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ENVIRONMENT RESEARCH

Ozone disinfection of treated wastewater for inactivation of *Cryptosporidium parvum* for agricultural irrigation

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Abstract

The reliance on agriculture in many nations has increased the use of treated wastewater for irrigation. However, reclaimed water still poses health risks from resistant pathogens like *Cryptosporidium* spp. Ozone, a strong disinfectant, has been used in water treatment. This study assessed the microbiological quality of treated wastewater for irrigation and evaluated ozone effectiveness in inactivating *C. parvum* oocysts. All samples contained *Cryptosporidium* spp., with 163 to 850 oocysts 100 L^{-1}, and 50% contained viable oocysts. When *C. parvum* was exposed to different ozone residual concentrations (0.1, 0.8, and 1.3 mg L^{-1}), oocyst viability reduction of 73%, 85%, and 99% and infectivity of 0.8, 1.36, and 2 Log_{10} was achieved. The predicted values for infectious oocysts were 4.19, 3.64, and 3.27, representing absolute counts of infective oocysts after ozone treatment. These findings demonstrate ozone's effectiveness in inactivating *C. parvum* in treated wastewater, supporting its potential for safe water reuse.

Practitioner Points:

- All wastewater samples contained *Cryptosporidium* spp., with 163 to 850 oocysts per 100 L.
- Wastewater had 50% contained viable oocysts.
- Ozone concentrations (0.1, 0.8, 1.3 mg/l) achieved oocyst viability of 73.33%, 85.0%, and 99.4%, respectively.
- The predicted values for infectious oocysts were 4.19, 3.64, and 3.27, respectively for each ozone concentration.

KEYWORDS

C. parvum, oocyst infectivity, oocysts inactivation, ozone, treated wastewater reuse

INTRODUCTION

Water is an indispensable resource for human activities, economic development, and social welfare (Goswami & Bisht, 2017). However, factors such as population growth, economic development, urbanization, and pollution have caused the overexploitation of water sources, promoting problems of water scarcity (Hassan Rashid et al., 2018; Ren et al., 2014). With agriculture responsible for a staggering 70% of worldwide freshwater withdrawals, the sector is disproportionately impacted by water scarcity, making it imperative to explore alternative water sources (Bank, 2022; OECD, 2023). In response to this challenge, several countries, including Singapore, Israel, China, Australia, Libya, Sweden, the USA, and Mexico, have turned to treated wastewater as a potential solution to alleviate water shortages for agricultural use (Almanza, 2000; Cifuentes et al., 1993; Matos et al., 2014; Tram Vo et al., 2014). Notably, the Atotonilco Wastewater Treatment Plant (WWTP) in the state of Hidalgo, Mexico, boasts one of the largest treatment capacities globally where treated wastewater is used for agricultural irrigation (Water Technology, 2023).

However, the utilization of treated wastewater for agricultural irrigation introduces potential health risks due to the presence of resilient forms of pathogens and parasitic agents (Adegoke et al., 2018; Matos et al., 2014). Among these, *Cryptosporidium* stands out as a significant concern, contributing to waterborne diseases globally. *C. parvum* significantly impacts public health due to its ability to cause waterborne outbreaks of severe diarrhea, especially in contaminated water sources. It is particularly dangerous for immunocompromised individuals, where infections can become life-threatening. With its global prevalence, especially in areas with poor sanitation, and limited treatment options, *C. parvum* remains a major concern for vulnerable populations and water safety (Franceschelli et al., 2022; van der Giessen et al., 2021).

Traditional wastewater disinfection methods include chlorination, which can struggle with *Cryptosporidium* and generate toxic by-products, and UV radiation, which inactivates microorganisms without by-products but depends on water quality. These limitations heighten public health concerns because cyst- and spore-forming pathogens, such as *Cryptosporidium*, are generally more resistant to chlorine due to their protective structures, which shield them from chemical disinfectants (EPA, 2001; Morrison et al., 2022; Nasser, 2016).

To address the limitations of conventional disinfection practices, an alternative in the form of ozone treatment has gained prominence in various treated wastewater plants worldwide, including those in the USA, Canada, Germany, and Japan (Burns et al., 2007; Gerrity et al., 2017). For almost half a century, ozone has played a crucial role in the disinfection of drinking water and treated wastewater. Additionally, it has been utilized in various applications related to health and industry (Loeb, 2023). Ozone is generally more effective and does not leave toxic residues, but its high cost and lack of stability can be disadvantages compared to chlorine. Ozone offers advantages such as superior oxidative power compared to chlorine and the ability to target a wide range of microorganisms, including the resilient oocysts of *Cryptosporidium*, as highlighted in studies by Khadre et al. (2006) and Morrison et al. (2022).

However, essential to note that while promising results have been demonstrated in studies using artificial wastewater, questions linger about the real-world efficacy of ozone-treated wastewater conditions.

Recent studies have demonstrated significant log reduction values for *Cryptosporidium* oocysts in reclaimed water following ozonation. Research indicates that ozone treatment can achieve reductions of up to 1–2 Log_{10}, depending on the ozone dose and contact time (Li et al., 2001; Morrison et al., 2022; Ran et al., 2010), providing a highly effective method for inactivating this resilient pathogen. Building on this foundation, this research aims to enhance understanding of the issue by assessing the microbiological quality of treated wastewater, evaluating the effectiveness of ozone in laboratory settings for inactivating *C. parvum*, and demonstrating these findings through an in vivo model.

