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Bromine and Chlorine Disinfection of *Cryptosporidium parvum* Oocysts, *Bacillus atrophaeus* Spores, and MS2 Coliphage in Water

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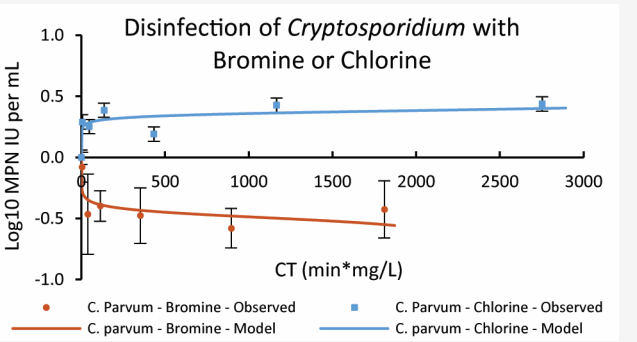
ABSTRACT: Conventional water treatment practices utilizing chemical disinfection, especially chlorination, are considered generally effective in producing microbiologically safe drinking water. However, protozoan pathogens such as oocysts of *Cryptosporidium parvum* are very resistant to chlorine, which has led to consideration of alternative disinfectants for their control. Free bromine, HOBr, has not been evaluated extensively as an alternative halogen disinfectant for inactivation of *Cryptosporidium parvum* in drinking water or reclaimed water for non-potable uses. Bromine is a versatile disinfectant consisting of different chemical forms with persistent microbicidal efficacy under varied water quality conditions and is effective against a range of waterborne microbes of health concern. The objectives of this study are to (1) compare the efficacy of free bromine to free chlorine at similar concentrations (as milligrams per liter) for disinfection of *Cryptosporidium parvum* oocysts, *Bacillus atrophaeus* spores, and MS2 coliphage in a model buffered water and (2) evaluate the kinetics of inactivation of these microorganisms using appropriate disinfection models. Overall, at a target concentration of ∼5 mg/L, bromine averaged 0.6 log (73.8%) reductions of *C. parvum* oocyst infectivity after 300 min (CT: 1166 min·mg/L) and produced up to a 0.8 log reduction disinfectant activity. An ∼5.0 mg/L chlorine dose increased oocyst infectivity by only 0.4 log (64%) after 300 min (CT: 895 min·mg/L). *Bacillus atrophaeus* spores and MS2 coliphage treated with bromine and chlorine were reduced by 4 $\log_{10}$ (99.99%) for both disinfectants over the duration of the experiments.



KEYWORDS: *Cryptosporidium, Bacillus spores, MS2 coliphage, bromine, chlorine, disinfection, water treatment*

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1. INTRODUCTION

Continuous chlorination of drinking water was first introduced in the U.S. in 1908 in Jersey City, New Jersey.[1] Since then, the importance of chemical disinfection of drinking water has become widely accepted as a significant public health achievement. Commonly used chemical disinfectants for drinking water include free chlorine, monochloramine, ozone, chlorine dioxide, and UV radiation. Of these, a disinfection residual to protect water during distribution and storage is provided by free chlorine, monochloramine, and chlorine dioxide. Of the conventional chemical disinfectants, chlorine is still the most widely used and is considered generally effective in producing microbiologically safe drinking water. However, oocysts of the protozoan *Cryptosporidium parvum* (*C. parvum*) are very resistant to chlorine.[2−4] Therefore, consideration of alternative disinfectants for the control of *C. parvum* in water is of interest for protection of public health against this chlorine resistant pathogen.

Most research on bromine for water and wastewater disinfection occurred before and during the 1970s, when a range of alternatives to chlorine were considered in the USA for the Safe Drinking Water Act[5−23] to address disinfection byproducts of health concern. Tables S1−S4 summarize inactivation of microorganisms by free bromine, bromamine, and bromine chloride. Bromine disinfection research for water and wastewater largely ended after brominated disinfection byproducts were reported to be detrimental to human health and were found present as a high percentage (39%) of total organic bromine in drinking waters.[24,25] There have been few recent investigations of bromine as a water disinfectant, although interest has recently renewed for its application as a point-of-use water treatment in the form of bromine as solid phase hydantoinylated polystyrene resin beads.[26,27]

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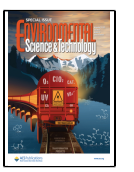

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There has been only one documented case of free bromine use in a municipal drinking water supply, which was in Irvington, California ca. 1938.[24] However, its use was discontinued because of the inability to maintain a residual due to the high reactivity of bromine with organic constituents in the water during distribution. Bromine is used to disinfect swimming pools[28,29] and cooling towers.[30] In recent years, bromine was found to be the second most common biocide used as a disinfectant in a survey of over 6000 cooling towers located throughout the United States.[31] In a comparison of *N*-halamine bromine and chlorine as solid phase disinfection media in canisters for point-of-use drinking water disinfection, Coulliette et al. (2010) reported that bromine consistently outperformed chlorine in reducing microcystin toxins as well as surpassing the U.S. Environmental Protection Agency virus reduction goal of 99.99%.[26] There is now renewed interest in bromine as more than just an industrial and engineered recreational water disinfectant, including its possible use for reclaimed water, agricultural irrigation water, and perhaps even drinking water. However, data are lacking on the efficacy of bromine for inactivation of key waterborne pathogens, such as *Cryptosporidium parvum* oocysts and biothreat agents such as *Bacillus anthracis* spores. The objectives of this study are to (1) compare the efficacy of free chlorine to free bromine for disinfection of *Bacillus atrophaeus* spores, *Cryptosporidium parvum* oocysts, and MS2 coliphage in water at an allowable concentration in drinking water and (2) evaluate the kinetics of inactivation of these microorganisms using suitable models.

