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Effect Factors of Chlorine Dioxide Inactivation *Cryptosporidium andersoni* Oocyst in Preoxidation Process

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The inactivation effects of chlorine dioxide (ClO_2) on inactivation of *cryptosporidium andersoni* oocysts in aqueous solution were studied systematically. Several influencing factors including ClO_2 dosage, contacting time, temperature, pH, turbidity and permanganate index of the solution were investigated. The results showed that the inactivation rates of *cryptosporidium andersoni* oocysts were affected by ClO_2 dosage and contacting time greatly, by temperature and turbidity slightly, and higher inactivation rate can be gotten at higher temperature. The effect of pH is slightly from 3.0 to 10.0. As to 10^5 cell/ml *cryptosporidium andersoni* oocysts, the inactivation rates of *cryptosporidium* oocysts can reach to 95% when prolonging reaction time to 120min at 5mg/L ClO_2; as to 50cell/ml *cryptosporidium andersoni* oocysts, contacting ClO_2 30min with 3mg/L ClO_2 or 60min with 2.0mg/L, the inactivation rate can reach 100%. All of these provide reference for engineering application.

Key words: Drinking water disinfection, Chlorine dioxide, Cryptosporidium oocyst, Inactivation effect.

Cryptosporidium can infect a wide range of fishes, amphibians, reptiles, birds and mammals (Xiao *et al*., 2001; Xiao *et al*., 2004) as a microbial pathogen. It was detected for the first time by Tyzzer (Tyzzer, 1907), and then found to widely exist in drinking water(Gaut *et al*., 2008), recreational water(CDC, 2007) and wastewaters (Ryan *et al*., 2005).

The most common genotypes of *Cryptosporidium* in surface waters were *C. parvum* and *C. hominis*, whereas the most common one in wastewater was *C. andersoni* (Xiao *et al*., 2001).

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Cryptosporidiosis, which is a common zoonotic illness caused by *Cryptosporidium* is discovered in 1976 by Nime(Nime *et al*., 1976). And the first cases in China were reported by Han(Han *et al*., 1987)and Zu(Zu and Du, 1987) independently in Nanjing and Anhui. The patients present watery diarrhea with abdominal cramps, possibly accompanied by fever, nausea, vomiting and myalgias, which may be lethal to AIDS patient and children(Yang and Wang, 2005). In the 1993 waterborne outbreak of cryptosporidiosis in greater Milwaukee, Wisconsin, estimated 403,000 residents fall ill and the total cost was $96.2 million(Corso *et al*., 2003). WHO added cryptosporidiosis as one of the suspicious indexes of AIDS in 1986(Zhang and Jiang, 2001), and cryptosporidium was included in the "WHO Neglected Diseases Initiative" in 2004(Saviol *et al*., 2006).

Cryptosporidium spreads in the natural water bodies as the type of oocysts, *Cryptosporidium* oocysts are extremely resistant

to most common used chlorination disinfections (Betancourt and Rose, 2004; Butz and Tauscher, 2002), whereas ozonation(Li and Haas, 2004), chlorine dioxide (ClO_2)(Clark *et al*., 2003) and UV irradiation(Clancy *et al*., 2004) can kill *Cryptosporidium* oocysts effectively.

ClO_2 has been widely used in water disinfection in consideration of its strong oxidizability. It can remove various contamination in water and produce no THMs comparing with chlorine and chloramine(Huang, 2002). In this study the effects of disinfectant dosage, contacting time, pH, turbidity, and permanganate index on ClO_2 inactivation *Cryptosporidium andersoni* oocysts was investigated at different levels using fluorescent dye permeability assay as viability measure, thereby provide reference basis for engineering application.

MATERIALS AND METHODS

Cryptosporidium andersoni oocysts were provided by School of Animal Husbandry and Veterinary Medicine, Jilin University and stocked in 2.5% potassium dichromate solution at 4°C avoiding light.

To prepare experimental samples, some *Cryptosporidium* oocysts suspension were centrifuged at 3000r/min for 10min and centrifuged twice at the same situation to wash away the potassium dichromate. Then the *Cryptosporidium andersoni* oocysts were diluted to 10^5cell/ml and 50cell/ml as the experimental samples. Stock solution of chlorine dioxide was prepared by the reaction of $NaClO_2$ and H_2SO_4 as (Ji *et al*., 2008), and calibrated as 160mg/L before using by sequential idiomatic method(Huang, 2000).