MATERIALS AND METHODS

C. parvum strain characteristics

For the experimental investigation into the effects of ozone treatment on the inactivation and infectivity of *Cryptosporidium* oocysts, we utilized a bovine-sourced Iowa strain P102C of *C. parvum* obtained from the Sterling Parasitology Laboratory at the University of Arizona. This choice was made to ensure the targeted impact on the specific microorganism. Additionally, we employed high concentrations to enable accurate visualization of the reductions achieved as a result of the treatment.

Study area

The descriptive study involved collecting treated wastewater samples from a specific WWTP located in Costa Rica, Culiacan, Sinaloa, Mexico (24° 36′ 02” N latitude and 107° 24′ 49” W). Conventional wastewater

treatment consists of pre-treatment, primary, secondary, and tertiary processes aimed at reducing microorganisms, with the use of chlorine and UV radiation to inactivate pathogens. This WWTP was chosen for analysis due to the urban nature of its wastewater source and its discharge into a side channel adjacent to a diverse food commodity growing area.

Sampling

Ten samples were collected per duplicate over a five-month period (February to June, encompassing winter and spring), with a frequency of twice a month, from WWTP. Each water sample (50 L) was placed in a polyurethane container, and 125 mL of a 2% sodium thiosulfate solution was added to neutralize any chlorine present in the sample. The samples were transported under refrigeration at 4°C and processed within 72 hours after collection. Sterile distilled water was used as the negative control.

Sample processing

The EPA method 1623 (EPA, 2005) was followed with minor modifications, which included replacing the immunomagnetic separation step with conventional separation using Percoll-sucrose (Supplementary Information 1).

Measurement of physical–chemical parameters

Simultaneously with sampling, the physical–chemical parameters of the wastewater were evaluated using a Hanna multiparameter, model HI9829–00041 (Woonsocket, RI, USA), which measures pH, temperature, turbidity, total suspended solids (TSS), conductivity, and oxygen demand. These tests were carried out according to the equipment instructions. Data were recorded using the meter's software for analysis.

For Biochemical Oxygen Demand (BOD): Samples were prepared according to the NMX-AA-028-SCFI-2021 protocol.

Viability of oocysts

To assess oocyst viability, the 1 mL tube was centrifuged at 1500 x *g* for 10 minutes at 4°C. The supernatant was aspirated down to 100 µL, and 100 µL of Acidified Hanks' solution (pH 2.75) was added, followed by a 1-hour incubation. Then, 10 µl of propidium iodide (PI, 1 mg mL^{-1}) and DAPI (2 mg mL^{-1}) were added and incubated for 90 minutes at 37°C. Monoclonal antibody (A100 FLK AquaGlo™ G/C Direct, Waterborne™ Inc, New Orleans, LA, USA was added and incubated for an additional 30 min. After three washes with non-acidified Hanks' solution, oocyst viability was determined using a LEICA DMI 6000 B inverted microscope (Wetzlar, HE, Germany) (Bukhari et al., 2000). Viable oocysts were identified based on specific criteria: those containing PI (propidium iodide) exhibited a bright red coloration and were categorized as non-viable. Conversely, oocysts containing DAPI (4′,6-diamidino-2-phenylindole) but excluding PI (DAPI+/PI-) displayed a sky-blue coloration, with all four nuclei of the oocyst visible, indicating viability. Oocysts lacking both DAPI and PI (DAPI-/PI-) were examined using Differential Interference Contrast (DIC) microscopy, and if all four nuclei were present, they were classified as viable oocysts (Campbell et al., 1992). Results were reported as the number of viable oocysts 100 L^{-1}. All experiments were replicated three times.

Oocyst inactivation with ozone

To investigate ozone-induced oocyst inactivation, the experiments were conducted as follows:

Preparation of oocyst inoculum

A 1.5 mL aliquot of the Iowa strain of *C. parvum* was washed three times with deionized water and then resuspended in 1 mL of Hanks solution. The oocyst concentration was determined using a LEICA DMI 3000 B phase-contrast microscope (Wetzlar, HE, Germany).

Ozone generation

Ozone was generated using an Apollo® corona discharge generator. The concentration of dissolved ozone was determined using AccuVac ampoules, employing the Indigo trisulphonate method as described by Widmer et al. (2002), and measured using the Hach® DR 3900 instrument (Loveland, CO. USA).

Ozone treatment

To evaluate the microbicidal effect of ozone, 4.1×10^6 oocysts were introduced into treated wastewater in a glass flask. Ozonized solution ranging from 8 to

10 mg L^{-1} was added to achieve the residual ozone concentrations (0.1, 0.8, and 1.3 mg L^{-1}) and allowed to act for 5 min. The mixture was then sampled to measure the remaining ozone which was neutralized using 3% sodium thiosulfate.

