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Pilot-Scale Ozone Inactivation of Cryptosporidium and Other Microorganisms in Natural Water

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Abstract

A pilot-scale study was conducted to evaluate the inactivation by ozone against *Cryptosporidium* oocysts, *Giardia* cysts, poliovirus, and *B. subtilis* endospores spiked into Ohio River water. The indigenous Ohio River populations of total coliform bacteria, heterotrophic plate count bacteria and endospores of aerobic spore forming bacteria were also evaluated. Endospores were the only organisms found to be more resistant to ozone than *Cryptosporidium* oocysts. Endospores may serve as an indicator of microbial treatment efficiency. *Cryptosporidium* oocysts were more resistant than *Giardia* cysts or poliovirus. Although HPC bacteria were less resistant than *Cryptosporidium* oocysts, variability limits their usefulness as an indicator of treatment efficiency. Ozone inactivation data generated in a pilot-scale study employing natural surface waters were comparable to inactivation data derived from previously published bench-scale studies using laboratory waters. The ozone requirements for inactivation of *Cryptosporidium* oocysts may produce elevated levels of bromate and ozone byproducts.

Introduction

Data that have shown *Cryptosporidium parvum* oocysts are virtually impervious to common drinking water disinfectants (1). The 1993 cryptosporidiosis outbreak in Milwaukee (2) has prompted many drinking water utilities currently chlorinating or

chloraminating their water supplies to consider using disinfectants with greater oxidation potential. Consequently, ozone has become a popular alternative to more conventional drinking water disinfectants. Researchers have generated a significant amount of data (3-7) to demonstrate the effect of ozone on various microorganisms in bench-scale batch reactors containing buffered laboratory waters. The following pilot-scale study was conducted to determine if the biocidal potential of ozone is comparable when it is used to disinfect natural surface water in a pilot-scale, continuous flow, countercurrent contactor.

Materials and Methods

Pilot-Scale Ozonation

This study was conducted at the U.S. Environmental Protection Agency's drinking water pilot plant in Cincinnati, Ohio (EPA). Either raw or filtered Ohio River water (ORW) was used. Filtered ORW was alum coagulated, clarified and sand filtered at the Cincinnati Water Works (CWW) before it was trucked to a 5000 gallon stainless steel storage tank at EPA. Raw ORW was collected at CWW's pump station, trucked to EPA, and transferred to the storage tank. Table I gives representative water quality data for filtered and raw ORW.

Table I. Ohio River Water Quality

Water	pH	Turbidity ntu	TOC mg/L	Alkalinity mg/L as $CaCO_3$
Filtered	a	0.49 ± 0.25 [b]	1.72 ± 0.42	59.0 ± 15.0
Raw [c]	7.10 ± 0.10	32 ± 6.0	2.10 ± 0.69	72.0 ± 14.8
Raw [d]	7.91 ± 0.36	10.5 ± 7.6	2.58 ± 0.50	62.5 ± 15.5

a see Table II
b before 1 um cartridge filtration; value reflects turbidity contributed by tanker truck
c TC and HPC tests
d endospore tests

The water was pumped to a single-stage, continuous flow, counter-current glass ozone contactor with a liquid depth of 2.65 meters and diameter of 0.15 meters. The mean operating conditions of the contactor were: 6.4 L/min liquid flow rate, 0.64 L/min gas flow rate, 0.1 gas-to-liquid ratio, with a transfer efficiency (TE) greater than 90 percent. Ozone was produced from oxygen by a 450 gram/day generator (model GL-1, PCI, Inc., W. Caldwell, NJ). Ozone concentrations applied to the contactor and in the off gas from the contactor were determined by UV absorbance (models HC and modified HC, respectively, PCI, Inc.). The amount of ozone transferred into water was determined by difference.

Simple CT products ($C_{avg} \times T_\theta$ = mg min/L) were used to quantify the intensity of each ozone test. The mean dissolved ozone concentration (C_{avg}) was derived from an ozone concentration profile based on measurements made at the influent, effluent, and four equally spaced (0.46m) intermediate ports using the indigo trisulfonate method (8). These empirical values were integrated over depth to obtain the mean dissolved ozone

concentration for the entire contactor. The mean theoretical contact time, T_{θ}, of the contactor was 7.4 minutes, based on flow rate of 6.4 L/min.

Preparation of Spiked Organisms

The RN66 strain of *C. muris* oocysts, originally obtained from M. Iseki (Osaka University Medical School, Japan), were propagated in female CF-1 mice (9). Each mouse was orally inoculated with approximately 2 x 10^5 oocysts in 200 uL phosphate buffered saline (PBS) and housed in suspension cages (10 mice/cage). Two weeks post-inoculation (PI), feces were collected and washed through a series of sieves (mesh sizes = 10, 20, 60, and 100) with 0.01% (v/v) Tween 20 (Tween). Approximately 150 mL of the sieved fecal slurry was underlaid with 75 mL 1.0 M sucrose and centrifuged at 1200 x g for 10 minutes at 4°C. Oocysts at the sucrose/Tween interface were isolated and washed two times. The oocyst prep was purified further by repeating this process using 0.85 M sucrose. Oocysts were stored in PBS with penicillin (100 units/mL) and streptomycin (100 ug/mL) at 4°C for up to 30 days.

