Evaluation of *Cryptosporidium* Disinfection by Ozone and Ultraviolet Irradiation Using Viability and Infectivity Assays

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In the ozone disinfection unit process of a piston type batch reactor with continuous ozone analysis using a flow injection analysis (FIA) system, the CT values for 1 log inactivation of *Cryptosporidium parvum* by viability assays of DAPI/PI and excystation were 1.8~2.2 mg/L·min at 25℃ and 9.1 mg/L·min at 5℃, respectively. At the low temperature, ozone requirement rises 4~5 times higher in order to achieve the same level of disinfection at room temperature.
In a 40 L scale pilot plant with continuous flow and constant 5 minutes retention time, disinfection effects were evaluated using excystation, DAPI/PI, and cell infection method at the same time. About 0.2 log inactivation of *Cryptosporidium* by DAPI/PI and excystation assay, and 1.2 log inactivation by cell infectivity assay were estimated, respectively, at the CT value of about 8 mg/L·min. The difference between DAPI/PI and excystation assay was not significant in evaluating CT values of *Cryptosporidium* by ozone in both experiment of the piston and the pilot reactors. However, there was significant difference between viability assay based on the intact cell wall structure and function and infectivity assay based on the developing oocysts to sporozoites and merozoites in the pilot study. The stage of development should be more sensitive to ozone oxidation than cell wall intactness of oocysts. The difference of CT values estimated by viability assay between two studies may partly come from underestimation of the residual ozone concentration due to the manual monitoring in the pilot study, or the difference of the reactor scale (50 mL vs 40 L) and types (batch vs continuous). Adequate IT value to disinfect 1 and 2 log scale of *Cryptosporidium* in UV irradiation process was 25 mWs/cm^2 and 50 mWs/cm^2, respectively, at 25℃ by DAPI/PI. At 5℃, 40 mWs/cm^2 was required for disinfecting 1 log *Cryptosporidium*, and 80 mWs/cm^2 for disinfecting 2 log *Cryptosporidium*. It was thought that about 60% increase of IT value requirement to compensate for the 20℃ decrease in temperature was due to the low voltage low output lamp letting weaker UV rays occur at lower temperatures.

Key words – *Cryptosporidium*, disinfection, ozone, UV, DAPI/PI, *in vitro* excystation, cell infection

Introduction

Cryptosporidium is a waterborne pathogen, which exists in fecally contaminated water. The Milwaukee outbreak in 1993, due to the *Cryptosporidium* contamination, is one of the well-known cases, with more than 400 thousand citizens infected through tap water inadequately processed after a heavy rain[5]. *Cryptosporidium* strongly resists against chlorine disinfection process. Therefore, the advanced oxidation processes such as ozone or UV for treatment of *Cryptosporidium* have been actively studied as the alternative processes of chlorine disinfection.

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In this study, disinfection of *Cryptosporidium* in the ozone unit process using a lab scale piston type reactor (50 mL, pyrex) and in the ultraviolet irradiation unit process using a low voltage UV lamp were evaluated. In addition, *Cryptosporidium* disinfection with a upscaled 40 L pilot reactor of continuous flow system installed in Incheon City Water Quality Research Institute was evaluated. *Cryptosporidium* was assayed with DAPI/PI and in vitro excystation as viability assays[1,2,6,11] and with cell culture-immunofluorescence assay (cell culture-IFA) using HCT-8 cell as an infectivity assay[8-10].