2. MATERIALS AND METHODS

2.1. Preparation of Reagents and Reaction Vessels. *2.1.1. Halogen-Demand-Free Water and Experimental Waters.* Halogen-demand-free (HDF) water was used in the preparation of all reagents and test waters and prepared by passing twice-deionized, activated carbon-filtered water through a macroreticular scavenging resin bed (Dracor Corp., Durham, NC). HDF 0.05 M phosphate-buffer at pH 7.5 was used as experimental test water.

2.1.2. Halogen Solutions and Measurements. Stock halogen solutions were prepared in amber bottles. Household bleach (6% w/v NaOCl) was diluted with HDF water to a stock concentration of ∼500 mg/L as Cl_2. Elemental bromine (Br_2) was purchased from Fisher Scientific (Pittsburgh, PA) and diluted prior to use with HDF water to a stock concentration of ∼580 mg/L as Br_2. Elemental bromine dissolved in water forms a pH dependent equilibrium of hypohalite anion and hypobromous acid, which is the species that is a more potent biocide.[32] Halogen concentrations were measured by the DPD colorimetric method with a Hach Pocket Colorimeter II (Loveland, CO) calibrated for chlorine measurement, per manufacturer instructions. The manufacturer's instruction manual provides a 95% confidence interval of ±0.2 mg/L Cl_2 for measured concentrations.[33]

2.1.3. Disinfection Reaction Vessels. Sigmacote (Sigma-Aldrich, St. Louis, MO) was applied to siliconize all reaction vessels and vials used for *C. parvum* oocysts to minimize oocyst adherence to interior container walls. A small volume (∼10% of the volume of the container being coated) of Sigmacote was dispensed into the container, and the container was mixed for 1 min to coat interior walls. Coated containers were autoclaved inverted with their caps loosely secured to sterilize and cure the Sigmacote. To minimize halogen demand exerted by test equipment, all reaction bottles, caps, and stir bars were soaked overnight in a 50 mg/L chlorine bath and then rinsed three times in HDF water prior to use in experiments.

2.2. Preparation of *C. parvum* Oocysts. Oocysts of *C. parvum*, Iowa strain, were commercially obtained as purified suspensions from two sources. One batch was received as a purified suspension from Bunch Grass Farm (Deary, ID). Briefly, two-day-old Holstein bull calves were orally dosed with 2×10^8 oocysts, and the shed stool was collected beginning 3 days postdosing. Oocysts were isolated and initially purified from the stool with diethyl ether sedimentation, followed by two-stage sucrose gradient (1:4 and 1:2) density centrifugation. Next, the oocysts were purified by centrifugation through a single layer of cesium chloride. Finally, the purified oocysts were suspended in 50 mL of PBS at a concentration of 2×10^7 oocysts/mL, supplemented with 1000 IU penicillin and 1000 μg streptomycin. The second batch of *C. parvum* oocysts was from the University of Arizona, Department of Veterinary Science and Microbiology (Tuscon, AZ). Oocysts were collected from the shed stool of infected calves, mixed with 0.1% TWEEN 80, and purified by differential sucrose and cesium chloride gradients. Finally, the purified oocysts were suspended in buffer amended with 1000 IU penicillin and 1000 μg/L streptomycin at a concentration of 2×10^7 oocysts/mL. For the purposes of analysis and modeling both batches of *C. parvum* oocysts were considered to be equivalent even though they were produced in bovine hosts of different ages.

For disinfection experiments, 10 mL aliquots of these purified stock oocyst suspensions were further purified by centrifuging at 2600*g* for 15 min at 4 °C. The supernatant was decanted, and the pellet was resuspended in 10 mL of PBS. Oocyst concentration was enumerated with a hemocytometer under a brightfield microscope. The purified stock suspension was diluted in test waters to a concentration of 1.7×10^5 oocysts/mL of test water to be able to document a 4-$\log_{10}$ (99.99%) reduction in infectious oocysts. The volume ratio of the purified oocyst suspension to test water was 1:100.

2.3. *B. atrophaeus* Spore Stock. *B. atrophaeus* spores were obtained commercially from SGM Biotech (Bozeman, MT) as a purified spore suspension with a concentration of 2.4×10^9 spores/mL, stored at 4 °C, and used for disinfection experiments within six months from the spore manufacture date. Pure spore stock was vortex mixed for 5 min prior to removing aliquots with a sterile syringe. Spore stock was diluted to levels in test waters to be able to document a 4 $\log_{10}$ (99.99%) reduction.

2.4. Batch Disinfection Experiments. Five 125 mL Teflon narrow-mouth bottles (Thermo Scientific) were used as reaction vessels. Two bottles were used for each halogen (chlorine and bromine), and one control bottle contained microbes in test water without halogens. For each pair of halogen bottles, one contained the microorganisms, and the other served as a microbe-negative control with halogen only. Treatment vessels contained a final volume of 100 mL of reaction mixture consisting of HDF 0.05 M phosphate-buffered water, pH 7.5, the *Cryptosporidium* oocysts, *B. atrophaeus* spores, MS2 coliphage, and halogen, added in order. Four separate replicate experiments were performed for each disinfectant.

Reaction vessels were placed in a 25 °C temperature-controlled water bath and stirred with a magnetic stirrer. The target halogen concentration in each vessel was ∼5.0 mg/L (31 μM Br_2, 71 μM Cl_2), with the exception of the halogen-negative control. Reaction time began with the addition of the

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halogen, and 2.7 mL aliquots were removed from the reaction vessels at 0, 1, 10, 30, 100, 300, and 1000 min, quenched with 0.3 mL of sodium thiosulfate (final concentration of 100 mg/L or 0.40 mM), and stored at 4 °C until microbial assays were performed. Halogen residual concentrations were measured immediately after sample collection. Microbial assays were typically performed within 12 h from the last time point of the experiment.

