Ozone Inactivation of *Cryptosporidium parvum* in Demand-Free Phosphate Buffer Determined by In Vitro Excystation and Animal Infectivity

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Inactivation of *Cryptosporidium parvum* oocysts by ozone was performed in ozone demand-free 0.05 M phosphate buffer (pH 6.9) in bench-scale batch reactors at 7 and 22°C. Ozone was added to each trial from a concentrated stock solution for contact times ranging from 5 to 15 min. The viability of the control and treated oocysts was determined by using in vitro excystation and infection in neonatal CD-1 mice. It was found that excystation consistently underestimated inactivation when compared with animal infectivity ($P \le 0.05$). As inactivations increased, the difference between excystation and infectivity also increased. The inactivation kinetics of *C. parvum* by ozone deviated from the simple first-order Chick-Watson model and was better described by a nonlinear Hom model. The use of the Hom model for predicting inactivation resulted in a family of unique concentration and time values for each inactivation level rather than the simple *CT* product of the Chick-Watson model.

Cryptosporidium spp. have become recognized as a cause of waterborne disease in humans (1). Cryptosporidiosis outbreaks from surface water supplies have been documented in the United States and Great Britain, and it has been speculated that many other cases of waterborne gastroenteritis may have been caused by *Cryptosporidium* spp. (11, 12, 16, 31–33).

The biology of *Cryptosporidium* spp. has been well documented in review articles (4, 5). The life cycle can be summarized by six events: excystation of the oocysts in the intestine of the host, replication within the host, gamete formation, fertilization, oocyst wall formation, and sporozoite formation (4). *Cryptosporidium parvum* appears to lack host specificity and has been shown to be able to cross-infect rodents, ruminants, and humans (5). Of most interest to the water supply industry is the thick-walled oocyst. LeChevallier et al. (23) detected *C. parvum* in 87% of raw water samples and 27% of drinking water samples during a survey of North American drinking water supplies.

There have been relatively few studies of ozone inactivation of *Cryptosporidium* oocysts to date (20, 22, 27, 28, 30). Most of these studies used animal infectivity as a measure of the degree of inactivation. Korich et al. (20) reported that in vitro excystation gave results similar to those of the animal model under the conditions examined. In addition, most of the previous work examined inactivation at room temperature and did not examine the kinetics of inactivation. The objectives of this study were to compare in vitro excystation with infectivity in neonatal CD-1 mice for determination of viability after disinfection, to determine the ozonation requirements at room temperature and at 7°C in a controlled laboratory water, and to determine the kinetics of inactivation.

MATERIALS AND METHODS

Phosphate buffer and ozone apparatus. Deionized water was obtained from a Milli-Q system (Millipore Corp. model OM-140) operated at a resistivity of at least 18 MΩ/cm. The 0.05 M phosphate buffer (pH 6.9) was prepared by using potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate (BDH Inc., AnalaR grade). The resulting solution was made ozone demand-free by using procedures described elsewhere (9).

A concentrated solution of ozone was prepared in Milli-Q water by using the procedure described in an earlier study (21). The concentration of ozone in aqueous solution was determined by using UV spectrophotometry at 260 nm and a molar absorption coefficient of 3,300 M^{-1} cm^{-1} (15). Residual ozone was neutralized with 1.0 M sodium formate. The amount of formate that was added varied depending on the ozone dose but was just sufficient to reduce the A_{260} to zero. The mass of sodium formate added to the test solution does not have an interfering A_{260} and has no adverse effect on the oocysts.

The reactor vessel was a 50- or 125-ml Erlenmeyer flask with a Teflon-coated magnetic stir bar for agitation. All reactors were made ozone demand-free prior to use by methods described elsewhere (9). Cold-temperature experiments were conducted by using an ice water bath around the reactor vessel.

Disinfection procedure. The *C. parvum* oocysts used in this study were obtained from Frank W. Schaefer III, Office of Research and Development, Risk Reduction Engineering Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. The oocysts were stored at 4°C in double-deionized water. When required for a disinfection trial, an aliquot of the stock oocyst suspension was washed and concentrated by centrifugation (Jouan model GR4.11 centrifuge) at 910 × *g* for 15 min before being resuspended in Milli-Q water. The number of oocysts in the preparation was determined by counting four grids of a hemocytometer. The counts were checked for consistency with a Poisson distribution by the Fisher index of dispersion (10). Oocysts were

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added to a 50- or 125-ml volume of phosphate buffer in an Erlenmeyer flask for a final oocyst concentration determined by hemocytometer counts of between 8×10^3 and 2×10^4 oocysts per ml (the target was approximately 10^4 oocysts per ml).