C. parvum oocysts, originally obtained from Harley Moon (National Animal Disease Center, Ames, IA), were propagated at EPA in experimentally infected neonatal Holstein bull calves infected with approximately 2 x 10^8 oocysts in milk replacer (10). Fecal collections began two days PI and continued for two to three weeks. Feces were sieved as described above and concentrated via centrifugation at 2500 x g for 10 minutes at 4°C. Pellets were resuspended in a volume of Tween that was three to five times greater than the volume of the pellet itself; 10 mL aliquots of the suspension were dispensed into 50 mL conical tubes and underlaid with sequential discontinuous Sheather's sucrose gradients (11). Tubes were spun at 1200 x g for 10 minutes at 4°C. Oocyst isolates were extracted from the Sheather's gradient, transferred to a fresh 50 mL conical tube, resuspended in Tween, centrifuged at 2500 x g for 10 minutes, and washed twice. Additional purification was accomplished by repeating this process one or two more times. Purified oocysts were stored in 0.01 M PBS with penicillin (100 units/mL) and streptomycin (100 ug/mL) at 4°C for up to 60 days.

Giardia muris cysts, originally obtained from David Stevens (University Hospital, Cleveland, OH), were propagated in female CF-1 mice as described by Roberts-Thompson et al. (12). Each mouse was inoculated with approximately 1 x 10^3 cysts suspended in 200 uL distilled water. Cyst-laden mouse feces were collected 7 days PI and sieved as described above. Cysts were isolated from the fecal slurry via centrifugation over 1 M sucrose at 1200 x g for 10 min at 4°C. These crude isolates were purified further by unit gravity velocity sedimentation on a Percoll density gradient of 1.01 to 1.03 g/mL (13). Purified cyst suspensions were stored at 4°C in distilled water and used within 5 days of the initial isolation.

A vaccine strain of poliovirus 1 (PV1) was cultured in a continuous African green monkey kidney cell line, designated BGM, initially grown in Earle's basic salt solution as described by Dahling et al. (14), and resuspended in PBS.

Bacillus subtilis endospores were obtained from Raven Biological Laboratories (Omaha,

NE).

Spiking and Sampling Conditions for Spiked Microorganisms

Step-dose tracer studies were used to determine that the time required to reach steady-state conditions in the contactor (T_{100}) was 21.25 minutes. Operating parameters, including the microbial injection rate, were held constant for at least two times T_{100} before sampling occurred in each test.

Each microorganism spiked into the ozone contactor was evaluated in an independent series of tests. Approximately 5 x 10^8 PV1, *G. muris* cysts, *C. parvum* oocysts, *C. muris* oocysts, or *B. subtilis* endospores were suspended in 150 mL PBS with a magnetic stir bar/plate in a 200 mL Erlenmeyer flask packed in ice. A peristaltic pump was used to transfer the suspensions through Tygon® tubing to an injection point ahead of an in-line static mixer that preceded the contactor. A spiral wound, nominal 1.0 um, filter cartridge (Cuno, Inc., Meriden, CT) was installed ahead of the injection point during *Cryptosporidium* tests to reduce the amount of particulate matter that could have potentially interfered with the microscopic analysis of oocysts.

Samples were collected at the influent and effluent ports of the contactor in appropriately sized flasks (required volume determined by the expected level of inactivation) containing sodium thiosulfate (final concentration = 0.0 1%, w/v) and packed in ice. *G. muris* cysts were concentrated with 5.0 um polycarbonate membrane filters (Millipore Corp., Bedford, MA) (15). PV1 were concentrated with 0.2 um Virozorb-1MDS filters (Cuno, Inc.) (16). *Cryptosporidium* oocyst samples were collected in flasks containing Tween® 20 (final concentration = 0.01%, v/v) and reduced to approximately 2 mL via centrifugation at 2500 x g for 10 minutes at 4°C.

Viability Assays for Spiked and Indigenous Microorganisms

The biocidal potential of ozone was evaluated by comparing the viability of microbial populations collected at the influent and effluent ports of the ozone contactor. The viability of PV1 and bacteria was determined by comparing the number of plaque or colony forming units (PFU or CFU) in influent samples to the number of PFU or CFU in effluent samples. PV1 viability was determined by plaque assay in BGM cell cultures at passage levels 134 to 142 by the method described by Dahling and Wright (17).

Viable TC bacteria, cultured in P-A broth and Colilert® (Idexx Laboratories, Westbrook, ME), were enumerated using the most probable number procedure (8). Heterotrophic plate count bacteria were quantified by the pour plate procedure using plate count agar (PCA) incubated at 35°C for 48 hrs and by the spread plate method using R2A media incubated at 28°C for 7 days (8). Indigenous or *B. subtilis* endospores were analyzed as described by Rice et al. (18).