For oocyst recovery, the water-pathogen mixture underwent centrifugation at 1500 x *g* for 15 min at 4°C. The supernatant was reduced to 5 mL, and 0.85% saline was added to reach a final volume of 20 mL. Next, 30 mL of Sheather's 1:2 solution (specific gravity = 1.104) was added slowly by pipetting to create an interface. These tubes were then centrifuged at 1050 x *g* for 15 min at 4°C, followed by two washes with saline solution. After combining the contents and reducing the volume to 5 mL through centrifugation, we assessed oocyst viability and infectivity following ozone treatment by counting 200 oocysts in triplicate for each sample. All experiments were replicated three times.

Infectivity assays in mice

To assess *C. parvum* oocyst infectivity after ozone treatment, aliquots containing different oocyst concentrations (1×10^2, 1×10^3, 1×10^4, 1×10^5) were resuspended in 100 µL of phosphate-buffered saline solution. These aliquots were then orally administered to groups of three CD-1 mice for each oocyst concentration, each aged 6 weeks and weighing 21–25 g, using a #22 esophageal cannula. To immunosuppress the mice, 6.25 µg of dexamethasone per gram of body weight was administered six days before inoculation (Rasmussen & Healey, 1992). As a negative control, 100 µL of phosphate-buffered saline solution was administered to a group of three mice.

Fecal samples were collected from mice on days 3, 6, and 9 post-administration to monitor oocyst content. The feces were filtered through #100 mesh using a 0.01% Tween 20 solution. A 500 µL aliquot was mixed with 20 mL of 0.85% saline solution in a centrifuge tube. Sheather's 1:2 solution (specific gravity = 1.104 mg L^{-1}) was slowly added to create an interface, followed by centrifugation at 1050 x *g* for 15 min at 4°C. Then, 5 ml was aspirated, filled to 50 mL with saline solution, and centrifuged again. Finally, 5 mL was used for staining, identification, quantification, and oocyst viability assessment.

To determine the inactivation of oocysts through infectivity assays, we utilized the following equation:

$$\mathrm{Log}_{10} = \frac{N}{N_0} \quad (1)$$

For the calculation of N_0, we employed the empirical non-linear regression model described below:

$$N_0 = 3.5 - exp^{(a+b*LogC)} \quad (2)$$

a, b = are values given by the empirical model.

LogC = quantified oocyst concentration in mouse feces expressed in logarithm base 10.

where:

N is the estimated infectious dose per animal after ozone treatment.

N_0 is the number of oocysts given to each mouse.

All experiments were replicated three times.

Statistical analysis

For inactivation, the statistical analysis of the experimental part consisted of evaluating three ozone concentrations (0.1, 0.8, and 1.3 mg L^{-1}) and one contact time (5 min), using a two-factor totally randomized design. The response variable was viability reduction in which the effect of the ozone concentration factor was measured using an ANOVA for a totally randomized 2-factor design. Mean differences in the treatments that were significant were performed using Tukey's test ($p < 0.05$).

For infectivity assays analysis were estimated from an empirical non-linear regression model with the MINITAB statistical package: $\mathrm{Log}_{10} = \frac{N}{N_0}$ was the dependent variable.

All experiments were replicated three times.

RESULTS

Detection and viability of Cryptosporidium spp. oocysts in treated wastewater

To assess the presence, quantify, and determine the viability of *Cryptosporidium* oocysts in treated wastewater,

TABLE 1 Physicochemical characterization of the effluent from the WWTP in Costa Rica, Sinaloa, Mexico.

Parameter	Value
pH	7.2 ± 0.2
Temperature	29.1 ± 0.3°C
Turbidity	5.2 ± 0.8 NTU
Total suspended solids	323 ± 12 mg L^{-1}
Conductivity	672 ± 21 µs cm^{-1}
BOD_5	4.4 ± 0.3 mg L^{-1}

BOD: Biochemical Oxygen Demand.
Values mean ± standard deviation. The values reported are average values of three measurements.

we conducted a descriptive study. The physicochemical characterization of the treated wastewater from WWTP of Costa Rica, Sinaloa, Mexico, is provided in Table 1. At this stage of the study, the presence of oocysts was identified based on morphological characteristics, including a spherical shape, size ranging from 4 to 6 μm, a bright apple-green color, and internal structures corresponding to *Cryptosporidium* spp. Oocysts were found in 100% (10/10) of the samples (Figure 1).

The concentration of *Cryptosporidium* spp. oocysts ranged from 163 to 850 oocysts 100 L^{-1} of treated wastewater. In addition to confirming the presence and concentration of oocysts in the WWTP, the viability of the oocysts was determined through fluorescence microscopy. It was found that 50% (50/100) of the samples contained viable oocysts. The values reported in Table 2 are the average values of three replicates.

Inactivation and viability of oocysts after exposure to ozone

In order to determine the percentage of oocyst reduction after exposure to different concentrations of ozone, we assessed the viability of the oocysts. Ozone concentrations of 0.1, 0.8, and 1.3 mg L^{-1}, with a contact time of 5 min, exhibited a directly proportional relationship between residual ozone concentration and the achieved reduction percentage (Figure 2). The analysis of variance revealed significant differences ($p = 0.000$) in the ozone concentrations used and the reduction of *C. parvum* oocyst viability.

