Inactivation of Cryptosporidium by ozone and cell ultrastructures

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Abstract

The fluorescence staining method and scanning electron microscopy (SEM) were used to study the effect of ozone (O₃) inactivating Cryptosporidium in water and cell ultrastructures variation to shed light on the mechanism of inactivation preliminarily. Results indicated that O₃ had a stronger inactivating capability. When the concentration of O₃ was above 3.0 mg/L and the contact time was up to 7 min, a significant inactivating effect could be achieved. The turbidity on inactivation effects was also found to be statistically significant in artificial water. With increases in turbidity, the inactivating effect decreased. Inactivation rate improved with a temperature increase from 5 to 25°C, but decreased beyond this. The inactivating capability of O₃ was found to be stronger under acidic than that under alkaline conditions. When the concentration of organic matter in the reaction system was increased, the competition between Cryptosporidium and organic with O₃ probably took place, thereby reducing the inactivation rate. In addition, the cellular morphology of Cryptosporidium varied with different contact times. At zero contact time, cells were rotundity and sphericity, at 60 sec they became folded, underwent emboly, and burst at 480 sec, the cell membrane of Cryptosporidium shrunk and collapsed completely.

Key words: ozone; inactivation; Cryptosporidium; ultrastructures

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Introduction

Cryptosporidium is the most common intestinal protozoan in domestic animals, including livestock, dogs and cats. It is also a common parasite of humans and wildlife (Fayer, 2004; Thompson and Monis, 2004). In particular, it can cause diarrhea in immuno-compromised hosts, such as infants, the elderly and AIDS patients, and possibly even threaten their lives (Xiao and Feng, 2008). Cryptosporidium has been found in most surface waters, where its concentration was related to the level of fecal pollution or the nature of human use of the water. Cryptosporidium oocysts are extremely persistent in water and greatly resistant to disinfectants commonly used in drinking-water treatment (Woodall, 2009). Thereby, non-exhaustively treatment of Cryptosporidium in drinking water would pose a serious threat to humanity (Castro-Hermida et al., 2008); In addition, recent outbreaks of waterborne cryptosporidiosis brought about by the contamination of drinking water have been a cause of concern.

Waterborne cryptosporidiosis existed not only in developing countries but also in developed countries, making it an urgent problem (Marion et al., 2009; Melanie et al., 2009). In fact, Cryptosporidium oocysts and Giardia cysts were reported to have been detected in 45.7% and 93.8%, respectively, of 162 river samples taken from around Paris (Céline et al., 2009). The United States Center for Disease Control (CDC), on the other hand, estimated that waterborne diseases were responsible for more than 2.5 million deaths in United States per year, with children under five years old accounting for most of the mortality (Karanis, 2006). In addition, a worldwide investigation by WHO found that there were at least 40 countries were contaminated with Cryptosporidium (Pond, 2005).

Cryptosporidium can survive a few months in the water and is a strong resistance to chlorine disinfectant (Eric et al., 2006). Therefore, it is important to prevent Cryptosporidium from entering and contaminating sources of drinking water. In addition, identifying an effective disinfectant and water treatment process is urgently needed.

A previous study by Ran et al. (2010) showed that O₃ effectively inactivates on Giardia. In a similar vein, this study investigated the mechanism of Cryptosporidium cell damaged by O₃. Fluorescent in vivo staining and scanning electron microscopy (SEM) were applied to demonstrate the O₃ inactivation of Cryptosporidium in drinking water.

1 Materials and methods

1.1 Preparation of oocysts

Cryptosporidium oocysts (Shenzhen isolated, Guangdong Province, China) were isolated from the feces
of naturally infected calf by performing discontinuous Sheather’s gradient centrifugation (Ewa and Daniel, 1999) and cesium chloride gradient centrifugation (Ruhangiz and Laila, 1987). The purified oocysts were resuspended in phosphate buffered saline (PBS) containing 0.01% Tween 20 (Solarbio, USA), 100 U/mL penicillin (Solarbio, USA), 100 µg/mL streptomycin, and 100 µg/mL gentamycin and then stored at 4°C. The total numbers of oocysts purified from feces were counted by a haemocytometer.

1.2 Experimental apparatus

O3 was produced from pure oxygen using a generator (CT-KG2, Yitian Environmental Protection Equipment Corporation Limited, China). O3 was bubbled through ultrapure water at pH 7.0 (adjusted by adding H2SO4) and 20°C in a 500 mL gas washing cylinder to produce a stock solution with the desired dissolved O3 concentration. A stirring plate was used to allow additional mixing inside the reactor.