Excystation assays were used to assess the viability of *G. muris* cysts (19) and *C. muris* oocysts (20). Excysted *G. muris* suspensions were transferred to depression slides, sealed under cover slips with vaspar, incubated at 37°C for 30 minutes, and observed with a inverted phase-contrast microscope (Axiovert, Zeiss, Thornwood, NY) at 400X (total

magnification). Concentrated *C. muris* oocyst samples were mixed with an equal volume of RPMI 1640 (Sigma Chemical, St. Louis, MO) and incubated at 37°C for 1 hour. Aliquots of these samples were placed on a microscope slide, sealed under a cover slip with a clear fixative, and viewed with an upright phase-contrast microscope (Axiophot, Zeiss) at 400X (total magnification). A minimum of 200 bodies [excysted cysts/oocysts (EC), partially excysted cysts/oocysts (PEC) and intact excysted cysts/oocysts (IC)] were scored to calculate *G. muris* and *C. muris* excystation. Percent excystation (EX) was calculated for each sample with the following equation:

$$EX = [(EC + PEC) / (IC + EC + PEC)] \times 100 \quad [1]$$

The effect of ozone on cysts and oocysts was determined by comparing the percentage of excysted cysts or oocysts in influent samples to the percentage of excysted cysts or oocysts in effluent samples the following equation:

$$\text{log inactivation} = \log_{10} [Xi / Xe] \quad [2]$$

where X is the percentage of viable oocysts, *i* is influent sample, and *e* is effluent sample.

C. parvum oocyst viability was evaluated using a modified neonatal mouse infectivity assay (12). The oocyst titer of each concentrated influent and effluent sample was based upon the mean of three hemacytometer counts. This value was used to create a series of oocyst inocula administered to five sets of neonatal BALB/c mice (comprised of 1 or 2 litters of 5-7 neonates caged with a lactating dam). The number of oocysts per 10 uL dose was determined using the following equation:

$$\text{\# of oocysts/dose} = 10^{N-0.4,\ N-0.2,\ N,\ N+0.2,\ N+0.4} \quad [3]$$

where $N = \log_{10}X$ and X = the estimated number of treated or untreated oocysts required to infect 50% of the exposed neonates (ID_{50}). For example, if the estimated number of oocysts required to infect 50% of exposed neonatal mice was 100, then inocula containing 40, 63, 100, 158, or 251 oocysts would be administered to each neonate in a given set. Each set of neonates that was inoculated with $10^{N\pm0.2}$ or 10^{N} oocysts was comprised of two litters; $10^{N\pm0.4}$ oocysts were administered to only one litter. Neonates were sacrificed 8 days PI to assess the number of infected mice in each set. The small intestine of each neonate was excised, placed in separately labeled 15 mL centrifuge tubes containing 3.0 mL PBS, and homogenized with a tissue grinder for 30 seconds. Oocysts were partially separated from debris via centrifugation over a Sheather's sucrose discontinuous gradient as described above and labled with Crypt-a-Glo (Waterborne, Inc., New Orleans, LA), a direct fluorescent antibody kit containing a fluorescein isothiocyanate labeled monoclonal antibody, according to the manufacturer's directions. Samples were reduced to approximately 50 uL via centrifugation in an Eppendorf microcentrifuge (model 5415C, Brinkmann Instruments, Inc., Westbury, NY) at 15,000 x g for 6 minutes at room temperature. Aliquots (20 uL) from each sample were mounted on individual slides under cover slips, and scanned with a Zeiss Axiophot under filtered ultraviolet light (450-490 nm excitation filter, 510 nm dichroic beam splitter, and 515-520 nm barrier filter) at 400X (total magnification). Each slide was scored positive or negative for oocysts. A logit dose response model (21) was used to predict the ID_{50} for effluent (treated) and influent

(untreated) oocysts. The biocidal impact of ozone in each exposure was calculated using the following equation:

$$\text{log inactivation} = \log_{10} [n/n_0] \quad [4]$$

where n is the ID_{50} for oocysts treated with ozone and n_0 is the ID_{50} for untreated oocysts (mean ID_{50} for unexposed oocysts = 128 oocysts/mouse).

Results and Discussion

Inactivation of Microorganisms

The level of inactivation achieved by the CT range of each organism is reported in Table II. The effect of ozone on each microorganism has been described by comparing the ozone CT product, based on integrated dissolved ozone concentration values (C_{avg}) and theoretical residence time (T_θ), to percent survival in Figures 1 through 4.

Table II. Inactivation of Organisms in Ohio River Water by Pilot-Scale Ozonation

Organisms	n	Temp C	pH	CT mg min/L	Log Inactivation range
Endospores	20	23.5 ± 1.2	7.91 ± 0.36	0.44 - 20.6	0.41 - 2.46
B. subtilis endospores	19	22.7 ± 1.0	7.93 ± 0.32	0.70 - 18.35	0 - 2.17
C. parvum oocysts	6	24.5 ± 1.6	8.24 ± 0.20	2.55 - 7.15	0.57 - 2.67
C. muris oocysts	7	23.6 ± 1.6	8.40 ± 0.11	0.98 - 10.7	0.36 - 2.56
G. muris cysts	5	25.2 ± 1.1	7.57 ± 0.29	0.28 - 1.04	1.52 - 2.70
Poliovirus 1	9	25.0 ± 1.0	8.05 ± 0.17	0.19 - 2.49	1.43 - 3.85
HPC, PCA	6	15.5 ± 2.0	7.10 ± 0.10	0.28 - 6.26	0.74 - 2.16
HPC, R2A	6	15.5 ± 2.0	7.10 ± 0.10	0.28 - 6.26	2.10 - 3.36
TC, P-A broth	6	15.5 ± 2.0	7.10 ± 0.10	0.28 - 6.26	2.63 - 3.95
TC, Colilert®	6	15.5 ± 2.0	7.10 ± 0.10	0.28 - 6.26	2.29 - 4.10