2.4.1. C. parvum Infectivity Assay. Infectious oocysts were assayed via cell culture infectivity in HCT-8 (human ileocecal colorectal adenocarcinoma) cells, a continuous line of epithelial cells shown previously to be an effective host for *C. parvum* infection.[34−36] HCT-8 cells were grown and maintained weekly by serial passage in RPMI 1640 medium (Gibco), supplemented with Fetal Clone 1 bovine serum (10% final concentration, Hyclone), HEPES buffer (15 mM, Mediatech), sodium pyruvate (1 mM, Gibco), and the antibiotics gentamicin and kanamycin at concentrations of 50 and 250 μg/mL, respectively. HCT-8 cells were seeded onto eight-well Lab-Tek II chamber slides (Fisher Scientific). Cells for the assay were observed periodically under a light microscope at a total magnification of 100×, and when the monolayer reached 80−90% confluency (typically 48−72 h postseeding), the medium was aspirated, and 100 μL of the undiluted and 10^{-1} diluted samples (in PBS) from the disinfection experiments were inoculated onto the monolayer. Inoculated cells were incubated at 37 °C (5% CO_2) for 1 h to allow for initial excystation and infection. Then, 0.5 mL of the modified RPMI 1640 medium was added to each well, and the cells were incubated at 37 °C (5% CO_2) for another 48 h.

After the incubation period, the culture medium was aspirated, and the cells were fixed with absolute methanol (0.5 mL per well) for 15 min at room temperature (RT). The methanol was aspirated, and the cells were washed with PBS (0.5 mL per well) three times. A blocking solution of PBS, supplemented with 1% bovine serum albumin (BSA), was applied for 1 h at RT (0.5 mL per well). The PBS-BSA was aspirated, and the monolayers were stained with 250−300 μL of purified fluorochrome-labeled C_3C_3-FITC monoclonal antibodies (initially provided by Mike Arrowood, US CDC);[37] then, they were diluted in PBS-BSA, to bind only to the living stages of *C. parvum* in infected cells. Slides were covered with foil and placed on a shaker platform for 90 min at RT. The immunofluorescent staining solution was aspirated, and the cells were washed with PBS three times (0.5 mL per well). Chamber walls were removed, and coverslips were mounted onto the surface of the slides and sealed onto the slides with PVA-DABCO mounting medium.

Cell monolayers on the slides were observed at a total magnification of 250× (oil immersion lens) with a Leitz Orthoplan 2 fluorescent microscope or at 200× (dry lens) with an Olympus BX61 fluorescent microscope. Microscopic viewing fields for each area of cells representing a well on the slide were scored as positive or negative for *C. parvum* living stages by looking for foci of infection that produced apple-green fluorescence. Each focus of infection was assumed to come from one infectious oocyst. The Thomas equation[38] was used to calculate a most probable number (MPN) of infectious units (IU) per mL:

$$\mathrm{MPN}\frac{\mathrm{IU}}{\mathrm{mL}} = \frac{(\text{number of positive fields})}{[(\text{mL sample in negative fields}) \times (\text{mL sample in all fields})]^{1/2}} \quad (1)$$

2.4.2. B. atrophaeus Spore Assay. Volumes of 100 μL of serially diluted samples from disinfection experiments were spread in duplicate onto tryptic soy agar (TSA) plates, allowed to dry at room temperature, and incubated aerobically at 36 °C for 18−24 h. TSA plates were predried at 36 °C for at least 24 h before the assay was performed to enhance absorption of the sample onto the hardened agar. Orange bacterial colonies compared against controls were considered to be progeny of *B. atrophaeus* spores, and the concentration was expressed as colony forming units per milliliter (CFU/mL). The countable range for spread plate assays is considered to be 20−200 CFU per plate.[39]

2.4.3. MS2 Coliphage Stocks and Assay. MS2 coliphage (ATCC# 15597-B1) stocks were prepared using an *E. coli* F-amp host as previously described.[40] Volumes of 100 μL of serially diluted samples from disinfection experiments were assayed by the double agar layer (DAL) plaque technique on the host bacterium, *E. coli* F-amp, using previously described standard procedures.[41] Plates were incubated aerobically at 36 °C for 18−24 h and observed for countable circular lysis zones (plaques). The countable range for DAL pour plate assays is considered to be 0−300 plaque forming units (PFU) per plate[41] and reported as PFU/mL.

2.5. Disinfection Modeling. Inactivation kinetics for *C. parvum* oocysts and *B. atrophaeus* spores were modeled using the following: Chick−Watson and Hom models, which do not account for decay of the disinfectant over the course of the experiment; efficiency factor Hom and Selleck models which do account for disinfectant decay over the course of the experiment.[42−45] The parameters and times to achieve various reductions (or increases) in microorganism survival were based on the results of the four replicate disinfection experiments.

As derived from the general differential rate law,

$$\frac{dN}{dt} = -kmN^{x}C^{n}t^{m-1} \quad (2)$$

where *m*, *n*, and *x* are empirical constants. The following disinfection kinetics models were used to describe inactivation kinetics:

The Chick−Watson pseudo-first-order rate law (3) for a disinfectant-demand-free system is

$$\frac{dN}{dt} = -kNC^{n} \quad (3)$$

where *N* is the number of viable organisms at time *t*, *k* is a first-order rate constant, *C* is the initial concentration of the disinfectant, and *n* is an empirical constant representing a coefficient of dilution that is indicative of the average amount of molecules of disinfectant bound to the organism before it loses viability,[46] which when integrated yields

$$\int_{N_0}^{N} \frac{dN}{N} = -kC^{n}\int_{0}^{T} dt \quad (4)$$

$$\ln\left(\frac{N}{N_0}\right) = -kC^{n}T \quad \text{or} \quad \log\left(\frac{N}{N_0}\right) = -KC^{n}T \quad (5)$$

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where $\left(\frac{N}{N_0}\right)$ is the survival ratio of the microorganisms being inactivated, $K = k/\ln(10)$, and T is the time to achieve a target reduction.

