Laboratory validation of an ozone device for recreational water treatment

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ABSTRACT

Obtaining an accurate assessment of a treatment system's antimicrobial efficacy in recreational water is difficult given the large scale and high flow rates of the water systems. A laboratory test system was designed to mimic the water conditions and potential microbial contaminants found in swimming pools. This system was utilized to evaluate the performance of an *in situ* ozone disinfection device against four microorganisms: *Cryptosporidium parvum*, bacteriophage MS2, *Enterococcus faecium*, and *Pseudomonas aeruginosa*. The sampling regimen evaluated the antimicrobial effectiveness in a single pass fashion, with samples being evaluated initially after exposure to the ozone unit, as well as at points downstream from the device. Based on the flow dynamics and log reductions, cycle threshold (Ct) values were calculated. The observed organism log reductions were as follows: >6.7 log for *E. faecium* and *P. aeruginosa*; >5.9 log for bacteriophage MS2; and between 2.7 and 4.1 log for *C. parvum*. The efficacy results indicate that the test system effectively functions as a secondary disinfection system as defined by the Centers for Disease Control and Prevention's Model Aquatic Health Code.

Key words | *Cryptosporidium parvum*, Ct value, disinfection, ozone, recreational water

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INTRODUCTION

Recreational water includes numerous water types, such as swimming pools, hot tubs, fresh water and salt water beaches, and water amusement parks. Of these many varieties, only swimming pools and spas are typically amended with biocides and/or antimicrobial treatment technologies to control microbial growth (Unhoch & Vore 2005). Recreational waters are subject to microbial contamination due to a variety of activities (Craun *et al*. 2005; Turgeon 2012). It is difficult to determine the levels of microbial contamination because of the variability of the sources and conditions (Castor & Beach 2004). Recreational water illness (RWI) can be spread by swallowing or just by having contact with the polluted water from swimming pools, spas, hot tubs, and lakes. The most common RWI is gastroenteritis, mainly diarrhea caused by microorganisms such as *Cryptosporidium*, *Giardia*, *Shigella*, and *Escherichia coli* O157:H7 (Craun *et al*. 2005; Turgeon 2012). The US Environmental Protection Agency (USEPA) has issued a number of water quality standards, such as the Interim Enhanced Surface Water Treatment Rule (IESWTR), to address microbial pathogen contamination in surface water and recreational water (USEPA 1998, 2001). However, the incidence of diarrhea related to RWI breakout in the United States has significantly increased over the past decade (Hlavsa *et al*. 2005). This is mainly due to the lack of effort to regulate public recreational water facilities.

Cryptosporidium parvum is one of the most important waterborne pathogens associated with RWI as it causes severe gastroenteritis (cryptosporidiosis) (Alden *et al*. 2007). *C. parvum* has been associated with rivers and lakes, most likely introduced via animal vectors. Traditional treatment technologies, such as chlorination,

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have been shown to have limited efficacy against *C. parvum* oocysts (Letterman 1999). Furthermore, many of the studies to determine the cycle threshold (Ct) values of treatment approaches for *C. parvum* have been performed under oxidant demand-free conditions. Ct values represent the product of *C* (residual disinfectant concentration in mg/L and *T* (disinfectant contact time in minutes). These Ct values may not be directly applicable to recreational water systems since the effect of organic contaminants present in a recreational water environment has not been evaluated (Carpenter *et al*. 1999). Due to the disadvantages associated with chemical treatment, researchers have used alternative methods, such as sand filtration, ultraviolet (UV) irradiation, and ozonation to control *C. parvum* contamination of recreational waters (Fayer *et al*. 1997; Clancy *et al*. 2000; Loganthan *et al*. 2012). Even though the USEPA has accepted UV disinfection as a method for inactivating *Cryptosporidium*, there are certain disadvantages associated with it. UV has poor penetration capability and therefore suspended particles cause a shielding effect to the smaller oocysts of the *Cryptosporidium* (Morita & Hirata 2005). In addition, UV treatment is flow-rate dependent. Since 1906, ozone has been used as a means of disinfection of drinking water. In comparison to chlorination, ozone produces less harmful by-products and is also effective against most of the waterborne pathogens, including protozoa and viruses (Shin & Sobsey 2003). It has been suggested that to optimize inactivation of *C. parvum* in recreational waters, a series of treatment technologies could be employed (Letterman 1999).

