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Effect of Cyanuric Acid on the Inactivation of *Cryptosporidium parvum* under Hyperchlorination Conditions

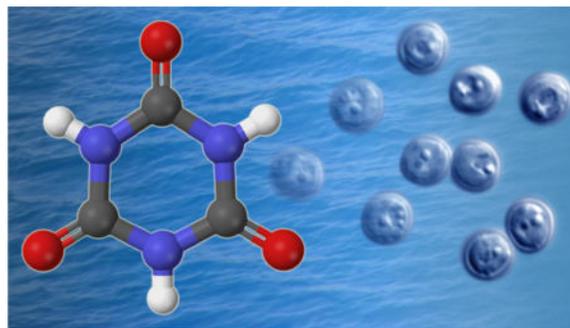
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Abstract

Cyanuric acid (CYA) is a chlorine stabilizer used in swimming pools to limit UV degradation of chlorine, thus reducing chlorine use and cost. However, CYA has been shown to decrease the efficacy of chlorine disinfection. In the event of a diarrheal incident, CDC recommends implementing 3- \log_{10} inactivation conditions for *Cryptosporidium* (CT value = 15 300 mg-min/L) to remediate pools. Currently, CYA's impact on *Cryptosporidium* inactivation is not fully determined. We investigated the impact of multiple concentrations of CYA on *C. parvum* inactivation (at 20 and 40 mg/L free chlorine; average pH 7.6; 25 °C). At 20 mg/L free chlorine, average estimated 3- \log_{10} CT values were 17 800 and 31 500 mg-min/L with 8 and 16 mg/L CYA, respectively, and the average estimated 1- \log_{10} CT value was 76 500 mg-min/L with 48 mg/L CYA. At 40 mg/L free chlorine, 3- \log_{10} CT values were lower than those at 20 mg/L, but still higher than those of free chlorine-only controls. In the presence of ~100 mg/L CYA, average 0.8- and 1.4- \log_{10} reductions were achieved by 72 h at 20 and 40 mg/L free chlorine, respectively. This study demonstrates CYA significantly delays chlorine inactivation of *Cryptosporidium* oocysts, emphasizing the need for additional pool remediation options following fecal incidents.

Graphical abstract



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Notes

The authors declare no competing financial interest.

INTRODUCTION

Cryptosporidium spp. are obligate, intracellular, protozoan parasites that infect the gastrointestinal tracts of humans and a wide variety of vertebrate mammalian hosts. *Cryptosporidium* is transmitted via ingestion of thick-walled, environmentally stable oocysts that are shed in the feces of infected persons or animals. In immunocompetent individuals, infection with *Cryptosporidium* can cause diarrhea which typically resolves in 2–3 weeks.¹ Severe diarrhea can lead to dehydration, which is of particular concern in vulnerable populations, such as young children and pregnant women. Immunocompromised patients, such as HIV-infected persons, might experience chronic, severe diarrhea, which can lead to life-threatening malabsorption and wasting.²

During 2001–2010, *Cryptosporidium* emerged as the leading etiologic agent of outbreaks associated with recreational water use in the United States. Due to the parasite's extreme chlorine tolerance, reported cryptosporidiosis outbreaks have largely occurred in chlorine-treated venues such as pools, interactive fountains, spas (or hot tubs), and waterparks.³ Currently, the U.S. Centers for Disease Control and Prevention (CDC) recommends hyperchlorination following a diarrheal incident (i.e., high-risk *Cryptosporidium* contamination event) or in response to at least a suspected recreational water-associated outbreak of cryptosporidiosis to achieve a CT value [chlorine concentration (mg/L) × time (min)] of 15 300 mg·min/L for 3- \log_{10} *Cryptosporidium* oocyst inactivation in the absence of cyanuric acid (CYA). This CT value can be achieved in pool water at pH 7.5 and 25 °C by increasing the free chlorine concentration to 20 mg/L and maintaining it at that concentration for 12.75 h.⁴ However, operational questions remain about the impact of additives, such as chlorine stabilizers, on the inactivation of *Cryptosporidium* by hyperchlorination.

CYA is a common chlorine stabilizer that has been used in many U.S. swimming pools since the late 1950s.⁵ CYA forms weak bonds with free chlorine in water, stabilizing the measured free chlorine level in the presence of UV light (e.g., sunlight). This stabilization reduces the amount of additional disinfectant required to maintain regulated levels during peak summertime use, which can provide substantial cost savings to pool operators. CYA can be added to chlorinated pools as a stabilizer in discrete amounts but is more typically added continuously as a chlorinated isocyanurate (e.g., dichloroisocyanuric or trichloroisocyanuric acid). CYA, including the cyanurate moiety of chlorinated isocyanurate disinfectants, is quite stable, and as it is added to a pool, it will accumulate to high levels unless the pool water is exchanged or diluted.

Research beginning in the 1960s has demonstrated that CYA decreases the disinfection efficacy of free chlorine against bacteria and viruses when compared with free chlorine alone.^{6–10} Shields¹¹ used a cell culture infectivity assay to provide the first evidence of reduced efficacy of free chlorine against *C. parvum* in the presence of CYA; however, their study reported CYA effects for a 1- \log_{10} inactivation at pH 6.5, not the 3- \log_{10} inactivation at pH 7.5 recommended by CDC in response to diarrheal incidents and suspected or confirmed cryptosporidiosis outbreaks. Data achieving 3- \log_{10} or more inactivation of

Cryptosporidium over various CYA concentrations typically found in public swimming pools are needed in order to revise current hyperchlorination recommendations.

