# Inactivation of *Cryptosporidium parvum* Oocysts and *Clostridium perfringens* Spores by a Mixed-Oxidant Disinfectant and by Free Chlorine

LINDA V. VENCZEL,[1]* MICHAEL ARROWOOD,[2] MARGARET HURD,[2] AND MARK D. SOBSEY[1]

*University of North Carolina, Chapel Hill, North Carolina 27599,[1] and Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30341[2]*

*Cryptosporidium parvum* **oocysts and** *Clostridium perfringens* **spores are very resistant to chlorine and other drinking-water disinfectants.** *Clostridium perfringens* **spores have been suggested as a surrogate indicator of disinfectant activity against** *Cryptosporidium parvum* **and other hardy pathogens in water. In this study, an alternative disinfection system consisting of an electrochemically produced mixed-oxidant solution (MIOX; LATA Inc.) was evaluated for inactivation of both** *Cryptosporidium parvum* **oocysts and** *Clostridium perfringens* **spores. The disinfection efficacy of the mixed-oxidant solution was compared to that of free chlorine on the basis of equal weight per volume concentrations of total oxidants. Batch inactivation experiments were done on purified oocysts and spores in buffered, oxidant demand-free water at pH 7 and 25°C by using a disinfectant dose of 5 mg/liter and contact times of up to 24 h. The mixed-oxidant solution was considerably more effective than free chlorine in inactivating both microorganisms. A 5-mg/liter dose of mixed oxidants produced a $>3$-$\log_{10}$-unit ($>99.9\%$) inactivation of** *Cryptosporidium parvum* **oocysts and** *Clostridium perfringens* **spores in 4 h. Free chlorine produced no measurable inactivation of** *Cryptosporidium parvum* **oocysts by 4 or 24 h, although** *Clostridium perfringens* **spores were inactivated by 1.4 $\log_{10}$ units after 4 h. The on-site generation of mixed oxidants may be a practical and cost-effective system of drinking water disinfection protecting against even the most resistant pathogens, including** *Cryptosporidium* **oocysts.**

**Introduction and background.** Conventional water treatment practices including chlorination are sometimes inadequate for effective control of *Cryptosporidium parvum* in water. A multiple-barrier approach, which includes source water protection, coagulation-flocculation, filtration, and disinfection, is recommended for drinking water derived from surface sources and is the basis of the Surface Water Treatment Rule (8, 16). A revision of this rule now in progress, the Enhanced Surface Water Treatment Rule, would establish source water quality and treatment requirements for *Cryptosporidium parvum*. However, the choice of treatment options for effective control of waterborne *Cryptosporidium* has not been adequately established.

Chlorine disinfection has been a key barrier in the prevention of waterborne diseases, but *Cryptosporidium parvum* is very resistant to chlorine and other commonly used drinking water disinfectants (10, 11). Concerns about the efficacy of chlorine against such resistant pathogens as *Cryptosporidium*, as well as its safety, cost, and potentially adverse health effects, have led to the consideration of alternative disinfectants and disinfection strategies, such as sequential treatment with two or more disinfectants (7).

Ideally, there should be a reliable surrogate or indicator organism for *Cryptosporidium* that could be routinely and reliably measured in water in order to determine *Cryptosporidium* presence and its response to disinfection and other treatment processes. Due to their observed resistance to chlorine and adverse environmental conditions, *Clostridium perfringens* spores have been suggested as a possible surrogate indicator of disinfectant activity against *Cryptosporidium* and other highly resistant pathogens. A membrane filtration method employing a selective medium, mCP, and simple anaerobic incubation has made possible the routine analysis of water for *Clostridium perfringens*. Payment and colleagues (13) evaluated the use of *Clostridium perfringens* and coliphages as indicators of treatment efficiency and found them to correlate with *Cryptosporidium*, *Giardia lamblia*, and human enteric viruses (12, 13).

As an alternative to free chlorine, ozone has been demonstrated to be an effective disinfectant against *Cryptosporidium* oocysts (6). However, the required ozone doses are relatively high, and ozone has the disadvantage of providing no residual protection in the distribution system. Recently, Finch et al. (7) demonstrated that the sequential use of either ozone or free chlorine followed by monochloramine was capable of inactivating *Cryptosporidium* oocysts. These results suggest that combinations of chemical disinfectants provide improved disinfection of *Cryptosporidium* oocysts through synergistic activity that may involve chemical action at different sites on or in the oocyst.