Materials and Methods

Ozone oxidation in a piston type batch reactor

A piston type reactor (50 mL, Pyrex) which does not have free headspace was used for the inactivation study. Concentrated ozone made with ozone generator (CFS-1, Ozonia Co., Switzerland) was introduced into a reactor and mixed immediately with a sample solution to attain an approximate 1~3 mg/L ozone concentration. The specially designed instrument where the flow injection analysis (FIA) technique applied was installed to continually and accurately measure the residual ozone concentration. A small fixed quantity of the ozonated samples was taken continuously and mixed rapidly with an indigo solution to determine the ozone concentration. In general, 4~6 samples of 1 mL for *Cryptosporidium* analysis were taken within 2~5 minutes. The 0.03 mL of $Na_2S_2O_3$ was used to stop the reaction with the residual ozone. The sand filtrate of a water utility in Seoul was used as the medium of this experiment and its pH was 7.1. Live *Cryptosporidium* (1×10^7/8 mL stock, Waterborne Inc.) was inoculated after being washed 3 times with distilled water and diluted to adequate concentrations.

Up-scaled ozone oxidation in a pilot reactor with continuous flow

A 40 L scale ozone reactor with continuous flow was used for this disinfection study. The sand filtrate of a water utility pilot plant in Incheon and ozone were mixed from the bottom to the top as sand filtrate and ozone generator (CF1A, Ozonia, USA) were connected at the bottom of the reactor. The CT values were controlled by varying ozone concentrations under the fixed water velocity of 8 L/min and contact time of 5 minutes. Live *Cryptosporidium* (5×10^8/10 L, Waterborne Inc.) was inoculated into the influx line with a metering pump at 16.67 mL/min to mix completely with the flowing water. Then the mixed water was agitated again to ensure the constant concentration of *Cryptosporidium* in water before it flowed into ozone reaction tank. The concentrations of the injected ozone were controlled by the generated ozone concentrations (6~18 g/m^3) and the injected ozone flow (0.25~1.5 L/min). The 1 L samples were taken from the T-valve installed at the outlet of ozone reactor, up to where the ozone and water had been mixed completely. The effluent water from the reactor was discharged into a sewer after pumping through two serial filters to remove remaining *Cryptosporidium*. The ozone oxidation reaction was stopped at the sampling in 1 L bottle contained 30 mL of 0.1 N sodium thiosulfate in advance. The residual ozone concentrations were measured by indigo method[3]. Water quality parameters of TOC, DOC, UV_{254}, pH, Temperature, turbidity were measured by TOC analyzer (Phoenix-8000, Tekmar, USA), UV-Visible spectrophotometer (Cary3E, Varian, USA), pH meter (920A, Orion, USA) and turbidometer (2100N, HACH, USA).

UV disinfection

Collimatric method and 15 W 253.7 nm germicidal lamp (15W×4, Philips Co., Netherlands) was used for inactivating *Cryptosporidium* in UV irradiation unit process. A petri dish (6 cm diameter, 1.5 cm depth, for cell culture) was used as a reactor where *Cryptosporidium* (washed and diluted as above) was irradiated with UV for reaction time. The experiments were performed under UV intensity of 0.2, 0.4, 0.9 mWs/cm^2 at 25℃, and 0.4 mWs/cm^2 at 5℃. The Samples (1 mL) were taken 5~7 times during the reaction times (50 sec to 120 min). The actual UV intensity irradiated was measured by UV detector (UVX radiometer, UVP Co., USA).

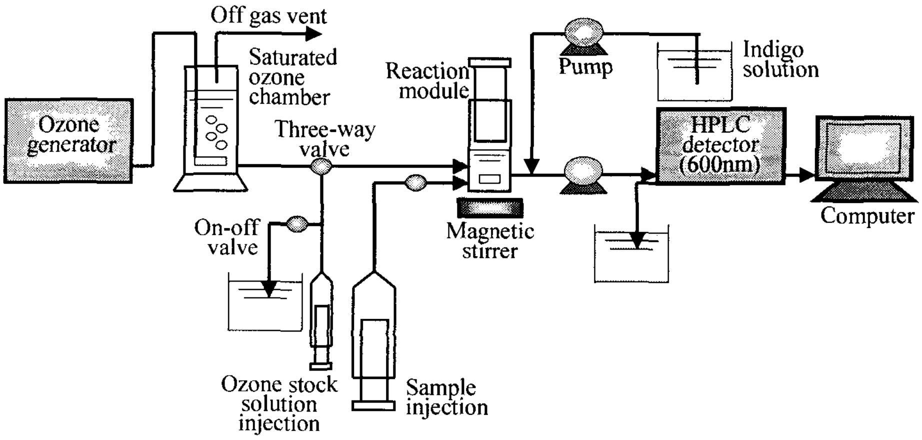

Fig. 1. Schematic diagram of ozone disinfection with a piston type batch reactor.