The Hom model (5), which is a generalization of the Chick−Watson rate law in which a new parameter m is introduced to address non-linear log survival over time, is

$$\frac{dN}{dt} = -kmNC^n t^{m-1} \tag{6}$$

$$\int_{N_0}^{N} \frac{dN}{N} = -kmC^n \int_0^T t^{m-1}\, dt \tag{7}$$

$$\ln\left(\frac{N}{N_0}\right) = -kC^n T^m \quad \text{or} \quad \log\left(\frac{N}{N_0}\right) = -KC^n T^m \tag{8}$$

where m is the Hom dilution coefficient.

Disinfectant decay was measured throughout the experiment and modeled by first-order reaction kinetics. The efficiency factor Hom (EF Hom) model (9) incorporates disinfectant demand, which accounts for decreases in disinfectant concentrations during the experiments, as a parameter in the Hom model, and provides for a satisfactory approximate solution for values of $m > 0.4$ and $m < 2$ if $nk'T > 0.7$[43] per

$$\log\left(\frac{N}{N_0}\right) = -KC^n T^m \eta \tag{9}$$

where

$$\eta = \left[\frac{1 - e^{(-nk'T/m)}}{\frac{nk'T}{m}}\right]^m \tag{10}$$

The Selleck model (11) under conditions where there is a disinfection demand exerted on the system, which assumes a first-order disinfectant decay rate, is

$$\frac{dS}{dt} = -\frac{nC_0 \exp(-k't)}{k} S^{(n+1)/n} \tag{11}$$

$$-n(S^{-1/n} - 1) = -n\frac{C_0}{kk'}(1 - e^{-k't}) \tag{12}$$

$$\log\left(\frac{N}{N_0}\right) = -n \log\left[1 + \frac{C_0}{kk'}(1 - e^{-k't})\right] \tag{13}$$

where $S = \left(\frac{N}{N_0}\right)$ is the survival ratio of the microorganisms being inactivated and disinfection decay is expressed by $C_0 \exp(-k't)$, where k' is a first-order disinfectant decay rate constant and C_0 is the initial disinfectant concentration in mg/L at time point zero.[47] Modeling and confidence intervals were performed in JMP Pro 14. As the disinfectant concentration decreases over the course of the experiment, the concentration × time (CT) values were calculated by integrating the modeled first-order disinfectant residual curve and expressed as min·mg/L.

3. RESULTS

The observed and modeled survival ratios as $\log_{10}(Nt/No)$ versus time are shown in Figure 1 for *C. parvum* oocysts and *B. atrophaeus* spores disinfected with chlorine and bromine in a buffered demand-free water for four separate replicate experiments. The concentrations of *Cryptosporidium parvum* oocysts, as infectious units per mL (IU/mL), and *B. atrophaeus* spores, as colony forming units (CFUs) per mL, were fitted to the Chick−Watson, Hom, EF Hom, and Selleck kinetic models and utilized to predict time to achieve 99% and 99.99% reductions.

In Figure 1A for *C. parvum* treated with a target ∼5 mg/L (range: 5.0−4.4 mg/L) free chlorine dose, there are apparent increases in average (n = 4) *C. parvum* infectivity of 0.29 $\log_{10}$ at 0−1 min (CT: 4.57 min·mg/L), 0.39 $\log_{10}$ at 30 min (CT: 135 min·mg/L), and 0.44 $\log_{10}$ by 1000 min (CT: 2890 min·mg/L). A key finding of the observed data is gradual increases in infectivity indicating that chlorine not only was ineffective for inactivation of *C. parvum* but in fact may have increased *C. parvum* infectivity by up to 63.7% as compared to the control. In contrast to free chlorine, the results of this study document bromine as effective for inactivation of *C. parvum* oocyst infectivity by as much as 0.80 $\log_{10}$ after 300 min of exposure. As shown in Figure 1B, average $\log_{10}$ reductions of *C. parvum* by a target dose of ∼5 mg/L (range: 4.3−3.4 mg/L) bromine were 0.40 (range: 0.29−0.58) $\log_{10}$ after 30 min (CT: 112 min·mg/L) and 0.58 (range: 0.45−0.80) $\log_{10}$ by 300 min (CT: 895 min·mg/L), after which there is a plateau and no further infectivity reduction over time.

Reductions of culturable *B. atrophaeus* spores were significantly greater compared to *C. parvum* oocysts. *B. atrophaeus* spores were reduced to non-detectable levels, which exceeded 4 $\log_{10}$ reductions, by ∼5.0 mg/L (4.3−3.4 mg/L) bromine after 1000 min (CT: 1870 min·mg/L) of exposure and after 100 min (CT: 433 min·mg/L) of exposure to ∼5 mg/L (5.0−4.4 mg/L) free chlorine. Figure 1C indicates average (n = 4) *B. atrophaeus* spore reductions by an ∼5 mg/L chlorine dose of 0.46 (range: 0.19−0.75) $\log_{10}$ at 10 min (CT: 45.5 min·mg/L), 3.6 (range: 3.1−3.9) $\log_{10}$ at 30 min (CT: 135 min·mg/L), and 4.9 (range: 4.6−5.1) $\log_{10}$ by 100 min (CT: 433 min·mg/L). *B. atrophaeus* spores treated with ∼5 mg/L of bromine were also reduced extensively but at a slower rate than observed for free chlorine. Figure 1D displays average (n = 4) inactivation of 0.24 (range: 0.010−0.85) $\log_{10}$ at 10 min (CT: 38.0 min·mg/L), 2.0 (range: 0.48−3.5) $\log_{10}$ at 300 min (CT: 895 min·mg/L), and 4.3 (range: 3.8−4.7) $\log_{10}$ by 1000 min (CT: 1870 min·mg/L).