1.3 Operation conditions

For each experimental trial, 1 × 10⁶ oocysts were washed with deionized water using Milli-Q deionizer and resuspended in 1.0 mL deionized water. The temperature was kept within 5–35°C range by immersing the batch reactor in a water bath. The pH was maintained within 6.0–9.0 range by adding either H2SO4 or NaOH immediately before starting an experiment to make sure carbonate re-equilibration with the atmosphere would be minimal. The dissolved ozone present in the sample was quenched by mixing it with indigo trisulfonate solution immediately upon collection (Bader and Hoigné, 1993) based on the morphology and the inclusion or exclusion of two vital dyes, 4’,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) and propidium iodide (PI) (Sigma, USA). The total numbers of oocysts in each subsample, the proportions of following categories of oocysts to the total were determined: (1) viable oocysts that included DAPI but exclude PI (DAPI+/PI−); (2) nonviable oocysts that included DAPI and PI (DAPI+/PI+); (3) potentially infectious oocysts that did not include either DAPI or PI but had “viable type” contents, as shown by differential interference contrast (DIC) microscopy (DAPI+/PI−, visible after further trigger); (4) empty oocysts that have no recognizable contents, as shown by DIC microscopy, and that include neither DAPI nor PI (DAPI−/PI−, nonviable). Three replicate counts were performed for each subsample. Oocysts with contents and which were either DAPI+/PI− or DAPI−/PI− were considered viable, thus, their total numbered represented the number of viable oocysts.

1.4.4 Microscopic observation

Scanning electron microscopy (SEM) was employed to observe the surface changes on Cryptosporidium with time. The experimental samples, together with the clean ones, were first fixed with 3.0% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 3 hr. This was followed by the dehydration in a graded ethanol series (50%, 70%, 80%, 90%, 100%, 100%, and 100%; 15 min each). After which the samples were gold-coated by a sputter and observed under SEM (Model 4700, HITACHI, Japan).

1.4.3 Data analysis and statistical approach

The differences in mortality among the treatments were statistically compared using Fisher’s least significant difference (LSD) multiple comparison test.

2 Results and discussion

2.1 Effects of disinfectant concentration and contact time on Cryptosporidium inactivation

O3 of various dosages were added into a given volume of Cryptosporidium solution; the contact time was also variable. O3 dosages ranged from 0.5 to 5.0 mg/L, and the contact time, from 0 to 10.0 min. Figure 1 shows the results obtained from the experiments performed to assess the effect of disinfectant concentration on the inactivation rate of Cryptosporidium at 20°C.

Notably, a significant elevation in the extinction rate of Cryptosporidium occurred with increasing contact times and disinfectant dosages. Overall, the inactivation effect of ozone on Cryptosporidium was obviously significant, and an exposure time of 7 min and O3 dosages above 3.0 mg/L achieved the desired inactivated results. With an O3 concentration at or below 2.0 mg/L, however, the contact time had to be increased to more than 10 min. With such an unsuitably long contact time, there emerged another...
problem – ozone decomposition (Gurrol and Singer, 1982). By keeping the contact time low at 7 min, a concentration of more than 2.0 mg/L was therefore needed to achieve Cryptosporidium inactivation.

The reactions of O$_3$ in water were as follows:

$$O_3 \rightarrow O + O_2$$

$$O + H_2O \rightarrow 2\cdot OH$$

Although oxidation of the hydroxyl radical, which had a high potential reaction speed, was more violent than that of O$_3$, the mechanism of Cryptosporidium inactivating by O$_3$ was not entirely clear. The absence of visible protein bands on polyacrylamide gels and immunoblots suggests that O$_3$ penetrated into the oocysts and degraded most of the oocyst wall and later, the trophozoite proteins. An alternative explanation was that O$_3$ only penetrated an outer cyst layer, and then induced the release of proteases from peripheral vesicles (Ward et al., 1997). Oocysts DNA and RNA were not affected by O$_3$, which suggested that the disinfectant did not deeply diffuse into the oocysts and cysts (data not shown). Regardless of whether the O$_3$ damaged the oocysts or was mediated via proteases, the immunoblot results were consistent with the absence of internal morphological features in cysts exposed to chlorine (Sauch and Berman, 1991).

2.2 Effects of turbidity on O$_3$ inactivation to Cryptosporidium

A variety of suspended solids and colloidal materials exists in natural water and inevitably affects the turbidity of water bodies. Falabi et al. (2002) studied the removal oocysts and influent turbidity which showed a significant correlation ($P \leq 0.05$ for Cryptosporidium and turbidity) between them. However, more data were needed to evaluate the significance of this supposed correlation.

Figure 2 presents oocyst inactivation at different turbidities of 0.1, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 NTU. The results suggested that the extinct rate decreased with an increase in turbidity values. With 7 min contact time the extinct rate fell from 99.2% to 86.2% when turbidities increased from 0.1 to 20.0 NTU. In addition, the effect of inactivation increased with time, if the contact time was raised to 12 min, the extinct rate increased to more than 99.0%. A reason may be that the formation of turbidity particles provided surface protection for the two insects, thus weakening the effect of disinfection. Another reason may be that the particles consumed a certain amount of disinfectant.

2.3 Effects of pH value on the inactivation of O$_3$

Wickramanayake et al. (1984) studied O$_3$ inactivation of protozoan cysts at 25°C in a semibatch reactor (continuous O$_3$ gas addition) and reported that pH was not a significant factor in the inactivation of Giardia cysts by O$_3$. Interestingly, existing literature reported conflicting findings on the effects of pH on O$_3$ activation efficiency. Farooq et al. (1977) studied the O$_3$ inactivation of Mycobacterium and reported that O$_3$ efficiency to be relatively stable within the range of pH 5.7–10.1 for a constant O$_3$ residual.