The concentration of the stock ozone solution was measured twice immediately before a calculated volume of ozone solution for the required applied dose was added to the reactor with a calibrated pipette. The reactor was continuously sampled in a closed loop at a flow rate of 8 ml/min. The sample was carried through a short piece of small-diameter Teflon tubing to a temperature-controlled, 35-μl flow cell with a light path of 1 cm. A diode-array spectrophotometer (Hewlett-Packard model 8452A) was used to continuously monitor the A_{260} of the solution. At the end of the predetermined contact time, any remaining ozone was removed by using sodium formate. The change in absorbance after adding formate was used to calculate the ozone residual at the end of the contact time. The applied ozone dose is the mass of ozone obtained from the stock solution divided by the final volume of the test liquid in the batch reactor. The initial ozone residual is the dissolved ozone observed in the vessel at time zero. The initial ozone residual is analogous to the transferred ozone dose in a system where ozone is added as a gas. The degree of immediate ozone demand can be estimated from the ratio of the initial ozone residual to the calculated applied ozone dose. Utilized ozone is the difference between the calculated applied ozone dose and the final ozone residual at the end of the contact time.

Oocyst recovery and animal infection procedure. After disinfection, 0.01% Tween 20 was added to the reactor vessel and mixed. The entire contents of the reactors were emptied into appropriately sized centrifuge tubes, rinsed with Milli-Q water and 0.01% Tween 20, and centrifuged at $900 \times g$ for 20 min. The supernatant was removed by aspiration, leaving approximately 5 ml of concentrated sample containing the oocysts. Oocysts were counted four times with a hemocytometer before 5×10^4 oocysts were removed for the excystation procedure. The remaining volume was concentrated further by centrifugation at $900 \times g$ for 15 min. The supernatant was removed by aspiration, leaving a 1-ml pellet that was transferred to a microcentrifuge tube. The original centrifuge tubes were washed with 0.01% Tween 20, and the wash water was saved. The wash water and pellet were centrifuged at $3,000 \times g$ for 3 min in an MSE Microcentaur centrifuge (Johns Scientific, Inc.). The supernatant was removed from all of the ultracentrifuge tubes, and the wash water pellet was resuspended in a small amount of 0.01% Tween 20, added to the remaining oocysts, and centrifuged again at $3,000 \times g$ for 3 min. The supernatant was removed, and 1 ml of 0.01% Tween 20 was added to the tube. The oocysts were counted again, and when necessary, the solution was further concentrated by centrifugation, and the appropriate amount was administered orally to the mice.

A neonatal mouse model described elsewhere was used to evaluate infectivity (7). Breeding pairs of CD-1 outbred mice were obtained from the Charles River Breeding Laboratories (St. Constant, Quebec, Canada). The animals were given food and water ad libitum and housed in cages with covers fitted with 0.22-μm-pore-size filters in a specific-pathogen-free (P-2 level) animal facility. Four days after birth, the mice were inoculated orally by using a pipette with a known number of oocysts suspended in 5 to 10 μl of Milli-Q water. The infectivity of the oocysts was determined 7 days after infection. The mice were killed by cervical dislocation, and the lower half of the small intestine, the cecum, and the

colon were removed and placed in 10 ml of Milli-Q water. The intestines were homogenized for 10 s in a Sorvall Omni-Mixer and washed three times in Milli-Q water containing 0.01% Tween 20 at 2,000 × g for 15 min. After centrifugation, the supernatant was discarded, 10 ml of Sheather's sugar solution was added to the pellet, and the mixture was centrifuged at 1,000 × g for 10 min. A few drops from the surface of the suspension were removed and examined with differential interference contrast microscopy at ×400 power. Mice were scored either positive or negative for oocysts after examination of the entire slide.

Oocyst viability. The excystation procedure was modified from that of Woodmansee (36). Approximately 5×10^4 oocysts were required for excystation. The oocysts were placed in 0.5 ml of phosphate-buffered saline (PBS), to which 0.5 ml of 2× excystation medium (0.05 g of trypsin and 0.15 g of sodium taurocholate in 5 ml of tissue-culture-grade PBS) was added. The oocysts were incubated in the excystation medium at 37°C in a water bath. After 60 min in the water bath, the tubes were brought to room temperature for 30 min. The numbers of oocysts, shells, and sporozoites were counted at a magnification of ×400 by using differential interference microscopy. A minimum of 100 shells plus intact oocysts were counted for each trial. Preparations were kept on ice until the counts were completed.

Excystation and theoretical sporozoite yield calculated by the Woodmansee (36) method is determined by:

$$\text{excystation} = \frac{\text{shells}}{\text{intact oocysts} + \text{shells}} \times 100 \quad (1)$$

The Woodmansee procedure is similar to that used for calculating excystation rates of *Giardia muris*. The Korich et al. (20) calculation is based on:

$$\% \text{ excystation} = \left(\frac{S}{S + \text{intact oocysts}} \right) \times 100 \quad (2)$$

where S is equal to the number of excysted sporozoites in the treated sample divided by the number of excysted sporozoites per shell in the control.

By using either method for the rate of excystation, the inactivation is estimated from:

$$\log \frac{N}{N_0} = \log \left(\frac{\% \text{ excystation}_{\text{treated}}}{\% \text{ excystation}_{\text{control}}} \right) \quad (3)$$

where N_0 is the initial concentration of bacteria and N is the concentration of surviving bacteria.