An alternative approach to the sequential use of multiple disinfectants for inactivation of *Cryptosporidium* oocysts is the use of disinfectant mixtures. Drinking water disinfection by the on-site production of a mixture of oxidants generated by the electrolysis of a solution of sodium chloride is such an alternative approach. Electrolysis converts the brine solution to a mixture of oxidants (free chlorine, chlorine dioxide, hydrogen peroxide, ozone, and other, short-lived oxidants). This technology has been developed over several decades, yet the chemical components of this mixture, as well as the efficacy of pathogen reduction, have not been adequately investigated. The objective of this study was to compare the inactivation kinetics of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores exposed at 25°C to a 5-mg total oxidant dose

---

* Corresponding author. Phone: (919) 966-7317. Fax: (919) 966-4711.

of mixed-oxidant solution or sodium hypochlorite per liter of pH 7, oxidant-demand-free (ODF) phosphate-buffered water.

**Disinfection experiments.** Experimental ODF water was prepared by previously described methods (15). A stock solution of mixed oxidants was produced by using a MIOX disinfection unit (6-V brine pump system) under the conditions specified in the manufacturer's technical guide (14). The mixed-oxidant solution was immediately captured from the polypropylene tubing containing the anolyte in demand-free vessels and analyzed prior to and during the experiments by the following standard methods (1): total oxidants, *N,N*-diethyl-*p*-phenylenediamine colorimetric method; chlorine dioxide, *N,N*-diethyl-*p*-phenylenediamine colorimetric method with the addition of glycine; ozone, indigo trisulfonate decolorization with addition of malonic acid. Sodium hypochlorite was prepared by dilution of commercial household bleach (5.25% free chlorine).

Field strains of *Clostridium perfringens* were isolated from raw sewage (Orange County Water and Sewage Authority, Chapel Hill, N.C.) by heating to 70°C for 20 min, serially diluting, and streaking onto mCP agar plates (AcuMedia, Inc., Baltimore, Md.). Presumptive *Clostridium perfringens* colonies were confirmed by using An-INDENT anaerobic test strips (Sherwood Medical, St. Louis, Mo.). Several confirmed *Clostridium perfringens* isolates were pooled and inoculated into Duncan-Strong sporulation medium (5) and incubated anaerobically overnight at 42°C to produce spore stocks. Gram staining and light microscopy were performed on dilutions of these suspensions to determine the amount of spore aggregation as well as the proportion of vegetative bacteria versus spores. Spore preparations used in disinfection experiments were free of visible aggregates and contained at least 95% spores. For disinfection experiments, stock spore suspensions were added to a final concentration of $10^4$ spores/ml in test disinfectant solutions. Control and disinfected samples were serially diluted for analyses by membrane filtration using mCP agar. Concentrations of *Clostridium perfringens* in disinfected and control samples were expressed as CFU per ml.

Fresh *Cryptosporidium* oocysts (Iowa strain) were recovered as fecal samples from experimentally infected Holstein calves. Oocysts were purified by filtration and then centrifugation in sucrose and isopycnic Percoll solutions (2). To reduce oxidant demand, the oocysts were further washed in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and then resuspended in 1,000 µl of ODF phosphate-buffered water. The concentration of oocysts in the suspension was determined microscopically in a hemocytometer.

Samples of either disinfected or control oocysts were supplemented with 200 µl of PBS-BSA, mixed, and centrifuged at 16,000 × *g*. The oocyst pellets were resuspended in 500 µl of PBS-BSA and centrifuged as before, and the oocyst pellets were resuspended in 250 µl of PBS-BSA. Resuspended oocyst suspensions were diluted, and 25-µl inoculum volumes were delivered to 4- to 6-day-old neonatal BALB/c mice by gastric intubation. Six days postinoculation, mouse terminal colon samples (~1 cm) were collected into 400 µl of $K_2Cr_2O_7$ at necropsy and homogenized with disposable wooden applicators. The colon suspensions were assayed by immunofluorescent flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) as follows. Colon homogenates were vortexed, and 200-µl supernatant aliquots were transferred to discontinuous sucrose microgradients and centrifuged at 1,500 × *g* for 25 min. Oocyst-containing gradient fractions were collected, washed by centrifugation at 16,000 × *g*, and suspended in PBS with 0.1% BSA. Samples were incubated with *Cryptosporidium parvum* monoclonal antibodies