PV1 and *Giardia* cysts were very susceptible to ozonation. Figure 1 indicates that 2-log inactivation of PV1 occurred at a CT value near 0.15 mg min/L, and 2-log inactivation of *G. muris* cysts required a CT near 0.5 mg min/L. The CTs required to reduce the viability of PV1 by 2 logs in ORW were comparable to results from a bench-scale studies in laboratory waters reported by Roy et al. (0.08 mg min/L at 20°C, pH 7.2) (22) and Evison (0.3 mg min/L at 25°C, pH 7.0) (23). *Giardia* cyst ORW inactivation data were within the range of bench-scale *G. muris* and *G. lamblia* cyst data reported by Finch et al. (2-log inactivation of *G. muris* and *G. lamblia* with 0.24 and 0.65 mg min/L, respectively at 22°C, pH 6.7, determined with animal infectivity) (24) and *G. lamblia* cyst data reported by Wickramanayake et al. (2-log inactivation with 0.25 mg min/L at 22°C, pH 7.0, determined with excystation) (25). The slopes of the inactivation plots for these two microorganisms suggested relatively slight increases in CT have a significant impact on their viability.

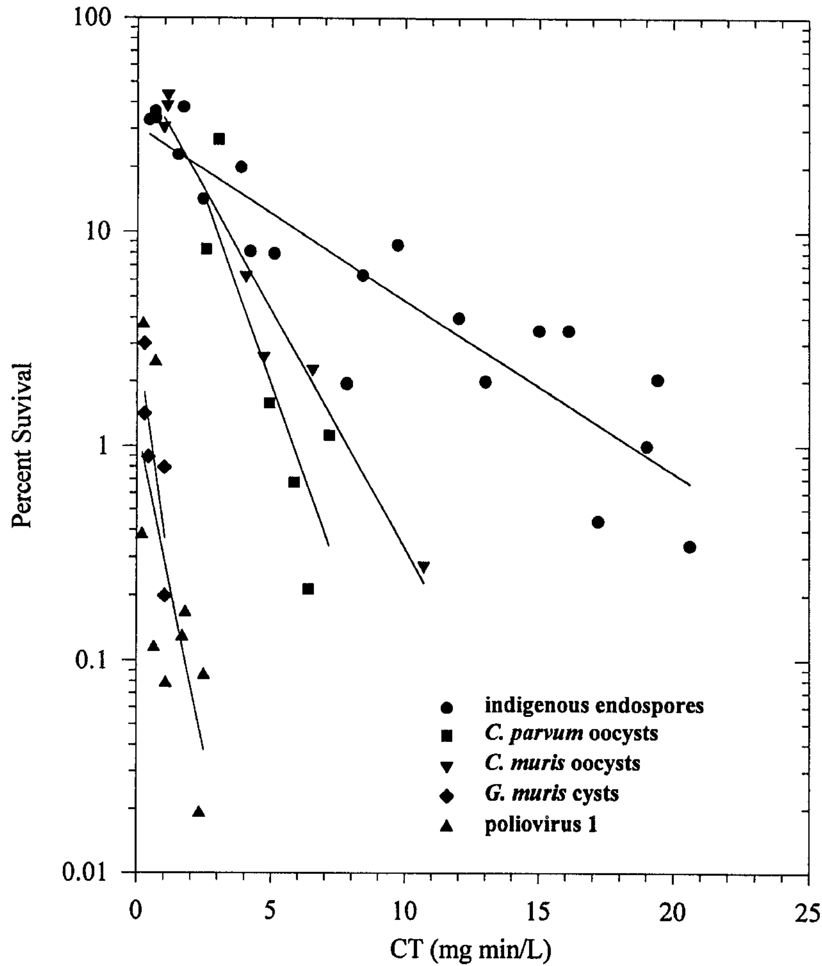

Figure 1. Comparison of inactivation of microbial populations exposed to ozone in filtered ORW. Temperature=23.6 to 25.2 C

C. parvum oocysts are more resistant to ozone inactivation than either PV1 or *G. muris* cysts. For 2-log inactivation of *C. parvum* oocysts, CT near 6 mg min/L was required, indicating that for the conditions in this study about 12 times higher CT was required for *Cryptosporidium* inactivation than for *Giardia* inactivation. While the range of empirical data did not allow for a similar comparison, the data suggest this ratio may be lower at 1-log inactivation.

Inactivation data for *C. parvum* oocysts exposed to ozone in ORW were comparable to bench-scale data in laboratory waters determined with animal infectivity reported by Korich et al. (2-log inactivation with 5 to 10 mg min/L at 25 °C, pH 7.0) (1), Finch et al.

(2-log inactivation with 3.5 mg min/L at 22°C, pH 6.9) (4), and Peeters et al. (2-log inactivation with 4.02 to 4.62 mg min/L at 25°C, pH 7.0) (6).