Annually, outbreaks of cryptosporidiosis in recreational water venues are reported to the Centers for Disease Control and Prevention (CDC) (Alden *et al*. 2007; Yoder *et al*. 2012). The frequency of these occurrences, coupled with the documented resistance of *C. parvum* to chlorination, suggest that alternative treatment technologies need to be researched and implemented (Bukhari *et al*. 2000). To assess the potential antimicrobial efficacy of various treatment technologies in recreational water, guidelines and standards have been developed by independent bodies, such as AOAC and NSF International, and have been subsequently adopted in regulations set forth by the USEPA (USEPA 1979; NSF 2009). These methodologies typically incorporate the physical conditions and surrogate organisms to mimic 'real world' conditions and public health risks. The AOAC method for evaluating swimming pool disinfectants and ANSI/NSF International's Standard 50 both utilize bacterial species as test surrogates. Additional organisms could be evaluated, but their inclusion into the protocol must be validated.

To guide local and state agencies in preventing and responding to RWIs in swimming pools the CDC has released the Model Aquatic Health Code, or MAHC (CDC 2012). Currently, no uniform standard is available on recreational swimming pool water quality or RWI response. The MAHC describes the operating criteria for different treatment technologies and classifies these approaches in three different categories: primary (chlorine and bromine), secondary (ozone and UV light disinfection), and supplementary (i.e., copper and silver ions). As a secondary treatment system, the MAHC states that the system must achieve a minimum of 3 log reduction of infectious *C. parvum*.

The objective of the present study was to validate the antimicrobial efficacy of a commercial ozone generator for the treatment of contained recreational water systems (i.e., swimming pools). To accurately assess the disinfection capabilities of the treatment device, a test rig was designed that incorporated the flow rates, water quality, and potential interferences expected in a swimming pool, rather than rely on a scaled-down bench scale evaluation. Antimicrobial efficacy, in terms of log reduction, was assessed using representative Gram-positive and Gram-negative bacteria, as well as a RNA bacteriophage, MS2. The impact on the infectivity of *C. parvum* was also evaluated. Ct values were then calculated for *C. parvum* within the test system.

MATERIALS AND METHODS

Cultures, cell lines, media, and reagents

The following bacteria were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): *Enterococcus faecium* strain PRD ATCC 6569, *Escherichia coli* ATCC 15597, and *Pseudomonas aeruginosa* ATCC 27313. Bacteriophage MS2 ATCC 15597-B1 stock suspensions were obtained from Biological Consulting Services of North Florida (BCS, Gainesville, FL, USA). The bacterial

strains were initially grown according to ATCC's instructions. The concentration of the bacteriophage stock was verified by using the double agar overlay method (DAL) with tryptic soy agar (TSA) serving as the base media and tryptic soy broth (TSB) + 1% agar serving as the top agar (Kropinski *et al*. 2009).

C. parvum (Iowa strain) was obtained from Sterling Parasitology Laboratories (Tucson, AZ, USA). The *C. parvum* stock possessed at least 90% viability (as determined by excystation) and was stored with 100 IU/mL penicillin and 100 μg/mL gentamicin at 4 °C. The stock was purified via discontinuous sucrose and cesium chloride centrifugation gradients and was used within 8 weeks of collection.

All media used for bacterial isolation and growth were from Difco (Becton Dickinson, Franklin Lakes, NJ, USA). Unless otherwise noted, an incubation temperature of 35 °C was used for all enrichment and growth plates and all reagents and chemicals were ACS (American Chemical Society) reagent grade or higher (Sigma-Aldrich Co., St Louis, MO, USA).

The human colon cancer cell line HCT-8 (ATCC CCL 244) was obtained from ATCC and cultured/propagated according to the supplier's specifications.