TABLE 1. Inactivation of *Cryptosporidium parvum* oocysts by a 5-mg/liter dose of mixed oxidants in buffer at pH 7 at 25°C

| Time (h) | Log₁₀ reduction of oocyst infectivity | | | | |
|---|---|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 | Expt 4 | Avg |
| 1 | 1.3 | No data | No data | No data | 1.3 |
| 4 | 2.3 | >3.6 | >3.6 | >4.6 | >3.5 |
| 8 | No data | >4.6 | >3.6 | >4.6 | >4.3 |
| 12 | No data | >4.6 | >4.6 | >4.6 | >4.6 |
| 24 | >3.6 | >4.1 | No data | No data | >3.8 |

conjugated with fluorescein isothiocyanate (OW50-FITC) for 30 min, diluted with PBS, and analyzed with the flow cytometer using logical gating of light scattering properties and fluorescence. Samples were categorized as positive or negative and evaluated by chi-square analyses.

The procedures for disinfection experiments were modified from those of Sobsey et al. (15). Parallel experiments were performed at 25°C with a mixed-oxidant solution or sodium hypochlorite at a target concentration of 5 mg/liter in 0.01 M phosphate-buffered ODF water, pH 7. *Clostridium perfringens* spores and *Cryptosporidium* oocysts were placed in separate Teflon reactor tubes at the target concentration, and the disinfectants were added at the same moment with continuous mixing. Samples were withdrawn from the reactors at 1, 4, 8, 12, and 24 h and quenched of residual disinfectant by the addition of an equal volume of 1%, wt/vol, sodium thiosulfate. Control samples included vessels with test organisms in buffered water minus the disinfectant (to measure microbial losses attributable to factors other than the disinfectant) and vessels with disinfectant in buffered water minus the microbes (to measure initial disinfectant levels and their stability throughout the experiment).

*Clostridium perfringens* concentrations in disinfected samples were determined for each time point, including zero time; $N_0$ was the mean of the *Clostridium perfringens* concentrations of the disinfectant-free control sample measured at zero time and at the end of the experiment. For each time point, the surviving fraction of *Clostridium perfringens* was calculated, $N_t/N_0$. These values were then $\log_{10}$ transformed, and mean $\log_{10} N_t/N_0$ values were calculated from the data of replicate experiments. Estimated times for 99, 99.9, and 99.99% (2-, 3-, and 4-$\log_{10}$-unit) inactivation were computed by linear regression on the $\log_{10}$ survival data of each experiment. Concentrations of infectious *Cryptosporidium* oocysts were calculated from the data on proportions of infected animals (number infected/number inoculated) at each dose by standard quantal methods. From these data, $\log_{10} N_t/N_0$ values at each contact time were calculated for disinfected samples, as they were for *Clostridium perfringens* spores.

**Inactivation kinetics.** Inactivation kinetics of *Cryptosporidium* oocysts exposed to a 5-mg/liter dose of mixed oxidants in 0.01 M phosphate buffer at pH 7 at 25°C are summarized in Table 1 as a function of contact time for four replicate experiments. These results indicate that a 5-mg/liter dose of mixed oxidants produced substantial inactivation of *Cryptosporidium* oocysts. Reductions of *Cryptosporidium* oocyst infectivity after 4 h of exposure were about 2.3 $\log_{10}$ units in one experiment and >3.6 $\log_{10}$ units in three other experiments.

For three replicate experiments on the inactivation of *Cryptosporidium* oocysts by a 5-mg/liter dose of free chlorine in 0.01 M phosphate buffer at pH 7 at 25°C, there was essentially no reduction of oocyst infectivity after 24 h of exposure (data not shown). These results are consistent with those of previous

TABLE 2. Inactivation of *Cryptosporidium parvum* spores by
a 5-mg/liter doses of mixed oxidants or free
chlorine in buffer at pH 7 at 25°C

| Time (h) | Log$_{10}$ reduction of spore infectivity[a] | |
|---|---|---|
| | Mixed oxidants | Free chlorine |
| 1 | 2.2 | 1.0 |
| 3 | 2.6 | 1.3 |
| 4 | 2.7 | 1.5 |
| 8 | 3.3 | 1.6 |
| 12 | 3.6 | 1.7 |
| 24 | 3.7 | 1.7 |

[a] Mean of four replicate experiments.

studies which indicate essentially no inactivation of *Cryptosporidium* oocysts by free chlorine (7, 9).