MS2 Coliphage was rapidly inactivated by ≥4 $\log_{10}$ by both free chlorine and free bromine. Disinfection by 5 mg/L free chlorine displayed no detectable MS2 coliphage plaques at the earliest 1 min time point (n = 4) with an average 4.6 $\log_{10}$ reduction. A target dose of 5 mg/L free bromine resulted in averaged reductions of 4.8 $\log_{10}$ after 1 min of treatment with no detectable plaques (n = 4). Positive controls were utilized for all time points and showed no significant reductions over the treatment period of 30 min. Modeling of MS2 coliphage inactivation kinetics was not plausible at target halogen concentrations due to disinfection occurring prior to the initial 1 min time point.

Although the experimental matrix was a halogen-demand-free test water, the introduction of microbes and then the extended length of experimental time necessitated monitoring of disinfectant concentrations. After 1000 min, chlorine and bromine were reduced from an initial target concentration of 5.0 mg/L to average concentrations of 2.0 and 1.4 mg/L, respectively, in the experimental vessels with microorganisms (Table S9). Chlorine and bromine in the control reactors decayed by an average of 0.8 and 0.6 mg/L, respectively, in the

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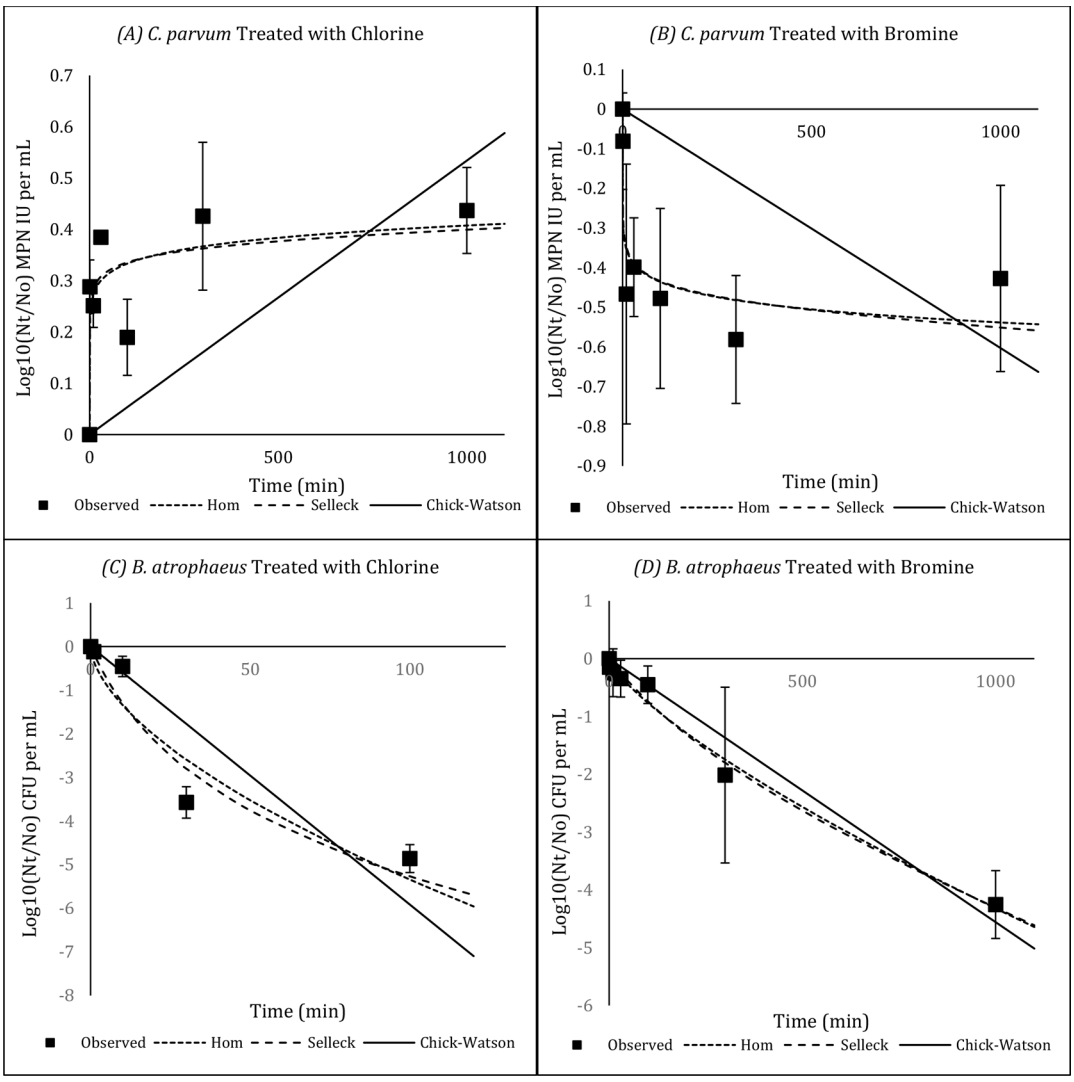

Figure 1. Disinfection of *C. parvum* and *B. atrophaeus* with chlorine or bromine: (A) *C. parvum* treated with chlorine; (B) *C. parvum* treated with bromine; (C) *B. atrophaeus* spores treated with chlorine; (D) *B. atrophaeus* spores treated with bromine. Average observed (square marker) $\log_{10}$ survival ratios as Nt/No vs time from four replicate experiments ($n = 4$) and modeled (lines) $\log_{10}$(Nt/No) reductions for *C. parvum* oocyst and *B. atrophaeus* spore disinfection with chlorine (~5.0 mg/L) and bromine (~5.0 mg/L) in buffered demand-free water. MS2 coliphage is not displayed, as complete disinfection occurred prior to the initial 1 min time point. Error bars denoted as the standard error of replicates per time point and Hom, Selleck, and Chick−Watson models displayed. Note the positive *Y*-axis in part A indicates an increase in *C. parvum* infectivity. Observed and modeled data tables are provided in Supporting Information Tables S4−S7.