In our study, it was observed that utilizing a residual ozone concentration of 0.1 mg L^{-1} with a 5-minute contact time (Ct = 0.5 mg-min L^{-1}) resulted in a viability reduction of 73% (0.81 Log_{10}) of the oocysts. In contrast, with a residual ozone concentration of 0.8 mg L^{-1} and 5 min of contact time (Ct = 4.0 mg-min L^{-1}), an 85% (0.94 Log_{10}) reduction in oocyst viability was achieved. Moreover, employing a residual ozone concentration of 1.3 mg L^{-1} for 5 min (Ct = 6.5 mg-min L^{-1}) achieved a remarkable 99% (2.4 Log_{10}) reduction in oocyst viability (Figure 2).

Infectivity of oocysts after exposure to ozone

Results obtained for 0.1 mg L^{-1} ozone and 5 min of contact (Ct = 0.5 mg-min L^{-1}) indicated that 100% of mice

TABLE 2 Concentration and viability of *Cryptosporidium* spp. isolated from treated wastewater.

Sampling date	Concentration*	Viability
February 14	272 ± 1.16	177 ± 1.30
February 28	363 ± 1.24	254 ± 1.75
March 15	400 ± 1.09	ND
March 30	387 ± 0.78	96 ± 0.92
April 14	480 ± 1.48	ND
April 30	423 ± 0.83	127 ± 0.88
May 14	850 ± 1.23	255 ± 1.00
May 30	794 ± 1.56	ND
June 15	163 ± 0.70	ND
June 30	206 ± 0.92	ND

*Oocyst100 L^{-1}; ND, non-detected.
Values mean ± standard deviation. The values reported are average values of three replicates.

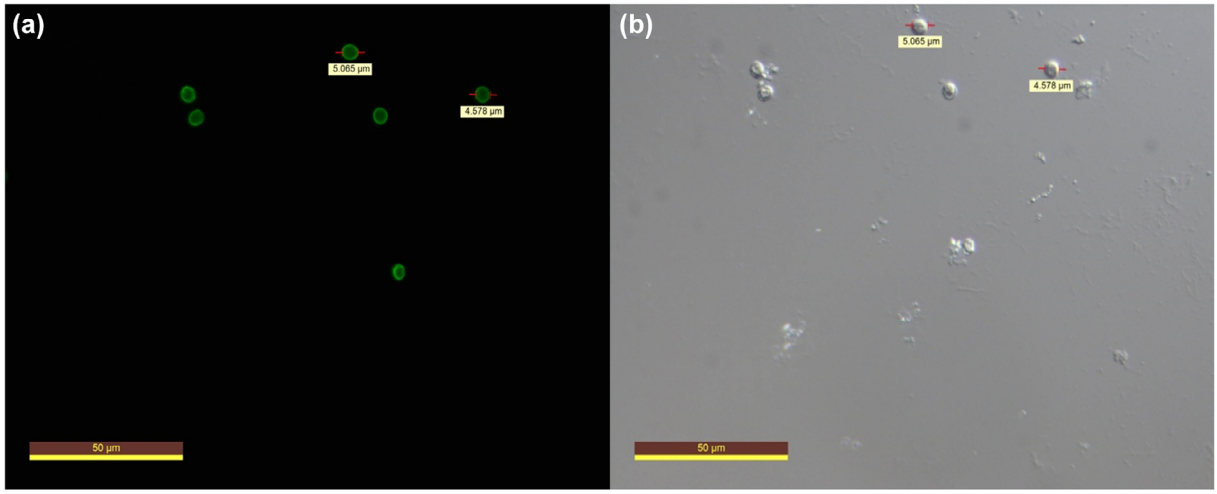

FIGURE 1 Morphological characteristics of *Cryptosporidium* spp. (A) Oocysts observed under fluorescence microscopy, (B) visualization of oocyst nuclei by differential interference contrast microscopy (DIC).

