The efficiency of ozonated water from a water treatment plant to inactivate *Cryptosporidium* oocysts during two seasonal temperatures

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ABSTRACT

We investigated the efficiency of residual ozone from an advanced water treatment plant with an applied dose of 2.5 mg l^{-1} to inactivate viable *Cryptosporidium* oocysts during summer (i.e. 24°C) and winter (i.e. 18.9°C) in Queensland, Australia. Containers for sample collection were inoculated with 1,000 oocysts l^{-1} and filled with ozonated water. Ozone residual concentrations were measured at 0, 5 and 10 min intervals. Viability was determined by excystation. Non-ozonated water from the plant, trip and laboratory controls were also analysed. The applied ozone dose of 2.5 mg l^{-1} produced an immediate residual concentration of 1.25 mg 1^{-1} at 24°C and 1.34 mg 1^{-1} at 18.9°C in unseeded samples. The initial ozone residual in seeded containers was 1.22 ± 0.03 mg 1^{-1} at 24°C and 1.37 ± 0.04 mg 1^{-1} at 18.9°C. There was a gradual increase in inactivation of oocysts, with 49% of oocysts inactivated at 0 min to 92% after 10 min at 24°C and 57% at 0 min to 92.8% at 10 min at 18.9°C.

Key words | *Cryptosporidium*, excystation, inactivation, ozone, temperature

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INTRODUCTION

Cryptosporidium parvum is a frequent cause of water-borne disease in humans in many parts of the world (Lisle & Rose 1995; Craun *et al.* 1998; Clancy & Hansen 1999). Transmission of oocysts may occur through consumption of, or contact with, contaminated drinking water (MacKenzie *et al.* 1994; Marshall *et al.* 1997; Thwaites 1997), swimming pools (Towner & Marshall 1999; Kebabjian 2001; Puech *et al.* 2001) and surface waters used for recreational activities (Fayer *et al.* 1997). LeChevallier *et al.* (1991) studied contamination levels of surface waters in the United States and showed that it ranged from 0.07 to 484 oocysts l^{-1}. A similar study by Thurman *et al.* (1998) has shown a contamination level of 0.6 oocysts l^{-1} in rivers and creeks in rural Australia.

Chlorine at concentrations routinely used for disinfection of microorganisms has been shown to be insufficient for inactivation of *C. parvum* oocysts. Korich *et al.* (1990) reported that a chlorine concentration of 80 mg 1^{-1} for 90 min was required to inactivate 99% of oocysts while an ozone concentration of 1 mg 1^{-1} for between 5 and 10 min achieved the same results. Ozone is a powerful disinfectant and is commonly used to disinfect drinking and waste-waters (Peeters *et al.* 1989). It has been shown that water containing 10^4 oocysts requires an ozone concentration of 1.11 mg 1^{-1} for 6 min to eliminate infectivity in mice and water containing 5×10^5 oocysts requires a concentration of 2.27 mg 1^{-1} for 8 min (Peeters *et al.* 1989).

Most of these studies assessing the effectiveness of ozone as a disinfectant have been performed under laboratory conditions (Korich *et al.* 1990; Finch *et al.* 1993; Bukhari *et al.* 2000). Ozone gas is unstable in water and, in addition to reacting with pathogens, it reacts with other constituents commonly present in natural waters and, if exposed to the atmosphere, decomposes back into oxygen within a short period of time after generation (US Environmental Protection Agency 1999). This causes the ozone residual concentrations to decay rapidly, and the decay rate is dependent on the temperature and contamination level of the water (Peeters *et al.* 1989; Gujer & von Gunten 2003). Due to this rapid decay, ozonation residual is not considered sufficient to provide ongoing protection and is never used as the sole disinfection procedure, but is used in conjunction with chemical disinfection, such as chlorine, to provide ongoing protection from other contaminants in the distribution system (Corona-Vasques *et al.* 2002).