The dose-response model used in this study to determine postdisinfection viability was developed for neonatal CD-1 mice (7):

$$\text{Response logit} = -6.738 + 3.547 \log_{10}(\text{inoculum}) \quad (4)$$

where logit is $\ln [P/(1 - P)]$ and P is the proportion of animals infected for the specified inoculum. The predicted oral inoculum was multiplied by the dilution factor for the group of animals responding to estimate the infective oocysts. The inactivation was determined by a comparison of the infective oocysts per animal after disinfection with the total number of oocysts given to each animal. The log inactivation is calculated from:

$$\log \frac{N}{N_0} = \log \left(\frac{n}{n_0} \right) \quad (5)$$

where n is the estimated infectious dose per animal after ozone treatment and n_0 is the number of oocysts given to each animal.

Data analysis. Chick (3) postulated that the disinfection reaction was similar to a chemical reaction, with the reaction rate dependent on the relative concentrations of microorganisms and disinfectant. If the disinfectant concentration was constant, the reaction would be first order with respect to surviving bacteria. Watson (35), by using Chick's data, expanded the model to account for changes in disinfectant concentration. The Chick-Watson model remains the dominant disinfection kinetic theory in use today. It has been expressed as the following (14):

$$\log\frac{N}{N_0} = -kC^nT \tag{6}$$

where T is time; k is the pseudo first-order reaction rate constant, C is the concentration of disinfectant, and n is the coefficient of dilution, an empirical factor frequently assumed to be unity. If a desired level of inactivation is set, then equation 6 reduces to a form frequently used by utilities to meet disinfection regulations:

$$CT = K \tag{7}$$

where K is a constant for each microorganism to obtain a desired level of inactivation at a given temperature. With some disinfectants, pH is also important in determining the constant. K also includes the inactivation rate constant k. Equation 7 suggests that for any combination of C and T that gives the K value, the appropriate level of inactivation will occur. For the purposes of further discussion in this article, the product of C and T will be referred to as the simple CT product.

The Chick-Watson model has been reported to be adequate for describing chlorine inactivation kinetics in chlorine demand-free waters (14). However, ozone behaves differently than chlorine. Several studies have noted that ozone disinfection had two distinct stages: a rapid initial stage followed by a much slower second stage (8, 17, 19). This type of kinetic plot deviates from first-order kinetics, suggesting that the Chick-Watson model was inadequate for describing ozone disinfection. Hom (18) proposed an alternate model to the Chick-Watson model to account for deviations from the Chick-Watson model encountered in practice. The model takes the form:

$$\log\frac{N}{N_0} = -kC^nT^m \tag{8}$$

where m is an empirical constant. Unlike the simple Chick-Watson model, where n equals 1 and the simple CT product is a constant for each level of inactivation irrespective of contact time or concentration, the Hom model has values of n and m that are not unity. Rather, the concentration and contact time change in a nonlinear fashion according to:

$$C^nT^m = K' \tag{9}$$

where K' is a constant for a given microorganism at the desired level of inactivation and incorporates the kinetic rate constant k. The empirical parameters k, n, and m can be estimated by using the experimental data. The solver function of Microsoft Excell (Microsoft Corporation, Mississauga, Ontario, Canada) was used to estimate the

parameters by a maximum-likelihood procedure that has been shown to be superior when some data exceed detection limits (13). Details of the analytical formulation of the maximum-likelihood approach are available elsewhere (6).

The assumption that underlies both the Chick-Watson and Hom models is that the disinfectant concentration remains approximately constant during the course of the contact time. In ozone studies, this is an incorrect assumption since ozone is reactive and continuously disappears during the contact time. There are several methods for defining the ozone concentration characteristic, C, depending on the method of adding ozone to the liquid and the type of reactor (24). Where ozone is added from a concentrated stock solution into a batch reactor, the reactor vessel can be considered as a reactive flow segment with ideal plug flow (24).

For purposes of gaining disinfection credit in an ozone disinfection process, several approaches have been suggested for estimating CT (26). The discussion here will focus on the reactive flow segment which is conceptually similar to the reactor used in this study. If there are no means of measuring ozone residual continuously in a reactive flow segment, it has been recommended that the ozone residual at the end of the contact time (outlet of the reactor) be used for CT calculations (24, 26). This approach is conservative since it provides no credit for the higher concentrations of ozone at the inlet to the reactor. A potentially better method is to estimate the CT product in a reactive flow segment by integrating the ozone residual concentration over the contact time of the reactor by measuring an average ozone residual for each short segment of contact time and then summing these over the total contact time of the reactor. Because ozone decay in demand-free water can be approximated by a first-order ozone consumption model, the integrated ozone residual, C, for each experimental trial can be estimated from:

$$C = \text{antilog}\left(\frac{\log C_0 + \log C_f}{2}\right) \tag{10}$$

where C_0 is the initial ozone residual at time zero and C_f is the ozone residual at the end of the contact time, T. Water quality has a major influence on ozone decomposition rates and reaction order. Ozone decomposition is affected by water quality parameters such as temperature, pH, dissolved organic carbon content, and alkalinity. To apply the approach presented in this study to other waters, prior knowledge of the ozone decomposition kinetics is necessary. If the kinetics are other than first order, the integrated ozone will need to be estimated by using an equation other than equation 10.