Currently, the Model Aquatic Health Code advises maintaining CYA levels at or below 100 mg/L,¹² but many pools likely exceed this level with unknown impact on the efficacy of *Cryptosporidium* hyperchlorination inactivation. The lack of 3-log₁₀ *Cryptosporidium* inactivation data in the presence of CYA, the wide range of CYA concentrations likely found in public pools, and the unknown impact of increasing levels of CYA on *Cryptosporidium* inactivation pose challenges to providing evidence-based updates to existing diarrheal incident and cryptosporidiosis outbreak response recommendations for public swimming pools. As a result, this study was initiated to better understand the impact of increasing CYA concentrations on *C. parvum* inactivation to inform revision of existing recommendations.

METHODS

C. parvum and Madin Darby Canine Kidney (MDCK) Stocks

C. parvum (Maine isolate) oocysts were produced at the CDC as previously described^{13,14} and stored at 4 °C. Madin Darby Canine Kidney (MDCK) cells (CDC Scientific Resources Program, Atlanta GA) were routinely subcultured and, for infectivity assays, were inoculated onto 2.1 cm² cover glass-bottom culture chambers (Nunc Lab-Tek, Rochester, NY), and incubated at 37 °C with 5% CO₂ to achieve confluent monolayers at 96 h. Dulbecco's Modified Eagle Medium (DMEM), High Glucose media supplemented to contain 0.1 mM MEM nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-Glutamine (Gibco, Long Island, NY), and 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO) was used for subculturing and infectivity assays.

Preparation of Oxidant Demand Free (ODF) Water and Glassware

Laboratory grade 5.65% sodium hypochlorite solution was used throughout the study. ODF water was prepared by buffering water treated by reverse osmosis to pH 7.5 using 1 M monobasic and dibasic sodium phosphate (final concentration 10 mM), then adding sodium hypochlorite to at least 5 mg/L free chlorine. Water was covered and remained at room temperature for at least 48 h before exposure to ultraviolet light in a biosafety cabinet to remove free chlorine. ODF glassware and stir bars were prepared by soaking them in deionized water containing at least 10 mg/L free chlorine for a minimum of 3 h, then rinsing with ODF water.¹⁵ Both ODF glassware and water were sterilized by autoclaving at standard sterilization conditions.

Preparation of Experimental Flasks

Each round of oocyst experimentation involved: (A) a flask containing only ODF water to measure natural oocyst die-off; (B) a flask containing ODF water and 20 or 40 mg/L free chlorine to measure oocyst inactivation in the absence of CYA; and (C and D) duplicate flasks containing ODF water; 20 or 40 mg/L free chlorine; and 8, 16, 50, or 100 mg/L CYA to measure oocyst inactivation in the presence of CYA. To prepare the CYA stock solution, 0.27 g of CYA (TCI America, Portland, OR) was added to 100 mL of ODF water, dissolved

completely, then filter-sterilized through a 0.2 μm filter. Once sodium hypochlorite and CYA were added to flasks to achieved target concentrations, water was allowed to stabilize for at least 30 min. Initial pH (Accumet AR25 Benchtop Meter, Fisher Scientific, Pittsburgh, PA), oxidation–reduction potential (ORP) (Orion APlus, Thermo Fisher Scientific, Waltham, MA), free chlorine concentration of a sample dilution in deionized water (DPD Free Chlorine Method, Hach, Loveland, CA), and CYA concentration (melamine turbidimetric method, Hach, Loveland, CA) were measured. Probes were calibrated or standardized according to manufacturer’s instructions prior to each experiment.

EXPERIMENTAL SECTION

The appropriate volume of *C. parvum* Maine isolate oocyst stock was added to each flask to achieve a final concentration of approximately 10^5 oocysts/mL. Flask openings were covered with foil and remained in an environmental chamber (Espec North America Inc., Hudsonville, MI) at 25 °C with magnetic stir bars stirring at 150 rpm, for the duration of the experiment. At select time points over the duration of experiments, a water sample was removed and pH, ORP, and free chlorine concentration were measured. Probes were rinsed thoroughly in deionized water between samples. Neither pH nor disinfectant concentrations were adjusted during the duration of the experiment. At set time points, 4-, 40-, or 120-mL (as three 40 mL aliquots) samples were taken and immediately quenched in 50 mL polypropylene conical tubes containing either 1 or 10 mL 0.01 M phosphate buffered saline (PBS; pH 7.4) plus 0.1% bovine serum albumin (BSA), respectively, and sodium thiosulfate (Fisher Scientific, Pittsburgh, PA) at 50 mg/L per 1 mg/L disinfectant. Samples were stored at 4 °C until all samples could be concentrated simultaneously. As previously described,¹² samples were centrifuged at 3290g for 10 min at 4 °C to pellet oocysts. Supernatant was carefully removed by aspiration and the pellet was resuspended and transferred into a nonstick 1.5 mL microcentrifuge tube (Phenix Research Products, Candler, NC) which was then centrifuged at 15 800g for 3 min at 4 °C. Supernatant was carefully removed by aspiration and rinsate from a 1 mL PBS (0.01 M, pH 7.2) rinse of the respective conical tube was layered onto the pellet before a final centrifugation step at 15 800g for 3 min at 4 °C. Supernatant was carefully removed by aspiration down to the 0.1 mL demarcation on the microcentrifuge tube and 0.9 mL of DMEM, High Glucose plus 0.75% synthetic sodium taurocholate was added. The solution was triturated and then incubated at ambient temperature (approximately 22 °C) for 10–15 min to initiate excystation (i.e., sporozoite release).