Test system design for *C. parvum* efficacy studies

The test setup for determining the efficacy of the ozonator device against *C. parvum* consisted of a test tank made of custom fabricated polypropylene (Imperial Industries, Inc., Belleville, MI, USA) and a pump (Model #3P663, Teel/Dayton Electric Manufacturing Co., Niles, IL, USA) that forced water through a main and branch line, a static mixer (Model #2-40C-4-6-2, Koflo Corporation, Cary, IL, USA) and 9.14 m of 50 mm hose downstream from the remix point of the main and branch lines. The ozone treatment system (Del Ozone Genesis CD-2, Del Ozone, San Luis Obispo, CA, USA) was located in the branch line of the setup. The system delivered 2 g/h at 2.5% by weight at 1.18 standard liters per minute gas flow. A schematic of the setup is illustrated in Figure 1. A volume of 3,028 L of de-ionized water was balanced to the specifications in Table 1 prior to the addition of challenge constituents. The system flow rate was set at 80 L/min. After the test water was conditioned, a 1 L sample was collected in triplicate from the test tank and served as the negative control sample. A branch water line was diverted to the ozone generator from the main

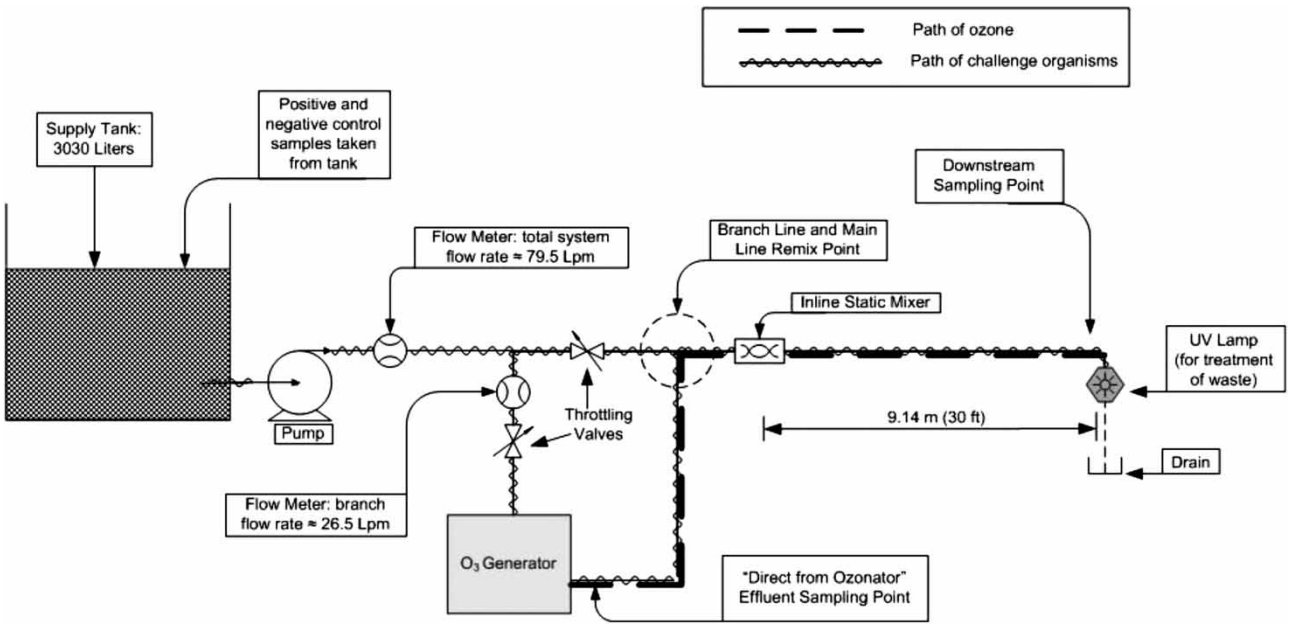

Figure 1 | Schematic and flow diagram for the recreational water test rig used for the *C. parvum* efficacy studies. Test water inoculated with the challenge organism is introduced to the commercial ozonator after a reduction in flow rate from 65 to 21 lpm. Samples were obtained directly after exposure to the treatment system (*C. parvum* studies only) as well as 9.14 m downstream (all studies) to assess the disinfection capability of residual ozone in the test system.