The effect of ozone on oocysts of both species of *Cryptosporidium* was similar. Data (Figure 1) indicate *C. muris* oocysts were slightly more resistant to ozone than *C. parvum* oocysts. Although the traditional excystation method used to assess *C. parvum* oocyst viability is known to underestimate the effect of ozone on *C. parvum* oocysts (4), results from this study suggest excystation could be used cautiously to determine the effect of ozone on *C. muris* oocysts. As such, *C. muris* oocysts assayed by excystation could be used to conservatively estimate ozone concentrations required to inactivate *C. parvum* oocysts without incurring the significant resources or health risks associated with the latter.

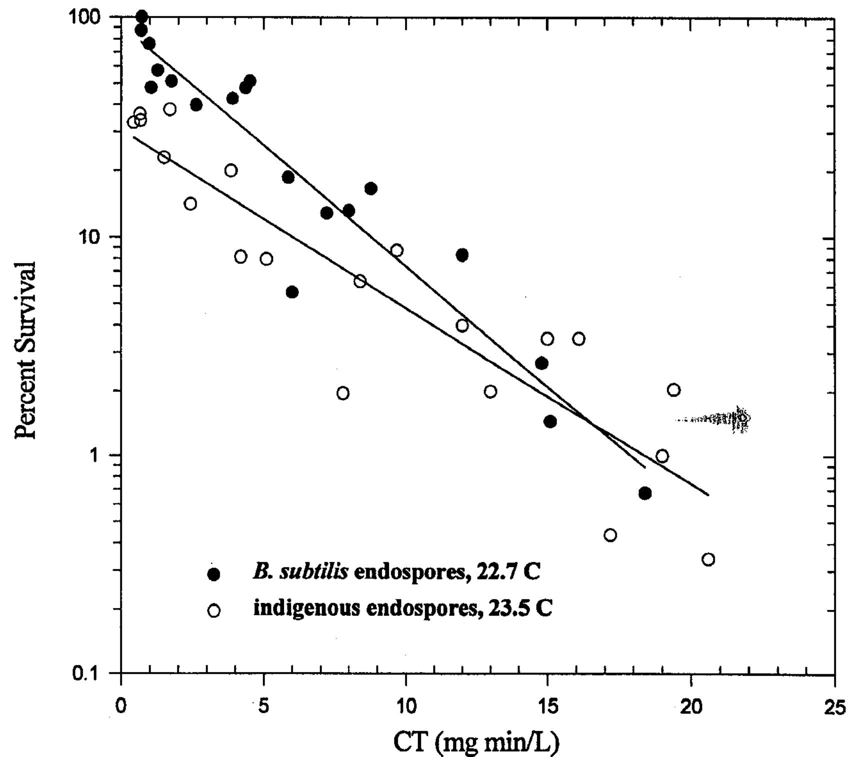

Figure 2. Ozone inactivation of indigenous and B. subtilis endospores in filtered ORW.

A method designed to evaluate the efficacy of primary drinking water treatment processes (18) with indigenous endospores of aerobic spore-forming bacteria was used to evaluate the biocidal potential of ozone. The method demonstrated that indigenous endospores were significantly more resistant to ozone than other microbial populations evaluated (Figure 1). CT required for 1- and 2-log inactivation of indigenous endospores was near 6

and 19 mg min/L, respectively. Coallier et al. (3), using a different method for endospore enumeration, evaluated ozonation of indigenous endospores in a full-scale plant treating surface water; the results for sampling in warmer months are similar to those reported here. These studies suggest indigenous endospores could be used by drinking water utilities as an indicator organism to monitor the efficacy of full-scale ozone contactors. The endospores are generally present in raw waters and clarified waters (18) influent to ozone contactors in plants treating the surface waters where *Cryptosporidium* oocysts and *Giardia* cysts are a concern, and may be quantitated with relative ease in less than 24 hours (18).

The effect of ozone on indigenous endospores was compared to its effect on *B. subtilis* endospores spiked into filtered ORW. Figure 2 shows *B. subtilis* endospores were more resistant to low ozone CTs than the indigenous population of endospores. Relatively low ozone CTs reduced the viable population of indigenous endospores by approximately 0.5 logs, while similar ozone CTs had little effect on *B. subtilis* endospores. This suggests some species among the indigenous population of endospores were considerably more susceptible to ozone than *B. subtilis* endospores. However, once the susceptible indigenous endospores were inactivated, ozone appeared to have a similar effect on both spiked *B. subtilis* and indigenous endopsores at higher CTs.

HPC bacteria appeared to have varying degrees of resistance to ozone. The apparent densities and resistance of naturally occurring organisms appeared to be related to the medium on which they were grown. Approximately one hundred times more CFU were detected in non-ozonated filtered ORW by R2A than by PCA. However, both media detected similar HPC concentrations in ORW following ozonation. Therefore, HPC bacteria detected by R2A appeared to be significantly less resistant to ozone than HPC detected by PCA (Figure 3). One possible explanation could be the presence of a relativlely large HPC subpopulation of fastidious bacteria that were very susceptible to ozone and only able to grow on R2A. In fact, ozone inactivation data generated for HPC bacteria on PCA in this study were similar to previously published R2A data (26). Regardless, results have shown R2A and PCA inactivation data are not necessarily interchangeable.

