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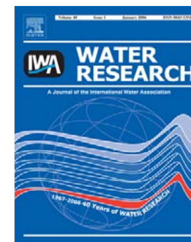

Investigating synergism during sequential inactivation of *Bacillus subtilis* spores with several disinfectants

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ABSTRACT

The sequential application of ozone, chlorine dioxide, or UV followed by free chlorine was performed to investigate the synergistic inactivation of *Bacillus subtilis* spores. The greatest synergism was observed when chlorine dioxide was used as a primary disinfectant followed by secondary disinfection with free chlorine. A lesser synergistic effect was observed when ozone was used as the primary disinfectant, but no synergism was observed when UV was used as the primary disinfectant. When free chlorine was used as the primary disinfectant (i.e., sequential application in the reverse order), the synergistic effect was shown only when chlorine dioxide was applied as the secondary disinfectant. The synergistic effect observed could be related to damage to the spore coat during primary disinfection, suggested by the loss of proteins from spores during disinfectant treatment. The greatest synergism observed by the chlorine dioxide/free chlorine pair suggested that common reaction sites might exist for these disinfectants. The concept of percent synergistic effect was introduced to quantitatively compare the extent of synergistic effects in the sequential disinfection processes.

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1. Introduction

Sequential disinfection is considered a viable treatment option to control pathogenic microorganisms such as oocysts of protozoan parasites (e.g., *Cryptosporidium parvum* etc.) and spores of vegetative bacteria (e.g., *Bacillus subtilis* and *B. anthracis*) that cannot be treated effectively by a single step application of common chlorine-based disinfectants. In the sequential disinfection processes, a primary disinfectant such as ozone, chlorine dioxide or UV is first applied to achieve a portion of the target inactivation level. A secondary disinfectant such as free chlorine is subsequently applied to attain further inactivation and to provide a residual disinfectant for a relatively long period of time during water distribution. Sequential application of certain set of disinfectants has been shown to result in a higher level of inactivation than the sum of inactivation levels achieved when each disinfectant is applied separately in a single step. This enhanced inactivation is often referred to as a synergism in the sequential disinfection processes. Synergism is beneficial since the disinfectant dose and the reaction time required for the same level of inactivation can be reduced, thus leading to reduced operating costs and decreased disinfection by-product formation.

Previous studies with *C. parvum* oocysts reported that synergism was observed when disinfection with ozone was followed by secondary disinfection with either free chlorine (Gyürék, 1997; Driedger et al., 2000; Rennecker et al., 2000) or

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monochloramine (Gyürék, 1997; Rennecker et al., 2000, 2001; Driedger et al., 2001a). On the contrary, when chlorine dioxide was used as the primary disinfectant, the secondary treatment with free chlorine or monochloramine did not show any synergism (Corona-Vasques et al., 2002). However, these observations on the synergistic effects do not appear to be consistent with other studies reported in the literature. Lewin et al. (2001) observed an absence of any synergistic effect in the sequential inactivation of *C. parvum* oocysts with ozone followed by free chlorine. Liyanage et al. (1997) observed a synergistic effect when ozone or chlorine dioxide was followed by free chlorine or monochloramine. Synergistic effects were also observed with other microorganisms. For example, sequential application of ozone followed by free chlorine to *B. subtilis* spores showed enhanced inactivation efficiency, according to Cho et al. (2003a). However, compared to *C. parvum* oocysts, studies on synergism in sequential inactivation of *B. subtilis* spores are relatively limited.

The objective of this study is to evaluate the synergistic effect when ozone, chlorine dioxide, or UV disinfection is followed by free chlorine disinfection. *B. subtilis* was used as a target microorganism since this species has often been used as a biological surrogate for *C. parvum* oocysts (Radziminski et al., 2002; Larson and Mariñas, 2003) as well as *B. anthracis* spores (Nicholson and Galeano, 2003; Armon et al., 2004). Synergism during sequential disinfection in the reverse order was investigated, and the rate of protein extraction during each disinfectant treatment was determined in order to explore the mechanism of synergism.

