The effect of cyanuric acid on the disinfection rate of *Cryptosporidium parvum* in 20-ppm free chlorine

Joan M. Shields, Michael J. Arrowood, Vincent R. Hill and Michael J. Beach

ABSTRACT

Cyanuric acid is used to stabilize free chlorine to reduce photodegradation in outdoor swimming pools. While there have been numerous studies examining its effect on the disinfection rates of bacteria and viruses, it is not known whether cyanuric acid can significantly impact the effectiveness of hyperchlorination for inactivating *Cryptosporidium* oocysts present in fecally-contaminated swimming pools. This study examined the effect of cyanuric acid on the disinfection rate of *Cryptosporidium parvum* under swimming pool hyperchlorination conditions (20 mg/ml free chlorine). When 50 mg/L cyanuric acid was present there was a 0.70-$\log_{10}$ reduction in oocyst viability after 10 hours as compared to a 3.7-$\log_{10}$ reduction without cyanuric acid. Aids to remediation, such as decreasing the pH to enhance the germicidal efficiency of the free chlorine and doubling the amount of free chlorine residual, were still unable to achieve a 3-$\log_{10}$ reduction. Current public health recommendations for hyperchlorination and pool remediation are insufficient for pools using cyanurate-stabilized chlorine to achieve a three log inactivation of the parasite.

Key words | chlorine, *Cryptosporidium parvum*, cyanuric acid, disinfection rate, recreational water, swimming pools

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INTRODUCTION

Cryptosporidium, an obligate, intracellular parasite of humans, is an important parasitic protozoan which has become the leading cause of recreational water-associated outbreaks of gastrointestinal illness. In 2003–2004, this parasite accounted for 61.1% (11/18) of gastrointestinal outbreaks associated with disinfected swimming venues (pools, water parks) (Dziuban *et al.* 2006). This is likely due to its high resistance to free chlorine (Korich *et al.* 1990), the main barrier to infectious disease transmission in pools. Current Centers for Disease Control and Prevention (CDC) recommendations for remediation of the parasite in swimming pools during suspected outbreaks is to use hyperchlorination conditions necessary to achieve a C*t* (concentration of disinfectant in milligrams per litre multiplied by time in minutes to achieve a desired log inactivation) of 15,300 (Shields *et al.* 2008); e.g., 20 mg/L free chlorine for 12.75 hours to achieve a 3-log reduction in oocyst viability and infectivity. This protocol does not account, however for the presence of cyanuric acid, a chlorine stabilizing compound used in pools throughout the world, which may decrease the inactivation rate of *Cryptosporidium*.

The chorine stabilizer, cyanuric acid, typically in the form of the isocyanurates dichloroisocyanuric (2,4,6-(1H,3Cl,5Cl)-s-triazinetrione; Dichlor) or trichloroisocyanuric (2,4,6-(1Cl,3Cl,5Cl)-s-triazinetrione, Trichlor) acids, has been used in outdoor swimming pools since 1958 to retard the rate of photochemical reduction of free chlorine

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(Canelli 1974). Many pool codes in the US set limits on the amount of cyanuric acid which can be present in swimming pools, on average between 100 and 150 mg/L (Johnston *et al.* 1999).

The effect of cyanuric acid on the disinfection of microorganisms with free chlorine has previously been explored, with numerous studies demonstrating a decrease in the rate of disinfection (Robinton & Mood 1967; Fitzgerald & DerVartanian 1969). Many investigators hypothesize that the stabilization mechanism which retards photochemical reduction also reduces the rate at which free chlorine is released to inactivate waterborne pathogens.

This study measured the effect of cyanuric acid on the disinfection rate of *C. parvum* under recommended swimming pool hyperchlorination protocols (CDC 2001). It also determined whether a reduction in pH or an increase in free chlorine could improve the disinfection efficacy of free chlorine in the presence of cyanuric acid.