C. parvum Infectivity Assay

The DMEM/taurocholate suspension was inverted three times to mix and 150 μL was inoculated in duplicate onto confluent MDCK cell monolayers in culture chambers containing 1.5 mL fresh DMEM medium. Slides were incubated at 37 °C and 5% CO₂ for 48–60 h. To visualize individual meronts and gamonts (life cycle stages that develop following sporozoite excystation and infection of MDCK cells if oocysts were viable and infectious), cell layers were fixed and labeled as previously described,¹³ with minor modification. Briefly, culture medium was removed and monolayers were washed three times with sterile 0.01 M PBS (pH 7.2), then fixed in Bouin’s solution (Ricca Chemical,

Arlington, TX) for 30 min. Bouin's solution was removed and monolayers were decolorized with five 10 min washes using 70% aqueous ethyl alcohol (prepared from anhydrous ethyl alcohol and deionized water), followed by overnight incubation in PBS with 0.1% BSA at 4 °C. The *Cryptosporidium*-specific monoclonal antibody C3C3 was bound to meronts and gamonts during a 1-h incubation. Unbound C3C3 was removed and monolayers were washed three times with sterile PBS and then fluorescently labeled by a 1-h incubation with FITC-Goat Anti-Mouse IgG (H + L) antibody (Invitrogen, Frederick, MD) diluted 1/100 (1.0%) in PBS/BSA supplemented with 2 mM sodium azide, followed by three washes with sterile PBS. All rinses and incubation periods were performed with gentle rocking at ambient temperature. Steps following use of fluorescent antibodies used covered chambers to protect the fluorochrome from quenching by exposure to light. After the final PBS wash, monolayers in each well were sealed using three drops of poly(vinyl alcohol) mounting medium with DABCO¹⁶ under an 18 mm² glass coverslip and stored covered at 4 °C.

Microscopy

Zeiss AxioVision software (Carl Zeiss, Thornwood, NY) systematically captured 72 adjacent immunofluorescent microscopical fields for each culture chamber at 100× magnification, representing approximately 20% of each monolayer. Images were captured using a Zeiss HRm digital camera (Carl Zeiss) on an AxioVert 200 M microscope (Carl Zeiss). Zeiss Vision Image (zvi) files were converted to JPEG files. More than 82 000 cell culture images were then visually assessed for defects (i.e., antibody labeling quality, lack of focus, missing monolayer), then analyzed by ImageJ software¹⁷ customized to enumerate individual meronts and gamonts based on size (~3–5 μm), shape (circularity), and labeling by fluorescent antibody (see Figure 1a,b). Microsoft Excel (Redmond, WA, U.S.A.) was used to calculate average number and size of developing life cycle stages per microscopical field. The number of developing stages in culture is directly related to the number of inoculated oocysts which contain infectious sporozoites.¹³ For each sample, an average count of developing stages in acceptable fields of duplicate wells was divided by the average number of microscopical fields counted; back-calculation provided expected counts for an entire well consisting of 350 microscopical fields. The original sample volume concentrated (e.g., 4 mL) and the volume of the concentrate applied to each well (i.e., 0.15 mL) were used to determine developing stages per mL in flasks. All in vitro parasite concentrations were log₁₀ transformed and compared to control flasks to determine log₁₀ inactivation.

CT Calculation

Free chlorine decay was defined as 20% decay in the free chlorine concentration from that at time 0 h; all *C. parvum* inactivation data associated with 20% decay in free chlorine were excluded. CT values were calculated for experiments that reached target log₁₀ inactivation; data from experiments that did not achieve the target level of *C. parvum* inactivation were excluded from CT calculation to avoid extrapolation. Free chlorine concentrations in each individual flask throughout the experimental contact time were arithmetically averaged to provide the overall concentration for that flask. *C. parvum* inactivation throughout the experimental contact time in each individual flask was determined by subtracting log₁₀[*C. parvum*] at each experimental time point from log₁₀[*C. parvum*] at time 0 h. A linear trend line was fit to a scatter plot of time versus log₁₀

inactivation; if R^2 values were > 0.9 , then the resulting equation was used to determine time required for the target level of log inactivation. Assuming first order kinetics, the CT value for each individual flask was calculated by multiplying the average free chlorine concentration by the time required to achieve the target level of inactivation. For each condition studied, these CT values were arithmetically averaged.

Statistical Analysis

To assess the association between CT values and oocyst age, the Pearson correlation coefficient was calculated between 3-log_{10} CT and oocyst age. To investigate water parameter measurements (e.g., pH, ORP, free chlorine concentration) between and across experimental groups, and \log_{10} inactivation across time, linear mixed models were implemented to account for the random effects arising from replicates with respect to the same CYA and free chlorine settings using SAS 9.3 (Cary, NC). Specifically, from modeling \log_{10} inactivation by time, linear trends and their corresponding 95% confidence intervals were estimated for each group and plotted in R software (Vienna, Austria).¹⁸ As these models focus on the inactivation trend rather than the 3-log_{10} CT value, experiments that did not achieve 3-log_{10} reduction but had minimal free chlorine decay were included in these analyses. The LOWESS (locally weighted scatterplot smoothing) method was also implemented to graphically illustrate a smooth trend line across time for CYA = 100 mg/L and ODF control groups. Comparisons of the \log_{10} inactivation among control and experimental groups were performed at selected time points. For all comparisons, Bonferroni correction, in which the original significance level is divided by the number of comparisons to derive the adjusted significance level for each individual comparison, was applied to adjust for the multiple comparisons.

