# INNOVATIVE TECHNOLOGIES FOR WASTE WATER DISINFECTION AND PATHOGEN DETECTION

**Mark D. Sobsey, Michael J. Casteel, Hyenmi Chung, Gregory Lovelace, Otto D. Simmons III and J. Scott Meschke, University of North Carolina, Department of Environmental Sciences and Engineering, CB# 7400, Rosenau Hall, Chapel Hill, North Carolina 27599-7400**

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## ABSTRACT

New and improved methods are needed to disinfect pathogens in treated wastewater, especially highly resistant ones like *Cryptosporidium parvum* oocysts. In addition, new and improved methods are needed to detect pathogens and indicators for them in wastewater in order to monitor the efficacy of disinfection and other waste water treatment processes. Spores of the anaerobic bacterium *Clostridium perfringens* and male-specific coliphages were evaluated as indicators of waste water treatment and chlorine disinfection. These indicators were easy to measure and were found to be more resistant to primary and secondary wastewater treatment and chlorine disinfection than were indicator bacteria such as fecal coliforms and enterococci. New cell culture methods were used to assay the infectivity of *C. parvum* oocysts in treated wastewater and water subjected to disinfection by electrochemically generated mixed oxidants. *C. perfringens* spores, coliphage MS2 and *E. coli* also were evaluated for their responses to mixed oxidants. Mixed oxidants effectively inactivated *C. parvum* oocysts in water and tertiary treated waste water, with infectivity reductions of >99.9%. *C. perfringens* spores were good indicators of *C. parvum* oocyst inactivation in water at mixed oxidant dose of 4 mg/l. In tertiary treated waste water, *C. parvum* oocysts were efficiently inactivated (>99.9%) by mixed oxidant doses of about 10-15 mg/l. Under the same conditions, *C. perfringens* spores, coliphage MS2 and *E. coli* were more sensitive to mixed oxidants than were *C. parvum* oocysts. Electrochemically generated mixed oxidants effectively inactivate *C. parvum* oocysts in water and treated waste water at practical doses and contact times. *C. perfringens* spores and male-specific coliphages are useful indicators of disinfection efficacy by mixed oxidants and chlorine, but they are not as resistant as *C. parvum* oocyts under some disinfection conditions.

## KEYWORDS

Mixed oxidants, disinfection, *Cryptosporidium parvum*, infectivity assay, *Clostridium perfringens* spores, male-specific coliphages

## INTRODUCTION AND BACKGROUND

Disinfection of pathogens in wastewater and water continues to be an essential barrier to reduce the risks of pathogen exposure and waterborne infectious diseases of humans and animals. Municipal raw sewage contains high concentrations of viral,

bacterial and protozoan pathogens, with estimated concentrations as high as a million of organisms per liter. Therefore, it is necessary that sewage treatment processes be effective in removing or destroying these pathogens before the resulting effluents and residuals are returned to the environment.

Conventional primary and secondary treatment processes are not likely to reduce the concentrations of enteric pathogens in raw sewage by more than 90 to 99%, especially the relatively resistant enteric viruses and protozoan cysts and oocysts. Secondary treated effluents sometimes contain more than 1,000 enteric viruses and protozoan cysts per liter. Therefore, disinfection becomes the most important barrier to destroy or remove enteric pathogens in treated sewage effluents. Historically, treated sewage effluents have been disinfected with chlorine. However, chlorination of sewage effluents has several important limitations. For one, chlorination of sewage effluents results in the formation of chlorinated compounds as by-products that are toxic to aquatic life and potentially carcinogenic to humans. When sewage effluents are chlorinated, they often have to be dechlorinated prior to discharge to the environment.

Furthermore, chlorination is relatively ineffective in reducing enteric viruses, bacterial spores and protozoan cysts and oocysts in sewage (Sobsey, 1989). Viruses are quite resistant to chloramines and protozoans such as *Cryptosporidium parvum* oocysts are very resistant to both free chlorine and monochloramine. Estimated CT-99 (product of disinfectant concentration and contact time to achieve 99% microbial reduction) values are in the thousands of mg-minutes per liter for enteric virus inactivation by combined chlorine and *Cryptosporidium parvum* oocyst inactivation by free or combined chlorine. Therefore, enteric virus and parasite concentrations of chlorinated sewage effluents can be quite high, even if fecal indicator bacteria (e.g., fecal coliform) levels have been appreciably reduced. Fecal coliforms, coliforms and other vegetative enteric bacterial indicators (fecal streptococci, enterococci, etc.) are much more sensitive to free and combined chlorine than are enteric viruses and parasites. Achieving extensive reductions of these conventional indicator bacteria by effluent chlorination give a false sense of security about the microbiological quality of the effluent with respect to enteric viruses and parasites.