HDF test water after 1000 min. First-order decay rates as k' (min^{-1}) were calculated as −0.00112 (R^2 = 0.97) for free chlorine and −0.00175 (R^2 = 0.95) for free bromine. Additional CT values were calculated utilizing first-order decay of each disinfectant based on the average measured concentration and reported in Tables S4−S7. Initial measured bromine concentrations after introduction to the reaction vessels ranged from 4.3 to 3.4 mg/L, as compared to the target 5.0 mg/L, indicating that there was a halogen demand in the approximately 3−6 min it takes to perform the colorimetric DPD bromine concentration assay.

As seen in Figures 1A and 2A, the models for Hom, Selleck, and Chick−Watson for this data represent increases in infectivity of *C. parvum* as a result of exposure to ~5 mg/L of chlorine. The EF Hom model also showed a good fit (MSE = 0.013), but after iterative fitting, the m parameter (m = 0.0008) fell well below the m > 0.4 cutoff for accurate approximation of the incomplete gamma function. Although all of the models depict increasing survival of *C. parvum*, the Hom and Selleck models show a gradual increase and then a plateau after less than 100 min. The Chick−Watson model was less effective in capturing the increasing infectivity at early time points that lie above the line of concurrence, as seen in Figure 2A where the increasing infectivity observed is not predicted. Based on model goodness of fit, the Hom model is the best-fit to these data with a mean squared error (MSE) closest to zero, of 0.0114, and a predicted time to reach a 50% infectivity increase after 10 min. Figures 1B and 2B display models for Hom, Selleck, and Chick−Watson for this data that depict decreased infectivity of *C. parvum* as result of exposure to ~5 mg/L of bromine. Although all the models represent decreased survival of *C. parvum*, the EF Hom model (m = 0.44, m StdErr = 0.45) is a best-fit to these data with a mean squared error (MSE) closest to zero, of 0.0316, and a predicted time to reach a 50% reduction in infectivity by 6 min. The Chick−Watson model failed to capture observed infectivity reductions at early

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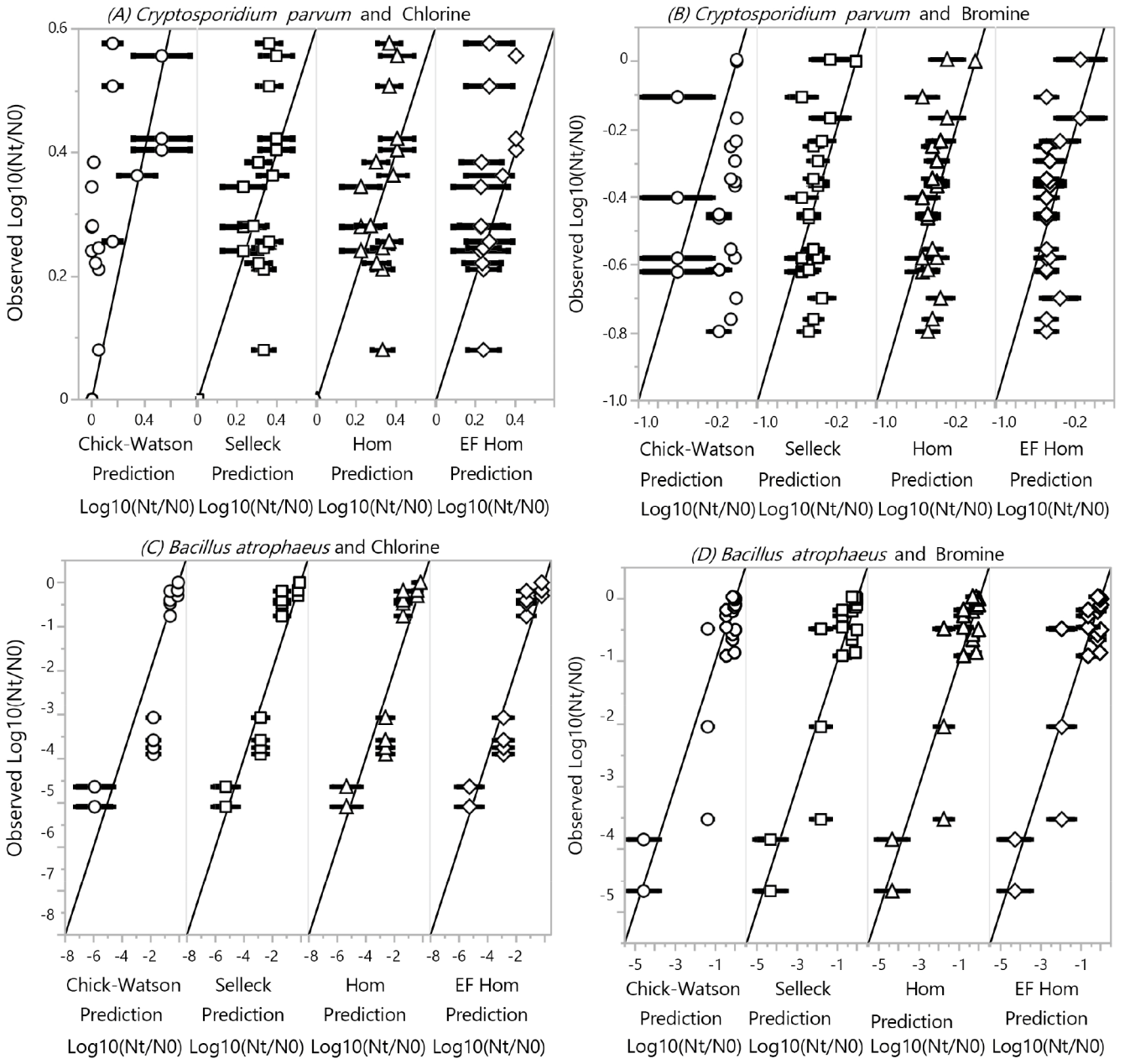