Table 1 | Water parameters utilized for the *Cryptosporidium parvum*, bacterial and viral efficacy studies using a commercial ozone water treatment device. Data presented for the *C. parvum* assays are averages of three studies. Standard deviations for the data are presented. Bacteriophage MS2, *Enterococcus faecium*, and *P. aeruginosa* were utilized as the challenge bacteria according to NSF Standard 50 (NSF 2009)

Parameter	*C. parvum* infectivity assay	Standard 50 bacterial and viral assays
Water temperature	25.0 ± 0.6 °C	19.4 °C
pH	7.30 ± 0.15	7.30
Total alkalinity	106 ± 30 mg/L	88.2 mg/L
Total hardness	293 ± 63 mg/L	389 mg/L
Free chlorine	<0.01 ± 0.00 mg/L	<0.01 mg/L
Turbidity	0.90 ± 0.07 NTU	0.54 NTU
Baby oil	Not added	20 mg/L
Urea	Not added	9 mg/L

line at a flow rate of 27 L/min then recombined with the mainline flow and directed to drain. Thereby only one-third of the flow was exposed to the ozone treatment system.

Water flow within the test system was discontinued for a period to allow for addition of *C. parvum*. The oocysts were added to the tank to achieve a target of 1.0×10^6 oocysts per 10 L. A circulating pump was employed for 10 min within the tank to achieve homogenous distribution of the challenge organism. To confirm the actual oocyst concentration in the test system, a 1 L sample was collected in triplicate from the test tank immediately after the mixing period. These samples served as the numbers control for subsequent log reduction calculations. Flow was then resumed to the ozone treatment system and the generator was turned on. At the time points of 4, 9, and 14 min following the initiation of the challenge water flow, individual 10 L samples were collected at a location 9.14 m downstream from the ozone generator/mainline remix point. The sampling duration for each time point lasted 3 min. At a time point of 19 min, a 10 L sample was collected directly downstream of the ozone generator before being remixed with the mainline. Following collection of the samples, the samples were allowed to aerate for more than 30 min before being capped and placed at 4 °C and transported overnight to BCS for infectivity processing.

For determining system variability, the *C. parvum* efficacy test was repeated on three separate occasions.

Cryptosporidium infectivity determination

To determine the concentration of the *C. parvum* stock, membrane filtration followed by immunofluorescent staining using the Crypt-a-Glo kit from Waterborne Inc. (New Orleans, LA, USA) was performed. The percent infectivity of the oocyst stock, controls, and experimental samples was determined using a modified foci detection-most probable number (MPN) method (Slifko *et al.* 1999; Aboytes *et al.* 2004; Johnson *et al.* 2012). The control and experimental water samples were transferred to 500 mL conical centrifuge tubes (Corning, Lowell, MA, USA) and centrifuged for 15 min at $3{,}000 \times g$. The supernatant of each centrifugation was aspirated gently by vacuum, and an additional water sample was added and re-centrifuged. The final concentrated pellet was washed and suspended in 50 mL of phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA), transferred to 50 mL tubes and re-centrifuged. The supernatant was aspirated and the pellet was suspended in 2 mL of acidified Hank's balanced salt solution (HBSS) supplemented with 0.001% trypsin (MediaTech, Manassas, VA, USA). The solution was then incubated for 60 min at 37 °C, with vortexing every 15 min, to trigger oocyst excystation and infectivity. The concentrates were then washed by centrifugation, and were suspended in HBSS. This wash was repeated in duplicate. Three 10-fold dilutions of the numbers control sample and three of the experimental samples were performed using HBSS as the diluent. Five 0.1 mL aliquots of each sample and dilution were used for each infectivity assay.

HCT-8 cells were cultured in Labtech II eight-well chamber slides (Nalgene Nunc, Naperville, IL, USA). All eight wells of each chamber slide were inoculated with 100 μL of each control or sample dilution. The oocysts in each sample were allowed to incubate onto the cell monolayer in each well for 120 min. RPMI media (MediaTech, Manassas, VA, USA) supplemented with 2% FBS (Atlanta Biologicals, Lawrenceville, GA, USA) was added to the wells. The well slides were then incubated in a 5% CO_2 atmosphere incubator at 37 °C for 72 h. Following the 72 h incubation, the cells were washed twice in PBS and

fixed in methanol. The infected cell monolayers were stained with fluorescent-labeled monoclonal antibody specific for the reproductive stages of the *C. parvum* life-cycle. The number of infectious oocysts were enumerated by evaluating each well for the presence of foci of infection by UV epifluorescence microscopy. These foci contain clusters of stained sporozoites that have disseminated from a central focus of infectivity. When a focus is identified, it is confirmed by differential interference contrast (DIC) microscopy so that sporozoite structure can be determined. Wells were scored as either positive or negative based on the presence or absence of sporozoite clusters. The number of infections per well is not relevant in the MPN method. Results were calculated using a MPN statistical analysis. Results were reported as MPN of infectious oocysts per unit volume.