Enteric viruses and protozoans are also more resistant than indicator bacteria (fecal coliforms, enterococci, etc.) and typical bacterial pathogens (*Salmonella*, *Shigella*, *E. coli*, etc.) to other wastewater and water disinfectants, including ozone, chlorine dioxide and UV radiation (Sobsey, 1989). These disinfectants are receiving more attention as alternatives to chlorine for disinfection of waste water and water. Ozone and chlorine dioxide are more effective than free or combined chlorine for disinfection of *Cryptosporidium parvum* oocysts. In clean water the approximate CT values for 99% inactivation of *C. parvum* oocysts are about 10 and 80 mg-minutes per liter for ozone and chlorine dioxide, respectively. However, the efficacy of these alternative disinfectants for inactivation of *Cryptosporidium* in sewage effluents is unknown or poorly documented. UV radiation has received increased attention and use as a wastewater effluent disinfectant because there is no addition of chemicals to leave toxic residuals or form toxic by-products. However, doses of UV to achieve

appreciable virus inactivation are excess of 100 mW-sec/cm$^2$ and to achieve appreciable inactivation of *Cryptosporidium parvum* oocysts they are in excess of thousands of mW-sec/cm$^2$. The doses required for inactivation of *Cryptosporidium* are much higher than typically applied and may substantially increase wastewater disinfection costs.

Recently, the disinfection technology of mixed oxidants electrochemically from brine solution has been shown to efficiently inactivate *C. parvum* oocysts and *C. perfringens* spores in buffered water (Venczel et al., 1997). A 5 mg/l dose of mixed oxidants reduced the mouse infectivity of *C. parvum* oocysts by >99.9 to >99.99% after 4 hours. Under the same conditions, the infectivity of *C. perfringens* spores was reduced by >99.5%. A 5 mg/l dose of free chlorine gave no inactivation of *C. parvum* oocysts, even after 24 hours of contact, and *C. perfringens* spores were reduced by <99%. Mixed oxidant disinfection technology is relatively simple, economical and especially suitable for small treatment systems. The chemical composition of the active microbiocidal species in electrochemically mixed oxidants is unclear at this time. Free chlorine is a major ingredient, but other oxidants must be present to account for its ability to inactivate the infectivity of *C. parvum* oocysts. Despite its promise as a disinfectant, mixed oxidant technology has not been carefully evaluated for disinfection of treated waste water.

The inability of conventional indicator bacteria to reliably predict the inactivation of enteric viruses and protozoan cysts and oocysts by wastewater chlorination and other disinfection processes has sparked interest in more reliable indicators of these pathogens. For enteric viruses indicators attention has focused on bacterial viruses of *E. coli* or so-called coliphages. Coliphages are plentiful in raw sewage with typical levels ranging from 100 to 10,000 per ml. This is about 1,000 times higher than the levels of human enteric viruses in sewage. Furthermore, coliphages are easily and rapidly measured by simple and economical methods; results can be obtained within 24 hours for <$100 per sample. In contrast, human enteric virus analysis may take weeks at costs exceeding $1,000 per sample. Like coliform bacteria, there are a number of different types of coliphages; that is coliphages are a heterogeneous group. Therefore, certain coliphages may be more reliable indicators of human enteric viruses than others.

Attention had focused mainly on the male-specific (F+) coliphages containing ribonucleic acid genetic information as indicators of human enteric viruses in water and wastewater (Sobsey et al., 1995). These so-called F+ RNA coliphages are plentiful in raw sewage (100-10,000 per ml), they superficially resemble some of the important enteric viruses (enteroviruses, caliciviruses, astroviruses and hepatitis A and E viruses) in size, shape and composition, and they appear to have only feces or sewage as sources. Furthermore, the F+ RNA coliphages consist of four major subgroups (I-IV), of which groups II and III occur primarily in human sewage and fecal wastes while groups I and IV occur primarily in non-human animal fecal wastes (Hsu et al., 1995).

Spores of *Clostridium perfringens* have received interest as indicators of protozoan cysts and oocysts primarily in water (Payment et al., 1993), and they have promise as indicators in wastewater, too (Bisson and Cabelli, 1979). In particular, the resistance of

the spores to disinfection and environmental processes makes them potentially good indicators for the removal and inactivation of hardy protozoan cysts and oocysts.

