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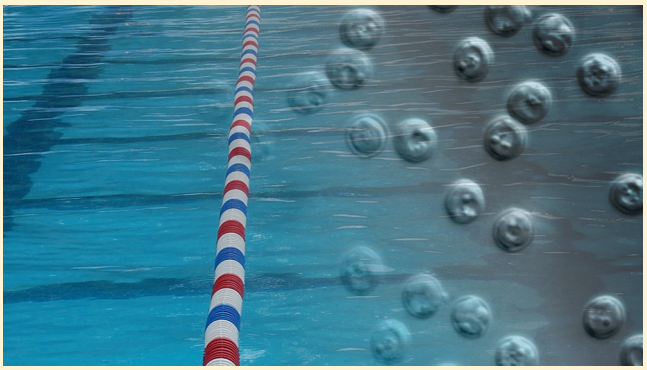
Efficacy of Chlorine Dioxide Tablets on Inactivation of *Cryptosporidium* Oocysts

Jennifer L. Murphy,*[,†] Charles N. Haas,[‡] Michael J. Arrowood,[†] Michele C. Hlavsa,[†] Michael J. Beach,[†] and Vincent R. Hill[†]

[†]Waterborne Disease Prevention Branch, Division of Foodborne, Waterborne, and Environmental Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30329, United States

[‡]Drexel University, Philadelphia, Pennsylvania 19104, United States

ABSTRACT: The ability of chlorine dioxide (ClO_2) to achieve 2-log inactivation of *Cryptosporidium* in drinking water has been documented. No studies have specifically addressed the effects of ClO_2 on *C. parvum* oocyst infectivity in chlorinated recreational water venues (e.g., pools). The aim of this research was to determine the efficacy of ClO_2 as an alternative to existing hyperchlorination protocols that are used to achieve a 3-log inactivation of *Cryptosporidium* in such venues. To obtain a 3-log inactivation of *C. parvum* Iowa oocysts, contact times of 105 and 128 min for a solution containing 5 mg/L ClO_2 with and without the addition of 2.6 mg/L free chlorine, respectively, were required. Contact times of 294 and 857 min for a solution containing 1.4 mg/L ClO_2 with and without the addition of 3.6 mg/L free chlorine, respectively, were required. The hyperchlorination control (21 mg/L free chlorine only) required 455 min for a 3-log inactivation. Use of a solution containing 5 mg/L ClO_2 and solutions containing 5 or 1.4 mg/L ClO_2 with the addition of free chlorine appears to be a promising alternative to hyperchlorination for inactivating *Cryptosporidium* in chlorinated recreational water venues, but further studies are required to evaluate safety constraints on use.



INTRODUCTION

Cryptosporidium spp. are coccidian protozoan parasites that infect the epithelial cells of the small intestine of humans and other mammals, causing self-limiting diarrheal illness in healthy individuals and sometimes contributing to death among those who are immunocompromised.[1] The parasite completes its entire life cycle within the host organism, forming small, environmentally hardy oocysts that are excreted in the stool fully sporulated and infectious. *Cryptosporidium* spp. exhibit an extreme tolerance of chemical disinfectants (notably chlorine) and ability for waterborne transmission. Since the first reported U.S. recreational water-associated cryptosporidiosis outbreak was identified in 1988, *Cryptosporidium* has emerged as the single most important etiologic agent of recreational water-associated outbreaks.[2−5] In 2009−2010, *Cryptosporidium* caused 24 (69%) of 35 treated U.S. recreational water-associated outbreaks with an identified infectious etiology.[2]

Hyperchlorination, or the increase in free chlorine concentration to achieve a CT value [disinfectant concentration (mg/L) × time (min) required for a 3-log inactivation of *C. parvum* oocysts] of 15 300 mg·min/L, is routinely performed when a chlorinated recreational water venue is at least suspected to be associated with a cryptosporidiosis outbreak or following a diarrheal incident (i.e., a high-risk cryptosporidiosis event) in such venues and requires closure of the recreational water venue to swimmers.[6] At 20 mg/L free chlorine, pH 7.5, and 25 °C, achieving this CT value requires 12.75 h of contact time. This time does not include the period required to raise the chlorine level to start inactivation nor reduce the chlorine level after inactivation to required state or local operational standards (e.g., 1−3 mg/L) before allowing patrons back into the water. As a result, hyperchlorination can cause lengthy closures, lost revenue, and increased operational costs that may influence compliance by aquatic facilities. Therefore, it is important to explore other potential disinfectants to improve the efficacy and reduce the burden of remediation procedures to improve compliance with state and local pool codes.