RESULTS

ODF Water Control

The average ORP in ODF water control flasks ($n = 12$) was 275 mV (range 178–390 mV) (Table 1). In ODF water at 25 °C, natural oocyst die-off was $<0.5 \log_{10}$ through approximately 96 h; there was an approximate 1-log_{10} reduction in viability by 172 h and at least a 2.4-log_{10} reduction by 360 h (Figure 2c).

20 mg/L Free Chlorine Control

The average free chlorine concentration in all 20 mg/L free chlorine control flasks ($n = 11$) was 20.9 mg/L (range 17.2–24.8 mg/L) and the average ORP was 770 mV (range 724–819 mV). A 3-log_{10} inactivation of oocysts was achieved in six 20 mg/L free chlorine control experiments after an average contact time of 8.2 h (range 6.9–9.5 h) (Figure 2a). The average estimated CT value for a 3-log_{10} inactivation of oocysts was 10,500 mg·min/L (range 9200–12 000 mg·min/L) (Table 2).

40 mg/L Free Chlorine Control

The average free chlorine concentration in all 40 mg/L free chlorine control flasks ($n = 8$) was 40.3 mg/L (range 36.6–43.8 mg/L) and the average ORP was 796 mV (range 753–831 mV). A 3-log_{10} inactivation of oocysts was achieved in seven 40 mg/L free chlorine

control experiments after an average contact time of 5.1 h (range 4.2–6.3 h) (Figure 2b). The average estimated CT value for a 3-log₁₀ inactivation of oocysts was 12 400 mg·min/L (range 10 300–15 200 mg·min/L).

Low CYA Concentration Experiments

8 mg/L CYA Experiments—At 20 mg/L free chlorine (average 21.1 mg/L, range 19.6–24.0 mg/L) and with an average of 8 mg/L CYA (range 7–9 mg/L) ($n = 4$ flasks), the average ORP was 770 mV (range 744–792 mV). A 3-log₁₀ inactivation of oocysts was achieved in three experiments after an average contact time of 14.1 h (range 13.0–16.3 h) (Figure 2a). The average estimated CT value for a 3-log₁₀ inactivation of oocysts was 17 800 mg·min/L (range 16 000–20 300 mg·min/L).

At 40 mg/L free chlorine (average 41.0 mg/L, range 34.8–43.2 mg/L) and with an average of 9 mg/L CYA (range 8–9 mg/L) ($n = 4$ flasks), the average ORP was 773 mV (range 750–786 mV). A 3-log₁₀ inactivation of oocysts was achieved in all four experiments after an average contact time of 6.2 h (range 6.1–6.4 h) (Figure 2b). The average estimated CT value for a 3-log₁₀ inactivation of oocysts was 15 300 mg·min/L (range 14 500–16 100 mg·min/L).

16 mg/L CYA Experiments—At 20 mg/L free chlorine (average 19.5 mg/L, range 17.3–21.6 mg/L) and with an average of 16 mg/L CYA (range 16–17 mg/L) ($n = 4$ flasks), the average ORP was 757 mV (694–777 mV). A 3-log₁₀ inactivation of oocysts was achieved in two experiments after an average contact time of 27.5 h (range 27.0–27.9 h) (Figure 2a). The average estimated CT value for a 3-log₁₀ inactivation of oocysts was 31 500 mg·min/L (range 30 900–32 100 mg·min/L).

At 40 mg/L free chlorine (average 37.4 mg/L, range 33.2–40.6) and with an average of 15 mg/L CYA (range 14–16 mg/L) ($n = 4$ flasks); no ORP data are available due to technical difficulties. A 3-log₁₀ inactivation of oocysts was achieved in two experiments after an average contact time of 8.4 h (range 8.4–8.5 h) (Figure 2b). The average estimated CT value for a 3-log₁₀ inactivation of oocysts was 19 400 mg·min/L (range 19 300–19 500 mg·min/L).

High CYA Concentration Experiments

50 mg/L CYA Experiments—At 20 mg/L free chlorine (average 20.7 mg/L, range 16.2–28.6 mg/L) and with an average of 48 mg/L CYA (range 45–52 mg/L) ($n = 4$ flasks), the average ORP was 653 mV (range 546–728 mV). A 1-log₁₀ inactivation of oocysts was achieved in all four experiments after an average contact time of 61.9 h (range 43.5–79.4 h) (Figure 2a). The average estimated CT value for a 1-log₁₀ inactivation of oocysts was 76 500 mg·min/L (range 58 500–91 000 mg·min/L) (Table 3).

At 40 mg/L free chlorine (average 38.5 mg/L, range 34.6–42.6 mg/L) and with an average of 46 mg/L CYA (range 41–52 mg/L) ($n = 4$ flasks), the average ORP was 737 mV (range 682–771 mV). A 1-log₁₀ inactivation of oocysts was achieved in two experiments after an average contact time of 17.2 h (range 13.5–23.4 h) (Figure 2b). The average estimated CT value for a 1-log₁₀ inactivation of oocysts was 40 000 mg·min/L (range 31 100–55 300 mg·min/L).

100 mg/L CYA Experiments—At both 20 and 40 mg/L free chlorine, 1- \log_{10} CT values could not be calculated for 100 mg/L CYA experiments due to the nonlinearity of the data. Instead, \log_{10} reduction values are provided.