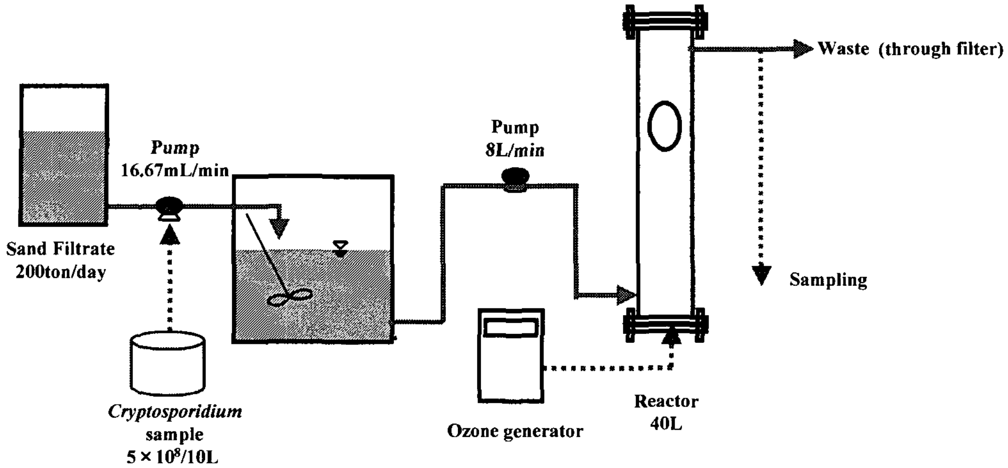

Fig. 2. Schematic diagram of ozone disinfection with a up-scaled pilot plant system.

Cryptosporidium assay

Cryptosporidium was assayed by DAPI/PI and in vitro excystation for the viability tests, and cell culture-IFA using HCT-8 cell for the infectivity test. The low concentrations of *Cryptosporidium* in 1 L samples from the pilot study were further concentrated to 1 mL by serial centrifugations (15 minutes at 1,500 g) before the assays.

DAPI/PI. A 50 μL of each concentrated samples was dropped in well slides, dried, and then fixed by adding 50 μL of 100% methanol. Afterwards, the samples were dyed by immunofluorescent detection reagent (Meridian diagnostics) and DAPI/PI (4′-6-diamidino-2-phenylindole/propidium iodide), and then observed with fluorescent microscope, as modified from Campbell method[2].

In vitro excystation. Acidified HBSS (pH 2.75) was added to 100 μL of each sample and was incubated for 1 hour under 37℃. After the incubation, sample was rinsed 3 times by centrifuging at 15,000 rpm for 5 minutes with HBSS (pH 7.2) and then adjusted to the original volume of 100 μL. After the 50 μL of 0.44% sodium bicarbonate and 200 μL of Hank's minimal essential medium containing 1% bile were added in the sample and incubated for 1 hour at 37℃, the sample was observed under the DIC microscope at 1,000x magnification.