Chlorine dioxide is a disinfectant with a number of traditional uses, including paper bleaching, surface disinfection, biofilm removal in water distribution systems, and increasingly, for treatment to produce potable water.[7] ClO_2 has a number of advantages over hyperchlorination for recreational water remediation following diarrheal fecal contamination or in response to a cryptosporidiosis outbreak, including a higher

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oxidative capacity; lack of trihalomethane (THM) and chloramine formation; and effective action under a wider range of pH values.[8] However, there are disadvantages of ClO_2 use, such as formation of chlorite and chlorate byproducts, decomposition in sunlight, explosive nature under certain conditions and other safety concerns, and high costs for user training, sampling, and testing.[8,9]

A number of studies of the efficacy of ClO_2 against *C. parvum* oocysts have been reported in the peer-reviewed literature.[10−19] While these studies report promising efficacy of ClO_2 against *C. parvum*, often reporting substantially lower contact times than those required for inactivation using free chlorine, it is difficult to generalize results as they varied in the methodologies used and means of reporting. Furthermore, previous studies often used a nonideal viability assay, such as *in vitro* excystation;[20−22] assessed sequential disinfection using chlorine dioxide and nonchlorine disinfectants; applied uncorrected disinfection models to the data (i.e., models that do not incorporate disinfectant decay); or primarily focused on development of CT values for use in the drinking water sector, in which only a 2-log (99%) inactivation of oocysts is required.

The aim of this research was to determine whether ClO_2 offers an advantage over hyperchlorination in remediation procedures for chlorinated recreational water venues. The ClO_2 tablet we used also contains 7% sodium dichloro-isocyanurate ("dichlor"), a stabilized form of chlorine; stabilized chlorine products are routinely used in chlorinated recreation water venues throughout the United States. To assess these ClO_2 tablets optimally, we used a quantitative tissue culture infectivity assay and performed kinetics analyses, including using the Hom model,[23] to yield contact time estimates for two concentrations of ClO_2 as the primary disinfectant, as well as with the simultaneous addition of a low level of free chlorine, required for a 3-log inactivation of *C. parvum* oocysts.

MATERIALS AND METHODS

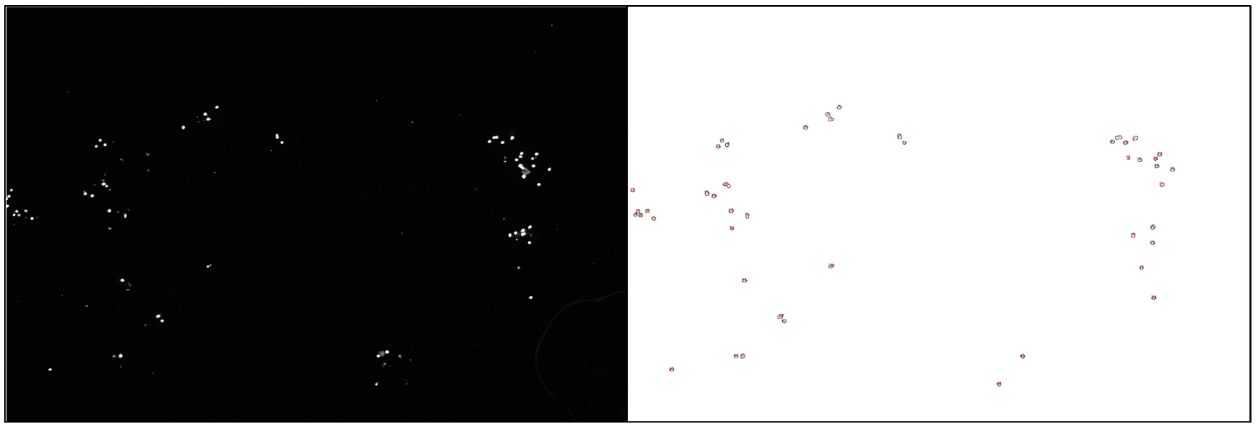
C. parvum and Madin Darby Canine Kidney (MDCK) Stocks. Two separate lots of *C. parvum* oocysts originating from a bovine source in Iowa (Harley Moon isolate) were purchased from Waterborne, Inc. (New Orleans, LA). Oocysts used in all experiments were less than three months old (14−91 days). MDCK cells (Scientific Resources Program, CDC, Atlanta, GA) were routinely passaged and were inoculated onto 2.1 cm^2 cover glass-bottom culture chambers (Nunc Lab-Tek, Rochester, NY) for infectivity assays to achieve confluent monolayers at 96 h. Passaging and infectivity assays were performed using Dulbecco's Modified Eagle Medium (DMEM), high glucose media supplemented to contain 0.1 mM MEM nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine (Gibco, Long Island, NY), and 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO).

Preparation of Oxidant-Demand-Free Water and Glassware. Oxidant-demand-free (ODF) water and glassware were used in all experiments. ODF water was prepared by buffering deionized water to pH 7.5 using 1 M monobasic or dibasic sodium phosphate (final concentration 10 mM) and then adding laboratory grade sodium hypochlorite to at least 5 mg/L free chlorine. Water was covered and remained at room temperature for at least 48 h before exposure to ultraviolet light in a biological safety cabinet to remove free chlorine.[24] ODF glassware and stir bars were soaked in deionized water containing at least 10 mg/L free chlorine for a minimum of 3 h and then rinsed with ODF water. Both ODF glassware and water were sterilized by autoclaving at standard sterilization conditions.