Staining agents 42 9,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) was bought from Hoffmann-La Roche Ltd. Before experiments it was dissolved to 1g/L in high-performance liquid chromatography-grade methanol and 0.1 M PBS (phosphate-buffered saline), respectively.

0.1 M pH7.2 PBS was prepared by 0.028 M $NaH_2PO_4 \cdot H_2O$, 0.072 M NaH_2PO_4 and 0.145 M NaCl.The other pH buffer solutions used was made up following Britton-Robinson method (Britton and Robinson, 1931). All the water used was distilled water if not been noted specially.

Experimental procedure

At first a 1ml *Cryptosporidium andersoni* oocysts sample was taken in 1.5ml centrifuge tube and pH buffer solution was added in to adjust pH value; then certain amount of ClO_2 stock solution was added after setting reaction parameters such as temperature, turbidity, permanganate index to conduct sterilization experiments; at last, reaction was stopped and the residual viable oocysts enumerated by microscopic procedures.

As is known, the particles forming turbidity in the natural water usually contains organic substance, so when investigating the effect of turbidity on *Cryptosporidium andersoni* oocysts inactivation, the effect of organic substance (denoted by permanganate index) should be considered as well. Prepare 3 series samples as follow: Series 1 was prepared by humus soils to make the turbidity as 2.20, 3.45, 4.00 and 5.02 NTU and the permanganate index as 2.80, 3.77, 4.85 and 5.56mg/L, respective; series 2 was prepared by kaolinite clay (containing no permanganate index) and make the turbidity as 2.0, 3.0, 4.0 and 5.0 NTU; series 3 was prepared by fulvic acid (containing no turbidity) and make the permanganate index as 1.0, 2.2, 3.2, 4.3 and 5.1. All the 3 series samples above reacted at pH 7.0, 15°C for 20min, and then recorded the number of vital oocysts.

Assessment of oocyst viability

The viability of *Cryptosporidium andersoni* oocysts was determined using fluorescent dye permeability assay based on (Campbell *et al*., 1992; Montemayor *et al*., 2005)with some modification. *Cryptosporidium* oocysts samples were centrifuging to 100μL at 3000r/min for 10min. Then 5μL DAPI and PI was added to the concentrated oocysts and incubated at 37°C for 2h. Superfluous dyes were washed in 0.1M PBS solution twice. Then the samples were concentrated by centrifuging again and examed under Olympus BX51 research microscope at 500nm with blue filter block, 350nm with UV filter block and white light. Because of the PI achromatophilia of viable oocysts (*VO*), the viable oocysts is observed as [DAPI+ PI-] seeing the azure fluorescence by 500nm exciting light. Empty oocysts can be picked out under white light as the refraction only occurrence on the outline of the residues; the oocysts with luminous red exciting light at 350nm are marked as PI+. Empty

oocysts, PI+ oocysts and DAPI- oocysts are recorded as inactive oocysts. The inactivaton rate is calculated as (1).

Inactivation rate(%) = $[(VO_0\text{-}VO_1)/VO_0]\times100$ ...(1)

In this formula, VO_0——the oocysts concentration before sterilization experiments (cell/ml); VO_1——the oocysts concentration after sterilization experiment (cell/ml) .

In terms of each 50cell/ml sample, the viability assessment was conducted for 10 times to eliminate the experimental error of no oocysts observed at a time.