Cell culture-IFA using HCT-8 cells. The HCT-8 cells were cultured in RPMI 1640 medium containing filtered 10% FBS (fetal bovine serum) in cell culture incubator (37℃, 5% CO_2 and constant humidity) for 2∼3 days to form monolayer in 75 m^2 tissue culture flasks. The cultured cells were transferred to 8-well chamber slide systems (Lab-Tek chamber slide system 177445) to be formed in monolayers. *Cryptosporidium* was inoculated in each well with cell monolayer, and incubated for 3∼4 days at the same condition mentioned above. After incubation, the supernatant medium in the well was removed by Pasteur pipette to dye *Cryptosporidium* with the fluorescent reagent, sporo-glo (Waterborne, Inc.), which bonds antibody against sporozoites and merozoites. The sporozoites and merozoites differentiated from infectious oocysts were observed under the fluorescent microscopy.

Results and Discussion

Inactivation of *Cryptosporidium* in a piston type ozone batch reactor

The ozone concentrations in water samples, as seen in Fig. 3, decreased immediately to 0.6, 1.7, 2.8 mg/L from initial 0.8, 2, 3.1 mg/L due to the instant ozone demand (IOD). The IOD is mainly due to the decomposition of organic substances dissolved in water[7]. To minimize the impact of *Cryptosporidium* inoculum on IOD, the inoculum had been washed 3 times thoroughly. Despite the effort, the ozone concentration dropped from 0.8 mg/L to 0.25 mg/L in 30 seconds when 0.8 mg/L of ozone was injected. Adequate CT value for disinfection could not be obtained after 3 minutes, when the ozone was diminished completely. When 2 mg/L of ozone was injected, inactivation of *Cryptosporidium* could be obtained after 1.5 minutes of reaction in both DAPI/PI and in vitro excystation. Injection of 3.1 mg/L of ozone led to 1 log scale of inactivation of *Cryptosporidium* after 0.8 minutes. 2 log scale of inactivation was obtained after 1.6 to 2 minutes in DAPI/PI and in vitro excystation, respectively.

As shown in Fig. 4, CT values to inactivate 1 log scale

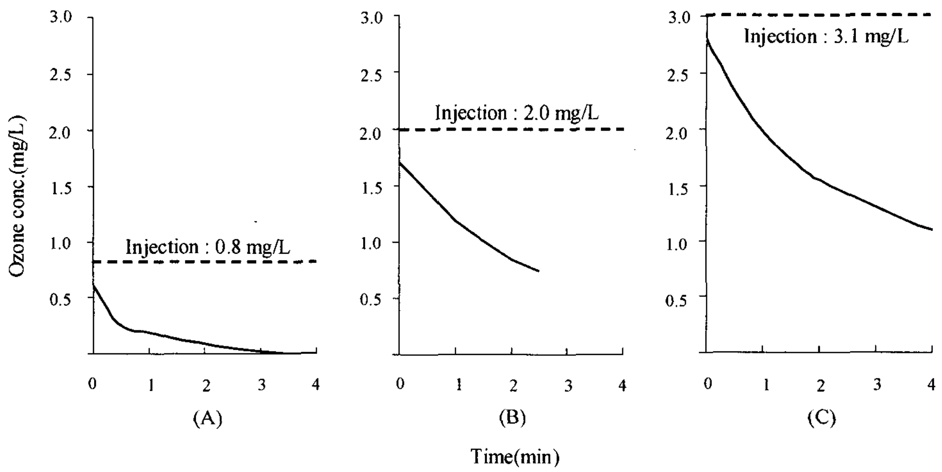

Fig. 3. Instant ozone demand (IOD) and the following decrement of ozone concentration as reaction time increment, when injected ozone were at 1 (A), 2 (B) and 3 mg/L (C).