Preparation of Disinfectant. Due to laboratory constraints and safety considerations, a ClO_2 tablet branded Aseptrol S−Tab10 ("Aseptrol") (BASF, Florham Park, NJ) was used as a proxy for using a generator to produce the gaseous form of ClO_2. When added to water, the Aseptrol tablet readily dissolves, producing a solution containing a high concentration of ClO_2. To prepare a ClO_2 concentrate for these experiments, 1 g of an Aseptrol tablet was added to 100 mL of ODF water, and the solution was loosely covered and allowed to stabilize in a chemical fume hood for at least 30 min on the day of each experiment. Select volumes of this concentrate were added to ODF water to achieve desired ClO_2 concentrations.

Preparation of Experimental Flasks. Laboratory grade sodium hypochlorite was used in all experiments. Each experiment included five target conditions: (A) ClO_2 experimental flask at either 5 or 1 mg/L; (B) 2 mg/L free chlorine experimental flask containing either 5 or 1 mg/L ClO_2; (C) control flask containing ODF water alone; (D) control flask containing 20 mg/L free chlorine (recommended hyperchlorination concentration); and (E) control flask containing 2 mg/L free chlorine. Control Flask E served to monitor the decay of free chlorine in the combined disinfectant Flask B, as neither ClO_2 nor free chlorine could be measured individually in this flask due to the ability of *N,N*-diethyl-*p*-phenylenediamine (DPD) to react simultaneously with both chemicals. The ClO_2 concentrate was added to experimental flasks containing ODF water (Flask A) and ODF water with 2 mg/L free chlorine (Flask B) to achieve either 1 or 5 mg/L ClO_2. The same volume of ClO_2 concentrate was added to equal volumes of test water in Flasks A and B; ClO_2 concentration in Flask A was measured by DPD, and the ClO_2 concentration in Flask B was considered equivalent. Initial pH (Accumet AR25 Benchtop Meter, Fisher Scientific, Pittsburgh, PA), oxidation−reduction potential (ORP) (Orion APlus, Thermo Fisher Scientific, Waltham, MA), and ClO_2 and FC concentrations were measured using DPD methodology according to manufacturer's instructions (Hach, Loveland, CA). If necessary, flasks were adjusted to pH 7.5 using sterile 1 M monobasic or dibasic sodium phosphate.

Experimental Protocol. *C. parvum* oocysts were added to each flask to achieve a final target concentration of 10^5 oocysts/mL. Flask openings were covered with foil, and flasks remained in a 25 °C environmental chamber with stirring by magnetic stir bars at 150 rpm for the duration of the experiment. Chlorine dioxide concentration, free chlorine concentration, pH, and ORP were measured throughout the experimental time period; neither pH nor disinfectant concentrations were amended after initial adjustment. At set time points, 4, 40, or 120 mL (as three 40 mL aliquots) samples were taken. Samples were immediately quenched in 50 mL polypropylene conical tubes containing either 1 or 10 mL of 0.01 M phosphate buffered saline (pH 7.4) plus 0.1% bovine serum albumin (PBS/BSA) and sodium thiosulfate (Fisher Scientific, Pittsburgh, PA) at 50 mg/L per 1 mg/L disinfectant (ClO_2 and/or free chlorine) for 4 and 40 mL sample volumes, respectively. Samples were stored at 4 °C until all could be concentrated simultaneously. As previously described,[25] samples were centrifuged at 3290*g* for 10 min at 4 °C to pellet oocysts. Supernatant was carefully removed by aspiration, and the pellet was resuspended and transferred into a 1.5 mL nonstick



(a) (b)

Figure 1. (a) Microscopical field with fluorescently labeled *C. parvum* living stages; (b) counterpart image with *C. parvum* life stages enumerated by ImageJ Software.

microcentrifuge tube (Phenix Research Products, Candler, NC) which was then centrifuged at 15,800*g* for 3 min at 4 °C. Supernatant was carefully removed by aspiration, and rinsate from a 1 mL PBS (0.01 M, pH 7.2) rinse of the respective conical tube was layered onto the pellet before a final centrifugation step at 15 800*g* for 3 min at 4 °C. Supernatant was carefully removed by aspiration down to the 0.1 mL demarcation on the microcentrifuge tube, and 0.9 mL of DMEM, high glucose plus 0.75% synthetic sodium taurocholate (Sigma, St. Louis, MO) was added; the oocyst pellet was triturated with a pipet tip, after which the samples were incubated at room temperature for 10−15 min to promote oocyst excystation and sporozoite release.