R2A and PCA were both monitored in raw and ozonated ORW in the first two months of a long-term, pilot-scale study (27, 28) involving the same ozone contactor used in the present study wherein gas and liquid flow rates, TEs, liquid depth, etc. were similar. Raw R2A densities ranged from 10^4 to 10^5 CFU/mL. Raw PCA densities ranged from 10^2 to 10^4 CFU/mL. At a transferred ozone/TOC dose near 0.8 mg/mg (CT near 1.5 mg min/L) and a mean temperature near 26°C, both R2A and PCA were inactivated by an average of 1.5 logs, unlike the difference between the two described in Figure 3. This variability in HPC densities and sensitivity should be considered before its inactivation data are used to assess the impact of ozone on other waterborne microorganisms.

The number of TC bacteria detected in both ozonated and non-ozonated ORW by P-A broth and by Colilert® were similar. Results shown in Figure 3 demonstrated TC bacteria were very susceptible to ozonation. This was consistent with previously published *E. coli* ozone inactivation data (29, 30). In the long-term, pilot-scale study (27, 28), TC were never detected in the ozone contactor effluent.

HPC and TC bacteria inactivation tests (Figure 3) were conducted at lower temperatures than other microorganisms (Figures 1 and 2). Therefore, inactivation data for HPC and TC bacteria should be adjusted for the effects of temperature on the efficacy of ozone inactivation when compared to the other microorganisms.

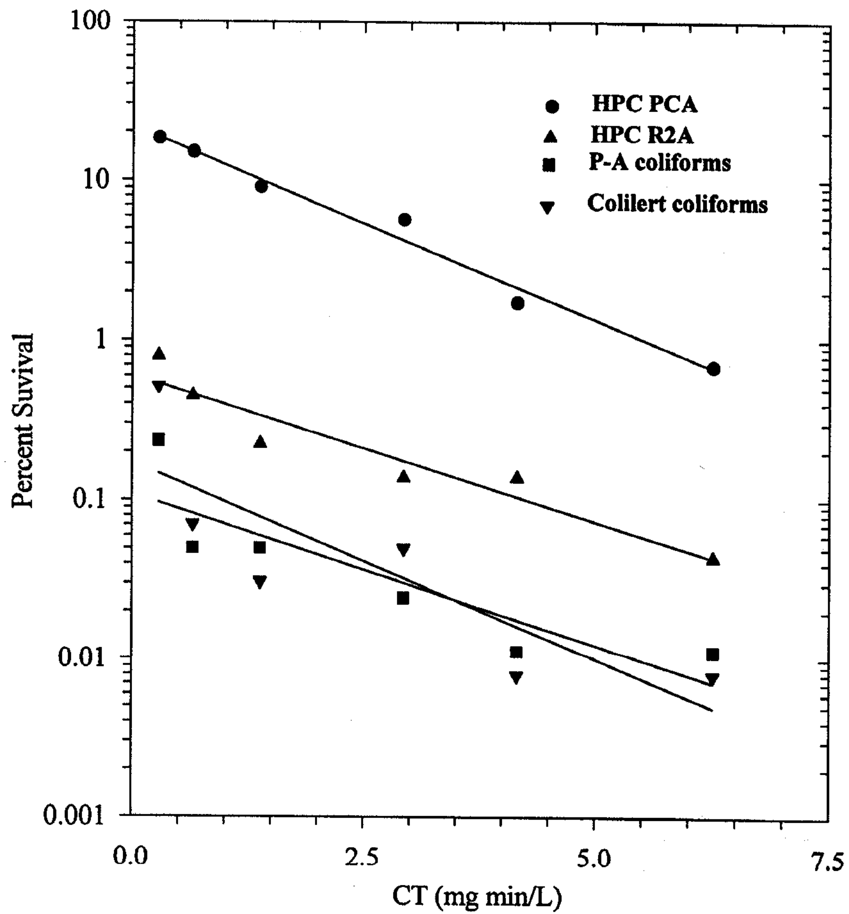

Figure 3. Comparison of inactivation of microbial populations exposed to ozone in raw ORW. Temperatures = 15.5 +/- 2 C.

Endospore inactivation tests were conducted at colder ORW temperatures to assess the effect temperature had on the biocidal potential of ozone (Figure 4). An increase in temperature from 9.4°C to 28.3°C reduced the ozone required for 1-log inactivation of indigenous endospores from 12.6 to 1.9 mg min/L.

CT values are often times determined differently in reported studies. For batch and semi-batch, bench-scale studies, C may be based on dissolved ozone measured over time, measured only at T = 0, assumed constant, or not well described. Inactivation has been based on excystation or on infectivity using different animal models. Consequently, comparing CT values and accompanying inactivation data from one study to another must be carried out cautiously. Because C is based on dissolved ozone and the ozone demand of a natural water does not enter into the calculation, results of these studies, involving a pilot-scale, counter-current contactor treating a natural water and operating at steady state with inactivation based on simple CT (C_{avg} x T_θ), were in generally good agreement with bench-scale, batch or semi-batch contactors treating buffered laboratory waters. Results from these studies suggest that bench-scale studies employing laboratory water can play a role in defining CT tables for ozone inactivation of *Cryptosporidium* oocysts and other regulated microorganisms.