In a continuous-flow system, a tracer study would need to be performed to determine the characteristic T for the reactor (25). When using a batch reactor such as was used in this study, the actual contact time in each experimental trial can be used as the characteristic T. Consequently, when the results of this study are used in a full-scale, dynamic system, the kinetic parameters can be used with whatever mixing regime is determined for the system.

RESULTS AND DISCUSSION

Viability determination after inactivation. Table 1 summarizes the ozonation conditions of each experimental trial.

TABLE 1. Summary of experimental conditions and inactivation of *C. parvum* oocysts in 0.05 M phosphate buffer (pH 6.9)

Trial code no.	Reactor vol (ml)	Temp (°C)	Contact time (min)	Applied ozone dose (mg/liter)	Initial ozone residual (mg/liter)	Final ozone residual (mg/liter)	Integrated ozone residual (mg/liter)	Reduction in infectivity (log units)	Reduction in excystation (log units)[a]	Reduction in excystation (log units)[b]
6	50	22	5	1.6	1.5	0.3	0.7	2.5	Not done	Not done
10	125	24	5	0.7	0.7	0.4	0.5	2.6	0.5	0.7
16	50	21	5	2.6	2.7	1.4	1.9	>2.4	1.6	1.4
17	50	21	5	1.8	1.8	0.9	1.3	2.1	1.1	1.1
30	50	22	5	2.1	2.0	0.9	1.3	0.8	0.3	0.4
35	125	22	10	2.9	2.9	1.3	2.0	>2.5	1.6	1.8
37	125	22	5	2.8	2.7	1.9	2.3	>2.5	1.7	1.5
43	50	22	10	1.6	1.7	0.3	0.8	>1.5	0.8	0.5
45	50	22	15	1.4	1.4	0.1	0.4	>1.9	1.1	0.6
56	50	22	10	1.7	1.7	0.3	0.8	3.6	0.8	0.9
58	50	22	5	2.4	2.4	1.2	1.7	3.7	0.3	0.5
62	50	7	5	1.6	1.5	0.9	1.2	1.6	0.6	0.8
64	50	7	10	1.5	1.5	0.5	0.8	>1.9	0.5	0.6
68	50	5	5	2.9	2.6	1.2	1.7	2.4	1.0	1.1
70	50	5	10	2.0	1.9	0.3	0.8	2.8	0.7	0.9
73	50	3	5	0.8	0.8	0.4	0.6	1.0	1.1	0.6
75	50	3	10	0.6	0.6	0.2	0.3	0.7	0.6	0.6
78	50	8	10	2.4	2.3	0.9	1.5	3.7	2.0	1.9
80	50	8	5	1.3	1.4	0.8	1.0	0.7	0.1	0.1
84	50	7	10	2.3	2.3	0.7	1.2	3.2	1.7	1.4
88	50	10	9	2.6	2.7	1.3	1.8	2.8	2.0	1.4
93	50	8	15	1.9	2.0	0.5	0.9	3.6	1.5	1.56
95	50	22	5	1.4	1.4	0.6	0.9	1.2	0.8	0.59
97	50	22	10	1.7	1.7	0.3	0.7	2.5	1.7	1.54
100	50	22	5	0.8	0.7	0.3	0.5	1.9	1.5	1.03
102	50	22	10	2.6	2.4	0.5	1.0	4.2	1.5	1.15
105	50	22	10	1.6	1.5	0.2	0.5	3.5	1.4	1.11
107	50	22	15	2.4	2.2	0.2	0.6	4.7	1.6	1.20

[a] Determined by the method of Korich et al. (20).
[b] Determined by the method of Woodmansee (36).

Table 2 summarizes *C. parvum* infection in neonatal CD-1 mice for each ozone trial. Inactivation data were estimated by using the two excystation calculation methods and the dose-response model for neonatal CD-1 mice. By using a paired *t* test (2), it was found that there was no significant difference between the two excystation calculation procedures (*P* ≤ 0.05). However, when examining the calculated inactivations from each method for outliers (2), it was found that the Woodmansee procedure had outliers whereas the Korich procedure did not. On this basis, it was decided that the Korich method would be used for calculating inactivations by in vitro excystation.