Test system design and assays for the antibacterial and antiviral efficacy studies

A total volume of 625 L of deionized test water was balanced to the specifications in Table 1 prior to the addition of challenge constituents (urea and baby oil) and microorganisms. A recirculation pump (Model #3P663, Teel/Dayton Electric Manufacturing Co., Niles, IL, USA) was utilized to maintain a flow rate of 25 L/min. Three 1 L water samples were obtained to serve as a background organism control. All of the samples for this analysis were collected using sterile bottles at a location immediately downstream of the mixing tower on the return line to the tank.

Challenge cultures of *E. faecium* and *P. aeruginosa* were prepared according to the protocol specified in ANSI/NSF Standard 50 (NSF 2009). A final concentration of 1.0×10^6 colony forming units (CFU)/100 mL for each organism was targeted for the test water. For bacteriophage MS2, a final concentration of 1.0×10^5 plaque forming units (PFU)/mL was targeted. To obtain a homogenous dispersion of the microorganism challenges, urea and baby oil within the test system, the water was circulated for 15 min with the ozone treatment system turned off. Three 1 L water samples were obtained to serve as a numbers control. The ozone treatment system was turned on and, following 6 min of circulation, three 1 L water samples were obtained. Additional samples were taken in triplicate every 6 min until an exposure duration of 30 min had been reached.

All samples were processed to determine microbiological content within 1 h of collection. The concentrations of *P. aeruginosa* and *E. faecium* were determined using the membrane filtration method (APHA 2002). For each sample, a volume of 100 mL was filtered through a 0.45 μm porosity cellulose filter (GN6, Pall Corporation, East Hills, NY, USA). The filters were aseptically transferred to M-PA-C agar and KF Streptococcus agar, then incubated at 35 °C for 48 h. The concentrations of bacteriophage MS2 within the samples were determined using the DAL procedure described previously.

Calculations

An individual geometric mean was obtained for all triplicate samples taken at each individual time point. The log reduction was determined at each sample time by using the following equation:

$$\text{Log reduction} = \log 10\,(\text{Ns}/\text{No}) \quad (1)$$

where Ns is the sample geometric mean for each exposure time and No is the geometric mean of the numbers control (NSF 2012).

Table 2 contains the physical parameters and flow rates of the test system used for the calculation of the Ct values within the branch line and branch/main mixing line. The Ct values were calculated by determining the time the test water was exposed to ozone based on test flow rates and the cross-sectional area of the piping setup. The concentration of ozone (mg/L) was then multiplied by the exposure time (minutes) to calculate the Ct. Calculations in the undiluted mix were made based on a certified ozone output rate of 2 g/h and a mass transfer efficiency of both 60 and 100%. This output rate calculates to an ozone concentration of 1.257 mg/L in the test water at 100% transfer efficiency. The calculated *C. parvum* Ct values for the branch line and branch/main mixing line are provided in Table 3.

Table 2 | Measured physical parameters and flow rates of the test system used for the calculation of Ct values within the branch line and branch/main mixing line

Piping description	Piping area cm² (in²)	Piping length cm (inches)	Flow rate L/min (gal/min)	Velocity cm/s	Time seconds
Branch line					
19 mm sch40 PVC	3.3 (0.51)	107 (42.0)	26.57 ± 0.45 (7.02 ± 0.12)	135.2 ± 2.3	0.8 ± 0.0
Contact chamber	165.2 (25.60)	82.6 (32.5)	26.57 ± 0.45 (7.02 ± 0.12)	2.69 ± 0.05	30.8 ± 0.6
19 mm sch40 PVC	3.3 (0.51)	87.6 (34.5)	26.57 ± 0.45 (7.02 ± 0.12)	135.2 ± 2.3	0.6 ± 0.1
19 mm PVC hose	2.1 (0.33)	383.5 (151.0)	26.57 ± 0.45 (7.02 ± 0.12)	206.9 ± 3.6	1.9 ± 0.1
Branch and main line mix					
50 mm sch80 PVC	19.0 (2.95)	20.3 (8.0)	79.9 ± 0.79 (21.1 ± 0.21)	70.00 ± 0.64	0.4 ± 0.1
50 mm static mixer	19.0 (2.95)	33.0 (13.0)	79.9 ± 0.79 (21.1 ± 0.21)	70.00 ± 0.64	0.5 ± 0.0
50 mm ID PVC hose	20.3 (3.14)	929.6 (366.0)	79.9 ± 0.79 (21.1 ± 0.21)	65.81 ± 0.61	14.0 ± 0.1