Recent evidence indicates that there may be two distinct genotypes of *Cryptosporidium parvum* (Peng et al., 1997), one primarily circulating in humans (type 1) and the other primarily circulating in cattle (type 2). Only the cattle genotype (2) infects mice and calves; the human genotype does not (1) The animal genotype may differ in infectivity for humans. Therefore, attention is shifting to domestic and municipal wastewater effluents as important sources of human *Cryptosporidium parvum* oocyst contamination of surface and ground waters. It is now becoming increasingly important to determine the ability of sewage treatment processes, and especially disinfection processes to remove or inactivate *C. parvum* oocysts.

Until recently, assays for *C. parvum* infectivity have required the use of mice as hosts for bioassays. Mouse bioassays for *C. parvum* infectivity are expensive, tedious and lacking in precision and accuracy. Typically, only a few mice can be inoculated per sample dilution, thereby giving only a rough estimate of infectivity titer. Mouse bioassays have considerable limitations for assaying *C. parvum* infectivity in field samples of water and wastewater, including limited availability, high cost, and potential for coinfection by other enteric pathogens in water and wastewater. Furthermore, mouse bioassays are possible only with the *C. parvum* strains of genotype 2 from cattle.

Because of the limitations of mouse infectivity assays, detection of *C. parvum* in environmental waters and wastewaters been based on estimates of viability. Viability assays for *C. parvum* oocysts include reactivity with vital dyes or stains, such as DAPI and propidium iodide, and in-vitro excystation. These assays have been criticized as being unreliable in predicting the infectivity of oocysts subjected to certain physical and chemical treatments, including disinfection. Chemical disinfectants may react with and alter the permeability of the *C. parvum* oocyst wall and thereby change reactivity with vital dyes or induce excystation, but not necessarily change infectivity to the same extent.

In this study the inactivation kinetics of *Cryptosporidium parvum* oocysts*, Clostridium perfringens* spores *Escherichia coli* B, and MS2 coliphage by an electrochemically generated mixed oxidant solution were determined in tertiary-treated wastewater effluent. The inactivation kinetics of *C. parvum* oocysts and *C. perfringens* spores by mixed oxidant disinfection of water also was determined. *C. perfringens* and male-specific coliphages were evaluated as indicators of wastewater treatment and disinfection. The mixed oxidant was generated by an electrochemical cell provided by the MIOX Corporation (Albuquerque, NM).

## METHODS AND MATERIALS

### Test microbes and assays

*C. parvum* oocysts (IOWA strain), coliphage MS2, *E. coli* B and spores of *C. perfringens* were used in mixed oxidant disinfection experiments in tertiary sewage effluent and

phosphate buffered water. *C. parvum* oocysts produced in experimentally infected calves were obtained from Pleasant Hill Farm, Troy, ID. as gradient purified suspensions in buffered water with antibiotics. Coliphage MS2 and *E. coli* B were obtained from the American type culture collection. *E. coli* Famp (a gift from Victor Cabelli, University of Rhode Island) and *Salmonella typhimurium* strain WG49 (a gift of Arie Havelaar, National Institute of Public Health and the Environment, The Netherlands) were used as hosts for F+ coliphages. *C. perfringens* spores were produced from sewage isolates of the bacterium as previously described (Venczel et al., 1997). Titers of each of microorganism for laboratory disinfection experiments were determined via hemacytometer counts (*C. parvum*), optical density (*E. coli* B) or prior infectivity assay (MS2 and *C. perfringens* spores). Suspensions of test microbes were washed or diluted in either PBS or oxidant demand free (ODF) buffer to reduce oxidant demand. *C. parvum* infectivity was assayed in 2-chamber well slides of confluent Madin-Darby Canine Kidney (MDCK) cell layers as described below. *E. coli* B was assayed by the spread plate method on MacConkey agar and *C. perfringens* spores were assayed by the membrane filter method on mCp agar. Coliphage MS2 was assayed by the double agar layer plaque technique on host *E. coli* F-amp using tryptic soy agar containing ampicillin and streptomycin (20 µg/ml each). In disinfection experiments treated and control samples (2 ml) were immediately added to 2 ml sodium thiosulfate solution (1%) to neutralize mixed oxidants in the treated samples.

*C. parvum* infectivity was assayed by inoculating 0.4 ml-volumes of undiluted or diluted sample into one chamber on well slide; the other chamber served as a negative control. Concentrations of oocysts per chamber ranged from $2 \times 10^5$ (undiluted samples) to $2 \times 10^3$ (100-fold dilution). Samples were diluted in Ultraculture medium (BIO Whittaker, Walkersville, MD). Stock *C. parvum* oocysts also were assayed for cell culture infectivity by inoculating 0.1 ml volumes of stock oocyst dilutions (in Ultraculture medium) into replicate culture chambers of MDCK cells per dilution.