Human defecation can release a range of 10^6 to 10^{11} of *Cryptosporidium* oocysts g^{-1} of faeces and sewage treatment plant failures could release these parasites into surface waters (Hayes *et al.* 1989; Suwa & Suzuki 2003). Presence of oocysts in raw sewage discharges have been recorded at levels as high as 1.3×10^2 oocysts l^{-1} (Rose *et al.* 2002). During a *Cryptosporidium* outbreak in the Sunshine Coast region of Queensland, Australia (Harper *et al.* 2002), 1.4×10^3 oocysts l^{-1} were detected in the untreated effluent of the sewage treatment plant (Wohlsen *et al.* 2006). It has been shown that, as the concentration of oocysts increases, the concentration of ozone required for effective inactivation also increases, as the extra demand on the ozone causes it to decay more rapidly (Peeters *et al.* 1989).

As waterborne contamination continues to occur due to water treatment plant failures (Mazounie *et al.* 2000; Stein 2000), ozone is considered the last line of defence in Australian water treatment plants. The Landers Shute advanced water treatment plant located in the Sunshine Coast region of Australia utilises an ozone generating system with a maximum capacity of 2.5 mg l^{-1} ozone to treat water from the Baroon Pocket dam, which includes the Maleny catchment area. In view of the past outbreak of *Cryptosporidium* in the region, we decided to evaluate the effectiveness of residual ozone from an applied dose of 2.5 mg l^{-1} used in the water treatment plant to inactivate

1.0×10^3 l^{-1} *C. parvum* oocysts during summer (i.e. 24°C) and winter (i.e. 18°C).

MATERIAL AND METHODS

Water treatment plant

The study was performed at the Landers Shute Advanced Water Treatment Plant, situated approximately 100 km north of Brisbane, on the Sunshine Coast, Queensland, Australia. The plant has the capacity to supply 140 Ml of treated water per day and utilises a multi-barrier approach to water treatment, including the use of ozone and biological activated carbon. Ozone was generated by passing a high voltage current through oxygen gas to produce ozone with a maximum applied dose of 2.5 mg l^{-1}. The ozone is continuously generated in the ozone contact tank (inter-ozone static mixer), where it comes into contact with the water for a minimum of 10 min. Excess ozone is removed by catalytic destruction. The first experiment was performed in December 2003 (summer in the southern hemisphere) with a water temperature of 24°C, an environmental temperature of 27°C and a pH of 7.1. This experiment was repeated in June 2004 (winter time in the southern hemisphere) with a water temperature of 18.9°C, an environmental temperature of 21°C and a pH of 6.9. Water samples for both experiments were drawn from the inter-ozone static mixer tank outlet into a sample container. Measurement of residual ozone was taken at 0, 5 and 10 min intervals with and without the oocysts present using a HACH Pocket Dissolved Ozone colorimetric device (Biolab, Queensland, Australia).

Oocysts and sampling procedure

Approximately 1.0×10^3 l^{-1} of viable *C. parvum* oocysts (BTF Pty. Ltd., New South Wales, Australia) were added to each of three 5–l sample containers on site at the water treatment plant. Containers labelled 0, 5 and 10 min were filled with ozonated water drawn from the inter-ozone static mixer tank outlet and all containers were recapped. Immediately upon filling the container (i.e. time 0), the ozone concentration was measured and 100 ml of 10% (w/v) sodium thiosulfate was added to inactivate the ozone. The other containers remained capped for the required

contact time. At 5 and 10 min, the ozone concentration was measured, respectively, after which 100 ml of 10% (w/v) sodium thiosulfate was added to each container. In order to assess the level of ozone decay due to the presence of oocysts, this procedure was also performed in parallel on unseeded water samples. Sample containers were placed on ice for transportation to the laboratory, were sub-sampled (5 1-l sub-samples from each container) and analysed within 24 h. The entire procedure was repeated twice more on the same day for both sets of experiments conducted during summer and winter.