At 20 mg/L free chlorine (average 20.3 mg/L, range 15.6–24.2 mg/L) and with an average of 98 mg/L CYA (range 90–100 mg/L) ($n = 4$ flasks), free chlorine concentrations did not decay 20% (compared with free chlorine concentration at time 0 h) up to a minimum of 144 h. The average ORP was 652 mV (range 614–688 mV). There was an average 0.8- \log_{10} reduction of oocysts at 72 h and 1.6- \log_{10} inactivation at 144 h (Figure 2c).

At 40 mg/L free chlorine (average 38.7 mg/L, range 30.4–42.6 mg/L) and with an average of 90 mg/L CYA ($n = 4$), the free chlorine concentration did not decay 20% up to a minimum of 72 h. The average ORP was 692 mV (range 660–729 mV). There was an average 0.8- \log_{10} reduction of oocysts at 24 h and 1.4- \log_{10} inactivation at 72 h (Figure 2c).

Statistical Comparisons

Oocyst Age—Across all experiments, the average age of oocysts at the time of experiment was 74 days (range 22–110 days). No systematic pattern was detected on a scatter plot between oocyst age and CT value in 20 and 40 mg/L free chlorine control groups (data not shown). Although a similar examination of oocyst age versus CT value among CYA experimental groups is restricted due to small sample sizes, this result suggests that oocyst age within the range tested was not associated with altered CT values for CYA experimental groups, as free chlorine controls utilizing the same oocyst lot were always conducted in parallel.

pH—Across all experimental conditions, the average pH over the duration of all experiments was 7.6 (range 7.4–7.7). pH was not statistically different between free chlorine controls and low CYA and ~50 mg/L CYA experimental groups ($p > 0.03$; adjusted significance level = 0.005); however, pH in ~100 mg/L CYA experiments was practically the same (average pH 7.5), but statistically lower ($p < 0.001$) in comparison.

Free Chlorine and ORP—Average free chlorine concentrations were consistent across flasks containing 20 mg/L free chlorine or flasks containing 40 mg/L. ORP was not statistically different between free chlorine controls and low concentration CYA experimental groups ($p > 0.02$; adjusted significance level = 0.005), however ORP in ODF controls and high concentration CYA experiments was statistically lower ($p < 0.001$ for all) in comparison.

Log₁₀ Inactivation—Comparisons of the control and experimental groups performed at select time points indicated that at both 20 and 40 mg/L free chlorine, \log_{10} inactivation was not significantly different early in the disinfection process (up to 1 h for 20 mg/L and up to 0.5 h for 40 mg/L). However, as early as 2 h, \log_{10} inactivation was significantly lower in the presence of low CYA concentrations and ~50 mg/L CYA (Table 4).

DISCUSSION

Fecal incidents in public pools are relatively common and have the potential to transmit infectious pathogens among swimmers.¹⁹ The extreme chlorine tolerance of *Cryptosporidium* led to public health recommendations to treat a diarrheal release as a potential high-risk *Cryptosporidium* contamination event requiring hyperchlorination remediation as well as in response to suspected or confirmed recreational water-associated outbreak of cryptosporidiosis. As a result, CDC recommends increasing the free chlorine concentration to 20 mg/L for 12.75 h (CT value = 15 300 mg·min/L) for a 3-log₁₀ inactivation of *Cryptosporidium* oocysts in the absence of CYA.⁴ In this study, we report increased CT values for inactivation of the *C. parvum* Maine isolate in the presence of low and high concentrations of CYA, at both 20 and 40 mg/L free chlorine (average pH 7.6, 25 °C). These data indicate that addition of CYA substantially delays *Cryptosporidium* inactivation as CYA concentration increases. Changes in disinfection efficacy are also suggested by the modest but statistically significant decrease in ORP readings observed between free chlorine controls and high CYA concentration experiments.

At 20 mg/L free chlorine, the presence of 8 and 16 mg/L CYA resulted in average estimated 3-log₁₀ CT values (17 800 and 31 500 mg·min/L, respectively), nearly 2–3X higher than CT values calculated for the 20 mg/L free chlorine control (10 500 mg·min/L). At 9 and 15 mg/L CYA, doubling the free chlorine concentration to 40 mg/L resulted in average estimated 3-log₁₀ CT values (15 300 and 19 400 mg·min/L, respectively); these CT values were lower than those calculated at 20 mg/L free chlorine, but still higher than that calculated for the 40 mg/L free chlorine control (12 400 mg·min/L). At 20 mg/L free chlorine, the presence of 48 mg/L CYA resulted in a nearly 22× increase in the average estimated 1-log₁₀ CT value (76 500 mg·min/L) as compared with the 20 mg/L free chlorine control (3500 mg·min/L). At 40 mg/L free chlorine and 46 mg/L CYA, the average estimated 1-log₁₀ CT value increase was nearly 10X that of the 40 mg/L free chlorine control (40 000 and 4100 mg·min/L, respectively). At both low CYA concentration and ~50 mg/L CYA, log₁₀ inactivation was significantly less than 20 and 40 mg/L free chlorine controls within an exposure time of 2 h.

In the presence of ~100 mg/L CYA, 1-log₁₀ CT values could not be calculated due to nonlinearity of the data; however, at 72 h, average 0.8- and 1.4-log₁₀ reductions were achieved with 20 and 40 mg/L free chlorine concentrations, respectively. ODF water control data indicate that natural oocyst die-off was 0.5-log₁₀ by 96 h and continued to increase to 2.4-log₁₀ by 360 h. This suggests that natural oocyst die-off played an increasingly substantial role in the reduction of infectious oocysts over extended experimental time periods when CYA was present.

