as methods for the detection of adenoviruses. Water Res. **22:**1547–1552.
25. **Irving, L. G., and P. A. Smith.** 1981. One-year survey of enteroviruses, adenoviruses, and reoviruses isolated from effluent at an activated-sludge purification plant. Appl. Environ. Microbiol. **41:**51–59.
26. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195:**51–61.
27. **Kapikian, A. Z., M. K. Estes, and R. M. Chanock.** 1996. Norwalk group of viruses, p. 783–810. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven, Philadelphia, Pa.
28. **Kaplan, J. E., R. A. Goodman, L. B. Schonberger, E. C. Lippy, and G. W. Gary.** 1982. Gastroenteritis due to Norwalk virus: an outbreak associated with a municipal water system. J. Infect. Dis. **146:**190–197.
29. **Keswick, T. K. Satterwhite, P. C. Johnson, H. L. DuPont, S. L. Secor, J. A. Bitsura, G. W. Gary, and J. C. Hoff.** 1985. Inactivation of Norwalk virus in drinking water by chlorine. Appl. Environ. Microbiol. **50:**261–264.
30. **Kukkula, M., P. Arstila, M. L. Klossner, L. Maunula, C. H. V. Bonsdorff, and P. Jaatinen.** 1997. Waterborne outbreak of viral gastroenteritis. Scand. J. Infect. Dis. **29:**415–418.
31. **Kukkula, M., L. Maunula, E. Silvennoinen, and C.-H. von Bonsdorff.** 1999. Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses. J. Infect. Dis. **180:**1771–1776.
32. **Lawler, D. F., C. R. O'Melia, and J. E. Tobiason.** 1980. Integral water treatment plant design from particle size to plan performance. *In* J. O. Leckie (ed.), Particulates in water. American Chemical Society, Washington, D.C.
33. **Lukasik, J., M. L. Bradley, T. M. Scott, M. Dea, A. Koo, W. Y. Hsu, J. A. Bartz, and S. R. Farrah.** 2003. Reduction of poliovirus 1, bacteriophages, *Salmonella montevido*, and *Escherichia coli* O157:H7 on strawberries by physical and disinfectant washes. J. Food Prot. **66:**188–193.
34. **Nuanualsuwan, S., and D. O. Cliver.** 2003. Capsid functions of inactivated human picornaviruses and feline calicivirus. Appl. Environ. Microbiol. **69:**350–357.
35. **Papapetropoulou, M., and A. C. Vantarakis.** 1998. Detection of adenovirus outbreak at a municipal swimming pool by nested PCR amplification. J. Infect. **36:**101–103.
36. **Prasad, B. V. V., M. E. Hardy, and M. K. Estes.** 2000. Structural studies of recombinant Norwalk capsids. J. Infect. Dis. **181:**S317–S321.
37. **Scott, F. W.** 1980. Virucidal disinfectants and feline viruses. Am. J. Vet. Res. **41:**410–414.
38. **Sharp, D. G., and J. Leong.** 1980. Inactivation of poliovirus 1 (Brunhilde) single particles by chlorine in water. Appl. Environ. Microbiol. **40:**381–385.
39. **Sharp, D. G., D. C. Young, R. Floyd, and J. D. Johnson.** 1980. Effect of ionic environment on the inactivation of poliovirus in water by chlorine. Appl. Environ. Microbiol. **39:**530–534.
40. **Shin, G. A., D. Battigelli, and M. D. Sobsey.** 1998. Reduction of Norwalk virus, poliovirus 1, and coliphage MS2 by free chlorine, chlorine dioxide, and ozone disinfection of water. *In* Proceedings of the Water Quality Technology Conference. American Water Works Association, Denver, Colo.
41. **Slomka, M. J., and H. Appleton.** 1998. Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. Epidemiol. Infect. **121:**401–407.
42. **Sobsey, M. D., T. Fuji, and R. M. Hall.** 1991. Inactivation of cell-associated and dispersed hepatitis A virus in water. J. Am. Water Works Assoc. **83:**64–67.
43. **Sobsey, M. D., T. Fuji, and P. A. Shields.** 1988. Inactivation of hepatitis A virus and model viruses in water by free chlorine and monochloramine. Water Sci. Technol. **20:**385–391.
44. **Thurman, R. B., and C. P. Gerba.** 1988. Molecular mechanisms of viral inactivation by water disinfectants. Adv. Appl. Microbiol. **33:**75–105.
45. **U.S. Environmental Protection Agency.** 1989. Guidance manual for compliance with the filtration and disinfection requirements for public water systems using surface water sources. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
46. **U.S. Environmental Protection Agency.** 2001. National primary drinking water standards. EPA816.-F-01–007. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
47. **White, G. C.** 1999. Handbook of chlorination and alternative disinfectants, 4th ed. John Wiley & Sons, Inc., New York, N.Y.