Non-ozonated control

The filtration module (including sand filtration) in the water treatment plant occurred immediately prior to the ozone module and non-ozonated samples were drawn from this outlet. A container with approximately 1.0×10^3 oocysts l^{-1} was filled with non-ozonated water. Sodium thiosulfate (100 ml of 10% (w/v)) was added to the container. This procedure was repeated three times at 24°C and twice at 18.9°C. Containers were returned to the laboratory for sub-sampling (5 1-l sub-samples from each container) and analysed within 24 h. These samples were treated and analysed in the same manner as the ozonated samples.

Trip control

A vial containing approximately 1.0×10^3 viable oocysts in 200 ml phosphate buffered saline (PBS, pH 7.4) was transported on ice to the water treatment plant. This vial was identical to the vials used to seed the samples on site at the plant and was returned to the laboratory and analysed in the same manner as the ozonated samples. These samples were compared with the laboratory controls to determine any variation in oocyst viability due to transport conditions. This procedure was repeated three times for both experiments (i.e. 24°C and 18.9°C).

Laboratory control

A vial containing approximately 1.0×10^3 viable oocysts in 200 ml PBS (pH 7.4), the same as used for seeding, was kept in the laboratory and stored at 4°C. This procedure was repeated three times. These samples were used to calculate the viability of the stock oocysts.

Excystation

On return to the laboratory, each 1-l sub-sample was centrifuged at $1,100\,g$ for 15 min. The supernatant was aspirated and the pellet was resuspended in 100 µl PBS (pH 7.4). A pre-incubation procedure was performed by mixing the sample with acidified Hank's balanced salt solution (HBSS) and incubated at 37°C for 30 min (Hirata *et al.* 2001). The washed pellet was resuspended in 100 µl of PBS and 10 µl of 1% (w/v) sodium deoxycholate in Hank's minimal essential medium and mixed with 10 µl of 2.2% (w/v) $NaHCO_3$ in HBSS. The samples were incubated at 37°C for 4 h as described previously (Robertson *et al.* 1993; Vesey *et al.* 1997; Hirata *et al.* 2001). When the excystation protocol was completed, 25 µl of sample was air-dried onto Spot-on-slides (Dynal Biotech Pty., Ltd., Victoria, Australia) and fixed in methanol. Slides were stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody and 4′, 6′-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) and examined under an epifluorescent microscope.

A minimum of 100 oocysts were counted in each sample. The percentage of oocysts inactivated after 0, 5 and 10 min was calculated based on the percentage of excysted oocysts in the non-ozonated water sample, as below:

$$\frac{\text{Mean excystation of ozonated water sample}}{\text{Mean excystation of filtered water sample from WTP}} \times 100$$

$$= \%\text{viable}.$$

Percentage inactivated was then calculated by taking away the percentage viable count from 100, as detailed below:

$$100 - \%\text{viable} = \%\text{inactivated}.$$

Monoclonal antibody

The EasyStain kit (BTF Pty. Ltd., New South Wales, Australia) containing FITC-conjugated anti-immunoglobulin monoclonal antibodies specific to *Cryptosporidium* IgG_1 (Ferrari *et al.* 1999; Weir *et al.* 2000) was used to stain the spot-on-slides. DAPI was also used to stain the slides to aid in the visualisation of sporozoites. The EasyStain kit was

used according to the manufacturer's instructions and a working strength concentration of DAPI (Sigma-Aldrich) was prepared by mixing $10\,\mu l$ of DAPI stock solution ($2\,mg\ ml^{-1}$) with $10\,ml$ of PBS (pH 7.4) and filter-sterilised using a $0.45\,\mu m$ pore sized membrane filter.