***C. parvum* Infectivity Assay.** The DMEM/taurocholate suspension was inverted three times to mix. and 150 μL was inoculated in duplicate onto MDCK cell monolayers in culture chambers containing 1.5 mL of fresh, supplemented DMEM. Slides were incubated at 37 °C for 48−60 h with 5% CO_2. To visualize individual meronts and gamonts (life cycle stages that develop if oocysts were infectious), cell layers were fixed and labeled as previously described,[25] with minor modification. Briefly, culture medium was removed, and monolayers were washed three times with sterile 0.01 M PBS (pH 7.2) and then fixed in Bouin's solution (Ricca Chemical, Arlington, TX) for 30 min. Bouin's solution was removed, and monolayers were decolorized with five 10 min washes using 70% ethyl alcohol (anhydrous), followed by overnight incubation in PBS with 0.1% BSA at 4 °C. The *Cryptosporidium*-specific monoclonal antibody C3C3[26] was bound to meronts and gamonts during a 1 h incubation. Unbound C3C3 was removed, and monolayers were washed three times with sterile PBS and then fluorescently labeled by a 1 h incubation with FITC-Goat Anti-Mouse IgG (H + L) antibody (Invitrogen, Frederick, MD) at a concentration of 0.5% in PBS/BSA supplemented with 2 mM sodium azide, followed by three washes with sterile PBS. All rinses and incubation periods were performed with gentle rocking at room temperature. Steps following use of fluorescent antibodies used covered chambers to protect the fluorochrome from quenching by exposure to light. After the final PBS wash, monolayers in each well were sealed using three drops of PVA-DABCO[26] under an 18 mm^2 glass coverslip and stored covered at 4 °C.

Microscopy. Zeiss AxioVision software (Carl Zeiss, Thornwood, NY) systematically captured 72 adjacent immunofluorescent microscopical fields for each culture chamber at 100× magnification, representing approximately 20% of each monolayer. Images were captured using a Zeiss HRm digital camera (Carl Zeiss) on an AxioVert 200 M microscope (Carl Zeiss). Zeiss Vision Image (zvi) files were converted to JPEG files and then analyzed by ImageJ software[27] customized to enumerate developing life cycle stages (individual meronts and gamonts) based on size (~3−5 μm), shape (circularity), and labeling by fluorescent antibody. Each individual image was manually compared with its software-analyzed counterpart image to assess reliability of the count (Figure 1). Approximately 17 000 images were captured and analyzed in the study. Microsoft Excel was used to calculate average number and size of developing life cycle stages per microscopical field. The number of developing stages in culture is directly related to the number of inoculated oocysts which contain infectious sporozoites.[25] Back-calculation provided an estimate of the $\log_{10}$ inactivation of oocysts in each condition over contact time as compared with the oocyst concentration in the control flask.

Kinetic Analysis. The disinfection kinetic data from the experiments with a target concentration of 5 mg/L ClO_2, both with and without the addition of free chlorine, were analyzed for fit to the Chick-Watson and Hom kinetic models using nonlinear regression as described by Haas and Kaymak.[28] Models accounted for ClO_2 decay throughout experimental time periods and therefore provided contact times for use in recreational water settings in which disinfectant levels should be constantly maintained using automated control systems. Computations were performed by a program written using the R platform.[29] As a preliminary step, the disinfectant decay rate (k') for 5 mg/L ClO_2 was determined using a linear least-squares fit with a log-transform of the ratio of residual relative to dose. The "y" intercept was indistinguishable from 0, indicating lack of bias in determining residual. The decay rate was determined under the assumption that the decay rate for all experiments in the data set was given by a first order decay law, with a single rate constant characterizing decay (this assumption was confirmed by visual assessment).

Given k', the fit of both the Chick-Watson and the Hom models with first order decay were determined for the ClO_2

data both with and without the addition of free chlorine. Log transformation of the parameters was used to constrain the underlying values of the parameters to positive values and to improve convergence behavior. The Chick-Watson and Hom models were each fitted to all data combined ("pooled") and to each of the data subsets separately (5 mg/L ClO_2 and 5 mg/L ClO_2 with 2.6 mg/L free chlorine) ("separate"). In the latter case, the log likelihoods of the fits to each of the separately fitted subsets were summed. The Akaike Information Criterion (AIC) was used to select among the different model alternatives; the alternative yielding the minimum value of AIC was regarded as the most appropriate.

Due to limited disinfectant decay in experiments with target concentrations of 1 mg/L ClO_2 and 20 mg/L free chlorine, the Hom model could not be used to analyze disinfection data. In both cases, the coefficient of dilution ("n") was indeterminate (i.e., an infinite combination of "k" and "n" values would provide the same fit, and only the lumped group kC^n can be determined from such data). Hence, in these experiments, contact times required for 1-, 2-, and 3-log inactivation of *C. parvum* were extrapolated from a line fit to data of *C. parvum* inactivation over contact time, using average initial ClO_2 or free chlorine concentrations for all experiments. Oocyst inactivation was not determined for the 2 mg/L free chlorine controls.