Figure 2. Comparison of observed log changes in concentration against predicted log changes in concentration from modeling: (A) *C. parvum* treated with chlorine; (B) *C. parvum* treated with bromine; (C) *B. atrophaeus* spores treated with chlorine; (D) *B. atrophaeus* spores treated with bromine. Observed and modeled $\log_{10}$ changes in concentration for *C. parvum* oocysts and *B. atrophaeus* spores disinfected with chlorine and bromine are plotted against each other with 95% confidence intervals. The line represents a perfect 1:1 concurrence between model prediction and observed values. For graphs B–D, points falling above the line of concurrence indicate the model predicted greater microbial concentration changes than were observed. Points falling below the line indicate that greater microbial concentration changes were observed than were predicted. For graph A, points falling above the line indicate greater observed increases in infectivity of *C. parvum* than was predicted by the models.

time points. Both the Hom and Selleck models had a similar fit with a MSE of 0.035 and a predicted time to reach a 50% reduction in infectivity by 2 and 3 min, respectively. These models may be more appropriate for this data as the *m* parameter for EF Hom is close to the 0.4 cutoff.

Modeling of *B. atrophaeus* spore reductions by an ~5 mg/L chlorine dose as seen in Figures 1C and 2C had a higher MSE, as the log reductions achieved were >4 $\log_{10}$. Hom, EF Hom, and Selleck all demonstrated tailing off kinetics out to 100 min of treatment. Of the three disinfection kinetics models tested, the EF Hom and Selleck models achieved the best fit with a MSE of 0.373 and 0.391, respectively, and best modeled the late tailing-off kinetics of *B. atrophaeus* treated with free chlorine. The initial inactivation through the first 10 min was best modeled by Chick–Watson (MSE = 0.94). The Hom, EF Hom, and Selleck models all predicted 2 $\log_{10}$ (99%) and 4 $\log_{10}$ (99.99%) reductions after 19 and 60 min, respectively, while the Chick–Watson model predicted 2 $\log_{10}$ and 4 $\log_{10}$ reductions after 35 and 70 min of exposure, respectively. *B. atrophaeus* treated with bromine was the only disinfection condition that approached log–linear reduction kinetics with a Chick–Watson model fit MSE of 0.338 as compared to the Hom, EF Hom, and Selleck model MSEs of 0.304, 0.309, and 0.302, respectively. The Hom, EF Hom, and Selleck models all

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predicted 2 $\log_{10}$ and 4 $\log_{10}$ reductions after 350 and 900 min, respectively, while the Chick−Watson model predicted 2 $\log_{10}$ and 4 $\log_{10}$ reductions after 450 and 900 min, respectively, of exposure.

4. DISCUSSION

From this evaluation of free chlorine and bromine for the disinfection of *C. parvum* oocysts in water, neither disinfectant achieved the US EPA target 2 $\log_{10}$ reduction for infectious oocysts. Both disinfectants failed to achieve even a 1 $\log_{10}$ reduction under the conditions studied. For *C. parvum* oocysts, free chlorine disinfection was not only ineffective but apparently increased oocyst infectivity by 63.7% (0.44 $\log_{10}$). In contrast, free bromine reduced *C. parvum* oocyst infectivity by 0.58 $\log_{10}$. Both free chlorine and free bromine achieved >4 $\log_{10}$ reductions of B. *atrophaeus* spores, and based on the rate of reduction, chlorine was a more rapid sporicidal disinfectant than bromine as either an equal weight or molar concentration.

While both chlorine and bromine achieved extensive (>4 $\log_{10}$) reductions of *B. atrophaeus* spores, chlorine disinfection was more rapid with a 2 $\log_{10}$ reduction achieved in about 19 min by free chlorine and 350 min by free bromine. Of the models that did not include a term for disinfectant decay, the Hom model gave the best fit for the observed tailing off microbial reductions over time. Overall, based on inactivation kinetics, the Selleck and EF Hom models had a better fit with inclusion of a term for disinfectant decay over time. However, the EF Hom model failed to optimize with $m > 0.4$ for *C. parvum* treated with chlorine and had an m value very close to 0.4 with a relatively high standard error for *C. parvum* treated with bromine. This may indicate that, for kinetic modeling of tailing data from a limited number of replicates with less than 1 log of change, the EF Hom model is not appropriate.

Halogen concentration measurements from one of the four bromine trials indicated higher observed halogen demand than in the other three trials. It is possible that either (1) a lower residual could have hindered the rate of or stopped inactivation and/or (2) greater aggregation of spores or oocysts could have occurred. These possible explanations were not evaluated in this study but are known to contribute to declines in microbial inactivation rates over time, as microorganisms in water and wastewater can become aggregated.[48,49] The relatively low disinfectant concentrations and observed disinfection kinetics results of this work may resemble real-world conditions of a drinking water system treating water from a pristine source water. However, these experimental conditions and results are less representative for bromine and chlorine disinfection of treated wastewaters, engineered recreational water, or waters used for crop irrigation for which higher disinfectant concentrations could be used resulting in different disinfection kinetics.