Microscopic examination

A Zeiss Axioskop2 epifluorescence microscope was used to examine the stained slides. The entire well was examined under $\times 20$ magnification and confirmation was performed at $\times 40$ magnification. Microscope filter cubes appropriate for FITC (wavelength 450–590 nm, FT 510, LP 515) and DAPI (BP 365 nm, FT 395, LP397) were used to examine oocysts (US Environmental Protection Agency 2001). Oocysts were located using the FITC filter set and identification was assisted by using the DAPI filter set and differential interference contrast (DIC) to visualise sporozoites. Oocysts were categorised into categories of whole, empty or partially empty. Empty or partially empty oocysts referred to cells where sporozoites were no longer visible within the cell wall. Cells with a disrupted wall to form a "pac man" appearance were considered to have excysted and were included in the "empty" count. A whole oocyst was considered to be unable to excyst and therefore was considered non-viable. For each water sample, the number of empty oocysts was divided by the total number of oocysts counted in that sample to obtain the percentage of excystation. Three microscope slides were prepared and examined for each control sample and five slides for each ozonated sample.

Statistical analysis

Analysis of variance (ANOVA) on ranks (Kruskal–Wallis non-parametric test) was used to compare the significance of difference between inactivation of oocysts in the water samples.

RESULTS

In oocyst-free water, the initial ozone concentration (at time $= 0$) in the sample container in summer (i.e. 24°C) was $1.25 \pm 0.04\,mg\ 1^{-1}$. There was a decrease in ozone concen-

tration over 10 min of measurement, reaching the minimum concentration of $0.31 \pm 0.18\,mg\ 1^{-1}$ (Figure 1(a)). In winter (i.e. 18.9°C), the immediate ozone concentration was $1.34 \pm 0.0\,mg\ 1^{-1}$, which reached a minimum of $0.12 \pm 0.0\,mg\ 1^{-1}$ after 10 min (Figure 1(b)). The rate of ozone decay over 10 min in unseeded samples did not differ significantly between two temperatures. However, it was significant for samples collected at 5 min ($p = 0.046$). In containers seeded with oocysts, the initial value of ozone concentration at 24°C was $1.22 \pm 0.03\,mg\ 1^{-1}$ reaching a minimum concentration of $0.04 \pm 0.03\,mg\ 1^{-1}$ after 10 min (Figure 1(a)). These figures for seeded samples at 18.9°C were $1.37 \pm 0.04\,mg\ 1^{-1}$ at 0 min and $0.13 \pm 0.01\,mg\ 1^{-1}$ for 10 min (Figure 1(b)). There were no significant differences in ozone decay between the two different temperatures.

The total number of oocysts (Count T) and number of empty oocysts counted (Count E) in each sample were used to calculate viability and are given in Table 1. Oocyst inactivation increased with time from 49% after initial exposure to ozone at 0 min to 66% at 5 min to 92% at 10 min in summer samples (i.e. 24°C) (Table 1). These
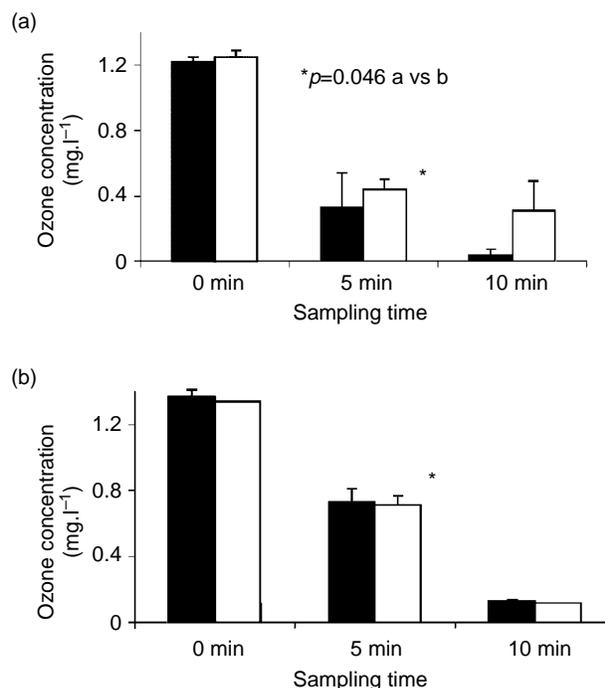

Figure 1 | Rate of ozone decay over 10 min in water samples collected from the ozone static mixer tank with ■ and without □ oocysts present at 24°C (a) and 18.9°C (b) water temperature.