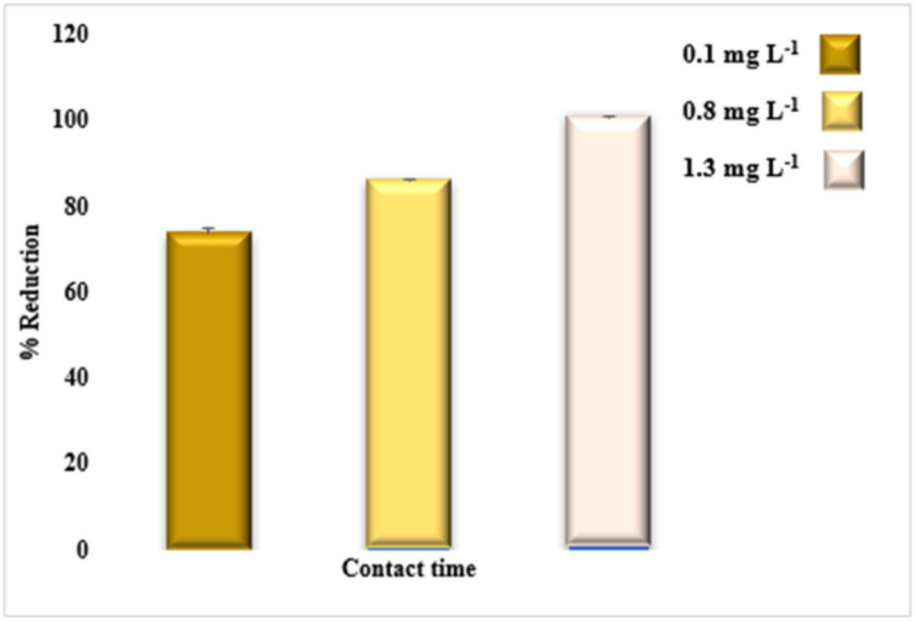

FIGURE 2 Percentage reduction in viability of *C. parvum* oocysts under different ozone concentrations, 5 min contact time, and 25°C. Values mean ± standard deviation.

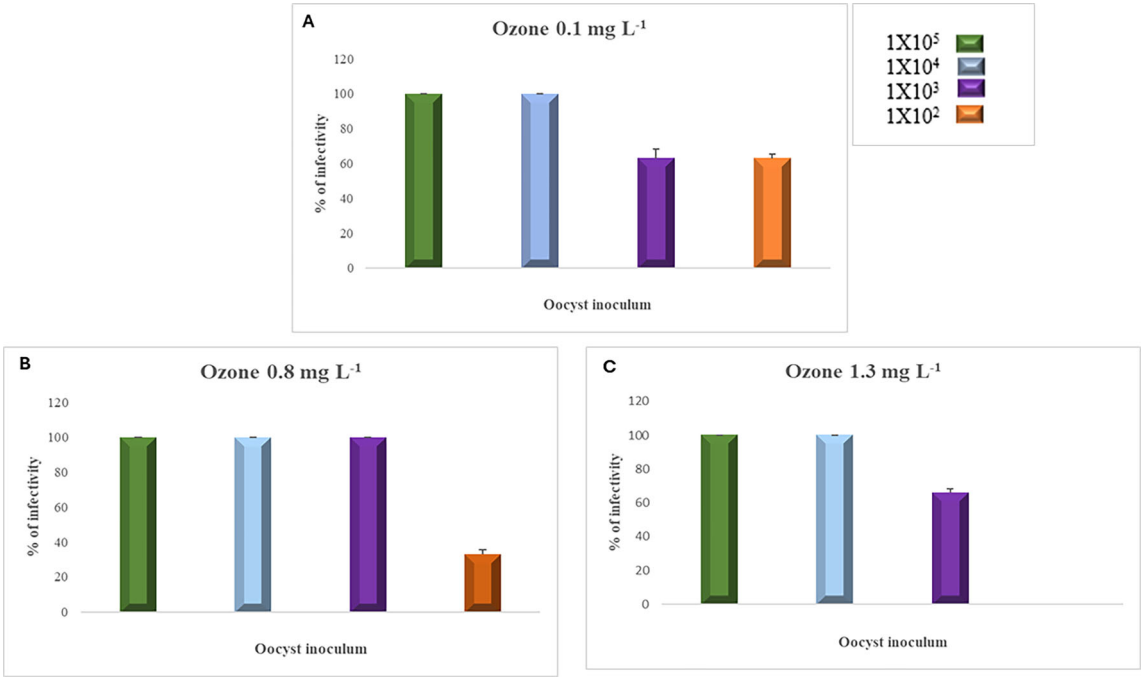

FIGURE 3 Infectivity in CD-1 mice inoculated with *C. parvum* oocysts exposed to different ozone residual and 5 min contact at 25°C. A) 0.1 mg L^{-1}, B) 0.8 mg L^{-1} and C) 1.3 mg L^{-1}. Values mean ± standard deviation.

inoculated with doses of 1×10^5 and 1×10^4 oocysts were infected, while for the 1×10^3 and 1×10^2 inoculum only 66% of mice were infected (Figure 3A). Nonlinear regression analysis for the inactivation of oocysts treated with this concentration showed that the N_0 value (prediction of infective oocysts after ozone treatment) was 4.19 oocysts (Figure 4A), and the decrease in survival of *C. parvum* oocysts was 0.8 Log_{10} (72% reduction).

For the concentration of residual ozone of 0.8 mg L^{-1} ozone and 5 min of contact (Ct = 4.0 mg-min L^{-1}). The results obtained showed that 100% of the mice inoculated with 1×10^5 and 1×10^4 oocysts were infected, indicating that the applied concentration was not sufficient to achieve at least a 4 Log_{10} inactivation. Similarly, as observed with the previous Ct, 100% of mice inoculated with 1×10^3 oocysts were infected; however, when a dose of 1×10^2 was administered, only 33% of mice were