MATERIALS AND METHODS

C. parvum oocyst source and purification

C. parvum oocysts (17–28 days old), originating from a bovine source in Iowa (Harley Moon isolate), were used for all experiments. All oocysts were purified from calf feces as described in Arrowood & Donaldson (1996). In short, feces were passed through stainless steel sieves and oocysts were purified through a series of two discontinuous Sheather's sucrose gradients (1.064 specific gravity over 1.103 sp. gr.) with final purification being made over a microscale cesium chloride (210.6 g/L, sp. gr. = 1.15) gradient. Oocysts were stored at 4°C in 2.5% aqueous potassium dichromate (w/v $K_2Cr_2O_7$) until used.

Chlorine demand-free water and glassware

Chlorine demand-free (CDF) water and glassware were prepared as described in *Standard Methods for the Examination of Water and Wastewater* (19th edition) (APHA 1995). Briefly, distilled de-ionized water was buffered to pH 7.5 with monobasic and dibasic sodium phosphate to a final concentration of 10 mM. Sodium hypochlorite (Fisher Scientific, Pittsburgh, PA) was added to a concentration of at least 5 mg/L and left covered for two days at room temperature. If after this time the free chlorine level had dropped by 50% or more, the water was discarded and the process begun again. The free chlorine was removed from the water by 48-hour exposure to ultraviolet light. Glassware was rendered chlorine demand-free by soaking it in water with at least 10 mg/L free chlorine for three hours or more followed by rinsing with CDF water.

Chemical-physical monitoring

The water in the inactivation vessels was monitored every two hours for temperature, pH, free chlorine and total chlorine. Adjustments were made if the pH and chlorine levels changed more than 5% from the starting values. Temperature and pH were measured using a Thermo Orion Model 290Aplus Portable Meter. Free and total chlorine were monitored using a Thermo-Orion (Beverly, MA) AQUAfast II photometer using DPD (*N*, *N*-diethyl-*p*-phenylenediamine) chemistry.

Disinfection experiments

The disinfection experiments were performed as previously described by Shields and colleagues (Shields *et al.* 2008). There were 5 control experiments performed (no cyanuric acid added) and 6 experiments with 50 mg/ml of cyanuric acid amended water. In short, 1-L glass beakers were used as reaction vessels. For all beakers, the CDF water (either amended with 50 mg/L cyanuric acid (TCI America, Portland, OR) or not) was brought to 20 mg/L free chlorine residual and adjusted to pH 7.5 or 6.5 using monobasic and dibasic sodium phosphate. The water was stirred at 200-rpm during the course of the experiment. After the free chlorine residual remained steady for at least an hour, 1×10^8 *C. parvum* oocysts were added. Free chlorine, total chlorine, pH and temperature were measured after one hour and every two hours afterwards. Ten hours after the addition of the oocysts, a 40-ml sample was taken and the chlorine was neutralized with the addition of 1 ml of 10% sodium thiosulfate (Sigma, St. Louis, MO). Samples were centrifuged at 3,290 × *g* for ten minutes to pellet the oocysts; the

supernatant was decanted and the pellet re-suspended with 1 ml of UltraCULTURE™ media (Lonza Group Ltd., Switzerland) and stored overnight at 4°C. The following morning, the oocysts were washed with CDF water and re-suspended again in 1 ml of UltraCULTURE™ media. An aliquot was removed for counting by hemocytometer; the final total was the average of at least three individual counts.

The experiments examining remediation efforts were as described above except that water adjusted to pH 6.5 was used to prepare CDF water, the water was brought to 40 mg/ml for that respective experiment and the experiments were monitored for 12 hours, left unmonitored for 11 hours, then monitored for the final hour before the 24 hour sample was taken. Samples were taken after 10 and 24 hours in disinfectant. After 24 hours there was little change in the pH or free chlorine level.