RESULTS

The average initial free chlorine concentration for target 20 mg/L free chlorine control (D) flasks was 21 mg/L; free chlorine concentrations remained relatively constant in these flasks throughout each experiment, except in one case in which the concentration decreased to 17.6 mg/L at 8 h. Across experiments, the average initial ClO_2 concentrations were 5.4 mg/L (for target 5 mg/L ClO_2 experiments) and 1.4 mg/L (for target 1 mg/L ClO_2 experiments). Chlorine dioxide concentrations decreased throughout the experimental time periods. Initial ClO_2 concentrations and the significant ClO_2 decay in 5 mg/L ClO_2 experiments were accounted for in analysis models. On the basis of measurements in free chlorine control (E) flasks, average initial free chlorine concentrations in ClO_2 with additional free chlorine (B) flasks were 2.6 mg/L (for target 5 mg/L ClO_2 experiments) and 3.6 mg/L (for target 1 mg/L ClO_2 experiments). Free chlorine concentrations decayed in the control (E) flasks over time; however, free chlorine concentrations did not fall below 1 mg/L over experimental time periods, except in one case. This decay was not accounted for in analyses; contact times presented for the combined disinfectants are considered relevant for chlorinated recreational water venues that are sustained within a free chlorine concentration of 1−3 mg/L.

The average initial pH for all conditions tested was pH 7.5 in 5 mg/L ClO_2 flasks and pH 7.6 in 1 mg/L ClO_2 flasks. The pH values remained relatively constant throughout all experimental time periods, with measurements decreasing at most by 0.1 at final time points. Across all experiments, the average initial ORP values were 373 mV for ODF controls, 760 mV for 20 mg/L free chlorine controls, and 674 for 2 mg/L free chlorine controls. ORP varied by an average of 1% in 20 mg/L free chlorine controls and decreased by an average of 35% in ODF controls over the course of experiments. In 2 mg/L free chlorine control flasks, ORP decreased by an average of 22% at final time points. In 5 mg/L ClO_2 flasks, the average ORP values were 721 mV with the addition of free chlorine (average concentration: 2.6 mg/L) and 725 mV without the addition of free chlorine. In 1 mg/L ClO_2 flasks, the average ORP values were 712 mV with the addition of free chlorine (average concentration: 3.6 mg/L free chlorine) and slightly lower at 625 mV without the addition of free chlorine. ORP values generally decreased consistently with a decrease in ClO_2 concentration, with the largest being a decrease of 384 mV at 735 min in one 1 mg/L ClO_2 experiment; ClO_2 concentration at this time point was 0.02 mg/L.

The results of the 5 mg/L ClO_2 kinetic analyses are shown in Tables 1 and 2. In Table 1, the goodness of fit of the Chick-Watson and Hom models are tested in two cases, where all data (regardless of the presence of additional free chlorine) are pooled into a single data set and where the fits are performed separately by the presence of additional free chlorine. The latter situation requires more fitting parameters (for each subset), and the Hom model requires more parameters than the Chick-Watson model. The most appropriate model was selected using the AIC, which penalizes more highly parametrized models for reduced parsimony. Using this approach, it was determined that the best model is one using the Hom relationship, but applied separately to the data with and without the addition of free chlorine. Using the Hom model applied separately to each subset by presence of additional free chlorine, Table 2 provides the set of parameters for the 5 mg/L ClO_2 data, both with and without the addition of 2.6 mg/L free chlorine. The mean squared errors (between the data and the model fits) are comparable to, or even less than, those which have previously been reported for other disinfection systems (including *Cryptosporidium*).[30] Table 3 presents contact times and corresponding CT values for the 21 mg/L free chlorine controls and solutions containing 5 mg/L ClO_2, both with and without the addition of free chlorine (2.6 mg/L), for 1-, 2-, and 3-log *C. parvum* inactivation. Target concentrations of 5 mg/L ClO_2, both with and without the addition of free chlorine, resulted in a substantially lower contact time required for a 3-log inactivation than for 21 mg/L free chlorine. There was a greater than 4-fold decrease in the contact time required for a 3-log inactivation with 5 mg/L ClO_2 with the addition of 2.6 mg/L free chlorine than that required for 21 mg/L free chlorine alone. Without the addition of free chlorine, there was a 3.6-fold decrease in the contact time required for a 3-log inactivation with 5 mg/L ClO_2. ODF-control flasks for all experiments indicated no appreciable reduction in oocyst infectivity over the course of experiments. Figure 2a,b provides

Table 1. *C. parvum* Inactivation Kinetics Model Selection for a Solution Containing 5 mg/L ClO_2 with and without the Addition of Free Chlorine

model	data stratification	log likelihood	# parameters	AIC[a]
Chick-Watson	pooled[b]	−102.9944	2	209.9888
	separate fits[c]	−87.841 76	4	183.683 52
Hom	pooled[b]	−99.015 06	3	204.030 12
	separate parameters[c]	−63.779	6	139.558

[a]Akaike Information Criterion: $2p - \ln(L)$, where p = number of parameters that are fit into the data and $\ln(L)$ is the log likelihood function. [b]5 mg/L ClO_2 (target) and 5 mg/L ClO_2 (target) + 2.6 mg/L additional free chlorine (average) data sets combined. [c]Sum of separate fits for 5 mg/L ClO_2 (target) data and 5 ClO_2 (target) + 2.6 mg/L additional free chlorine (average) data.