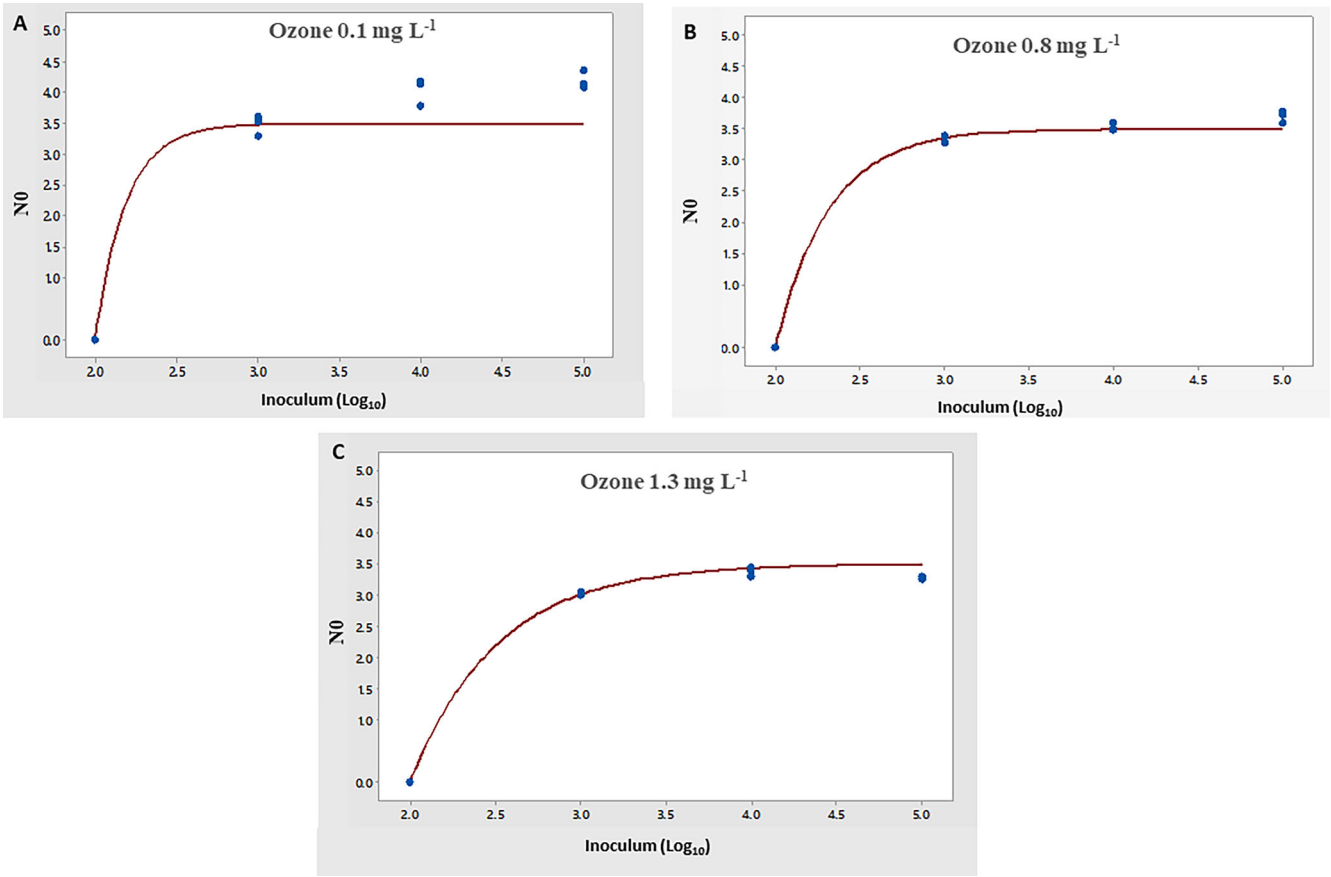

FIGURE 4 Prediction of infective oocysts (N_0) to different ozone residuals and 5 min contact at 25°C. **A**) 0.1 mg L^{-1}, **B**) 0.8 mg L^{-1} and **C**) 1.3 mg L^{-1}.

infected (Figure 3B). The N_0 value was 3.64 oocysts (Figure 4B), and the decrease in *C. parvum* oocyst survival was 1.36 Log_{10} (94% reduction).

Finally, the residual ozone concentration of 1.3 mg L^{-1} with 5 min of contact showed in the infectivity tests that mice inoculated with 1×10^5 and 1×10^4 oocysts resulted in 100% of animals infected. For an inoculum of 1×10^3 oocysts, 66% of individuals presented infection, while no infection was observed in individuals inoculated with 1×10^2 oocysts (Figure 3C).

The application of the non-linear regression model to this remaining ozone concentration resulted in an N_0 value of 3.27 (Figure 4C) and a *C. parvum* oocyst survival decrease of 1.72 Log_{10} (98% reduction).

DISCUSSION

In this study, the presence of oocysts was identified in 100% of the samples collected from the WWTP under investigation. This finding suggests that effluents of treated wastewater in Sinaloa could serve as a pathway for the dispersion of this pathogen because the disinfection methods such as chlorination and UV radiation were not efficient in eliminating this microorganism. The highest concentration of oocysts was detected in May. Some studies have reported a high presence of *C. parvum* oocysts during the autumn months; however, others studies have indicated that spring and autumn are the months with the highest incidence of *Cryptosporidium* which coincides with our research (Balderrama et al., 2012; Pepper et al., 2015).