**Wastewater (WW) effluent for disinfection experiments**

Tertiary treated sewage effluent (activated carbon-filtered, secondary effluent) was obtained from the South Durham Reclamation plant, Durham, NC. The effluent had the following quality: total suspended solids = 1 mg/l, BOD-5 = 1 mg/l, total N = 5 mg/l, ammonia N <0.05 mg/l, $NO_3$+$NO_2$ ~ 5 mg/l and pH = 7.0.

**Mixed oxidant (MO) disinfectant**

Mixed oxidants were generated from a MIOX Water Disinfection Unit as previously described (Venczel et al., 1997). Briefly, fresh brine (10 g/L NaCl solution in reagent grade water) was pumped through the MIOX Unit at a pump speed of 20-25 L/hr and at 7.5 to 7.6 amps. Mixed oxidants were collected from the anode flow of the electrochemical cell in oxidant demand-free (ODF) water in amber bottles. The collected anolyte was analyzed for total oxidants and applied at a dose of 10-15 mg/l to waste water seeded with test microbes.

**ODF water, glassware, buffers and buffered test water for disinfection experiments**

Twice-deionized, activated carbon-filtered water passed through a macroreticular scavenging resin bed (Dracor Corp., Durham, NC) was oxidant demand-free. Glassware was made ODF by soaking for 24 hours in a >30 mg/L free chlorine solution and then rinsing 4X with ODF water. Glassware was covered with aluminum foil and heated at 200$^{o}$C for 4 hours. Phosphate buffer stocks of 0.1 M, pH 4.5 or pH 9.5, were in made in ODF water according to the protocol in Standard Methods for the Examination of Water and Wastewater (APHA, 1992). Buffers were diluted appropriately in ODF water to make 0.01 M buffered water, pH 8, for disinfection experiments. Mixed oxidant disinfection of *C. parvum* oocysts and *C. perfringens* spores in water was done in 0.01 M phosphate buffer at pH 8 and room 25$^{o}$C.

**Determination of oxidant concentration**

Total oxidants were determined by the N,N,-Diethyl-p-phenylenediamine (DPD) colorimetric method (4500-Cl, Standard Methods for the Examination of Water and Wastewater) (APHA, 1992)

**Field studies of indicator reductions by conventional wastewater treatment and disinfection**

Twenty samples of raw sewage and final effluent were collected regularly over 14 months from the Morehead City, NC. sewage treatment plant. Treatment was primary sedimentation, trickling filtration and chlorination. Samples were analyzed for F+ coliphages by the double agar layer method on hosts *E. coli* F-amp and *Salmonella typhimurium* WG49 (Sobsey et al., 1995). Fecal coliforms were analyzed by membrane filter method on mFC medium using 2-5 hour resuscitation at 35$^{o}$C prior to incubation at 44.5$^{o}$C for 17-20 hours. *E. coli* were analyzed by transferring membranes from mFC to nutrient agar plates with 100 µg/ml 4-methylumbelliferon glucuronide (MUG), incubating 4 hours at 35$^{o}$C and scoring for blue-fluorescing colonies under long wavelength UV light (Mates and Shaffer, 1989). Enterococci were analyzed my membrane filter method on modified mE medium with incubation at 41$^{o}$C for 24 hours (Dufour, 1980). *C. perfringens* were analyzed by membrane filter method on mCp medium with incubation at 44.5$^{o}$C for 18-24 hours (Bisson and Cabelli, 1979).

***Cryptosporidium parvum* infectivity assay**

A MDCK cell culture infectivity assay for production of living stages of *C. parvum* from oocysts was used (Arrowood et al., 1994; 1996). Assay steps are shown below.

Centrifuge sample at 13K for 3 minutes; decant supernatant; resuspend pellet in PBS/BSA; repeat 2X (optional: leave in pelleted form)

Resuspend pellet in 100-200 ml of prewarmed (37$^{o}$C) Ultraculture medium

Transfer volume to each well of a 2-chamber slide

Incubate slides at 37$^{o}$C (5% $CO_2$) for 3 hours

Decant fluid from slides; replace with fresh Ultraculture medium

Incubate slides for 48 hours at 37°C (5% $CO_2$)

Decant fluid from slides; wash with T-PBS; repeat 3X

Fix cell layers with 0.5 ml Bouin's solution per well for 2 hours @ room temperature

Decant fixative; replace with 1 ml 70% ethanol per chamber well; place slides on shaker platform for 5 minutes