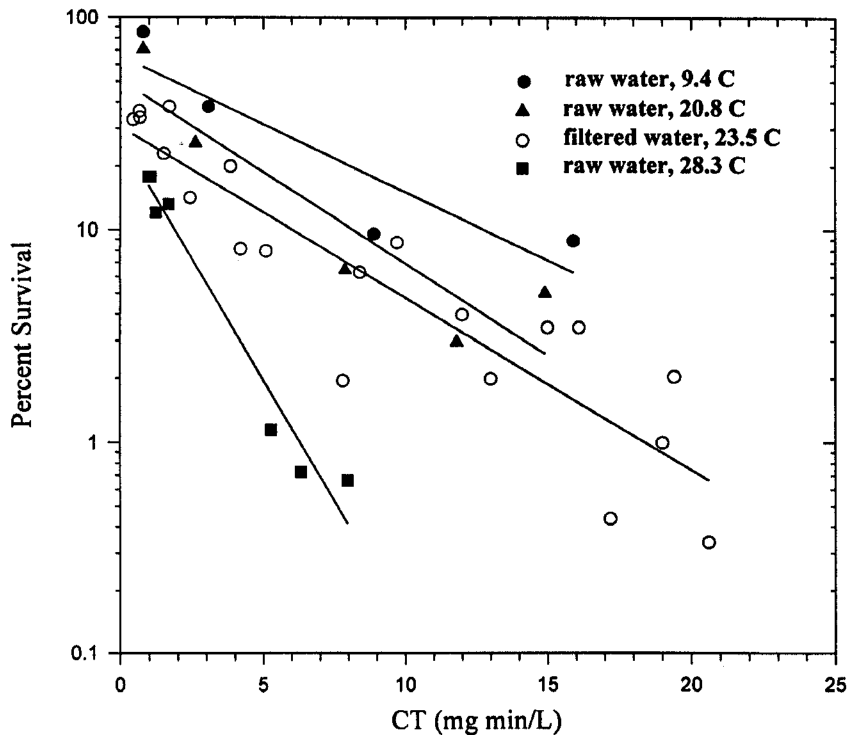

Figure 4. Effect of temperature on ozone inactivation of indigenous endospores in ORW.

Treatment Trade Offs

Ozonation offers many advantages in drinking water treatment in addition to inactivation (taste and odor (T&O) control, oxidation of disinfection byproduct precursors, iron and manganese control, etc.). But employing ozonation to achieve the CT required to control

Cryptosporidium oocysts may require utilities to operate at higher ozone doses than they would otherwise. Higher ozone doses may present trade offs in ozonated water quality. The formation of aldehydes, keto acids, carboxylic acids, assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC) are examples. Bromate will be regulated at a maximum contaminant level (MCL) of 0.01 mg/L under the Disinfectant/Disinfection By-Product (D/DBP) Rule. In the D/DBP Rule, the EPA has expressed concern over the control of AOC and BDOC and the role they play in potential bacterial regrowth in distribution systems, and has discussed the importance of biologically active filtration downstream of ozonation for their control.

In these studies with *Cryptosporidium*, a CT of 6 mg min/L, which provided 2-log inactivation of the oocysts, was equivalent to a transferred ozone-to-TOC ratio of near 1.8 mg/mg. The transferred ozone-to-TOC is ratio is generally below 1 mg/mg for purposes of DBP precursor oxidation, T&O control, etc. Thus, for purposes of *Cryptosporidium* oocyst control, higher ozone doses and higher dissolved ozone residuals may be employed. As bromate, AOC and BDOC concentrations are, in part, dependent on ozone concentrations, some utilities may face trade-offs in control of these ozone by-products and control of microbial contamination.

The ozone contactor employed in this inactivation study was used in other studies (31, 32) treating ORW. The gas and liquid flow rates, TEs, liquid depth, etc. were similar; the gas-phase ozone concentration was varied. Tables III and IV show CT increasing with increasing transferred ozone-to-TOC ratio. Because the ozone demand of each ORW is different and because CT is based on the dissolved ozone concentration, the relationship between this ratio and CT will be different for each ORW. These data indicate that at CTs near 3.5 mg min/L where 1-log inactivation of *Cryptosporidium* oocysts would occur, the formation of bromate approached 10 ug/L, the bromate MCL. At 6 mg min/L where 2-log inactivation of *Cryprosporidium* oocysts would occur, the formation of bromate exceeded the MCL. Similarly, Table IV shows that at 6 mg min/L, the formation of AOC had approximately tripled and the formation of BDOC more than quadrupled. Thus, a water utility using ozone to inactivate *Cryptosporidium* oocysts may be faced with MCL compliance for bromate, and may be faced with problematic levels of AOC and BDOC that suggest elevated levels of distribution system nutrients.