Inactivation of *Clostridium perfringens* spores by a 5-mg/liter dose of mixed oxidants or free chlorine is summarized in Table 2. The inactivation of *Clostridium perfringens* spores by mixed oxidants exhibited declining rates with increasing contact time or so-called "retardant die-off" kinetics. These retardant die-off kinetics are not accounted for by major losses in mixed-oxidant concentration, because the average concentration throughout the experiment was 3.8 mg/liter. *Clostridium perfringens* spores exposed to a 5-mg/liter dose of free chlorine exhibited much slower inactivation rates than those exposed to mixed oxidants. *Clostridium perfringens* spores also exhibited retardant die-off kinetics for free-chlorine inactivation, despite minimal loss of disinfectant residual over the 24-h contact time (mean free-chlorine concentration, 4.15 mg/liter).

Within the limits of the precision and accuracy of the infectivity assay methods, especially for *Cryptosporidium* oocyst infectivity, the mixed-oxidant solution produced similar inactivation of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores. For inactivation by free chlorine, *Clostridium perfringens* spores appear to be somewhat more susceptible than *Cryptosporidium* oocysts. After 24 h of contact, a 5-mg/liter dose of free chlorine produced an approximately 1.7-log$_{10}$-unit reduction of *Clostridium perfringens* spores, but there was essentially no reduction of *Cryptosporidium* oocyst infectivity.

An electrochemically produced solution of mixed oxidants at a dose of 5 mg/liter inactivated both *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores in pH 7.0 buffered water at 25°C, with >2.3-log$_{10}$-unit (>99.5%) inactivation in 4 h. The same organisms exposed to a 5-mg/liter dose of free chlorine for the same period were inactivated less extensively, with only about 1.5-log$_{10}$-unit (97%) inactivation of *Clostridium perfringens* spores and essentially no inactivation of *Cryptosporidium* oocysts at 4 h. Korich et al. (9) reported that *Cryptosporidium* oocysts exposed to a free-chlorine dose of 80 mg/liter were inactivated by 1 log$_{10}$ unit in 90 min; such a high concentration of chlorine would never be used in the disinfection of drinking water. In this study we applied a relatively high but allowable dose of 5 mg of either free chlorine or mixed-oxidant solution per liter.

**Applications.** There are several advantages in using the MIOX system over other disinfectants. It is generated on site and therefore can be installed in small water treatment utilities, remote communities, and emergency and disaster areas. The equipment requires very little time in terms of operation and maintenance. Once the salt solution is prepared, and the initial amperage and flow rate are confirmed, the MIOX unit can run without further supervision. Furthermore, the raw

materials, common salt and water, are found virtually everywhere. The main stimulus for performing these experiments was to determine the ability of a mixed-oxidant solution to inactivate parasites such as *Cryptosporidium parvum* and other waterborne pathogens in stored household drinking water in developing countries. The MIOX unit was field tested in 1995 in a periurban area of Bolivia. With minimal training, community members successfully operated and maintained the equipment over a 6-month period and continue to use the unit today. Experience from pilot field studies indicated that when a 5-mg/liter dose of a mixed-oxidant solution was applied to untreated surface and ground waters, it was beneficial to apply the disinfectant and leave the water undisturbed overnight in order to reduce any taste or odor imbued by disinfection. This approach not only overcame people's rejection of the water due to their perception of a change in taste but also provided an adequate contact time for microbial inactivation by the disinfectant. MIOX units have also been installed in Boston Bar, British Columbia, Canada, in a small-community drinking water system serving Native Americans, in the Sandia ranger station of the U.S. Forest Service, and in the city of Albuquerque sewer lines to control odor.

The generally similar inactivation kinetics of *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores by mixed oxidants indicates the potential utility of *Clostridium perfringens* spores as an indicator of disinfection efficacy for this highly resistant waterborne parasite. In order to fully establish the reliability of *Clostridium perfringens* spores as indicators of *Cryptosporidium* oocyst inactivation by mixed oxidants, additional studies with different mixed-oxidant doses are needed under a variety of water quality conditions.