Table 3 | Calculated Ct values for the branch line and branch/main mixing line for the *C. parvum* infectivity assay. Data presented for the contact times and ozone concentrations are averages of three studies. Standard deviations for the data are presented. Ozone concentrations are presented in mg/L. Ct values are calculated using the following equation: Ct = (Ozone mg/L) * [(Contact time seconds)/(60 s)]. To calculate the NSF rated Ct value, a safety factor of 2 was incorporated

Location	Contact time (seconds)	60% Ozone concentration	100% Ozone concentration	60% Ct (calculated)	100% Ct (calculated)
Branch line	34.1 ± 0.6	0.74 ± 0.01	1.24 ± 0.02	0.42 ± 0.01	0.70 ± 0.01
Branch and main line mix	14.9 ± 0.1	0.61 ± 0.42	0.66 ± 0.36	0.05 ± 0.04	0.06 ± 0.03

Total Ct = 0.76 ± 0.04.
NSF rated Ct value = (0.76 × 2) = 1.52.

RESULTS AND DISCUSSION

Evaluation of the microbial efficacy of various water treatment and disinfection technologies at the bench-scale level is well documented (Letterman 1999). The test conditions usually involve well-defined water quality parameters and may lack built-in interferences for the disinfection technology being evaluated. This may lead to an inaccurate estimate of the Ct value associated with that technology. This is especially true for recreational water treatment systems where the initial Ct studies may have involved only oxidant demand-free water and may not be representative of actual recreational water (Carpenter *et al.* 1999). During scale-up or implementation of the treatment technology at the field level, safety factors are often added to ensure that public health is protected. This typically involves increasing the dosage of the disinfectant treatment to account for untested or unexpected water quality conditions (Rakness 2005). This paper details a test system designed for assessing the extent of microbial disinfection by a commercial ozonator intended for the treatment of recreational swimming pool waters. The test system and challenge water were designed specifically to include the interferences that may be expected in a 'real world' situation. The assessment of the antimicrobial performance of the device was carried out at the same flow rates that would be used in a swimming pool application. The water temperature, chemical constituents, and additives employed in the study were selected to mimic both human contamination and the water quality of the pools (NSF 2009). The goal of this study was to obtain representative Ct values, relative to *C. parvum*, for a commercial ozonator when treating microbiologically contaminated swimming pool water.

Four challenge organisms were selected for use in the study: *C. parvum*, bacteriophage MS2, *E. faecium*, and *P. aeruginosa*. Their inclusion was based on historical epidemiological data associated with acquired waterborne illness in recreational waters. *E. faecium* and *P. aeruginosa* were also selected to serve as representatives for other pathogenic Gram-negative and Gram-positive bacteria. For determining system variability, the *C. parvum* efficacy test was repeated on three separate occasions.

Prior to initiation of the study, the actual infectivity of the stock oocysts was determined. The MPN infectivity assay revealed that the *C. parvum* stock was approximately 7% infective (culture MPN assay compared to direct counts via hemocytometer). The initial test was performed at a lower challenge concentration (target 1.0×10^3 MPN/10 L). This concentration was targeted given the MAHC's requirement of secondary disinfection systems achieving a minimum 3 log (99.9%) reduction of infective *Cryptosporidium* oocysts (CDC 2012). Additionally, the lower concentration helped to contain costs since this was an exploratory run. Results for the first run demonstrated >3 log reduction in infectivity at all points. Once the results were available, the influent concentrations were increased in the two subsequent test runs. The target for infectious cysts in the test system for the second and third assays was 1.0×10^6 per 10 L. These test achieved an average of 2.9×10^5 infectious cysts per 10 L. Theoretically, if no infectious cysts were present in the treated samples, the treatment system would have achieved greater than 5.5 log reduction in infectious cysts. The results of the *C. parvum* infectivity reduction studies are presented in Table 4. All samples exposed to the ozone treatment displayed greater than a 1 log reduction in infectivity. The greatest reduction (average 3.4 log for runs 2 and 3) in infectivity observed was in the branch/main mixing line, immediately after exposure to the ozone treatment system. Moderate reduction in *C. parvum* infectivity (average 1.4 log for runs 2 and 3) was observed for all of the samples obtained downstream of the treatment device. This demonstrated that the residual ozone remaining in the system was at a concentration that was efficacious.