Decant ethanol; wash chamber cells with TPBS; repeat until no yellow color from cell layer

Decant T-PBS; add 1 ml PBS/BSA per well

Incubate slides for 30 minutes at 37°C (5% $CO_2$)

Decant PBS/BSA

Stain cell layers with 300 ml fluorochrome (Cy3)-labeled C3C3 MAb (diluted 1:500 in PBS/BSA) per well

Cover slides with foil and incubate for 90 minutes at room temperature on shaker platform

Decant staining solution; wash wells with T-PBS; repeat 3X

Decant T-PBS; add 1-2 drops of polyvinyl alcohol / 1,4-Diazabicyclo-[2.2.2] octane (DABCO) per cell layer

Mount 18 mm coverslip on cell layers

Microscopic observation

For experimental and control preparations, 100 random microscope fields (Leitz Orthoplan 2, Germany) were viewed at a total magnification of 400X and scored either positive or negative for the presence of red-fluorescing objects (asexual and sexual intracellular developmental stages of *C. parvum)*. The number of microscope fields positive for living stages of *C. parvum* out of 100 fields observed was converted to a percentage and expressed in Probit units. The relationships between Probit units and oocyst concentrations were determined by linear regression analysis for treated and control samples. For treated and control samples containing the same number of oocysts, the Probit value of the untreated sample was used to estimate the decrease in infectivity of the untreated sample. This difference in $\log_{10}$ concentration of infectious oocysts was taken as the $\log_{10}$ reduction due to disinfection.

**Experimental procedures for mixed oxidant disinfection experiments**

The experimental procedures for disinfection experiments are outlined below.

| TEST SAMPLE | DISINFECTANT CONTROL | BIOLOGICAL CONTROL |
|---|---|---|
| water or WW (9 parts) containing MO (1 part) and ~$10^7$ oocysts and $10^6$ of each other test microbe (total volume 10 ml) | water or WW containing MO disinfectant (two reactors) and MO plus microbes (4 reactors) (10 ml volume each) | water or WW containing ~$10^7$ oocysts and ~$10^6$ of each of the other test microbes (total volume 10 ml) |
| Sample 2 ml at 1, 10, 30, and 90 minutes | Sample 10 ml at beginning and end of experiment (MO reactors) and at 1,10,30, and 90 minutes (MO + microbes reactors); analyze for total oxidants | Sample 2 ml at beginning and end of experiment |
| Neutralize sample disinfectant with equal volume of 1-3% w/v sodium thiosulfate | Mean disinfectant concentration based on MO residuals at 1, 10, 30, and 90 minutes | Add samples to equal volume of 1-3% w/v sodium thiosulfate |
| Assay for test microbes | | Assay for test microbes |

**Analyses of Microbial Inactivation Data**.

Microbial reductions are calculated by dividing the mean number of microbes (as units per ml) surviving at time t ($N_t$) by the original microbe titer ($N_o$), and expressing as a $\log_{10}$ value, $\log_{10}$ ($N_t/N_o$) or percentage reduction. The average results for three or more replicate experiments are used to compute an average $\log_{10} N_t/N_o$ value.

**RESULTS AND DISCUSSION**

**Field studies on reduction of microbial indicators by conventional wastewater treatment and chlorination**

The concentrations of *C. perfringens*, F+ coliphages and indicator bacteria in raw and treated sewage are summarized in Table 1 below. As expected, the conventional indicator bacteria of fecal coliforms, *E. coli* and enterococci were detected at high concentrations in raw sewage, with geometric mean concentrations of 58,000,000, 6,000,000 and 370,000 per 100 ml, respectively. *C. perfringens* levels in raw sewage were lower, with a geometric mean concentration of 80,000 per 100 ml. Concentrations of F+ coliphages were similar to those of *E. coli* and enterococci, with geometric mean concentrations of 2,000,000 and 1,000,000 on hosts *S. typhimurium* WG49 and *E. coli* F-amp, respectively.