RESULTS AND DISCUSSION

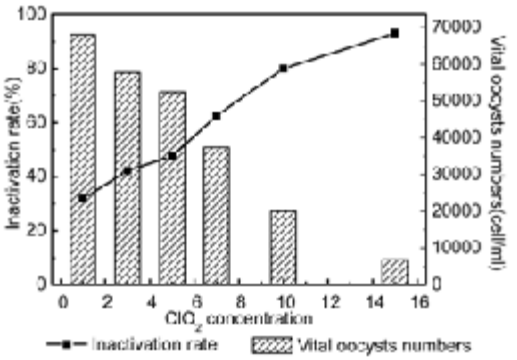
The effect of ClO_2 concentrations on *Cryptosporidium andersoni* oocyst inactivation

Six groups 1ml 10^5cell/ml *Cryptosporidium andersoni* oocysts samples (each group include 2 parallel samples) were put in 1.5ml centrifugal tubes before adding ClO_2 to make ClO_2 concentrations as 1, 3, 5, 7, 10 and 15mg/L, and then assessing the viability of oocysts after reacting 20min under the condition of pH 7.0 and 15°C. The inactivation rates are showed in fig. 1(a). The inactivation curve indicates the effect of ClO_2 concentration on *Cryptosporidium* oocysts inactivation is obviously. The inactivation rates rise along with the increasing of ClO_2 concentrations, and they are in positive correlations. The inactivation rate is 31.61% when ClO_2 concentration is 1mg/L, and then it increased fast to 80.40% until 10mg/L. The inactivation rate can get to 92.9% at 15mg/L ClO_2 and the remaining viral oocysts were 6.8×10^3 cell/ml.

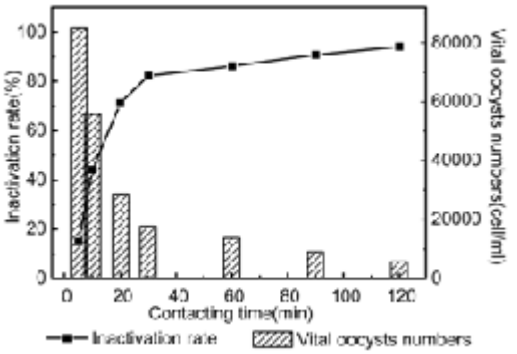
The concentration of *Cryptosporidium andersoni* oocysts in natural water body usually range from several dozen per liter(Fan *et al*., 2001), in particular, it can reach up to 10^4 cell/L in the slaughterhouse wastewater and even remain still as high as 10^3 cell/L(Qian *et al*., 2000). Therefore, we can adjust the ClO_2 dosage when treating different contamination water to maintain good treatment effect.

The effect of contacting time on *Cryptosporidium andersoni* oocyst inactivation

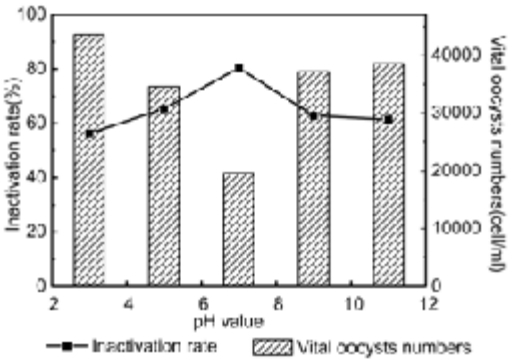
Take 7 groups of 10^5 cell/ml samples, of which the ClO_2 concentrations were set as 10 mg/L, pH as 7.0 and temperature as 15°C. Vital oocysts were assessed at 5, 10, 20, 30, 60, 90 and 120 min. From the data presented in fig.1(b) we can see that the inactivation rates are positively correlated with the contacting time, and are impacted highly by the time at the initial 30min. Then the inactivation rate curve becomes smooth between 30 and 120min. The inactivation rate can reach to 82.26% at 30min,



a) (pH 7.2, T=15°C, t=20min, $C_{AO}=10^5$ cell/ml)



b) ([ClO_2] =10mg/L, pH=7.0, =15°C, $C_{AO}=10^5$cell/ml)