As in this study, Shields¹¹ reported a significant reduction in *C. parvum* inactivation in the presence of CYA. However, several notable differences exist between the Shields study and the current study. Shields extrapolated data and suggested that in the presence of 50 mg/L CYA, increasing the free chlorine concentration to 40 mg/L and decreasing the pH to 6.5 would be needed to achieve a 3-log₁₀ inactivation (extrapolated CT value of 67 000 mg·min/L, based on a 2.7-log₁₀ inactivation). Such a response protocol would require

substantial amendment of pool chemistries, which could pose challenges to pool operators depending on their level of training. In the current study, experiments were conducted at pH 7.5 (considered a more realistic option for typical pool operation and remediation) and lower CYA concentrations were investigated to enable estimation of CT values based on measurable 3-log₁₀ reductions. Data from this study showed that 62 h were required to inactivate 90% (1-log₁₀) of *Cryptosporidium* in the presence of approximately 50 mg/L CYA at 20 mg/L free chlorine; however, Shields reported a 0.7-log₁₀ reduction of *C. parvum* Iowa oocysts at 10 h under these same conditions. These differences might be due to this study's use of improved methodological and analytical procedures, such as automated image analysis. In addition, the improved methods used in this study suggest that the lower CT value derived for *Cryptosporidium* inactivation in the absence of CYA (10 500 mg-min/L), when compared to the Shields data (15 300 mg-min/L),¹⁴ should be evaluated for inclusion in future updates to diarrheal fecal incident remediation recommendations.

This study was subject to several notable limitations. First, water in recreational water venues can contain materials introduced by bathers and the environment, including nitrogenous wastes (e.g., sweat, urine), sunscreen and other personal care products, organic debris, disinfection byproducts, and other chemicals that make it a complex and ever-changing chemistry. Furthermore, uncovered outdoor pools receive exposure to ultraviolet (UV) light which affects free chlorine chemistry both in the presence and absence of CYA. This study was conducted in oxidant-demand-free water under ideal swimming pool water conditions without exposure to UV light to minimize and control experimental variability. Further research is warranted to evaluate disinfection differences that might exist in waters more representative of actual recreational swimming venues. Second, due to the complexity, labor-intensity, and high cost of experiments, the sample size for each CYA concentration was low. Data for additional replicates and CYA concentrations might help further elucidate relationships between CYA, free chlorine concentrations, *C. parvum* inactivation, and resulting CT values. Additional research evaluating variations in temperature and pH as well as research further evaluating the complex chemistry of these matrices,²⁰ including the relationship between molar ratios and disinfection efficacy, is also warranted. Third, 3-log₁₀ CT values were not able to be calculated in high CYA concentration experiments due to excessive free chlorine decay over extended experimental time periods (up to 360 h). Maintaining the target free chlorine concentration throughout entire experimental time periods, perhaps by means of an automated chlorine feed system, would have provided improved data for calculating CT values.

This study demonstrates that cyanuric acid significantly reduces the efficacy of chlorination for inactivating *Cryptosporidium* oocysts. Data from low CYA experiments indicate that hyperchlorination can still be effective in achieving 3-log₁₀ inactivation of *Cryptosporidium* when CYA concentrations are low (~8 mg/L), but higher chlorine concentrations (e.g., 30–40 mg/L) might be needed to achieve such reductions within a time frame typically used for hyperchlorination (e.g., 12.75 h) (Figure 2). On the basis of the results of this study, existing responses to diarrheal fecal incidents and suspected or confirmed recreational water-associated outbreaks of cryptosporidiosis that rely on hyperchlorination are inadequate when used in pools that contain higher levels of cyanurate (50–100 mg/L), including concentrations currently acceptable in many state pool codes and

the Model Aquatic Health Code. Such pools would need to reduce CYA levels to achieve 3-log₁₀ inactivation conditions or employ an alternate disinfectant technique for remediation. While data for alternatives to hyperchlorination are not robust, some scientific literature is available that suggests that using disinfection technologies such as chlorine dioxide,²¹ UV irradiation systems,²² or ozonation²³ could be effective for achieving 3-log₁₀ inactivation of *Cryptosporidium* in aquatics facilities that use CYA-based water treatment products. In addition, facility operators also have the option of removing *Cryptosporidium* oocysts from affected pool systems by enhanced filtration or removing and replacing contaminated water in the systems. These findings suggest that implementable changes or alternatives to hyperchlorination remediation following diarrheal fecal incidents should be further investigated for use in recreational water venues that utilize CYA-based chlorine stabilization products.