Table 4 | Log reductions observed for *Cryptosporidium parvum* when exposed to ozone-treated test water at varying time intervals. The system evaluation was repeated on three separate occasions and the results for each are provided. The samples designated as 4, 9, and 14 min post-startup were collected at a location 9.14 m downstream from the ozone generator/mainline remix point. The system blank was collected from the test tank prior to inoculation. Total *C. parvum* concentrations were determined via microscopy. MPN infectious *C. parvum* concentrations were determined via the foci detection-most probable number method (Slifko *et al.* 1999). All concentrations for *C. parvum* are presented in MPN/10 L

Sample description	Experiment 1	Experiment 2	Experiment 3
Initial total *C. parvum*	6.6×10^3	2.8×10^6	4.5×10^6
Initial MPN infectious *C. parvum*	2.4×10^3	3.5×10^5	3.2×10^5
Log reduction at 4 min post-startup	>3.0	1.3	<1.3
Log reduction at 9 min post-startup	>3.0	1.6	1.5
Log reduction at 14 min post-startup	>3.0	1.3	1.5
Log reduction immediately after ozonator	>3.0	2.7	4.1
System blank *C. parvum*	<1.0	<1.0	<1.0

The antibacterial and antiviral efficacy assays indicated that the ozone dosage allowed for a significant decrease in viability for both *E. faecium* and *P. aeruginosa*, as well as for bacteriophage MS2. The actual bacteria and virus count data and the log reductions are presented in Table 5. The target of 1.0×10^6 CFU/100 mL was achieved for the bacterial studies. Greater than a 6 log reduction was observed for both organisms at 6 min post-startup, and for each subsequent sampling event. The observed 6 log kill achieved through the ozone device at less than 10 min

Table 5 | Log reductions observed for *Enterococcus faecium, Pseudomonas aeruginosa*, and MS2 bacteriophage when exposed to ozone-treated test water at varying time intervals. The post-startup samples were collected at a location 9.14 m downstream from the ozone generator/mainline remix point. The system blank was collected from the test tank prior to inoculation. Following addition of the challenge organisms and 15 min mixing period, the numbers control samples were collected from the tank. The post-startup concentrations represent the observed results for the five different sampling times (6, 12, 18, 24, and 30 min). All concentrations for *E. faecium* and *P. aeruginosa* are presented in colony forming units (CFU) per 100 mL. All concentrations for bacteriophage MS2 are presented in plaque forming units (PFU) per mL. The results presented are means of triplicate replicates. Standard deviations are provided in parentheses

Challenge organism	Numbers control concentrations	Post-startup concentrations	Log reduction
E. faecium	4.5×10^6 (3.6×10^5)	$<1.0 \times 10^0$	>6.7
P. aeruginosa	4.5×10^6 (1.9×10^6)	$<1.0 \times 10^0$	>6.7
MS2 bacteriophage	7.1×10^5 (2.1×10^5)	$<1.0 \times 10^0$	>5.9
System blank	$<1.0 \times 10^0$	$<1.0 \times 10^0$	Not applicable

compares favorably to alternative treatment technologies. Anipsitakis *et al.* (2008) detailed a 4 log kill of *E. coli* after 60 min of treatment with activated potassium peroxymonosulfate. The viability of bacteriophage MS2 was reduced by greater than 5.9 log at all exposure points. The susceptibility of MS2 to active ozone in this test system is consistent with previously published data. In demand-free water, Shin & Sobsey (2003) observed greater than a 7.5 log reduction in infective MS2 following a 10 s exposure to test water at pH 7 and possessing an ozone concentration of 0.37 mg/L.