Determining the viability/infectivity of *C. parvum* oocysts

Cell culture

Madin-Darby canine kidney (MDCK) cells were inoculated onto cover glass-bottomed culture chambers (Nunc Lab-Tek, Rochester, NY) with UltraCULTURE™ media as previously described (Arrowood 2002). After 48 hours, oocysts were inoculated in triplicate onto the cell monolayers and incubated at 37°C for 48 hours with 5% CO_2. The number of oocysts inoculated was dependent upon the time point and whether or not it was a control or an experimental sample (Shields *et al.* 2008). As a baseline, 10,000 untreated oocysts and 500,000 oocysts from the 10-hour control vessel were inoculated onto cell monolayers. This was to prevent over-inoculating the monolayer with untreated oocysts and to be certain of capturing any viable oocysts still present after 10 hours.

After 48 hours, the inoculated monolayers were removed from the incubator and stained as described in Arrowood (2002). In short, the culture media was removed and the monolayers washed with sterile phosphate buffered saline (PBS). The monolayers were fixed in Bouin's solution for 1 hour. The Bouin's solution was subsequently removed and the monolayers decolorized with 70% ethanol (5 washes) followed by an overnight incubation in PBS supplemented with 0.1% bovine serum albumin (PBS + BSA) at 4°C. *C. parvum* sexual stages were stained using the *Cryptosporidium*-specific monoclonal antibody C3C3 conjugated with indocarbocyanine (Research Organics, Cleveland, OH) for at least one hour at 4°C in the dark. After the removal of any unbound antibody with PBS + BSA, the monolayers were sealed using two drops of PVA-DABCO (2.4 g polyvinyl alcohol (Sigma, St. Louis, MO), 6.0 g glycerol, 6.0 ml H2O, 12.0 ml 0.2 M Tris buffer (pH 8.5) and 2.5% (wt/vol) of the antiquenching agent DABCO (1,4-diazabicyclo-[2.2.2]-octane) (Arrowood 2002)) under an 18-mm^2 glass cover slip. After overnight storage at 4°C in darkness, the cell culture slides could be examined and the developmental stages counted.

Microscopical enumeration of parasite survival in cell culture preparations

Developing life cycle stages of *C. parvum* (meronts and gamonts) were defined by their size (~3–5 μm), shape and labeling by fluorescent antibody. The number of stages per slide was determined by dividing the slide into 30 rows and counting five individual rows equally spaced along the length of the slide at 250× magnification then multiplying this number by six. The number of stages per sample was calculated from the average of the triplicate slides.

RESULTS

Disinfection efficacy in the presence of 50 mg/L cyanuric acid

The average physical-chemical conditions for the experiments are listed in Table 1. The pH ranged from 7.52 to 7.56 across all experiments with pH 7.52 being the average of experiments containing cyanuric acid and pH 7.54 the average for the controls. The temperatures ranged from 21 to 24°C across all experiments with an average of 23.2°C for the cyanuric acid experiments and 23.0°C for the controls. Free chlorine levels, except for rare minor (<1.5 mg/L) adjustments, remained steady.

When comparing disinfection reactions in which no cyanuric acid was added to those amended with 50 mg/L

Table 1 | The average physical chemical conditions of the experiments

Experiment set	Number of individual experiments	Cyanuric acid (mg/L)	pH	°C	Chlorine		
					Free	Total	Combined
Control	5	0	7.5	23.0	20.7	21.4	0.8
Cyanuric acid added	6	50	7.5	23.2	21.0	21.5	0.7
pH lowered	2	50	6.5	23.7	20.5	20.9	0.7
pH lowered and free chlorine raised	2	50	6.5	23.8	40.9	42.2	1.2

cyanuric acid, a 3.7-$\log_{10}$ reduction in oocyst viability was measured after 10 hours for the control samples (20 mg/L free chlorine, no cyanuric acid, $n = 5$) and a 0.70-$\log_{10}$ reduction in oocyst viability after 10 hours for the samples containing cyanuric acid ($n = 6$) (Figure 1). Using linear interpolation, the data shown in Figure 1 for the "no cyanuric acid" condition indicate that the 3-$\log_{10}$ chlorine inactivation C*t* value for *C. parvum* oocysts was 10,400. A 3-$\log_{10}$ C*t* value for *C. parvum* oocysts for chlorine inactivation in the presence of cyanuric acid was not attained.