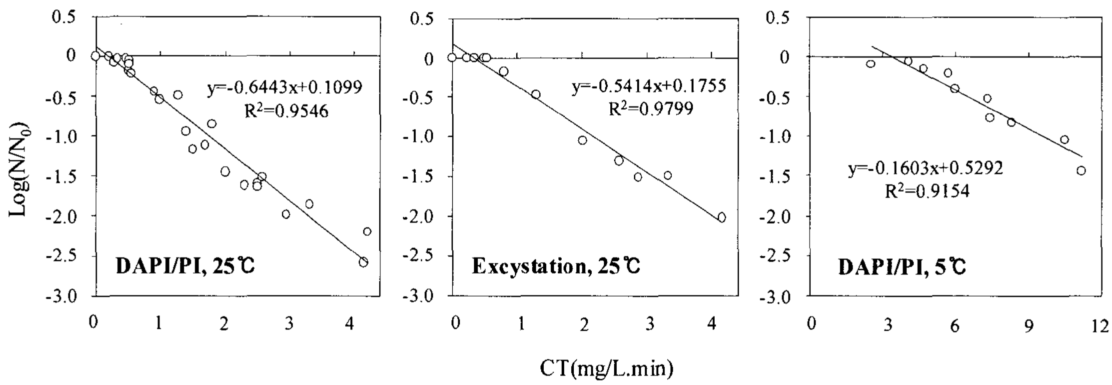

Fig. 4. Comparison of CT curves of *Cryptosporidum* by DAPI/ PI and in vitro excystation at reaction temperature of 25℃ and 5℃.

of *Cryptosporidium* at 25℃ were estimated as 1.8 mg/L · min by DAPI/PI method, and 2.2 mg/L · min by in vitro excystation. The CT values required to inactivate 2 log were 3.2 mg/L · min by DAPI/PI and 3.8 mg/L · min by in vitro excystation. According to these results, ozone could be much more effective for *Cryptosporidium*, when compared to the CT value of 7,200 mg/L · min for 1 log inactivation with chlorine[4]. At low temperature of 5℃, CT value for 1 log inactivation was measured to be 9.1 mg/L · min in comparison to the 1.8 mg/L · min at 25℃ by DAPI/PI method. The CT value for 2 log inactivation was 14.8 mg/L · min at 5℃ in comparison to 3.2 mg/L · min at 25℃. It showed that the ozone disinfection requirement should be stronger at lower temperature in order to achieve the same level of disinfection.

Inactivation of *Cryptosporidium* in a up-scaled ozone pilot with continuous flow

Water quality of the influent water of the ozone pilot reactor, which was the sand filtrate of the water treatment plant in Incheon, was measured to find the effect on ozone disinfection and oxidation. The influent water quality measured with 0.07~0.12 NTU in turbidity, 1.035~1.387 mg/L in TOC, 0.758~1.044 mg/L in DOC, and 0.0081~0.0119 in UV_{254}, had not changed much in the effluent as measured as shown 0.07~0.11 NTU in turbidity, 1.066~1.603 mg/L in TOC and 0.933~1.099 mg/L in DOC, respectively. Only UV_{254} showed a decrease by ozonation (Fig. 5). The water temperature of this water was about 9℃.

The CT value of *Cryptosporidium* derived from dissolved ozone concentration (measured) and contact time was plotted as shown in Fig. 6. In this experiment, live *Cryptosporidium* was measured by both viability and infectivity assays from the same samples. CT values and log reduction of *Cryptosporidium* were expressed as linear relationships. At maximum CT value of 8 mg/L · min, *Cryptosporidium* assayed with DAPI/PI and in vitro excystation appeared to be inactivated about 0.2 log. On the other hand, *Cryptosporidium*

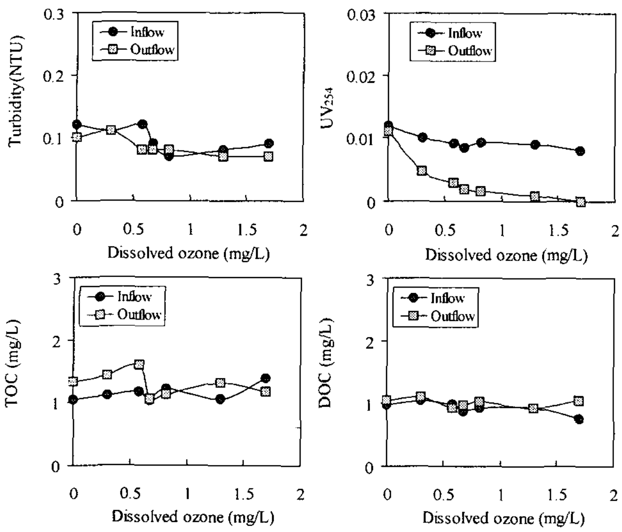

Fig. 5. Variation of turbidity, UV254, TOC and DOC by ozone treatment.