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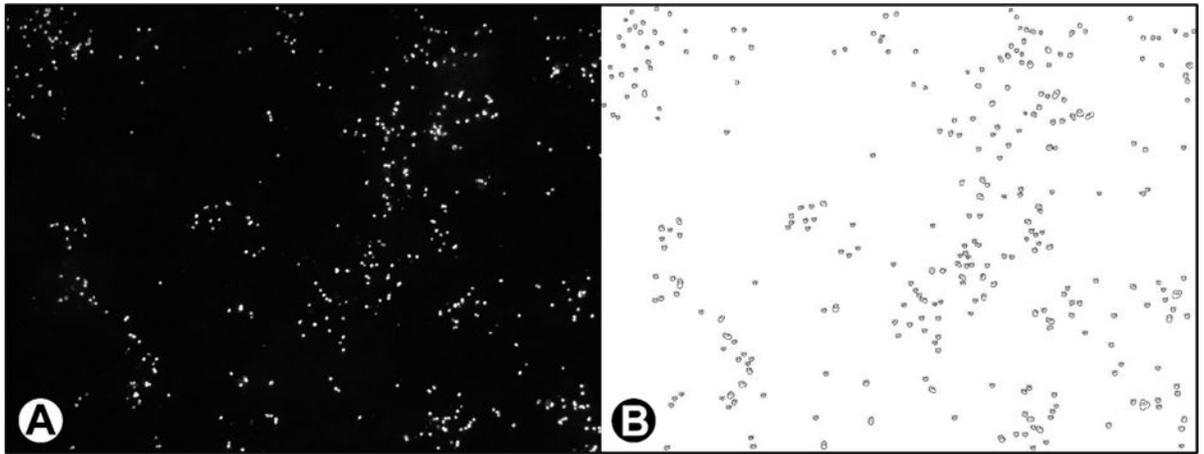


Figure 1.

(a) Microscopical field with *C. parvum* developing stages labeled with a parasite-specific fluorescent monoclonal antibody; (b) counterpart image with *C. parvum* life stages enumerated by ImageJ Software.

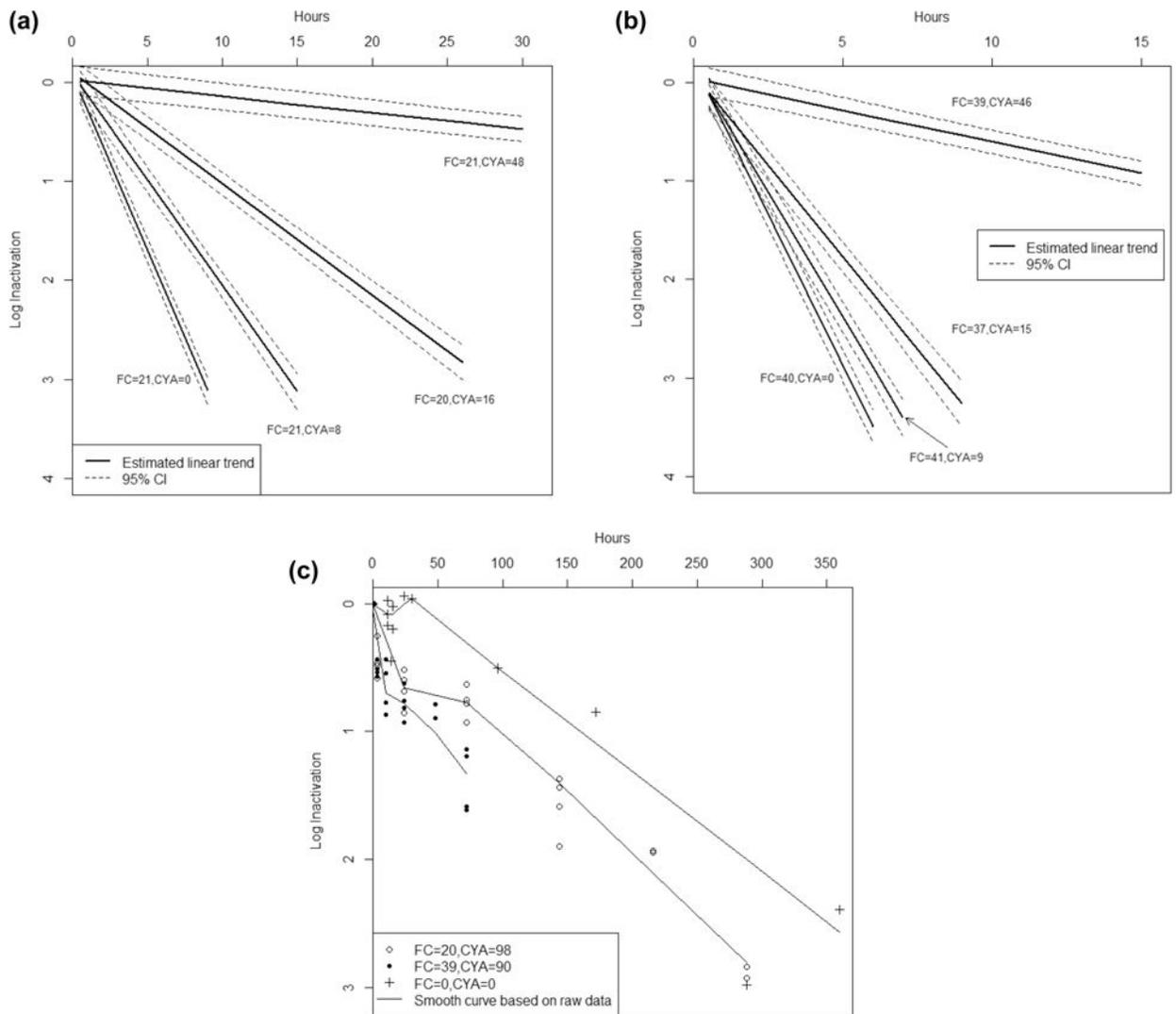


Figure 2.