Table 1 | Percentage of excysted and inactivated oocysts in ozonated water and non-ozonated controls and at 0, 5 and 10 min intervals. Dates are shown for samples collected during the summer at 24°C and the winter at 18.9°C

Sample	n	Count T	Count E	% excysted (Mean ± SD)	% inactivated (Mean ± SD)
24°C					
Non-ozonated samples:					
Laboratory control (LC)	3	300	0	89 ± 5.5	NA
Trip control (TC)	3	300	270	91 ± 7.2	NA
Filtered water from WTP	3	956	747	79 ± 8.2*	NA
Ozonated samples:					
0 min	3	899	360	41 ± 10.3*	49 ± 10.4
5 min	3	617	171	27 ± 6.3*	66 ± 9.1
10 min	3	1058	72	7 ± 1.9*	92 ± 0.6
18.9°C					
Non-ozonated samples:					
Laboratory control (LC)	2	1000	594	59 ± 8.5	NA
Trip control (TC)	2	1000	504	61 ± 18.4	NA
Filtered water from WTP	2	1000	495	50 ± 10.6[+]	NA
Ozonated samples:					
0 min	2	1000	215	22 ± 4.9[+]	57 ± 10.0
5 min	2	1000	90	9 ± 0.0[+]	82 ± 0.0
10 min	2	1000	35	4 ± 0.7[+]	93 ± 1.4

WTP: water treatment plant, NA: not applicable, *n*: number of sample sets, Count *T*: total number of oocysts counted, Count *E*: total number of empty oocysts.

[*]Filtered water from WTP *versus*: 0 min ($p = 0.0142$); 5 min ($p = 0.0027$); 10 min ($p = 0.0017$) for excysted oocysts at 24°C.

[+]Filtered water from WTP *versus*: 0 min ($p = 0.007$); 5 min ($p = 0.0009$); 10 min ($p = 0.0001$) for excysted oocysts at 18.9°C.

figures for winter samples varied from 57% at 0 min to 81.8% at 5 min to 92.8% at 10 min (Table 1). There were no significant differences in inactivation of oocysts between the two temperatures except for 5 min samples ($p = 0.0397$). There were also no significant differences between control groups.

At 24°C, there were significant differences in oocyst excystation when comparing the filtered water from water treatment plant to the 0 ($p = 0.0142$), 5 ($p = 0.0027$) and 10 min ($p = 0.0017$) measurements. At 18°C, these figures were $p = 0.007$ for 0, $p = 0.0009$ for 5 and $p = 0.0001$ for 10 min (see Table 1).

DISCUSSION

In this water treatment plant and under normal operational circumstances, the concentration of ozone would

be higher within the ozone contact tank than within the water drawn from the outlet tap used in this study. The plant has been designed so that water will take 10 min to travel through the tank, receiving a continuous dose of 2.5 mg l^{-1} at a maximum flow rate of 140 Ml per day. Longer contact times are achieved at this plant with slower plant flows. In our study, although all necessary precautions were taken to minimise exposure of samples to the atmosphere while drawing water from the ozonated tank, and despite an immediate capping of the sample containers, there was a rapid decline in the ozone concentration in all samples to almost half the value in mixing tank. Despite this, there was up to 92% inactivation of oocysts in summer and 92.8% in winter temperatures. Due to this reason, it is conceivable that the higher efficiency of ozone mixing in the main treatment plant (i.e. 2.5 mg l^{-1}), combined with a total contact time of 10 min at this constant concentration, will result in higher inactivation rates of oocysts than demonstrated in our experiment.