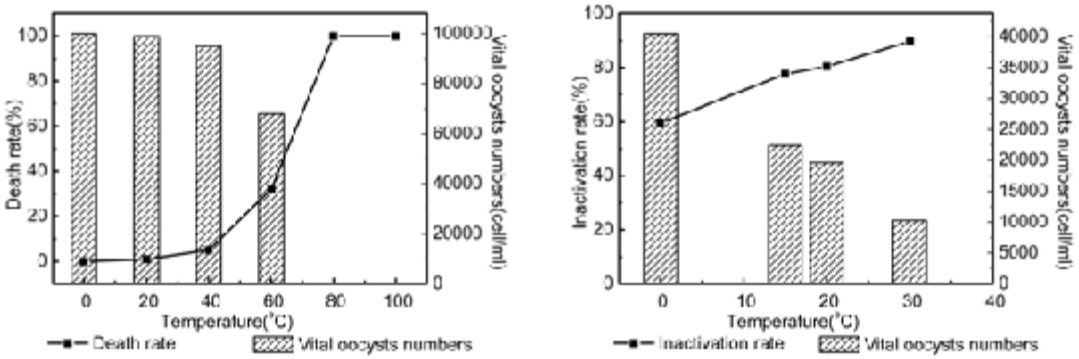
c) ([ClO_2]=10mg/L, T=15°C, t=20min, $C_{AO}=10^5$ cell/ml)

Fig. 1. Effects of contacting time, ClO_2 dosage and pH value on *Cryptosporidium andersoni* oocysts inactivation

and then rise to 86.30% at 90min and beyond 90% after then. So we can get the conclusion that the contacting time is a key parameter of ClO_2 inactivation *Cryptosporidium andersoni* oocysts, and affects the inactivation greatly especially in the initial 30min.

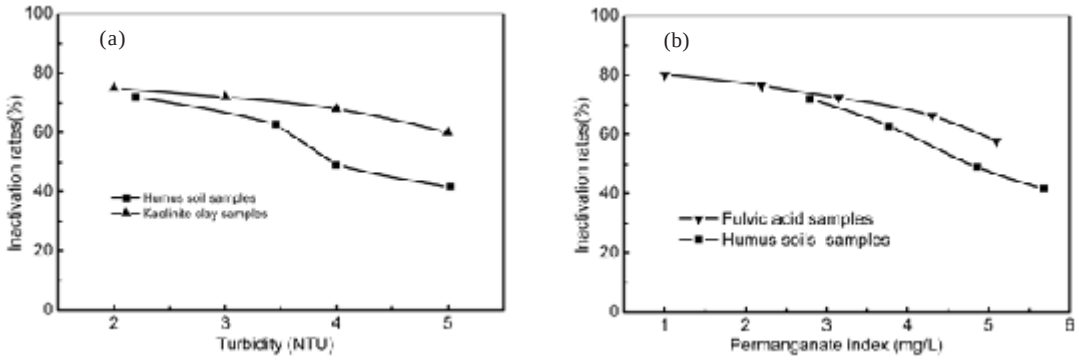
The effect of pH values on *Cryptosporidium andersoni* oocyst inactivation

Different pH buffer solutions were added into 5 groups 10^5 cell/ml *Cryptosporidium* oocysts



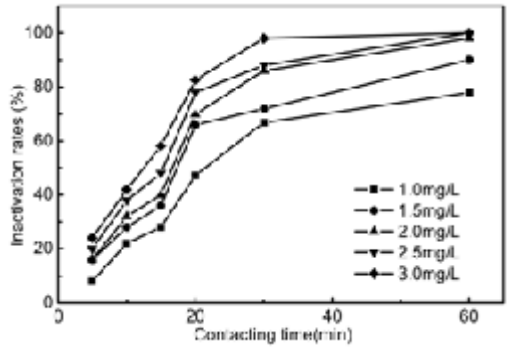
a) (t=5min, pH=7.0,$C_{AO}=10^5$ cell/ml) b) ([ClO_2]=10mg/L, pH=7.0, t=20min, $C_{AO}=10^5$cell/ml)

Fig. 2. Effects of temperature on *Cryptosporidium andersoni* oocysts inactivation



([ClO_2]=10mg/L, pH=7.0, T=15°C, t=20min, $C_{AO}=10^5$cell/ml)