Effect of reducing pH and increasing free chlorine residual on disinfection efficacy

Decreasing the pH to 6.5 in the cyanuric acid-amended water at 20 mg/L free chlorine (Figure 2) did not result in a disinfection rate that was appreciably different (0.60-$\log_{10}$) from the rate observed at pH 7.5 (Figure 1, 0.70-$\log_{10}$). After 24 hours at pH 6.5 and 20 mg/L free chlorine, a 1.0 $\log_{10}$ inactivation was measured; this corresponds to a C*t* for 1-$\log_{10}$ reduction of 28,800. Increasing the free chlorine level also had little effect, resulting in a 0.80-$\log_{10}$ reduction after 10 hours. However, after 24 hours of doubling the amount of free chlorine residual and using a lower pH, a 2.7-$\log_{10}$ reduction in oocyst viability was attained.

DISCUSSION

Cryptosporidium oocysts are extremely difficult to inactivate by chlorine disinfection in recreational waters even under hyperchlorination (20 mg/L free chlorine) conditions. The current study shows that the presence of a relatively low concentration of cyanuric acid (i.e. 50 mg/L) was associated with significantly reduced *C. parvum* oocyst inactivation (3-$\log_{10}$ lower) over a 10-hour period compared to *C. parvum* inactivation measured without cyanuric acid. Lowering the pH to 6.5, to increase the amount of active hypochlorous acid available, did not appreciably change the oocyst inactivation rate when compared to inactivation at pH 7.5 in the presence of cyanuric acid. Using linear

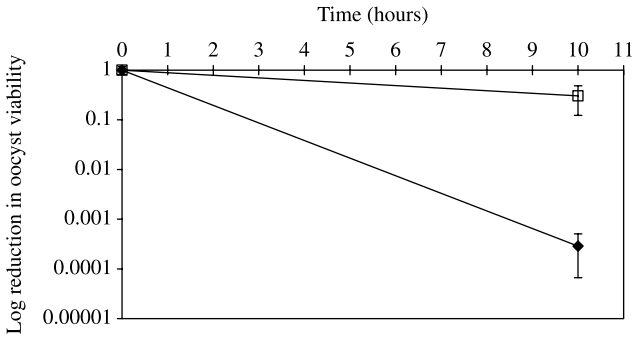

Figure 1 | Log reduction in oocyst viability after 10 hours in 20 mg/L sodium hypochlorite with (□, $n = 6$) and without (◆, $n = 5$) the addition of 50 mg/L cyanuric acid. Vertical bars indicate two standard deviations.

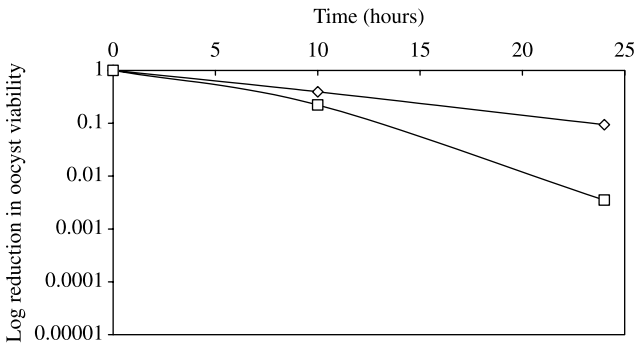

Figure 2 | Log reduction in oocyst viability with an increase in free chlorine and decrease in pH. Experiments incldued 20 mg/L free chlorine, pH 6.5, 50 mg/L cyanuric acid (◇, $n = 2$) and 40 mg/L free chlorine, pH 6.5, 50 mg/L cyanuric acid (□, $n = 2$).