Table 2. Hom Model Parameters for *C. parvum* Inactivation Using a Solution Containing 5 mg/L ClO_2 with and without the Addition of Free Chlorine (FC)[a]

disinfectant	k'	ln(k)	ln(n)	ln(m)
5 mg/L ClO_2	0.0041465***	−9.64418***	0.10804*	0.70190***
5 mg/L ClO_2 + 2.6 mg/L FC[b]	0.0041465***	−6.6914***	−0.2919*	0.4669**

[a]Note: this table presents the natural log transforms of the kinetic parameters; exponentiation can be used to obtain the normally presented k, n, and m parameters. Model applied separately to 5 mg/L ClO_2 (target) data and 5 ClO_2 (target) + 2.6 mg/L additional free chlorine (average) data. Significance codes: 0.05 "*", 0.001 "**", and 0 "***". [b]Concentration (mg/L) of added free chlorine.

Table 3. Time (Minutes) Required to Achieve 1-, 2-, and 3-log *C. parvum* Inactivation and 3-log CT Values for Hyperchlorination Controls and for a Solution Containing 5 mg/L ClO_2 with and without the Addition of Free Chlorine (FC)

ClO_2 conc (mg/L)	average FC conc (mg/L)	n	1-log inactivation (min)	2-log inactivation (min)	3-log inactivation (min)	3-log CT value (mg·min/L)
0	21[a]	4	152	303	455	9555
5	0	4	74	104	128	640
5	2.6[b]	3	53	81	105	525

[a]Data shown for 4 of 6 hyperchlorination controls (average 21 mg/L) from all ClO_2 experiments combined. [b]Concentration (mg/L) of added free chlorine.

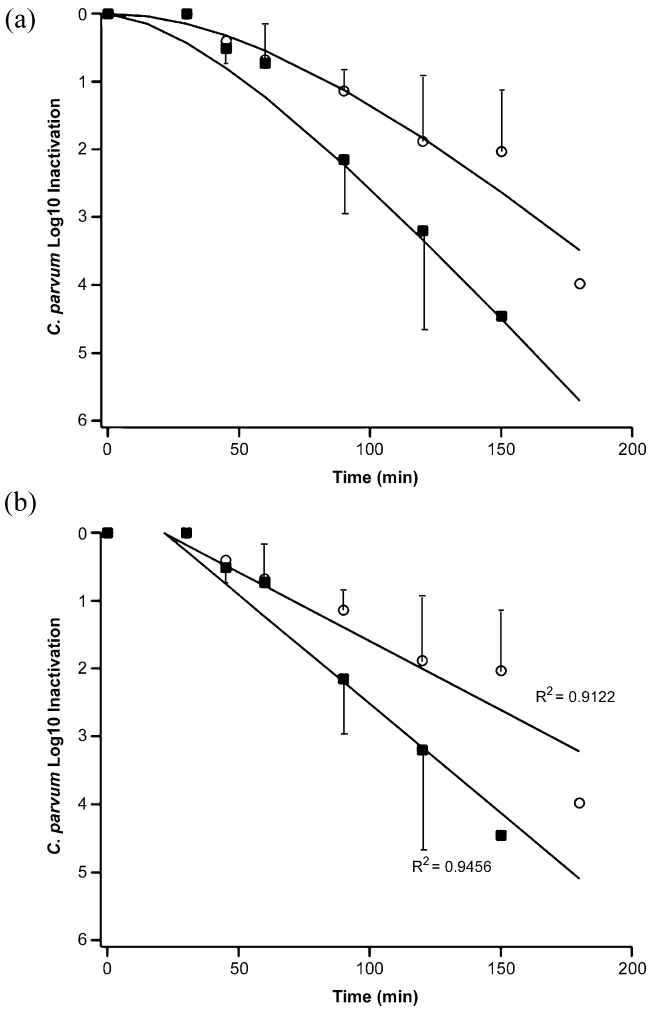


Figure 2. (a) Observed *C. parvum* inactivation (2 SD) over contact time for a solution containing 5 mg/L ClO_2 (○) and 5 mg/L ClO_2 with the addition of 2.6 mg/L free chlorine (■). (b) Fitted *C. parvum* inactivation (2 SD) over contact time for a solution containing 5 mg/L ClO_2 (○) and 5 mg/L ClO_2 with the addition of 2.6 mg/L free chlorine (■).

a graphical representation of observed *C. parvum* inactivation over contact time (during which ClO_2 decay occurred) (a) and inactivation over contact time when ClO_2 decay was accounted for by the Hom model (b).