A paired *t* test of inactivations using animal infectivity and the Korich excystation calculation revealed that the excystation procedure significantly (*P* ≤ 0.05) underestimated inactivation when compared with infectivity. Examination of the variance between animal infectivity and the Korich excystation calculation at each ozone dose level revealed that the difference between means increased as the degree of inactivation (or ozone dose) increased. Figure 1 summarizes the inactivation results determined by excystation as a function of the integrated ozone residual and contact time product. Figure 2 summarizes inactivation data determined by infectivity as a function of the integrated ozone residual and contact time product. Inactivation of *C. parvum*, measured by animal infectivity and excystation at 7°C, is shown in Fig. 3.

The underestimation of oocyst inactivation by in vitro excystation has important implications for interpretation of *Cryptosporidium* disinfection data collected in this study and

reported in other studies. Some studies regarding disinfection of *C. parvum* by using common water disinfectants have been published but they have used in vitro excystation as a measure of inactivation (30, 34). The simple *CT* values reported in these articles may be misleading partly because of the shortcomings of the excystation procedure. Figure 3 illustrates the difference in survival ratios determined by excystation and infectivity at 7°C. More research is required before a consensus can be reached regarding the best measure of *C. parvum* viability. In the meantime, it appears that the use of an animal infectivity model remains the best choice for determining *C. parvum* inactivation by chemical means.

Ozonation requirements. A discussion of the ozonation design criteria for controlling *C. parvum* in water must include a comparison with other ozone disinfection studies involving *Cryptosporidium* oocysts. However, such a comparison is difficult for three reasons: a suitable kinetic model has not been reported in previous studies, there is a potential disparity in viability measurements between animal infectivity and excystation, and the ozonation protocol varies from study to study. Only one previous study published information that could be adapted to the data analysis approach used in this study (28). The first part of the discussion will focus on comparing simple *CT* products. Following this, a more rigorous comparison will be made by using the kinetic model developed in this study. Table 3 summarizes the pertinent experimental conditions and findings from the present and other studies.

The simple *CT* products for 99 and 99.9% inactivation in

TABLE 2. Summary of *C. parvum* infection in neonatal CD-1 mice for each ozone trial

Trial code no.	Inoculum (oocysts/animal)	No. of live animals in cohort	No. of infected animals in cohort	Response ratio[a]	Estimated infectious dose (oocysts/animal)[b]	N/N_0[c]	Reduction in infectivity (log units)
6	8.84×10^4	7	6	0.86	254	2.87×10^{-3}	2.5
10	8.31×10^4	5	4	0.80	195	2.35×10^{-3}	2.6
16	6.00×10^3	8	0	0.00	<23	$<3.83 \times 10^{-3}$	>2.4
17	2.88×10^3	9	1	0.11	21	7.14×10^{-3}	2.1
30	2.00×10^2	5	1	0.20	32	1.61×10^{-1}	0.8
35	7.29×10^3	6	0	0.00	<23	$<3.15 \times 10^{-3}$	>2.5
37	8.13×10^3	6	0	0.00	<23	$<2.83 \times 10^{-3}$	>2.5
43	6.95×10^2	6	0	0.00	<23	$<3.31 \times 10^{-2}$	>1.5
45	2.03×10^3	4	0	0.00	<23	$<1.13 \times 10^{-2}$	>1.9
56	1.33×10^5	5	1	0.20	32	2.43×10^{-4}	3.6
58	1.54×10^5	5	1	0.20	32	2.10×10^{-4}	3.7
62	1.30×10^3	5	1	0.20	32	2.48×10^{-2}	1.6
64	1.39×10^3	3	0	0.00	<23	$<1.65 \times 10^{-2}$	>1.8
68	2.17×10^4	4	2	0.50	79	3.66×10^{-3}	2.4
70	2.19×10^4	4	1	0.25	39	1.78×10^{-3}	2.8
73	1.06×10^3	5	3	0.60	103	9.70×10^{-2}	1.0
75	6.80×10^2	6	4	0.67	124	2.19×10^{-1}	0.7
78	1.52×10^5	6	1	0.17	28	1.84×10^{-4}	3.7
80	1.81×10^2	5	1	0.20	32	1.79×10^{-1}	0.7
84	1.19×10^5	6	3	0.50	79	6.68×10^{-4}	3.2
88	1.36×10^5	5	4	0.80	195	1.44×10^{-3}	2.8
93	1.21×10^5	6	1	0.17	28	2.31×10^{-4}	3.6
95	1.08×10^3	5	2	0.40	61	5.63×10^{-2}	1.2
97	1.10×10^4	5	1	0.20	32	2.95×10^{-3}	2.5
100	1.65×10^4	5	4	0.80	195	1.18×10^{-2}	1.9
102	1.49×10^6	5	3	0.60	103	6.91×10^{-5}	4.2
105	2.02×10^5	5	2	0.40	61	3.02×10^{-4}	3.5
107	1.57×10^6	5	1	0.20	32	2.06×10^{-5}	4.7

[a] Response ratio is the number of mice infected mice divided by the number of mice inoculated with *C. parvum*.
[b] Determined from the CD-1 neonatal mouse dose-response model (37).
[c] N/N_0 is the estimated number of infectious oocysts divided by the number of oocysts given to the mice.

the present study were estimated from Fig. 2 and Tables 4 and 5. Simple *CT* values of about 3.5 and 7 mg · min/liter were required for 99% inactivation at 22 and 7°C, respectively. For 99.9% inactivation, simple *CT* values of about 5 and 10 mg · min/liter were required at 22 and 7°C, respectively.