The in situ generation of mixed oxidants may provide a reliable and inexpensive method of drinking water disinfection that is applicable under a variety of circumstances. The raw materials for mixed-oxidant production, salt (sodium chloride) and water, are widely available in nearly all parts of the world. Because the mixed-oxidant generator can operate on solar power or other alternative energy sources, the disinfectant can be produced in remote locations lacking electricity. The cost per kilogram of total oxidants produced varies with the size of the unit. The smaller MIOX units are less efficient at converting the sodium chloride solution to disinfectant. Calculations were made for the cost per cubic meter of water treated by the mixed-oxidant solution, which included cost estimates for salt, electricity, water, labor, amortization of equipment, and floor space for equipment. The total amount estimated in U.S. $ was 0.018 per cubic meter, with most of the cost due to labor. This cost is comparable to that of chlorine gas and more affordable than that of calcium hypochlorite. When one factors in the costs (and danger) of transporting disinfectants such as chlorine gas, the benefits of on-site generation are even greater. Generation of a mixed-oxidant gas renders an even more potent disinfectant, with higher conversion efficiency of the salt water to disinfectant. Research is under way to evaluate the safety and efficacy of the gas unit under various water quality conditions.

In other studies we have found that the mixed oxidants also are very effective in inactivating enteric viruses, such as coliphage MS2, and other bacteria, such *Vibrio cholerae* and *Escherichia coli* (17). Also, the chemical composition of the mixed-oxidant solution is being investigated, as well as the by-products formed by the reaction of the mixed oxidants with constituents of natural water. Dowd (4) found that the mixed-oxidant solution produced essentially the same levels of trihalomethanes in model and real waters tested as free chlorine. Other studies conducted by Bradford (3) indicated a reduction

of more than 50% in total trihalomethane production for mixed oxidants versus free chlorine. Further research is needed to clarify these discrepancies. Based on the results of this study, we conclude that the mixed-oxidant system is an effective drinking water disinfectant at practical and economical doses.

### REFERENCES

1. **American Public Health Association.** 1995. Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association, Washington, D.C.
2. **Arrowood, M. J., and C. R. Sterling.** 1990. Isolation of *Cryptosporidium* oocysts and sporozoites during discontinuous sucrose and isopycnic Percoll gradients. J. Parasitol. **73:**314–319.
3. **Bradford, W. L.** 1993. Design, fabrication and testing of a laboratory test electrolytic water disinfection unit (LTEWDU). Los Alamos technical report LATA/MX-93/0003. Los Alamos Technical Associates, Inc., Los Alamos, N.Mex.
4. **Dowd, M.** 1994. Assessment of THM formation with MIOX. Master's thesis. University of North Carolina, Chapel Hill.
5. **Duncan, C., and D. Strong.** 1967. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16:**82–90.
6. **Finch, G. R., E. K. Black, L. Gyurek, and M. Belosevic.** 1993. Ozone inactivation of *Cryptosporidium parvum* in demand-free phosphate buffer determined by in vitro excystation and animal infectivity. Appl. Environ. Microbiol. **59:**4203–4210.
7. **Finch, G. R., E. K. Black, and L. L. Gyurek.** 1995. Ozone and chlorine inactivation of *Cryptosporidium*, p. 1303–1318. *In* Proceedings of the 1994 Water Quality Technology Conference. American Water Works Association, Denver, Colo.
8. **Geldreich, E. E.** 1987. Drinking water microbiology—new directions toward water quality enhancement. *In* Proceedings of the International Food and Water Microbiology Conference, Halkidiki, Greece, 5 to 9 October 1987.
9. **Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:**1423–1428.
10. **Lisle, J. T., and J. B. Rose.** 1995. *Cryptosporidium* contamination of water in the USA and UK: a mini-review. Aqua **44**(3)**:**103–117.
11. **MacKenzie, W., N. M. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. R. Rose, and J. P. Davis.** 1995. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:**161–167.
12. **Payment, P., and E. Franco.** 1993. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl. Environ. Microbiol. **59:**2418–2424.
13. **Payment, P., M. Trudel, and R. Plante.** 1985. Elimination of viruses and indicator bacteria at each step of treatment during preparation of drinking water at seven water treatment plants. Appl. Environ. Microbiol. **49:**1418–1428.
14. **Robson, M. (ed.).** 1984. MIOX operations user's guide. MIOX Corp., Albuquerque, N.Mex.
15. **Sobsey, M. D., T. Fuji, and P. A. Shields.** 1989. Inactivation of hepatitis A virus and model viruses in water by free chlorine and monochloramine. Water Sci. Technol. **20**(11/12)**:**385–391.
16. **U.S. Environmental Protection Agency.** 1989. National primary drinking water regulations. Filtration and disinfection: turbidity, *Giardia lamblia*, viruses, *Legionella*, and heterotrophic bacteria. Final rule. Fed. Regist. **54:**27486.
17. **Venczel, L. V., et al.** Unpublished data.