Table 4 presents contact times for the 21 mg/L free chlorine controls and for 1.4 mg/L ClO_2 (average initial ClO_2 concentration for all experiments) with and without the addition of 3.6 mg/L free chlorine for 1-, 2-, and 3-log *C. parvum* inactivation. The combination of this lower concentration of ClO_2 and addition of free chlorine resulted in substantially lower contact time for *C. parvum* inactivation than for 21 mg/L free chlorine. However, a low dose of ClO_2 alone appeared to be less effective than free chlorine at concentrations typically used for hyperchlorination. In low-dose experimental flasks, ClO_2 concentration decreased to nondetectable levels before 3-log inactivation of *C. parvum* was achieved. In these low dose experiments, the differential ClO_2 decay could not be analyzed using the Hom model. Contact times for 1-, 2-, and 3-log inactivation were determined using extrapolation from lines fit to observed inactivation data (R^2 = 0.9299 for 1.4 mg/L ClO_2 data and R^2 = 0.9087 for 1.4 mg/L ClO_2 + 3.6 mg/L additional free chlorine data).

Table 4. Time (Minutes) Required to Achieve 1-, 2-, and 3-log *C. parvum* Inactivation for Hyperchlorination Controls and for a Solution Containing 1.4 mg/L ClO_2 with and without the Addition of Free Chlorine[a]

ClO_2 conc (mg/L)	average FC conc (mg/L)	n	1-log inactivation (min)	2-log inactivation (min)	3-log inactivation (min)
0	21[b]	4	152	303	455
1.4	0	2	286	571	857
1.4	3.6[c]	2	98	196	294

[a]3-log CT values not reported because data were not able to be modeled to account for ClO_2 decay. [b]Data shown for 4 of 6 hyperchlorination controls (average 21 mg/L) from all ClO_2 experiments combined. [c]Concentration (mg/L) of added free chlorine.

DISCUSSION

We used a quantitative tissue culture infectivity assay and performed kinetics analyses to provide contact time estimates for two concentrations of ClO_2 as the primary disinfectant, as well as with the addition of a low level of free chlorine, required for a 3-log inactivation of *C. parvum* oocysts. The Hom model

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yielded contact time estimates of 128 and 105 min, for a solution containing 5 mg/L ClO_2 and 5 mg/L ClO_2 with the addition of 2.6 mg/L free chlorine, respectively, required for a 3-log inactivation of oocysts. These contact times were substantially lower than the contact time (455 min) for equivalent inactivation of oocysts in 21 mg/L free chlorine controls. At these contact time estimates, corresponding ClO_2 CT values were 640 and 525 mg·min/L, respectively. These CT values are more than an order of magnitude lower than the CT value of 9555 mg·min/L determined for the 21 mg/L free chlorine controls that were run in parallel. The free chlorine control CT value is consistent with that reported in the literature for the *C. parvum* Iowa strain (10 400 ± 587 mg·min/L) and lower than that for the *C. parvum* Maine strain (15 300 mg·min/L).[6] For solutions containing 1.4 mg/L ClO_2 and 1.4 mg/L ClO_2 with the addition of 3.6 mg/L free chlorine, contact times of 857 and 294 min, respectively, were required for a 3-log inactivation of *C. parvum* oocysts. For a solution containing 5 mg/L ClO_2 alone and concentrations of 5 and 1.4 mg/L ClO_2 with the addition of low levels of free chlorine, contact times required for a 3-log inactivation presented here are lower than those for CDC's hyperchlorination recommendations (i.e., 765 min using 20 ppm free chlorine or a CT value of 15 300 mg·min/L based on inactivation of the *C. parvum* Maine isolate).[31]

Previous studies provide a foundation of information on the enhanced efficacy of ClO_2 against *C. parvum*, but used different approaches for assessing viability and analyzing data.[10−19] The quantitative tissue culture assay utilized in these studies is a preferred alternative to more traditional *C. parvum* disinfection assays; *in vitro* excystation can underestimate oocyst viability,[20−22] and mouse infectivity assays, although considered the gold standard, are expensive, difficult, and even more time-intensive. Earlier research has shown comparable results between tissue culture and mouse infectivity assays.[22,25,32] In addition, while prior studies generally utilized the Chick-Watson model to analyze ClO_2 disinfection data, we were able to apply the Hom model[23] to the 5 mg/L ClO_2 data, allowing ClO_2 decay to be accounted for in calculations for contact times required for 1-, 2-, and 3-log inactivation. For the 5 mg/L ClO_2 presented here, the contact time required for a 2-log inactivation time results in a CT value of 520 mg·min/L. This value falls within the previously reported CT values of 75−1000 mg·min/L (depending on oocyst source) also derived using a cell culture infectivity assay.[10] However, the latter study and other research assessing sequential disinfection were focused on drinking water disinfection and are therefore less relevant to chlorinated recreational water venues in which a 3-log *C. parvum* inactivation is required due to an elevated potential exposure level following a diarrheal incident in a small volume recirculated system.[33]