Korich et al. (20) used a semibatch reactor containing 0.01 M phosphate buffer (pH 7) with continuously added ozonated gas to maintain a constant ozone residual of 1 mg/liter. Neonatal BALB/c mice were used to determine viability of the oocysts. A constant ozone residual of 1.0 mg/liter was reported to effect 99% inactivation after 5 min of exposure (a simple *CT* of 5 mg · min/liter). A potential problem with this study was the use of a semibatch ozone contactor. The ozone dose and the decay of ozone during the course of the experiments were not reported, which resulted in the underestimation of the actual ozone dose used in the experiments to effect the inactivation.

Langlais et al. (22) used immune-suppressed male Sprague-Dawley rats to determine the infectivity of *Cryptosporidium baileyi* oocysts after ozonation. They reported that a simple *CT* product of 3.2 mg · min/liter inactivated between 3 and 4 log units (99.9 and 99.99%, respectively) of oocysts. They also observed that a simple *CT* product of 4.4 mg · min/liter inactivated between 4 and 5 log units of oocysts (99.99 and 99.999%, respectively).

Peeters et al. (28) followed an experimental protocol very similar to the one used in this study. Neonatal Swiss OF1 mice were used to determine the viability of the oocysts. An estimated 99% inactivation was obtained for an integrated
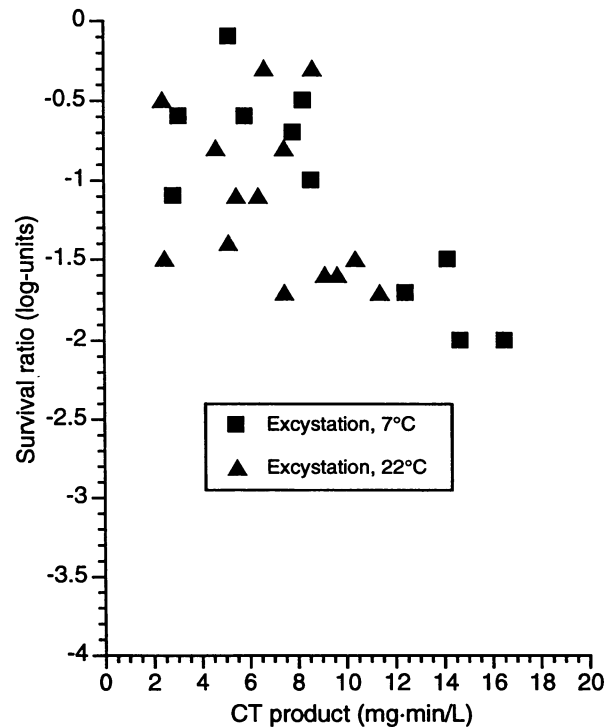

FIG. 1. *C. parvum* inactivation determined by excystation at 7 and 22°C as a function of the product of the integrated ozone residual and contact time.
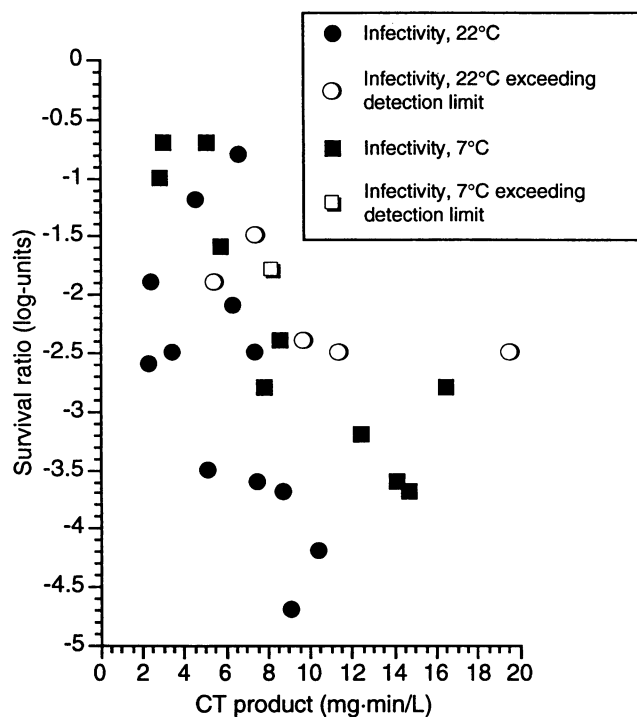
FIG. 2. *C. parvum* inactivation determined by animal infectivity at 7 and 22°C as a function of the product of the integrated ozone residual and contact time.
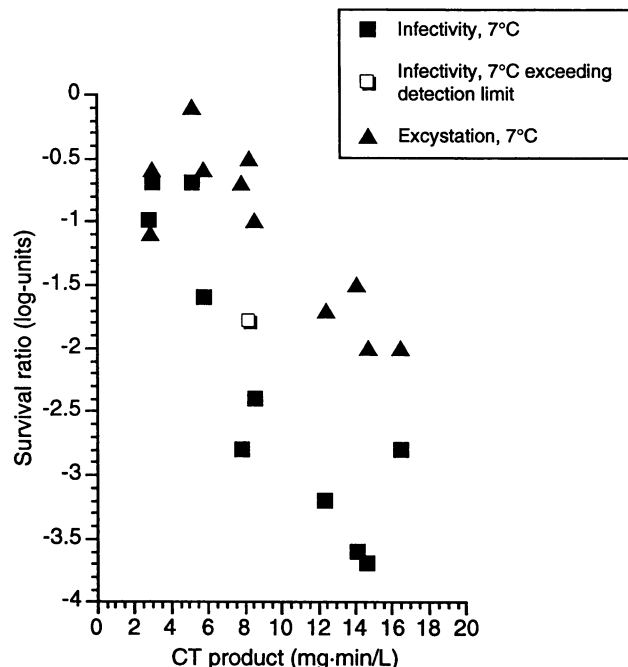