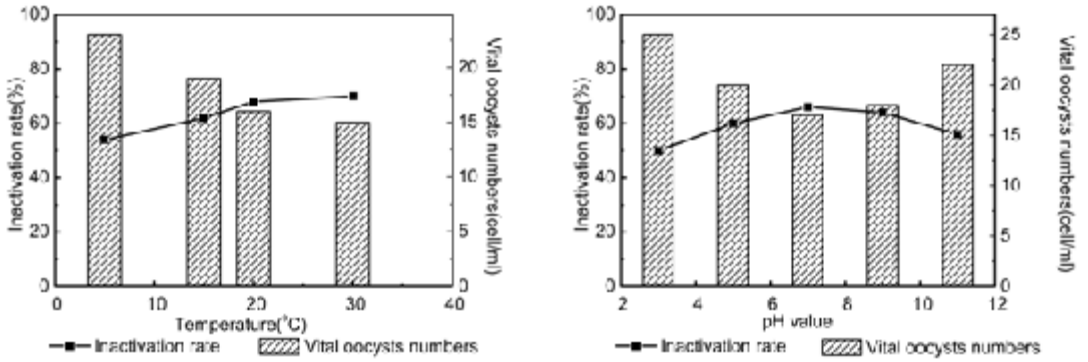
Fig. 3. Effects of turbidity and permanganate index on *Cryptosporidium* oocysts inactivation



(pH=7.0, T=15°C, C_{AO}=50cell/ml)

Fig. 4. Inactivation effect of ClO_2 dosage and time on low concentration *Cryptosporidium andersoni* oocysts

samples described above to make the pH value of reaction systems as 3, 5, 7, 9 and 11. The concentration of ClO_2 ([ClO_2]) in the samples was 10mg/L. The reaction was conducted at 15°C for 20min to investigate the effect of pH value on ClO_2 inactivation *Cryptosporidium* oocysts. From the results in Fig. 1(c), we can know pH value affects the inactivation a little. The inactivation rate curve rises up at first and then goes down when the pH value varies from 3 to 11, and it is 80% at 7.0 that is higher than that at 3 and 11. This can be explained by the transformation of ClO_2 at different pH value in water.



a) ([ClO_2] =2mg/L, pH=7.0, t=20min, C_{AO}=50cell/ml) b) ([ClO_2]=2mg/L, T=15°C, t=20min, C_{AO}=50cell/ml)

Fig. 5. Effect of temperature and pH value on low concentration *Cryptosporidium andersoni* oocysts inactivation

When pH ≤ 2, ClO_2 can be reduced to Cl^- by obtaining 5 mol electrons:

$$ClO_2 + 5e^- + 4H^+ = Cl^- + 2H_2O \quad ...(2)$$

When pH>9, ClO_2 will conduct disproportionating reaction:

$$2ClO_2 + 2OH^- = ClO_2^- + ClO_3^- + H_2O \quad ...(3)$$

As we know the pH value of general resource water is neutral, so ClO_2 is suitable to kill *Cryptosporidium andersoni* oocysts in drinking water.

The effect of temperature on *Cryptosporidium andersoni* oocyst inactivation

At first, the temperature endurance of *Cryptosporidium andersoni* oocysts was studied at 0, 20, 40, 60, 80 and 100 °C by 6 groups 10^5 cell/ml samples for 5min. The results in fig. 2(a) shows that the death rates of *Cryptosporidium* oocysts rised sharply from 40 °C to 80 °C, which indicated that *Cryptosporidium* oocysts can't endure high temperature, and can be totally killed by heating water to a rolling boil.

The effect of temperature on *Cryptosporidium* oocysts inactivation was studied with 4 groups of 10^5 cell/ml oocysts samples at pH 7.0, ClO_2 concentration of 10mg/L and temperatures of 0, 15, 20 and 30°C. The viable oocysts were counted after reacting 20min, the inactivation curve and the numbers of residual oocysts are showed in fig. 2(b), from which we can see that the inactivation rates go up with the temperatures and especially fast beyond 40°C. The inactivation rate is 59.48%, 77.61%, 80.40% and 89.75% at 0, 15, 20 and 30°C, respectively. Synthesizing the results above, the conclusion can be drawn that the *Cryptosporidium* oocysts can't resist high temperature, and ClO_2 can kill the oocysts more easily at high temperature.

The effect of turbidity and permanganate index on *Cryptosporidium andersoni* oocysts inactivation

Fig.3(a) indicated that turbidity has an indistinctive negative impact on the inactivation. The inactivation rates decrease from 75.2% to 60.1% when the turbidities rise from 2.0 to 5.0NTU in the kaolinite clay samples, while they decrease from 72.0% to 41.6% as the turbidities rise from 2.2 to 5.0NTU in the humus soil samples. These declines are caused not only by the turbidity, but also more by the organic matters which can consume ClO_2.