Low levels of free chlorine are considered ineffective against *C. parvum* because of the extremely long contact times required. For example, at 2 mg/L free chlorine, a 3-log inactivation of *C. parvum* is estimated to take 127.5 h.[31] The results of this study demonstrate that the combination of low levels of free chlorine with ClO_2 results in lower contact times for *C. parvum* inactivation than those for ClO_2 without the addition of free chlorine. These results suggest synergistic effects between the two disinfectants. Cho et al.[34] reported 145% and 100% synergistic effects for inactivation of *Bacillus subtilis* spores when ClO_2 dosing was followed by free chlorine at pH 5.6 and 8.2, respectively. Son et al.[35] also report a 45% enhancement of *B. subtilis* spore inactivation using a mechanically prepared mixture of low levels of ClO_2 and free chlorine as compared to free chlorine alone. Additionally, a synergistic effect was found for inactivation of *Escherichia coli* using sequential dosing of ClO_2 and free chlorine. The authors suggest that ClO_2 increases the *E. coli* cell wall permeability which results in accelerated movement of chlorine and ClO_2 into the cytoplasm where inner cell components are degraded, ultimately resulting in cell death.[36] However, mechanisms for *Cryptosporidium* oocyst inactivation using chlorine and/or ClO_2 are not well understood. To our knowledge, this is the first report of *C. parvum* oocyst inactivation in which ClO_2 tablets and free chlorine were dosed simultaneously, with the additional advantage that the combination of these two disinfectants is likely to represent a situation encountered by aquatics staff responding to a diarrheal fecal incident or cryptosporidiosis outbreak.

This study was subject to some notable limitations. First, due to the unsafe nature of ClO_2 production, we were required to use a product that also contained other components in low concentrations, including dichlor, a stabilized form of chlorine. Under the conditions used, we cannot ascertain how the presence of dichlor impacted our results. However, Shields[37] found that cyanuric acid substantially decreased the efficacy of chlorine against *C. parvum* inactivation. As a result, dichlor would be expected to have a negative impact on inactivation in this study; in the absence of dichlor, ClO_2 may inactivate *Cryptosporidium* more rapidly than reported here. The finding that *Cryptosporidium* is still rapidly inactivated by ClO_2 and ClO_2/free chlorine mixtures in the presence of dichlor is encouraging since dichlor and other stabilized chlorine products are routinely used in chlorinated recreation water venues throughout the United States. Second, experiments were conducted in oxidant-demand-free water under ideal conditions to minimize and control experimental variability; however, water in chlorinated recreational water venues is more complex due to the presence of bather and environmental loads (e.g., sweat, urine, personal care products).[38] Further research is needed to evaluate disinfection differences that might exist in more representative conditions. Third, uncovered outdoor venues receive constant exposure to ultraviolet light; the experimental setup did not allow for assessment of how UV light exposure affects ClO_2 and free chlorine concentrations and the impact that this might have on *C. parvum* oocyst inactivation. In addition, the rapid rate of decay in 1 mg/L ClO_2 flasks limited the ability to produce a more robust data set for this ClO_2 concentration. Lastly, the size of the data set does not allow for quantitative assessment of ClO_2 concentration differences or synergistic effects of free chlorine and different concentrations of ClO_2 on *C. parvum* oocyst inactivation; additional studies are needed to elucidate these effects.

Each year, there are approximately 4000 visits to U.S. emergency departments due to health events caused by pool chemicals.[2,39] Human error is the primary contributing factor. The addition of ClO_2 to the *Cryptosporidium* inactivation arsenal is likely to increase the complexity of safe chemical storage and handling and require additional staff training. Moreover, the maximum residual disinfectant level of ClO_2 in drinking water as set by the U.S. Environmental Protection Agency (USEPA) is 0.8 mg/L,[40] although ClO_2 concentrations exceeding 0.4−0.5 mg/L contribute to taste and odor problems.[41] Disinfection of water with ClO_2 does not produce halogenated, and potentially carcinogenic, byproducts as

generated by other chlorine compounds. However, it does form chlorite, chlorate, and chloride ions, which may accumulate and be toxic at high concentrations thereby posing a potential human health threat.[42] Under the USEPA Disinfection Byproduct Rule, the legal threshold limit or maximum contaminant level of chlorite allowed in drinking water is 1 mg/L.[43] These health impacts and limits of exposure must be evaluated when assessing the use of ClO_2 in chlorinated recreational water venues.

Free chlorine is commonly used to disinfect water in U.S. pools and interactive fountains, despite its reduced efficacy against *C. parvum* oocysts, even under hyperchlorination treatment conditions. Use of a solution containing 5 mg/L ClO_2 and solutions containing 5 and 1.4 mg/L ClO_2 with the addition of low concentrations of free chlorine appears to be promising alternatives to hyperchlorination to achieve 3-log inactivation of *C. parvum*. The use of ClO_2 could potentially reduce the burden on aquatic facilities and improve compliance with existing diarrheal fecal incident remediation and cryptosporidiosis outbreak response protocols. However, recommendations for dosage to remediate chlorinated recreational water venues are currently unavailable and safety concerns do exist;[42] more research is needed before development of a ClO_2-based *C. parvum* remediation procedure that optimizes occupational and swimmer safety (i.e., an alternative to hyperchlorination) could be considered.

AUTHOR INFORMATION

Corresponding Author

*Tel: +1-404-718-4155; fax: +1-404-718-4197; e-mail: iod7@cdc.gov.

Notes

The use of trade names and names of commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services.

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