FIG. 3. Comparison of excystation and infectivity as measures of viability of *C. parvum* oocysts after ozone inactivation at 7°C as a function of the product of the integrated ozone residual and contact time.

ozone residual of 0.51 mg/liter and contact time of 8 min for a simple *CT* of 4.1 mg · min/liter. The same inactivation was obtained from an integrated ozone residual of 0.77 mg/liter and a contact time of 6 min or a simple *CT* of 4.6 mg · min/ liter.

Perrine et al. (29) reported that a constant ozone residual of 0.44 mg/liter for 6 min effected 99% inactivation of *C. parvum* oocysts. The resulting *CT* value was 2.6 mg · min/ liter. However, the authors used a continuous ozonation system to maintain the resulting residual and thereby under-estimated the actual ozone dose. Langlais et al. (22) used a modified batch ozonation protocol with *C. baileyi* oocysts. Rather than adding ozone to a solution containing oocysts, they added oocysts to an ozonated solution containing the desired residual ozone after satisfying the initial ozone

demand of the test water. They reported a simple *CT* product that was lower than that obtained in this study.

When comparing the studies based on simple *CT* products, it can be seen that there is a wide range of reported *CT* values to achieve 99 or 99.9% inactivations. The results of the present study in conjunction with those of the other studies suggest that a simple *CT* product of 3 or 4 mg · min/ liter is required for 99% inactivation of oocysts in ozone demand-free laboratory water at room temperature. Data from the present study suggest that colder water tempera-tures decrease the rate of reaction, resulting in a larger simple *CT* value.

To apply the more rigorous kinetic approach to the data, the maximum-likelihood estimates for the parameters k, n, and m in the Hom model (equation 8) were obtained with the animal infectivity data. The resulting equations were:

TABLE 3. Summary of reported ozonation requirements for inactivation of *Cryptosporidium* sp. oocysts by using conventional linear *CT* products

Species	Ozone protocol	Water type	Ozone residual (mg/liter)	Contact time (min)	Temp (°C)	Conventional linear *CT* for ≥99% inactivation (mg · min/liter)[a]	Reference
C. parvum	Batch liquid, batch ozone	0.05 M Phosphate buffer	0.16–1.3	5, 10, 15	7	7	This study
	Batch liquid, batch ozone	0.05 M Phosphate buffer	0.17–1.9	5, 10, 15	22	3.5	This study
	Batch liquid, batch ozone	Deionized water	0.77	6	Room	4.6	28
	Batch liquid, batch ozone	Deionized water	0.51	8	Room	4	28
	Batch liquid, continuous gas	0.01 M Phosphate buffer	1.0	5,10	25	5–10	20
	Batch liquid, continuous gas	Distilled water	0.44	6	20	2.6	29
C. baileyi	Batch liquid, modified batch gas	Distilled water	0.6, 0.8	4	25	2.4–3.2	22

[a] Interpretation depends on protocol and method for reduction of ozone data used by authors.

TABLE 4. Summary of ozonation design criteria required to achieve 99% inactivation of *Cryptosporidium* oocysts