It can be known from fig. 3(b), the inactivation rates decrease from 81.1% to 57.7% when the permanganate index rise from 1.0 to 5.1mg/L, while they decrease from 72.0% to 41.6% when the permanganate index rises from 2.8 to 7.7mg/L. True, in the humus soil samples the influencing factors are both turbidity and permanganate index, but the effect of turbidity is slighter than it of permanganate index comparing with the data in fig. 3(a).

From the above discussion, the conclusion can be reached that the influence of turbidity on ClO_2 disinfection results from two points: one is the tiny particles in water proving sheltering for the oocysts so that reduce the effect of ClO_2, the other is the permanganate index of the particles that can react with the disinfection and

consume it. By analysis the data, the effect of permanganate index is more important than that of turbidity.

The effect of ClO_2 dosage and time on low concentration *Cryptosporidium andersoni* oocyst inactivation

The effects on low concentration oocysts inactivation were also been investigated followed by the previously experiments in order to providing reference to engineering application considering the actual situation of water bodies.

ClO_2 stock solution was added in to 6 group 50cell/ml *Cryptosporidium andersoni* oocysts samples to make the ClO_2 concentration as 1.0, 1.5, 2.0, 2.5 and 3.0mg/L. The reaction conducted at pH 7.0 and 15°C. Vital oocysts were assayed at 5, 10, 15, 20, 30 and 60min, respectively. The results are shown in fig. 4. We can see that the inactivation rates rise along the increasing of ClO_2 dosage, which is consistent with the result of high concentration above. Small amount of ClO_2 can kill oocysts effectively as the inactivation rate can reach to 90% by 2.0mg/L ClO_2 at 30min.When the reaction time is 20min, the inaction rates are 48%, 66%, 70%, 78% and 82% at ClO_2 dosages of 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L, respectively, and they rise to 78%, 90%, 98%, 100% and 100% when prolong the reaction time to 60min. The tendencies of inactivation rates with the time are similar under different ClO_2 dosages and they increase fast in the first 20min, and then slow down between 30 and 60min, so set the contacting time as 30min is suitable when use ClO_2 to control *Cryptosporidium andersoni* oocysts in the practical application.

A conclusion can be induced from these experiments that ClO_2 dosage and contacting time are important parameters to affect ClO_2 inactivation *Cryptosporidium andersoni* oocysts. Satisfaction effect can be obtained by more ClO_2 dosage and longer contacting time.

The effect of temperature and pH on low concentration *Cryptosporidium andersoni* oocyst inactivation

The ClO_2 dosage of 4 groups 50 cell/min oocysts samples were setted as 2.0mg/L and pH value of which was 7.0. The experiments were conducted at 5, 15, 20 and 30°C for 20min to investigate the effect of temperature on ClO_2 inactivation *Cryptosporidium andersoni* oocysts. The results (Fig.5(a)) show that the inactivation rates rise along with the temperature, higher inactivation rates can be gotten at higher temperature, it increase from 54% to 70% when the temperature rise from 5°C to 30°C, which is in accordance with the results above.

Five groups 50 cell/ml vital oocysts were prepared under the condition of 2mg/L ClO_2 and 15°C as described above. pH value was adjusted as 3, 5, 7, 9 and 11. The reaction was stop by sodium thiosulfate at 20min. From the results in fig.5 (b), we can see that the effect of pH value is not obvious and it is higher at neutral condition, which is in accord with the results of the previous study.

CONCLUSION

ClO_2 can inactivate *Cryptosporidium andersoni* oocysts in water effectively, and mostly affected by contacting time and ClO_2 dosage; better inactivation efficacy can be get at longer reaction time and higher ClO_2 dosage. pH affects the inactivation slightly, and the inactivation rates keep high at natural situation between pH 5-9. The influence of temperature on the inactivation is obvious, the inactivation rates rise along with the increasing of temperature, which may be explained as the oocysts can't resist high temperature. Both turbidity and organic matters can affect the oocysts inactivation by ClO_2 in water, and the influence of the latter is greater.

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