interpolation, the free chlorine C*t* for a 1-$\log_{10}$ reduction of *C. parvum* oocysts at pH 6.5 in the presence of cyanuric acid (28,800) suggests that the C*t* for a 3-$\log_{10}$ reduction in viability under these conditions could be on the order of 86,400, which is 8 times higher than the measured C*t* for a 3-$\log_{10}$ reduction in *C. parvum* viability (Iowa, 10,400) with 20 mg/L free chlorine alone. This difference would likely be markedly higher if *C. parvum* isolates that have higher C*t* values were investigated (i.e. the Maine isolate has a C*t* of 15,300 for a 3-$\log_{10}$ reduction; (Shields *et al.* 2008)).

Doubling the free chlorine residual to 40 mg/L (at pH 6.5) did not appreciably affect the disinfection rate (0.60 and 0.80-log reduction, respectively) after 10 hours. However, after 24 hours, a 2.7-$\log_{10}$ inactivation was achieved when 40 mg/L free chlorine was used (in the presence of 50 mg/L cyanuric acid at pH 6.5) as compared to a 1.0-$\log_{10}$ inactivation when 20 mg/L was used under otherwise similar pH and cyanurate levels. The 40 mg/L free chlorine inactivation result suggests that the free chlorine C*t* for a 3-$\log_{10}$ reduction in *C. parvum* viability in the presence of 50 mg/L cyanuric acid would be on the order of 67,000, a level which is approximately 6.5 times higher than the C*t* for a 3-$\log_{10}$ value for free chlorine alone. The data reported in this study suggest that at least 28 hours of exposure to 40 mg/L free chlorine would be needed to achieve 3-$\log_{10}$ inactivation of *C. parvum* oocysts (Iowa) when 50 mg/L cyanuric acid is present in a swimming pool.

Our findings are in agreement with other studies examining the effect of cyanuric acid on the disinfection capacity of stabilized chlorine solutions (Robinton & Mood 1967; Fitzgerald & DerVartanian 1969; Sommerfeld & Adamson 1982; Engel *et al.* 1983; Yamashita *et al.* 1988; Golaszewski & Seux 1994; Saita *et al.* 1998). The addition of cyanuric acid increased the time needed for disinfection of 12 virus types by a factor of 4.8–28.8 compared to free chlorine alone (Yamashita *et al.* 1988). The addition of cyanuric acid similarly impaired the inactivation of poliovirus (Saita *et al.* 1998). Likewise, algaecidal activity was reduced in the presence of cyanuric acid (Sommerfeld & Adamson 1982). There is scant data regarding protozoa and cyanuric acid. The disinfection rate for *Naegleria gruberi* was reduced by cyanuric acid (Engel *et al.* 1983). The present study supports and extends these previous findings by demonstrating that cyanuric acid significantly decreases the rate of inactivation for *C. parvum* oocysts.

Swimming pool disinfectant products containing chlorine stabilized with cyanuric acid are popular because of their ability to reducing photodegradation of free chlorine in outdoor swimming pools. However, the data from this study indicate that emergency disinfection and remediation of swimming pools containing cyanuric acid-based chlorine disinfectants will likely require increased exposure time, and likely higher concentrations of free chlorine (e.g., $>$ 40 mg/L), to achieve the same level of oocyst inactivation that can be expected for hyperchlorination when cyanuric acid is not present. Further investigations into the effect of higher and lower concentrations of cyanurate on *Cryptosporidium* inactivation are needed to determine the conditions necessary for achieving a 3–$\log_{10}$ inactivation. Additional research on the use of other remediation alternatives (e.g., flocculants, ozone, ultraviolet light systems) may be prudent for providing guidance to pool operators using chlorine stabilizers. Given the deleterious effect of cyanuric acid on *C. parvum* disinfection rates, current CDC guidelines for responding to a diarrheic stool contamination of a swimming pool (http://www.cdc.gov/healthyswimming/fecal_response.htm#feces) cannot be applied when 50 mg/ml cyanuric acid is present in the swimming pool.

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