Integrated ozone residual (mg/liter)	Required contact time (min)		Conventional linear CT		Nonlinear $C^n T^m$	
					$n = 0.68$, $m = 0.95$	$n = 0.23$, $m = 0.64$
	7°C	22°C	7°C	22°C	7°C	22°C
0.25	20.6	6.6	5.1	1.7	6.9	2.4
0.50	12.5	5.2	6.3	2.6	6.9	2.4
0.75	9.4	4.5	7.0	3.3	6.9	2.4
1.00	7.6	4.0	7.6	4.0	6.9	2.4
1.25	6.5	3.7	8.1	4.6	6.9	2.4
1.50	5.7	3.5	8.6	5.2	6.9	2.4
1.75	5.1	3.3	8.9	5.8	6.9	2.4
2.00	4.6	3.1	9.3	6.3	6.9	2.4
2.25	4.3	3.0	9.6	6.8	6.9	2.4
2.50	4.0	2.9	9.9	7.2	6.9	2.4
2.75	3.7	2.8	10.2	7.7	6.9	2.4
3.00	3.5	2.7	10.4	8.1	6.9	2.4

at 7°C

$$\log\frac{N}{N_0} = -0.29 C^{0.68} T^{0.95} \tag{11}$$

at 22°C

$$\log\frac{N}{N_0} = -0.82 C^{0.23} T^{0.64} \tag{12}$$

The required contact time for a predetermined integrated ozone residual to achieve 99 and 99.9% inactivation of *C. parvum* at 7 and 22°C has been summarized in Tables 4 and 5. The model results in 22°C $C^n T^m$ values of 2.4 and 3.7 for 99 and 99.9% inactivation, respectively. The 7°C values are 6.9 and 10.3 for 99 and 99.9% inactivation, respectively. A kinetic model was also estimated from the room temperature inactivation data of Peeters et al. (28). Before making this estimation, two assumptions were necessary to adapt their data to the present analysis. First, the decay of ozone residuals was assumed to be first order. This assumption was likely very close to the true situation because Peeters et al. used deionized water, which typically supports a first-order

TABLE 5. Summary of ozonation design criteria required to achieve 99.9% inactivation of *Cryptosporidium* oocysts

Integrated ozone residual (mg/liter)	Required contact time (min)		Conventional linear CT		Nonlinear $C^n T^m$	
					$n = 0.68$, $m = 0.95$	$n = 0.23$, $m = 0.64$
	7°C	22°C	7°C	22°C	7°C	22°C
0.25	31.6	12.5	7.9	3.1	10.3	3.7
0.50	19.2	9.7	9.6	4.9	10.3	3.7
0.75	14.4	8.4	10.8	6.3	10.3	3.7
1.00	11.7	7.6	11.7	7.6	10.3	3.7
1.25	10.0	7.0	12.5	8.8	10.3	3.7
1.50	8.8	6.6	13.1	9.8	10.3	3.7
1.75	7.8	6.2	13.7	10.9	10.3	3.7
2.00	7.1	5.9	14.2	11.8	10.3	3.7
2.25	6.5	5.7	14.7	12.8	10.3	3.7
2.50	6.1	5.5	15.2	13.6	10.3	3.7
2.75	5.7	5.3	15.6	14.5	10.3	3.7
3.00	5.3	5.1	16.0	15.3	10.3	3.7

ozone decay. The second assumption was that the dose-response model for the neonatal mice developed for the present study could be used with their neonatal mice. This assumption has the most uncertainty, but it is necessary for the subsequent analysis. The kinetic model parameters were estimated by the maximum-likelihood procedure and resulted in the following model:

$$\log\frac{N}{N_0} = -1.25 C^{0.092} T^{0.43} \tag{13}$$

The resulting $C^n T^m$ values from this equation were 1.6 and 2.4 for 99 and 99.9% inactivation, respectively. An important difference between the work of Peeters et al. (28) and the present study is the value of the parameter n. Equation 13 has an n of 0.092, whereas in the present study, n was 0.23 (equation 12). The Peeters et al. (28) model suggests that ozone concentration was of reduced importance when compared with contact time, whereas in the present study, ozone concentration and contact time were both important. While these apparent differences are of interest, it must be recognized that these analyses are preliminary attempts to quantify the disinfection requirements for *C. parvum* by using ozone. More data are needed before more-definitive statements can be made regarding the relative importance of contact time and ozone concentration.

Tables 4 and 5 illustrate the differences in the design conditions for ozone contact time and integrated ozone when compared with the simple CT values for the same ozonation conditions. For example, in Table 4, an integrated ozone residual of 0.25 mg/liter requires a contact time of 6.6 min for 99% inactivation (simple CT of 1.7 mg · min/liter). If the integrated ozone residual is doubled to 0.50 mg/liter, the required contact time drops to 5.2 min for 99% inactivation (simple CT of 2.6 mg · min/liter). Note that the simple CT value has increased. This example illustrates the nonlinearity of the model. Also, the designer of an ozone disinfection system needs to decide whether the cost of providing extra contact time can be justified by the savings in ozone generation. Extrapolation of these preliminary findings to full-scale natural water systems is not recommended at this time. When more data have been collected from natural water systems and analyzed by using the methods described here, more rigorous design criteria can be established.

The findings from this study will help the water industry and regulators gain insight into the requirements for chemical inactivation of *C. parvum* by using ozone. The results of this study show that ozone effectively inactivates *C. parvum* in ozone demand-free laboratory water. Further research is needed to evaluate the efficacy of ozone for the disinfection of drinking water when natural water rather than laboratory water is used.

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