Ct values for the bacterial and protozoal studies were calculated based on the observed flow rates of the test system as well as the measured ozone concentration present in the branch line and branch/main mixing line (Figure 1). For the studies, the concentration of ozone in the water was measured just before the dilution/mixing point to be an average of 1.07 mg/L. This value was used for calculations after the main line and the branch line were remixed (diluted mix) since the ozone concentration in water at that point was significantly lower and measured an average of 0.18 mg/L. The NSF rated Ct value of 1.56 includes a safety factor of 2 and assumes 100% transfer efficiency. This Ct value and corresponding log reductions for *C. parvum* and the two bacterial challenge organisms presented in this study vary from those published in the literature. At the furthest sampling point present in the system (9.14 m downstream of the ozone introduction), an average of 1.4 log inactivation of *C. parvum* oocysts was observed for runs 2 and 3. The log infectivity reduction for *C. parvum* was much greater at a sampling point immediately following the ozone treatment device (average 3.4 log for runs 2 and 3). Kanjo *et al.* (2000) demonstrated that *C. parvum* infectivity was reduced by 2 log and 3 log via ozone Ct values of 3 and 8 mg/min/L, respectively. Finch *et al.* (1993) utilized a nonlinear Hom model for calculating the Ct values for *C. parvum* infectivity reduction in a demand-free test water. Log reductions of 2 and 3 were observed at Ct values of 1.6 and 2.4, respectively (Finch *et al.* 1993). At colder temperatures (1 °C), it has been observed that Ct values of 7.2 and 15 mg.min/L were required to inactivate *C. parvum* oocysts to 0.7 and 1.3 logs, respectively (Finch & Hanbin 1999). In addition, studies carried out in natural waters showed increased Ct values and log inactivations compared with studies performed at the bench-scale level or in demand-free water. Owens *et al.* (2000) observed the following log reductions at a pilot-scale ozonation treatment project performed using Ohio River water: 2.67 log reduction for *C. parvum* at a Ct of 7.15 mg.min/L; 4.1 log reduction for coliform bacteria at a Ct of 6.26 mg.min/L; and 3.36 log reduction for heterotrophic bacteria at a Ct of 6.26 mg.min/L.

The current study differs from the aforementioned *C. parvum* investigations in that a cell culture-based assay was utilized for infectivity determinations rather than an animal infectivity model. *In vitro* excystation assays and vital dye approaches, such as those described by Bukhari *et al.* (2000), were considered, but due to their unreliability to assess inactivation as compared to neonatal mouse studies, an alternative method was sought. The cell culture-based assay described by Slifko *et al.* (1999) and Aboytes *et al.* (2004) showed close correlation to the gold-standard neonatal mouse infectivity standard and were thus selected for the current investigation. The *in vitro* cell culture technique is more cost efficient, it does not rely on sacrificing live animals, and the results are obtained in half the time than animal infectivity assays. Johnson *et al.* (2012) have recently reported on a study comparing the HCT-8 cell culture immunofluorescent antibody and microscopy assay (IFA) method and an approach that coupled cell culture to quantitative polymerase chain reaction (CC-qPCR). The researchers observed that the HCT-8 IFA cell culture method generated the lowest number of false positives and had a lower limit of detection. Thus, it was concluded that the HCT-8 IFA cell culture method is most suitable for use by the drinking water industry in detecting infective *C. parvum* oocysts.

CONCLUSIONS

This study demonstrates that a laboratory test system can be constructed to simulate the conditions expected at a recreational water facility (i.e., inorganic and organic interferences, flow rates, exposure times) and be effectively utilized to evaluate the antimicrobial capabilities of a given water treatment system. In this particular study, a commercial ozone device was the focus. The test system was constructed in a manner such that different treatment systems could be incorporated, such as membrane filtration

or UV light disinfection. The presented method is based on a controlled system with all components well defined, leading to a robust determination of Ct values. These controlled conditions do not negate the validity of the Ct values when applied to another system, but do place importance on knowledge of critical process parameters when applied. Accurate data for piping system characteristics and accurate measurements of ozone concentrations and flow rates are required. The level of water chemistry control and actual water quality in real world systems may introduce interference to the implementation of the Ct values determined in this study. However, the water chemistry used in the study was adjusted to levels found in well-balanced and maintained pool water to mimic real world application. NSF also applied a conservative factor of safety to the final Ct value to address unknown system variables so that the final Ct value might be used in application. Extrapolation of system size and Ct could be done, but requires accurate knowledge of the proposed system parameters, such as piping characteristics, water volume, flow rates, and disinfectant concentrations.

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