2. Materials and methods

2.1. Preparation and viability assessment of *B. subtilis* spores

Suspensions of *B. subtilis* spores were prepared following the procedure by Nakayama et al. (1996), except for minor modifications such as using an 1/10 diluted nutrient agar and providing an extended incubation period of 5–7 days (Cho et al., 2003a). In brief, a freeze-dried pellet of *B. subtilis* (ATCC 6633) obtained from the American Type Culture Collection (Manassas, VA) was first rehydrated aseptically using a Nutrient Broth (Difco Co., USA) and incubated at 37 °C for 18 h. Bacterial cells were then harvested from the broth by repeating centrifugation at $3500 \times g$ for 10 min and resuspension in a 50 ml of 150 mM phosphate buffered saline (PBS) at pH 7.2 twice. Several 47-mm sterile Petri dishes containing 1/10 diluted nutrient agar ($0.8\,g\,l^{-1}$ Difco Nutrient Broth+$15\,g\,l^{-1}$ Bacto Agar, Difco Co., USA) were subsequently inoculated and incubated at 37 °C for 5–7 days to induce sporulation. After incubation, *B. subtilis* spores were collected into 50 ml conical tubes by rinsing the agar with PBS. The spores were cleaned by repeated centrifugation at $3500 \times g$ for 10 min and resuspended in PBS three times. The recovery of spores from each centrifugation/resuspension procedure was over 99%. In order to inactivate any remaining vegetative cells, the stock solution was heat treated at 80 °C for 20 min before each experiment. The stock solution was then diluted with 20 mM PBS at pH 5.6 or 8.2 to prepare a test solution containing an initial spore population of 10^6–$10^7\,cfu\,ml^{-1}$. During disinfection experiments, 1 ml aliquots of samples were withdrawn from the reactor at various reaction times and immediately quenched using sodium thiosulfate ($Na_2S_2O_3$). The viability of spores in each sample was assessed by a spread plate method (Standard Method, 18th ed., 1992). For each sample, a ten-fold serial dilution was performed up to 1/10,000 dilution ratio using 150 mM PBS at pH 7.1. A 0.1 ml aliquot of each diluted solution was inoculated onto three replicate 47-mm sterile Petri dishes containing nutrient agar ($8\,g\,l^{-1}$ Difco Nutrient Broth+$15\,g\,l^{-1}$ Bacto Agar, Difco Co., USA). Colony forming units were counted after incubation at 37 °C for 24 h (Cho et al., 2003a, b). Selection of statistically meaningful plate counts was carried out according to Standard Methods (1992).

2.2. Preparation and analysis of disinfectants

Deionized/distilled water purified by a Barnstead NANO pure system (Barnstead Co., USA) and analytical reagent grade chemicals were used to prepare all experimental solutions. All glassware was cleaned by distilled water and further sterilized by autoclaving at 121 °C for 15 min. A concentrated ozone solution ($>40\,mg\,l^{-1}$) was prepared from pure oxygen using a CFS-1 Ozone Generator (Ozonia Co., Switzerland). Dissolved ozone concentrations were determined by the indigo colorimetric method (Bader and Hoigné, 1981). The absorbance of indigo trisulfonate at 600 nm was measured by a HP 8452A UV-VIS Spectrophotometer (Hewlett-Packard Co., Germany). A free chlorine stock solution ($300\,mg\,l^{-1}$) was prepared by diluting a reagent sodium hypochlorite solution (13%, Junsei Co., Japan). Free chlorine concentrations were determined by the DPD colorimetric method (Rennecker et al., 2000) using a DR/2010 Spectrophotometer (HACH Co., USA). A stock solution of chlorine dioxide ($200\,mg\,l^{-1}$) was prepared by oxidizing $NaClO_2$ (Aldrich Co., USA) with H_2SO_4 (Aldrich Co., USA) following the procedure by Radziminski et al. (2002). Chlorine dioxide concentrations were determined by measuring the UV absorbance at 385 nm (Aieta and Berg, 1986). The UV intensity at 254 nm was measured using a radiometer and a UV 254 detector (UVX Radiometer, UVP Co., USA) (Shin et al., 2001).