Table 1. **Indicator Bacteria, C. perfringens F+ Coliphages in Raw Sewage and Treated Effluent**

| Indicator | Organism Concentrations per 100 ml | | |
|---|---|---|---|
| | Geom. Mean | Median | Range (Minimum-Maximum) |
| Raw Sewage | | | |
| Fecal coliforms | 58,000,000 | 61,000,000 | 9,500,000-500,000,000 |
| *E. coli* | 6,000,000 | 6,100,000 | 500,000-91,000,000 |
| enterococci | 370,000 | 380,000 | 220,000-730,000 |
| *C. perfringens* | 80,000 | 100,000 | 2,100-730,000 |
| F+ coliphages, S.t.* | 2,000,000 | 2,000,000 | 470,000-7,000,000 |
| F+ coliphages, F-amp* | 1,000,000 | 1,000,000 | 190,000-4,800,000 |
| Treated Sewage Effluent | | | |
| Fecal coliforms | 1,100 | 3,000 | 9-530,000 |
| *E. coli* | 90 | 28 | 2.5->30,000 |
| enterococci | 9.9 | 8 | <0.5-2,500 |
| *C. perfringens* | 2,900 | 3,400 | 250-13,000 |
| F+ coliphages, S.t.* | 13,000 | 19,000 | <100-250,000 |
| F+ coliphages, F-amp* | 7,700 | 9,400 | <100-190,000 |

*S.t. = bacterial host *Salmonella typhimurium* WG49; F-amp = bacterial host *E. coli* F-amp.

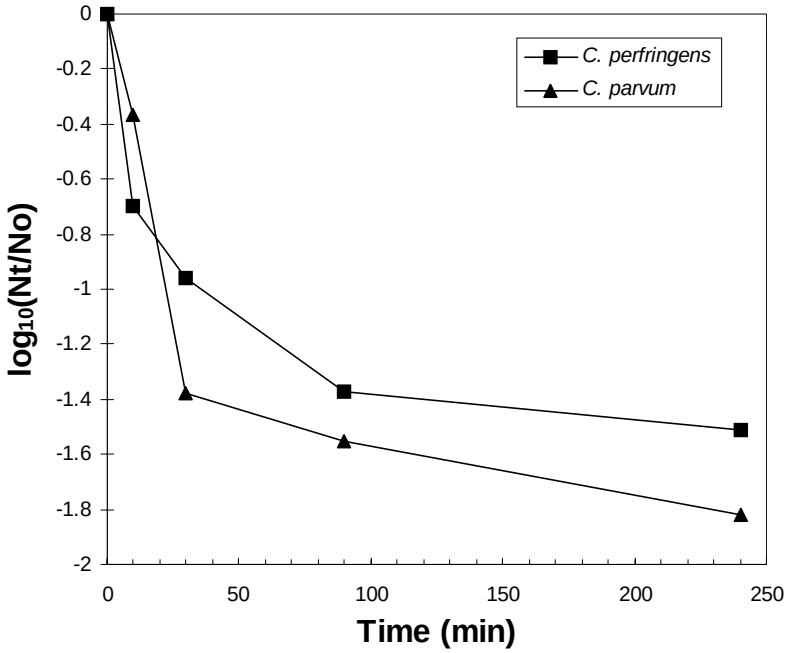
As expected, indicator levels were lower in the treated sewage effluent. For conventional indicator bacteria, geometric mean effluent concentrations were 1,100, 90 and 9.9 per 100 ml, for fecal coliforms, *E. coli* and enterococci, respectively. Hence, average $\log_{10}$ reductions by sewage treatment were about 4.7 for fecal coliforms, 4.8 for *E. coli*, and 4.2 for enterococci. Despite relatively low concentrations in raw sewage, *C. perfringens* levels in treated effluent exceeded those of the other indicator bacteria, with a geometric mean concentration of 2,900 per 100 ml. The mean reduction of *C. perfringens* by sewage treatment and chlorination was only was only 1.9 $\log_{10}$. This is far less than the reductions of the conventional indicator bacteria. Concentrations of F+ coliphages in treated effluent also exceeded those of the conventional indicator bacteria, with geometric mean values of 13,000 and 7,700 per 100 ml on hosts *S. typhimurium* WG49 and *E. coli* F-amp, respectively. The mean reduction of F+ coliphages by conventional sewage treatment and chlorination was only 2.2 $\log_{10}$. This reduction for F+ coliphages is far less than the reductions of the conventional indicator bacteria and is only somewhat greater than the reduction of *C. perfringens*.

Overall, it appears that *C. perfringens* and F+ coliphages are much more resistant indicators than conventional indicator bacteria to conventional sewage treatment processes, and they are present in treated and chlorinated effluents at higher concentrations than conventional indicator bacteria. For these reasons, *C. perfringens* and F+ coliphages may be better indicators of sewage treatment and disinfection efficacy and the impact of wastewater discharges on receiving waters with respect to enteric viruses and protozoan cysts and oocysts.