Table III. Bromate Formation [a] in Ohio River Water [b]
(from reference 31)

Transferred O_3/DOC mg/mg	CT mg min/L	Bromate ug/L
0	0	<0.3
0.53	0.96	1.1
0.81	2.15	4.1
1.11	3.85	10.5
1.78	7.18	24.1
2.54	10.9	40.7

a pH = 7.65, temperature = 23 - 24 C
b DOC = 1.68 mg/L, bromide = 50.7 ug/L

Table IV. Ozone Byproduct Formation [a] in Ohio River Water [b]
(from reference 32)

Transferred O_3/TOC mg/mg	CT mg min/L	Bromate ug/L	Total AOC [c] ug Ceq/L	BDOC mg/L
0	0	<5	199	<0.1
0.20	0.10	<5	324	<0.1
0.44	0.43	<5	423	0.14
0.76	1.56	<5	435	0.18
1.39	3.61	11.9	573	0.18
2.10	4.76	21.1	606	0.30
2.81	7.09	34.7		0.42

a pH = 7.8 - 8.1, temperature = 22.5 - 23.5 C
b TOC = 1.42 mg/L, bromide = 35.7 ug/L
c P17 as acetate and NOX as oxalate

Levels of AOC and BDOC can be lowered during biological filtration wherein the nutrients are utilized by the heterotrophic bacteria established on the filter media. In the long-term, pilot-scale study (27, 28) involving the same ozone contactor used in the present study (gas and liquid flow rates, TEs, liquid depth, etc. were similar) multiple parallel biological filters were evaluated. A biological activated carbon (BAC) filter with nondisinfected backwash was the most efficient. Table V shows 51 and 67 percent removal of AOC-NOX and BDOC, respectively, by the BAC filter. Because the TOC was relatively low and the ozone application was not targeted at conditions that would control *Cryptosporidium* oocysts, the BAC effluent AOC-NOX and BDOC levels were relatively low.

In another long-term, pilot-scale study (33) involving the same ozone contactor used in the present study (gas flow rates, TEs, liquid depth, etc. were similar, but liquid flow rate was lower thereby increasing CT) multiple parallel biological filters were evaluated. In this study, however, the TOC of the ozonated water was higher and ozone application that would provide near 2-log inactivation of *Cryptosporidium* oocysts was targeted. As a result, the formation of AOC and BDOC was significant. See Table VI. A BAC filter with nondisinfected backwash was again the most efficient. Even though the BAC filter provided 75 and 80 percent removal of AOC and BDOC, respectively, the BAC effluent levels were relatively high. Thus, some water utilities using ozone to inactivate *Cryptosporidium* oocysts may encounter problematic levels of AOC and BDOC, even after biological filtration.

Conclusions

The comparative inactivation of several microorganisms in natural water by ozone was evaluated in a pilot-scale contactor operating at steady state. The only organisms that were found to be more resistant to ozone than *Cryptosporidium* oocysts were endospores of aerobic spore-forming bacteria. Indigenous endospores, relatively simple to assay, may

serve as a good indicator of overall treatment effectiveness, including the control of *Cryptosporidium* oocysts with ozone. *Cryptosporidium* oocysts were more resistant than *Giardia* cysts and poliovirus. Results indicated *C. muris* oocysts assayed via excystation could be used to conservatively predict CT requirements for *C. parvum* oocyst inactivation. HPC bacteria appeared to be less resistant to ozone than *Cryptosporidium* oocysts. HPC results were variable and appeared to depend on the media on which they were grown, indicating caution should be exercised when HPC are used as an indicator of treatment effectiveness. Ozone inactivation data generated at the pilot-scale employing natural surface water were comparable to inactivation data derived from previously published bench-scale studies employing laboratory waters. At the ozone requirements for inactivation of *Cryptosporidium* oocysts, a trade off may exist between control of the oocysts and the production of elevated levels of regulated bromate and elevated levels of the ozone byproducts associated with possible distribution system regrowth of heterotrophic bacteria.

Table V. Mean Ozone [a] Byproduct Formation and Control in Ohio River Water
(from references 27 and 28)

Ozone Byproducts	Ozone Influent	Ozone Effluent	Settled BAC Influent	BAC [b] Effluent
AOC-NOX, [c] ug Ceq/L	129	594	321	157
BDOC, mg/L	0.39	0.71	0.36	0.12
Total aldehydes, [d] ug/L	3.2	33.4	29.6	3.1
Total keto acids, [e] ug/L	0.6	47.0	27.1	0.3

a ozone applied to raw water with TOC = 2.02 mg/L
a 0.8 mg/mg transferred ozone/TOC, 1.5 mg min/L CT
b Filtrasorb 400 at steady state, temperature = 17.8 - 27.3 C
c as oxalate
d formaldehyde, acetaldehyde, glyoxal and methyl glyoxal
e glyoxalic and pyruvic acids

Table VI. Mean Ozone [a] Byproduct Formation and Control in Harsha Lake Water
(from reference 33)

Ozone Byproducts	Ozone Influent	Ozone Effluent BAC Influent	BAC [b] Effluent
Total AOC, [c] ug Ceq/L	203	1314	329
BDOC, mg/L	0.53	1.21	0.24
Total aldehydes, [d] ug/L	8.0	40.6	4.0
Total keto acids, [e] ug/L	0.9	61.7	2.8

a ozone applied to coagulated water with TOC = 3.21 mg/L
a 0.9 mg/mg transferred ozone/TOC, CT = 7 mg min/L
b Filtrasorb 400 at steady state, temperature = 17.8 - 22.2 C
c P17 as acetate and NOX as oxalate
d formaldehyde, glyoxal and methyl glyoxal
e glyoxalic and pyruvic acids

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Keywords

Ozone; *Cryptosporidium*; *Giardia*; Endospores; *Bacillus Subtilis*; Poliovirus; Heterotrophic Bacteria; Coliform Bacteria; Bromate; Biological Activated Carbon Filtration; Aldehydes; Keto Acids; Ohio River Water