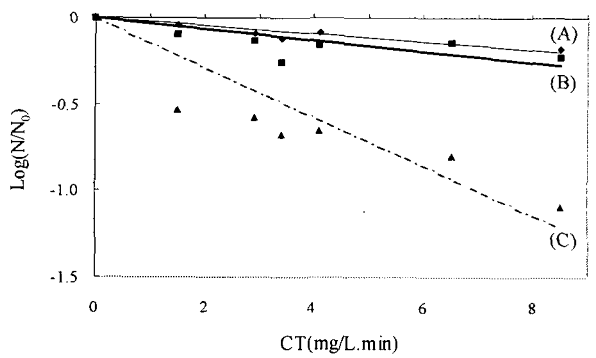

Fig. 6. Evaluation of *Cryptosporidium* inactivation using in vitro excystation(A), DAPI/PI (B) and cell culture-IFA method using HCT-8 cell (C) by ozone disinfection.

assayed with cell culture-IFA assay was inactivated at about 1.2 log at the same CT of 8 mg/L · min. The viability assays are based on the intactness of the cell wall structure and function of live cells. Infectivity test are based on the developing stage from oocysts to sporozoites and merozoites in the life cycle. Therefore, ozone was thought to damage the substances relating to the developing stages more seriously than the oocyst cell wall components.

When compare to the CT values of a piston type batch reactor study, which showed 9.1 mg/L · min of CT value to disinfect 1 log at 5℃ with DAPI/PI assay, the estimated CT values were about 4~5 times lower. The difference between the two studies of the piston type batch reactor and the continuous flow pilot reactor may partly come from the ozone monitoring, real-time versus manual monitoring. Because of strong oxidation agents diminishing quickly,

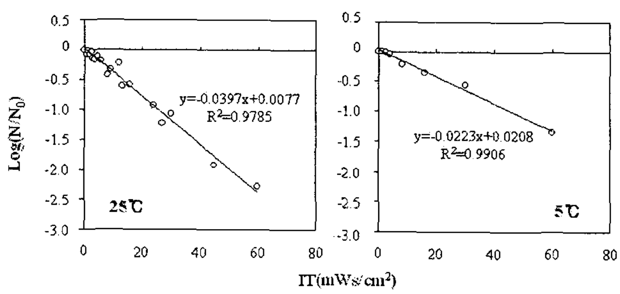

Fig. 7. IT value of *Cryptosporidum* by DAPI/PI from the experiment with UV intensity of 0.2, 0.4, 0.9 mWs/cm^2 at reaction temperature of 25℃ and 5℃.

manual monitoring of ozone concentration could underestimate the real residual concentrations. In addition, the up-scale continuous type reactor may have constantly high IOD keeping to the high disinfection requirement, which was not able to be measured.

Inactivation of *Cryptosporidium* in UV irradiation

The IT values of inactivating *Cryptosporidium* according to UV intensity of 0.2, 0.4, 0.9 mW/cm^2 were analyzed with DAPI/PI method. As a result, IT values for 1 and 2 log disinfection were 25 and 50 mWs/cm^2, respectively, at room temperature of 25℃. At low temperature of 5℃, 40 mWs/cm^2 was required for disinfecting 1 log *Cryptosporidium*, and 80 mWs/cm^2 for disinfecting 2 log *Cryptosporidium*. It showed that about 60% IT value was increased to compensate for the 20℃ decrease in temperature. It is thought that the property of low voltage low output lamp lets weaker UV rays occur at lower temperatures.