It is noteworthy that *C. parvum* oocysts used in this study had low infectivity for cell cultures. As oocysts are produced by infecting calves, the specific infectivity of oocysts can vary by batch among calves. From assays of non-halogen-containing reaction vessel samples, for which excystation and infectivity were not influenced by halogen exposure, *C. parvum* oocyst excystation and infectivity was about 0.1% (i.e., only about 1 out of every 1000 oocysts excysted and successfully infected the monolayer of HCT-8 cells). Excystation is often attributed to the weakening of the oocyst outer wall that facilitates the release of internal infectious sporozoites. Additionally, the results of this study are somewhat different from those of a previous study in which there was no observed reduction of *C. parvum* oocyst infectivity based on mouse bioassay when using a 5 mg/L dose of free chlorine in water after 24 h of exposure.[50] No apparent increase in oocyst infectivity was observed.

The infectivity results of this study are different from those of a previous study where *C. parvum* oocysts experienced almost 90% reduction based on *in vitro* excystation assays when treated with 4.96−8.48 mg/L of free chlorine.[51] However, an *in vitro* excystation "viability" assay is not a direct measurement of infectivity in either cell cultures or experimental animals. Therefore, a direct comparison of excystation results from those experiments to results from the current experiments based on oocyst infectivity is not appropriate.

In contrast to chlorine, an ∼5 mg/L dose of bromine reduced the infectivity of *C. parvum* oocysts. It may be that the oocyst wall is altered by bromine exposure that is not beneficial for future excystation in the presence of susceptible host cells. Perhaps brominated oocysts excyst earlier than those treated with chlorine, resulting in early release of sporozoites into test waters resulting in their inactivation prior to inoculation into cell cultures for infectivity assay. For example, King et al. found that the infectivity of *C. parvum* sporozoites decreased dramatically if they did not encounter host cells in time to infect them.[52] If sporozoites are "leaking" out of damaged oocysts during bromine disinfection (i.e., prematurely), they will not have the opportunity to find a host cell to infect before they are inactivated, or are otherwise destroyed during this susceptible life stage.

It is also possible that there are two populations of oocysts that differ in response to bromine and chlorine. One population is inactivated relatively quickly by bromine, and another population slowly becomes more infectious over time. Perhaps chlorine induces excystation enhancement in one population by weakening the oocyst wall, while bromine reduces infectivity by excessive early damage to the oocyst wall that causes premature release of sporozoites. By using vital dyes that are specific to living stages of *Cryptosporidium* and phase contrast or differential interference contrast microscopy, it should be possible to visually identify the physical nature of the oocysts and their sporozoites at different exposure times to better characterize excystation and sporozoite release phenomena in water and in the presence of mammalian host cells. Such experiments are recommended for future studies.

The findings from this research have important implications for municipalities that have *Cryptosporidium* oocysts in their source waters and use chlorine disinfection. The ability to meet the 2 $\log_{10}$ (99%) reduction requirement of *Cryptosporidium* by filtration (Long-term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) − 40 CFR 141.700-141.722) could be impacted for plants using free chlorine. Recognizing that halogen disinfection is not the primary treatment barrier in conventional water treatment for removing *C. parvum* oocysts from drinking waters, when the LT2ESWTR was stipulated, it was not anticipated that oocysts could become more infectious in chlorinated drinking water treatment systems. Results from the present cell culture infectivity study suggest that conventional treatment plants that chlorinate their drinking water could potentially be enhancing the infectivity of *C. parvum* oocysts that are not removed through conventional coagulation-flocculation and filtration and possibly result in the passage of infectious oocysts through their systems. Additional research is needed to determine the public health impact at

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conventional water treatment plants as findings from a controlled lab setting using cell culture infectivity may not be representative of real-world conditions where source waters vary in temperature, pH, halogen demand, *C. parvum* oocyst strain, and duration of time in the environment after shedding from a host.

The findings of this research also have specific implications for municipalities that have bromide-impacted source waters, such as in coastal areas where saltwater intrusion is common and other areas with brackish groundwater. If these municipalities are utilizing free chlorine as their drinking water disinfectant, chlorination will rapidly oxidize the bromides to free bromine and possibly alter the kinetics of microbial inactivation, with free bromine now present as an active disinfectant. Depending on the relative concentrations of bromide ions and free chlorine, free bromine may either be working in concert with free chlorine or predominating as the operative disinfectant. The latter is likely according to Westerhoff et al., who found that "bromine reacts faster and substitutes more efficiently than chlorine".[53] This is consistent with the finding that, even at low concentrations, the bromide ion has profound effects on speciation of byproducts in chlorine-disinfected water.[54] Bromide is commonly present in source waters. Amy et al. (1994) report that the bromide concentration in source waters in the U.S. averages between 61 and 64 μg/L, while Krasner et al. (1996) report bromide concentrations in the range of 100−500 μg/L in source water samples from the California State Water Project, an area with historically high bromide concentrations.[55,56] Chlorination plant managers of drinking water systems that have modeled their disinfection process based on chlorine inactivation kinetics may want to consider reviewing their chlorination process if their influent water is bromide-impacted. Finally, we recommend further evaluation of free bromine for inactivation of *Cryptosporidium ssp.* oocysts for non-potable uses, such as water reclamation for agricultural irrigation, as well as use in engineered recreational water systems such as pools and spas.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c00536.

Supporting figures and tables and a summary from the review of literature on bromine inactivation of microorganisms, observed and modeled experimental results, and disinfection modeling parameters (PDF)

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Author Contributions

C.K.C. performed the modeling and data analysis and contributed to the manuscript. J.K. performed the disinfection experiments and contributed to the manuscript. E.S.B., L.S.A., and J.B. contributed to the manuscript for publication. O.D.S. contributed to the disinfection experiments and study design. M.D.S. obtained the initial funding, led the project, designed the study, and directed the work of the other project team members.

Notes

The authors declare no competing financial interest.

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