The concentration of oocysts detected in effluent in our study ranged from 163 to 850 oocysts 100 L^{-1} of wastewater (equivalent to 1.6 to 8.5 oocysts and 2.5 viable oocysts L^{-1}). These results, however, differ in terms of concentration from previous studies, which reported a range of 16.6 to 200 oocysts 100 L^{-1} from the Cedritos Drain (Chaidez et al., 2005). It is important to note that water samples taken from a drain differ in concentration from those taken from a WWTP, as constant exposure to UV rays affects *C. parvum* oocysts (Morita et al., 2002; Soliman et al., 2018).

The concentrations of viable oocysts detected in this study (ranging from 4 to 21.2) exceed the infective dose of this pathogen, which is reported to be less than

10 oocysts per ingestion (Fayer et al., 2000). This is significantly higher than the concentrations reported during the 1993 Milwaukee, outbreak in the USA, where the oocyst concentration was less than 0.4 oocysts L^{-1}. The Milwaukee outbreak, which remains the largest worldwide, affected over 400,000 people and resulted in about 100 deaths; this was caused by the contamination of drinking water with wastewater (Corso et al., 2003). In comparison, Spain has reported high levels of *Cryptosporidium* oocysts in WWTPs (1–80 oocysts L^{-1}) (Castro-Hermida et al., 2010). These findings highlight the importance of continuous monitoring for the disinfection treatment used for this type of water, such as chlorination and UV radiation. Various countries have reported the presence of *C. parvum* in treated wastewater with various disinfection methods, including Rumania, China, Australia, Brazil, and Greece, among others; thus, increasing the statistics on waterborne diseases (Zahedi et al., 2021).

For the inactivation and viability reduction of oocysts after exposure to ozone with a Ct = 0.5 mg-min L^{-1}, there was a viability reduction of 73% (0.81 Log_{10}). These findings are consistent with other studies reporting a 67% reduction (0.74 Log_{10}) with a Ct = 0.8 mg-min L^{-1} (Bukhari et al., 2000). For a Ct = 4.0 mg-min L^{-1}, an 85% (0.94 Log_{10}) reduction in oocyst viability was achieved. Another study reported a Ct = 2.5 mg-min L^{-1}, achieving an 86% (0.95 Log_{10}) reduction in oocysts. The tests conducted in that study were carried out in water with recreated physicochemical conditions (Ran et al., 2010). Notably, under these conditions, the percentage of oocyst reduction is similar to our results. This outcome was expected, given the proximity of their Ct value to that of our research under similar conditions.

For a Ct = 6.5 mg-min L^{-1}, a 99% (2.4 Log_{10}) reduction in oocyst viability was achieved. In contrast, a study by Ran et al. (2010) reported a 92% (1.22 Log_{10}) reduction with a Ct = 5 mg-min L^{-1}. Although the residual ozone concentrations and contact times are similar. Ran et al. (2010) reported a lower percentage reduction which might be due to their tests being conducted at 20°C, whereas our study was conducted at 25°C. The higher temperature in our study, closer to real-world water conditions, likely contributed to the greater reduction in oocyst viability. Previous research indicates that lower temperatures require higher Ct values for effective oocyst inactivation (Li et al., 2001). Specifically, for every 10°C decrease in temperature, the Ct value must triple to achieve the same reduction, necessitating higher ozone concentrations or longer contact times (Rennecker et al., 1999).

In addition to the Ct values, it is important to note that the initial levels of *C. parvum* oocysts seem to play a significant role in the ability of treated samples to cause infection. Although ozone dosing is effective in reducing oocyst viability, recent studies and our own findings suggest that the residual infectivity may depend more on the initial concentration of *C. parvum* than on increasing the ozone dose. This aligns with previous research showing that even low levels of *C. parvum* are sufficient to cause infection, which may explain the observed discrepancy between viability and infectivity in some of our results.

Cryptosporidium oocysts were inoculated at various concentrations to systematically evaluate their capability to infect mice under different experimental conditions. For 0.1 mg L^{-1} ozone and 5 min of contact (Ct = 0.5 mg-min L^{-1}) no significant reduction in oocyst infectivity is achieved. In this regard. Bukhari et al. (2000) reported the application of 0.3 mg L^{-1} of ozone with 2 min of contact (Ct = 0.6 mg-min L^{-1}) on oocysts of *C. parvum*. The minimum inoculum dose applied to mice was 246 oocysts (2.46×10^2), resulting in 46% of the inoculated mice showing infection. When comparing the Ct value in this research with the one reported in our study, we notice similar values. This similarity explains why similar infectivity percentages were obtained.