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초록 : 크립토스포리디움의 활성/감염성 판별법을 이용한 오존 및 자외선 소독능 평가

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크립토스포리디움은 염소내성이 매우 강해 일반적인 표준정수처리공정의 소독으로는 제거가 불가능하다. 따라서 본 연구에서는 오존 및 UV를 이용한 단위소독공정에서 DAPI/PI 및 in vitro excystation을 이용하여 크립토스포리디움 불활성화를 평가하였으며, 또한 오존을 이용한 고도산화처리 파일럿에서는 세포배양법을 이용하여 크립토스포리디움 불활성화를 평가하였다.

오존 소독연구는 50 mL 용량의 piston type batch reactor에서 용존오존을 자동적으로 측정해주는 flow injection analysis (FIA) 시스템을 이용하여 실험한 결과, 1 log 제거에 필요한 CT값은 25℃에서 DAPI/PI 및 in vitro excystation에 의해 각각 약 1.8, 2.2 mg/L · min으로 나타났으며, 2 log 제거에 필요한 CT값은 각각 약 3.2, 3.8 mg/L · min으로 나타났다. 또한 5℃에서 크립토스포리디움 1 log 제거에 필요한 CT값은 DAPI/PI 방법에 의해 약 9.1 mg/L · min으로 나타났으며, 2 log 제거에 필요한 CT값은 14.8 mg/L · min로 나타나, 같은 소독효과를 나타내기 위해서 저온에서는 상온에서보다 요존 요구량이 약 4~5배 정도 증가하여야 함을 확인하였다.

40 L 규모의 오존 반응조를 이용한 파일럿 실험에서는 정수처리공정상 모래여과를 거친 물에 살아있는 크립토스포리디움을 접종한 것을 시료로 하여 연속적으로 흐르게 한 다음, 오존량을 변화시키고 체류시간은 5분으로 고정하여 불활성화를 평가하였다. 실험결과, 8 mg/L · min의 CT값에서 DAPI/PI 및 excystation과 같은 생사판별법을 이용하였을 경우에는 약 0.2 log 정도의 불활성화를 나타내었으며, 세포감염시험법을 이용하였을 경우에는 약 1.2 log 정도의 불활성화를 나타냈다. 오존에 의한 크립토스포리디움의 소독능 평가에 단위공정 및 파일럿 실험 모두 2 가지 생사판별법(DAPI/PI와 excystation) 사이에는 큰 차이를 나타내지 않았으나, 생사판별법과 세포감염시험법 사이에는 현저한 차이를 나타내었는데, 이는 세포감염시험법으로 측정하는 sporozoite 및 merozoite로의 분화과정이 생사판별법이 근거한 세포벽의 구조와 기능 유지 보다 더 오존 소독에 더 민감함을 알 수 있었다. 파일럿 실험에서의 CT값이 piston batch reactor에서의 CT값 보다 낮게 나타난 것은 파일럿 실험에서 수작업으로 인한 용존 오존 측정이 정밀하지 못하여 IOD가 농도에 반영되지 않았고, 반응조 규모(50 mL vs 40 L) 및 형태(회분식 vs 연속식)의 차이에 기인하는 것으로 여겨진다.

한편, UV를 이용한 단위공정에서는 크립토스포리디움 1, 2 log 제거에 필요한 IT값은 25℃에서 각각 DAPI/PI 방법에 의해 약 25, 50 mWs/cm^{2}로 나타났으며, 5℃에서의 크립토스포리디움 1, 2 log 제거에 필요한 IT값은 약 40, 80 mWs/cm^{2}로 나타났다. 온도 20℃ 감소 시 약 60% 정도의 IT값이 더 필요한데, 이것은 저온에서는 약한 자외선을 발산하는 저압저출력 UV 램프의 특성 때문인 것으로 사료되었다.