The effects of variations in temperature on inactivation of oocysts are well documented (Hirata *et al.* 2001; Clark *et al.* 2002). In our study, it was not feasible to cool the water inflow to the ozonator for this experiment, nor was it suitable to chill the water post-ozonation, as the chilling procedure would cause a lag phase in the experiment and residual ozone to decay further. Rose *et al.* (2002) showed that, for every 10°C decrease in temperature, the contact time per mg l^{-1} min^{-1} required for inactivation of oocysts increased by a factor of three. Despite this, we did not observed a significant difference between excystation percentages in the summer and winter samples. This suggests that the mild environment with a 5°C variation between the summer and winter temperatures, as is the case at this region, was not severe enough to verify their finding.

Finch *et al.* (1993) applied a level of ozone ranging from 0.6–2.9 mg l^{-1} from 5 to 15 min at temperatures ranging between 7°C and 24°C. The results obtained concluded that, at the average temperature of 22°C, 99.9% of oocysts were inactivated after 5.5 min of exposure to 2.5 mg l^{-1}, while 7 min was required at 1.25 mg l^{-1} and 12.5 min at 0.25 mg l^{-1}. Previous results from Korich *et al.* (1990) also demonstrated that ozone at a concentration of 1 mg l^{-1}

inactivated up to 99.9% of oocysts after 10 min, demonstrating some small degree of variability between studies. In our study, we obtained 92.8% inactivation after exposing oocysts to an initial dose of ozone at 1.37 mg l^{-1} over 10 min duration. This slight difference could be partly due to the ozone not being delivered at a constant dose throughout the experiments, as was the case in other laboratory based-studies. It has also been demonstrated that a pH range of 6–10 had no effect on inactivation with ozone (Rennecker *et al.* 2001). In our study, the pH of water ranged between pH 7.1 at 24°C and 6.9 at 18.9°C. These values remained constant throughout both experiments.

Animal infectivity studies have long been regarded as the "gold standard" for measuring oocyst viability and infectivity. However, due to animal ethical considerations, many laboratories prefer to use other methods such as cell culture (Slifko *et al.* 2002), vital dye assays (Smith *et al.* 2002), and excystation (Robertson *et al.* 1993; Vesey *et al.* 1997). Some comparative studies have shown that excystation overestimates oocyst viability (Bukhari *et al.* 2000). However, Hirata *et al.* (2001) has shown that viability, as determined by excystation with an additional 5-min preincubation step in acidified Hanks Balanced Salt Solution (HBSS) at 37°C, is a suitable substitute for animal infectivity in evaluating the disinfection efficacy of ozone. In this study, we used excystation with this pre-incubation procedure as a proxy measure of viability. Other researchers have also successfully used excystation to determine viability of ozonated oocysts (Rennecker *et al.* 1999; Driedger *et al.* 2001). Although DAPI was not used to indicate viability in this experiment, excystation can improve the permeability of oocysts to DAPI staining, enhancing visualisation of the sporozoites and nuclei in addition to the observation of excysted oocysts using DIC microscopy (Campbell *et al.* 1992).

We also measured the inactivation of oocysts in relation to ozone decay throughout the experiment. Contrary to other studies (Peeters *et al.* 1989), the variations noted in the decay of ozone were not significantly affected by the presence of oocysts at the concentrations used for this study. This could be due to modern improvements in the purification procedures used to isolate oocysts from the source, i.e. cell sorting flow cytometry.

CONCLUSIONS

The applied ozone dose of $2.5\,mg\,l^{-1}$ in containers seeded with oocysts, produced an initial ozone concentration of $1.22 \pm 0.03\,mg\,l^{-1}$ at 24°C and $1.37 \pm 0.04\,mg\,l^{-1}$ at 18.9°C, approximately half of the applied dose in the WTP container. We obtained a 92% inactivation at 24°C and 92.8% inactivation at 18.9°C over 10 min of measurement. Based on previous studies (Korich *et al.* 1990; Finch *et al.* 1993) and the constant supply of ozone at $2.5\,mg\,l^{-1}$ within the static mixer tank, we postulate that there would be higher inactivation of oocysts exposed to this constant concentration for a total of 10 min than that obtained in this study.

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