**Inactivation of *C. parvum* oocysts and *C. perfringens* spores in buffered water by mixed oxidants**

The results for bench laboratory experiments on inactivation of *C. parvum* oocysts and *C. perfringens* spores in 0.01 M buffered water at pH 8 and 25°C by a 4 mg/l dose of mixed oxidants over a total of 240 minutes of contact are summarized in Figure 1. The oxidant residual after 240 minutes was 0.5 mg/l, indicating the loss of most but not all of the oxidant dose over the course of the experiments. The infectivity of C. Parvum oocysts was reduced by about 0.4, 1.4, 1.6 and 1.8 $\log_{10}$ (57, 96, 97, and 98%) after 10, 30, 90 and 240 minutes, respectively. Most of the inactivation occurred within the first 30 minutes of contact., and the rate of inactivation declined over time. This may be due to the decline in mixed oxidant concentration over the time course of the experiment, to changes in the composition of the mixed oxidants over time, or differences in the resistance properties of the *C. parvum* oocysts (e.g., aggregation). Spores of *C. perfringens* showed similar rates and extents of inactivation by a 4 mg/l dose of mixed oxidants, with $\log_{10}$ reductions of about 0.7, 1.0, 1.4 and 1.5 (80, 90, 96 and 97%) after 10, 30, 90 and 240 minutes, respectively. As observed for *C. parvum* oocysts, the rate of inactivation of *C. perfringens* spores also decreased over the time course of the experiment. Overall, the rate and extent of inactivation of *C. perfringens* spores was very similar to that of *C. parvum* oocysts. This suggests that *C. perfringens* spores may be a reliable indicator of mixed oxidants inactivation of *C. parvum* oocysts in water.

**Figure 1. Inactivation of *C. parvum* and *C. perfringens* by 4 mg/l Mixed Oxidant Dose**



It is noteworthy that a 4 mg/l dose of mixed oxidants electrochemically generated from brine was capable of achieving >95% inactivation of *C. parvum* oocysts in water in 30 minutes. A 4 mg/l dose of free chlorine would produce no inactivation of *C. parvum* oocysts in water, as has been previously documented (Korich et al., 1990; Venczel et al., 1997). Because mixed oxidants were an effective disinfectant for *C. parvum* oocysts in water they were evaluated for inactivation of *C. parvum* oocysts in treated waste water.

**Laboratory studies on inactivation of *C. parvum* oocysts, *C. perfringens* spores and other indicators by mixed oxidant disinfection of tertiary treated waste water.**

Inactivation of test microbes in tertiary waste water effluent by a 13 mg/l dose of mixed oxidants is summarized in Table 2. *E. coli* and coliphage MS2 were rapidly and dramatically inactivated, with >5 log10 (>99.999%) inactivation after 1 minute. Spores of *C. perfringens* also were inactivated quite rapidly and extensively, with 3.1 $\log_{10}$ reduction after 1 minute and >4.7 log10 reduction by 10 minutes. Oocysts of *Cryptosporidium parvum* also were inactivated quite rapidly and extensively with $\log_{10}$ reductions of about 1.8, 2.2 and 3.3, after 10, 30 and 90 minutes, respectively.

Table 2. **Inactivation of *C. parvum* Oocysts, *C. perfringens* Spores, Coliphage MS2 and *E. coli* in Tertiary Effluent by a 13 mg/l Dose of Mixed Oxidants**

| | $\log_{10}$ Infectious Units Remaining at:[1] | | | | |
|---|---|---|---|---|---|
| **Microbe** | **Zero Time** | **1'** | **10'** | **30'** | **90'** |
| *E. coli* | 6.5 | 1.3 | < 0.5 | < 0.5 | < 0.5 |
| *C. perfringens* | 4.8 | 1.7 | < 1.1 | < 1.1 | < 1.1 |
| Coliphage MS2 | 5.51 | < 0.4 | < 0.4 | < 0.4 | < 0.4 |
| *C. parvum* | 5.5 | 3.7 | 3.9 | 3.2 | 2.3 |

[1]Less than (<) value indicates the detection limit of the method was reached .

The disinfectant residuals throughout the course of the experiment are summarized in Table 3.

The disinfectant dose was 13.4 mg/L and in the disinfectant control reactor with buffered ODF water there was essentially no loss of residual, with a 13.3 mg/l concentration after 90 minutes. In the disinfectant control reactor containing the waste water sample, the disinfectant residual also was relatively stable, with a 10.8 mg/l residual (81%) after 1 minute and an 8.8 mg/l residual (66%) after 90 minutes. In the reactor with test microbes the disinfectant residual was less stable, with 37 , 17, 10 and 5% of the initial 13.4 mg/l concentration remaining after 1, 10, 30 and 90 minutes, respectively. Apparently, the test microbes suspensions added to the reaction mixture

still had considerable oxidant demand despite centrifugation, washing and other purification measures. Even more rapid and extensive inactivation of test microbes would be expected if the mixed oxidant residual was maintained at higher concentrations through the time course of exposure.