For understanding the role of pH in the inactivation of Cryptosporidium, bench scale experiments were conducted to evaluate the impact of pH (pH range 6.0 to 9.0). The O$_3$ dosage used was 3.0 mg/L, which was the required O$_3$ concentrations, at neutral pH conditions for attaining an inactivation above 99.0%. The effect of water pH on O$_3$ inactivation was observed in Fig. 3.

As shown in Fig. 3, the inactivation rates at pH 6.0 and 7.0 were higher than those at pH 8.0 and 9.0. However, this difference was not very significant ($P > 0.05$). The concentration of hydroxyl radicals at pH 9.0 in the presence of dissolved O$_3$ at a concentration of 0.5 mg/L, despite being higher than that estimated at pH 6.0 with the same dissolved ozone concentration, did not seem to have an effect on the inactivation rate. Similarly, at lower O$_3$ concentration levels, the rate of inactivation was not affected by the concentration of hydroxyl radicals. The same conclusion is assumed to be reached with natural waters, in which carbonate and organic impurities, serving as hydroxyl radical scavengers, result in the formation of comparable or even lower hydroxyl radical concentrations.
2.4 Effects of temperature on the inactivation of Cryptosporidium

The results in Fig. 4 suggested that the effect of water temperature on O$_3$ inactivation was not significant. For example, at low temperature of 5°C, Cryptosporidium parvum was in a dormant state (inactivation rate was 92.4%), with self-protection capabilities; when the temperature was increased to 20°C, the inactivation rate gradually increased and reached 99.0%; finally, as the temperature was further elevated to 35°C, the capability of O$_3$ to inactivate Cryptosporidium was reduced (inactivation rate was 96.9%). The higher the temperature, the easier the decomposition of ozone became, it diminishing inactivating capacity. Likewise, as the temperature dropped, the increasing solubility of O$_3$ in water led to a lower inactivation rate.

2.5 Effects of dissolved organic matter on the inactivation of O$_3$

The rate of transformation of O$_3$ into OH radicals was influenced by the types and concentrations of dissolved organic matter (DOM) present in natural waters. Various experiments were performed to investigate the inactivation effect of O$_3$ on Cryptosporidium under different DOM concentrations (Fig. 5).

It was found from Fig. 5 that the DOM had a significantly effect on the inactivation of Cryptosporidium with O$_3$ ($P \leq 0.05$). At a contact time of 7.0 min, the inactivation rate significantly decreased from 99.1% to 98.3%, following an increase in DOM concentrations from 0 to 1.0 mg/L. When the DOM concentration was raised from 2.0 to 10.0 mg/L, the inactivation rate rapidly decreased from 84.9% to 62.1%. Given these results, the conclusion that the DOM competes with Cryptosporidium for O$_3$ can be made, such that an increase in DOM concentration decreases the inactivation effect of O$_3$.

2.6 SEM examination of control and O$_3$-treated oocysts

The surface structure of Cryptosporidium was investigated by scanning electron microscopy. The results revealed that when exposed to O$_3$, oocysts exhibited certain morphological alterations. The extent of these morphological changes appeared to be dependent on the exposure time.

The first photo (Fig. 6a) shows oocysts that received no O$_3$ exposure, the surfaces of the cell membranes were intact and smooth. The second photo (Fig. 6b), shows oocysts exposed to O$_3$ for 60 sec; the cells appeared to have folded. Photo (Fig. 6d) shows oocysts exposed to O$_3$ for 480 sec the cells were completely destroyed, and the cell membranes had shrunk.

After treatment with O$_3$, the changes in and the damage to the Cryptosporidium cell wall indicated that the main mechanism involved in Cryptosporidium inactivation was contact action. By causing damage to the cellular structure of Cryptosporidium, O$_3$ interfered with the physiological activities of the organism. As it is known that the cell was the basic functional unit of a living body, damage its structure leads to its impairment.

3 Conclusions

This work examined the inactivation efficiency of O$_3$ on Cryptosporidium using bench-scale experiments. The following conclusions can be drawn. (1) O$_3$ has better inactivation efficiency at a dosage of 3.0 mg/L and a contact time of 7 min under the conditions of pH 7.0, 20°C and 1.0 NTU. (2) Generally, a higher inactivation rate
could be achieved with lower turbidity. (3) The inactivation efficiency of O$_3$ on Cryptosporidium was not significantly affected within the pH range 6.0–9.0, and the capability of O$_3$ to inactivate Cryptosporidium was stronger under acidic than alkali conditions. (4) A lower inactivation rate can be achieved with a higher organic matter concentration. Inactivation efficiency improves with an increase in temperature from 5 to 25°C, but decreases beyond this. (5) The cellular morphology of Cryptosporidium varies with the contact time.

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