For the concentration of residual ozone of 0.8 mg L^{-1} ozone and 5 min of contact (Ct = 4.0 mg-min L^{-1}), 2 Log_{10} oocyst reduction is not achieved. Korich et al. (1990) reported that 1 mg L^{-1} of residual ozone for 5 min (Ct = 5 mg-min L^{-1}) reduces the infectivity of *C. parvum* oocysts inoculated into BALB/c mice by 2 Log_{10} (99%). In this work, achieving a similar percentage reduction was not attained under the experimental conditions described above. It is crucial to note that the inoculated oocysts in a phosphate buffer solution, which is free of or has low levels of turbidity, organic matter, and suspended solids. In such a solution, the disinfectant agent, ozone in this case, will not be consumed in oxidizing water compounds. In other words, the ozone applied only acted on oocysts and not on some other organic components as can be found in wastewater (Korich et al., 1990). In contrast, in this research, the oocysts were resuspended in treated wastewater containing a concentration of total suspended solids of 323 mg L^{-1}, turbidity of 5.15 NTU (Nephelometric Turbidity Unit), and a biochemical oxygen demand (BOD_5) of 4.4 mg L^{-1}, suggesting a rich content of organic matter. In other studies, different levels of turbidity and organic matter in the water were simulated to resuspend oocysts. The results consistently show that the effectiveness of ozone is directly proportional to the concentration of these two factors (Ran et al., 2010). For example, using 3 mg L^{-1} of residual ozone with 7 min of contact, the reduction in viability decreased from 99% to 86% as turbidity increased from 0.1 to 20 NTU.

Undoubtedly, organic matter had the greatest impact on the action of ozone, as the percentage of viability reduction decreased from 99% to 98% when the concentration of organic matter increased from 0 to 1 mg L^{-1}.

Other studies conclude that turbidity, color, and organic matter in the water play crucial roles in the impact of ozone on *C. parvum* oocysts. These studies found that the higher the concentration of these parameters, the lower the level of inactivation (Biswas et al., 2003; Owens et al., 2000). It is important to note that both studies were conducted in environmental source water, not under simulated conditions like Ran et al. (2010), making these results potentially more representative of real-world scenarios.

The U.S. Environmental Protection Agency (USEPA) published different ozone Ct values in 2003 required to achieve reductions of 0.5–3.0 Log_{10} for *C. parvum* over a temperature range of 1–25°C. To achieve a 2 Log_{10} reduction at 25°C, a Ct value of 4.9 mg-min L^{-1} is needed (Davis, 2010). In our study, with a concentration of 0.8 mg L^{-1} and a contact time of 5 min, a Ct value of 4.0 mg-min L^{-1} was generated. According to the EPA data, this Ct value would achieve a reduction of at least 1.5 Log_{10}. In comparison, our results showed a decrease in *C. parvum* oocyst survival of 1.36 Log_{10} (94% reduction). Based on these findings, we can conclude that the results of our research meet with EPA recommendations.

According to the Ct value of 6.5 mg-min L^{-1} for this residual ozone concentration (1.3 mg L^{-1}) and contact time (5 min), at least a 2.5 Log_{10} reduction should be achieved based on in vivo studies, as per data published by the EPA (Davis, 2010). The Ct value reported by this agency is 6.2 mg-min L^{-1}. However, both the non-linear regression model and the infectivity test results are below this value, with a decrease in *C. parvum* oocyst survival of 1.72 Log_{10} (98% reduction). In this sense, it is known that the EPA applies a correction factor to its Ct values, which may account for the difference between the theoretical values and those obtained here (Schulz et al., 2005). To reach a 2 Log_{10} reduction, according to Korich et al. (1990), Ct values of 5 to 10 mg-min L^{-1} are referred to, while others indicated a Ct of 7.15 mg-min-L^{-1} (Oppenheimer et al., 2000); both coincide with our results even though wastewater was used in those studies.

It is important to note that, for all ozone concentrations used (0.1, 0.8, and 1.3 mg L^{-1}), the N_0 value (prediction of infective oocysts after ozone treatment) was 4.19, 3.64, and 3.27 oocysts, respectively. This represents a predictive value through nonlinear regression, and to our knowledge, it is the first study that reports this type of analysis for the conditions mentioned.

CONCLUSIONS

The presence of *Cryptosporidium* spp oocysts was confirmed in the WWTP located in Costa Rica, Sinaloa. The concentration and viability of detected oocysts exceeded the infective dose reported for *Cryptosporidium* spp., indicating the ineffectiveness of the chlorine-based disinfection system currently in use. Recognition of the significance of this microorganism by authorities is crucial, along with the implementation of necessary measures for its control.

This study, conducted at the laboratory level, evaluated the use of ozone as a method for disinfecting wastewater inoculated with *C. parvum*, serving as an indicator of the process's effectiveness. With a dissolved ozone concentration of 1.3 mgL^{-1} and a contact time of 5 min, substantial elimination of 2 Log_{10} of oocysts was achieved. This represents a promising alternative for the disinfection process, complementing existing wastewater treatments.

AUTHOR CONTRIBUTIONS

María B. Contreras-Soto: formal analysis, methodology, writing, editing. **Nohemí Castro-del Campo:** methodology, funding acquisition. **Cristobal Chaidez:** supervision; writing, review, editing. **Flavio E. Velázquez-García:** writing, review, editing. **Jean P. González-Gómez:** writing, review, editing. **Célida I. Martínez-Rodríguez:** methodology, formal analysis. **Joel Gaxiola-Montoya:** methodology, formal analysis. **Nohelia Castro-del Campo*:** conceptualization, funding acquisition, supervision, writing original draft, editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FUNDING INFORMATION

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY MATERIAL

Supplementary Information 1. Sample Processing. EPA method 1623 and minor modifications.

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SUPPORTING INFORMATION

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