(a) Time (hr) versus log₁₀ inactivation of *C. parvum* (95% confidence interval) at 20 mg/L free chlorine (FC) for cyanuric acid (CYA) concentrations of 0–48 mg/L. Note: solid lines represent the linear trend estimated by a linear mixed model; graphical representations of log₁₀ inactivation utilize linear interpolation to communicate experimental results and should not be viewed as suggesting a kinetic model for the actual oocyst disinfection process. (b) Time (hr) versus log₁₀ inactivation of *C. parvum* (95% confidence intervals) at 40 mg/L FC for CYA concentrations 0–46 mg/L. Note: solid lines represent the linear trend estimated by a linear mixed model; graphical representations of log₁₀ inactivation utilize linear interpolation to communicate experimental results and should not be viewed as suggesting a kinetic model for the actual oocyst disinfection process. (c) Time (hr) versus log₁₀ inactivation of *C. parvum* (95% confidence intervals) at 20 and 40 mg/L FC for 100 mg/L CYA and oxidant demand-free (ODF) water control. Note: points represent the observed raw data and solid lines represent the approximated smooth curved based on raw data.

Table 1
Descriptive Characteristics for Oxidant Demand-Free (ODF) Water Control, Free Chlorine (FC) Controls, and Low and High Concentration Cyanuric Acid (CYA) Experiments

experimental type	no. experiments	avg oocyst age (days)	avg pH ^a	avg FC (mg/L) ^a	avg ORP (mV) ^{a,c}	avg CYA (mg/L) ^b
ODF control	12	74	7.6	0.0	275	0
21.6 mg/L FC	11	72	7.6	20.9	770	0
21.1 mg/L FC8 + mg/L CYA	4	52	7.6	21.1	770	8
19.1 mg/L FC + 16 mg/L CYA	4	90	7.6	19.5	757	16
21.2 mg/L FC + 48 mg/L CYA	4	70	7.6	20.7	653	48
21.2 mg/LFC + 98 mg/L CYA	4	40	7.5	20.3	652	98
40.6 mg/L FC	8	75	7.6	40.3	796	0
40.9 mg/LFC + 9 mg/L CYA	4	107	7.6	41.0	773	9
38.3 mg/L FC + 15 mg/L CYA	4	86	7.6	37.4	ND ^d	15
38.5 mg/L FC + 46 mg/L CYA	4	70	7.6	38.5	737	46
39.4 mg/L FC + 90 mg/L CYA	4	40	7.5	38.7	692	90

^a Average of measurements taken at each sampling time point (generally 4–5 time points per experiment).

^b Average measurement at time 0 h.

^c ORP: oxidation reduction potential.

^d ND: no data.

Table 2

Average Estimated 3- \log_{10} Inactivation CT Values for *C. parvum* in Free Chlorine (FC) Controls and Low Concentration Cyanuric Acid (CYA) Experiments; Average pH 7.6

average FC conc. (mg/L)	average CYA conc. (mg/L)	average time 3- \log_{10} inactivation (hr)	average estimated 3- \log_{10} CT value (mg-min/L) (range)
21.6	0	8.2	10 500 (9200–12 000)
21.1	8	14.1	17 800 (16 000–20 300)
19.1	16	27.5	31 500 (30 900–32 100)
40.6	0	5.1	12 400 (10 300–15 200)
40.9	9	6.2	15 300 (14 500–16 100)
38.3	15	8.4	19 400 (19 300–19 500)

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Table 3

Average Estimated 1- \log_{10} Inactivation CT Values for *C. parvum* in Free Chlorine (FC) Controls and 50 mg/L Cyanuric Acid (CYA) Experiments; Average pH 7.6

average FC conc. (mg/L)	average CYA conc. (mg/L)	average time 1- \log_{10} inactivation (hr)	average estimated 1- \log_{10} CT value (mg-min/L) (range)
21.6	0	2.7	3500 (3100–4000)
21.2	48	61.9	76 500 (58 500–91 000)
40.6	0	3.7	4100 (3400–5100)
38.5	46	17.2	40 000 (31 100–55 300)

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Table 4 Statistical Comparisons of Log₁₀ Inactivation at Different Cyanuric Acid (CYA) Concentrations and Select Time Points

FC ^c = 20 mg/L	CYA (mg/L)	p value			
		0.5 h	1 h	2 h	3+ hr
0 vs 8	0.355	0.080	<0.001 ^a	<0.001 ^a	<0.001 ^a
0 vs 16	0.268	0.056	<0.001 ^a	<0.001 ^a	<0.001 ^a
0 vs 48	0.837	0.328	0.012 ^b	<0.001 ^a	<0.001 ^a
8 vs 16	0.369	0.108	0.003 ^a	<0.001 ^a	<0.001 ^a
8 vs 48	0.910	0.424	0.031 ^b	<0.001 ^a	<0.001 ^a
16 vs 48	0.946	0.528	0.044 ^b	<0.001 ^a	<0.001 ^a
		p value			
FC ^c = 40 mg/L	CYA (mg/L)	0.5 h	1 h	2 h	3+ hr
0 vs 9	0.282	0.011 ^b	<0.001 ^a	<0.001 ^a	<0.001 ^a
0 vs 15	0.569	0.037 ^b	<0.001 ^a	<0.001 ^a	<0.001 ^a
0 vs 46	0.893	0.072	<0.001 ^a	<0.001 ^a	<0.001 ^a
9 vs 15	0.362	0.024 ^b	<0.001 ^a	<0.001 ^a	<0.001 ^a
9 vs 46	0.590	0.050 ^b	<0.001 ^a	<0.001 ^a	<0.001 ^a
15 vs 46	0.306	0.015 ^b	<0.001 ^a	<0.001 ^a	<0.001 ^a

^a significant ($p < 0.008$) (adjusted significance level).

^b marginally significant ($p = 0.009-0.05$).

^c FC: free chlorine.