Table 3. **Mixed Oxidant Concentrations in Test and Control Reactors Over Time**

**Residual disinfectant concentration as mg/L (%) remaining at:**

| Sample | Time Zero | 1' | 10' | 30' | 90' |
|---|---|---|---|---|---|
| Disinfectant only | 13.4 | - | - | - | 13.3 (99) |
| Disinfectant + test water | 13.4[2] (100) | 10.8 (81) | 9.8 (73) | 8.9 (66) | 8.8 (66) |
| Disinfectant + test water + microbes | 13.4[2] (100) | 4.90 (37) | 2.30 (17) | 1.30 (10) | 0.70 (5) |

[1]Measured immediately after preparation and after addition.
[2]Average disinfectant concentration over the course of the experiment.

## SUMMARY AND CONCLUSIONS

Spores of *Clostridium perfringens* and male-specific coliphages appear to be useful indicators of waste water disinfection and waste water quality because they are plentiful in raw sewage and in conventionally treated sewage effluents. Spores of *C. perfringens* are smaller in size (~2 µM diameter) than *Cryptosporidium* oocysts (~5 µM diameter) and are relatively resistant to sewage treatment and conventional disinfection processes. Therefore, they may be useful indicators of the physical removal and disinfection *C. parvum* oocysts in sewage by treatment processes. Male-specific coliphages are similar in size, shape and composition to important human enteric viruses and they show similar persistence in sewage treatment processes and in the environment. Therefore, they may be reliable indicators of human enteric viruses in disinfected wastewater and in receiving waters. Spores of *C. perfringens* and male-specific coliphages are more persistent in treated and disinfected sewage and at higher concentrations in treated sewage effluents than are conventional indicator bacteria. Therefore they may be better indicators of human enteric viruses and protozoan cysts and oocysts.

A new cell culture assay for the infectivity of *C. parvum* oocysts now makes it possible to determine with precision and accuracy the inactivation of *C. parvum* oocysts by waste water and water disinfection processes. This assay was used to determine the inactivation of *C. parvum* oocysts in buffered water at pH and 25$^{o}$C by a 4 mg/l dose of mixed oxidants electrochemically generated from brine. This disinfection technology is simple, economical and easy to use, and is especially appropriate for small treatment systems. Mixed oxidants produced >95% inactivation of *C. parvum* infectivity after 30 minutes and nearly 99% inactivation after 4 hours. Inactivation of *C. perfringens* spores was similar to that of *C. parvum* oocysts, with about 90% inactivation

in 30 minutes and >95% inactivation in 4 hours. *C. perfringens* spores may be a useful indicator of the inactivation of *C. parvum* oocysts by disinfection processes.

The observation that a dose of about 13 mg/l of mixed oxidants in tertiary treated waste water produced *C. parvum* inactivation of >99% after 30 minutes and >99.9% after 90 minutes indicates the considerable potential of this innovative and emerging disinfection technology to achieve a high degree of waste water disinfection for all enteric pathogens. These results suggest that public health concerns about potentially high concentrations of infectious *Cryptosporidium parvum* oocysts in municipal wastewater discharges on beneficial uses of receiving wastes may be adequately controlled by mixed oxidant disinfection. However, more disinfection studies of mixed oxidant inactivation of *C. parvum* oocysts in wastewater effluents of different quality are needed to better characterize the rate and extent of inactivation that can be achieved. *Clostridium perfringens* spores were inactivated more rapidly and extensively than *C. parvum* oocysts in these waste water disinfection studies. However, the rate and extent of *C. perfringens* spore inactivation was less than that of *E. coli* and coliphage MS2. Because of the technical difficulties in monitoring wastewater discharges for infectious or viable *C. parvum* oocysts in wastewater and treated effluents, and the simplicity, speed and low cost of *C. perfringens* assays, it may be possible measure the latter to monitor disinfection efficacy for the former. However, further studies are needed to determine if there are consistent relationships between *C. parvum* and *C. perfringens* reduction by mixed oxidants and other treatment processes to provide a basis for using *C. perfringens* spores as an indicator of waste treatment efficacy for *C. parvum* oocysts. Neither coliphages such as MS2 (F+ coliphage) or coliform bacteria such as *E. coli* appear to be suitable indicators for *C. parvum* reduction by wastewater disinfection because they are not nearly as resistant as *C. parvum* oocysts or even *C. perfringens* spores.

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