2.3. Single and sequential disinfection procedure

Single step disinfection experiments were performed at two pH levels (5.6 and 8.2) at a constant temperature of 25 ± 0.5 °C. The experiments with ozone, chlorine dioxide, and free chlorine were performed in a head-space-free Pyrex piston-type reactor that could hold 50 ml test solution. The experiments were initiated by instantly transferring the concentrated disinfectant stock solution into the reactor containing PBS (pH 5.6 or pH 8.2, 20 mM) and *B. subtilis* spores. During each experiment, the solution was mixed using a magnetic stir bar placed inside the reactor. Samples were taken from the sampling outlet of the reactor and collected into sampling bottles without contact to the atmosphere. The residual disinfectants were instantaneously quenched using excess sodium thiosulfate ($Na_2S_2O_3$). The UV disinfection experiments were performed with a bench-scale collimated-beam UV reactor that was equipped with a 15-W low-pressure UV

lamp (Philips Co., Netherlands), emitting a nearly monochromatic UV radiation at 253.7 nm. This equipment produced a parallel irradiation of UV by means of a 60×50 cm long collimating tube placed below the UV lamps as described by Huffman et al. (2002). The experiments were conducted following the procedure described by Shin et al. (2001). Prior to the initiation of the experiment, the UV lamp was turned on for at least 30 min to obtain a constant UV intensity output. A 60×15 mm sterile Petri dish filled with the 40-mL experimental solution was immediately placed normal to the incident light for the initiation of the experiment. All disinfections experiments were performed at a fixed initial chemical disinfectant concentration ($2\,mg\,l^{-1}$ for ozone and chlorine dioxide and $3\,mg\,l^{-1}$ for free chlorine) and UV intensity ($0.4\,mW\,(cm^2)^{-1}$). Three to six samples were taken during each experiment to assess viability of *B. subtilis* spores and measure residual disinfectant concentrations.

The sequential disinfection experiments were performed by first applying ozone, chlorine dioxide, or UV as the primary disinfectant and free chlorine as the secondary disinfectant. Another set of experiments was performed by reversing the order of disinfectant application, i.e., free chlorine as the primary disinfectant followed by ozone, chlorine dioxide or UV as the secondary disinfectant. Experiments were conduced to achieving each 1 log inactivation as primary and secondary disinfection, since typical level of inactivation would require around 2 log inactivation where protozoa, such as *C. parvum*, are concerned, and the maximum synergistic effect was observed on control test.

Primary disinfection was performed to achieve 1 log inactivation of *B. subtilis* spores at pH 5.6 and 8.2 following the procedure described above. The test solution was then centrifuged at $3700 \times g$ for 10 min and re-suspended in PBS at the same pH at which the primary disinfection was carried out. Secondary disinfection was conducted following the same procedures used for primary disinfection.

2.4. *Protein extraction and assay during disinfection*

Experiments were performed to quantify the amount of proteins extracted from *B. subtilis* spores during single-step application of each disinfectant. The initial disinfectant concentration and the light intensity were adjusted by trial and error such that the same level of inactivation, i.e., 1 log, would be achieved for the same exposure time of 60 s at 25 °C. The initial spore concentration in the experimental solution (20 mM PBS at pH 5.6) was also carefully adjusted to $1.0 \times 10^7\,cfu\,ml^{-1}$ for each experiment. At different reaction times (15, 30, 45 and 60 s), the *B. subtilis* spores were separated by centrifugation at $3500 \times g$ for 10 min, and the supernatants of the samples were collected for protein concentration measurement. The viability of *B. subtilis* spores was assayed at the same time. The viability of *B. subtilis* spores after 60 s exposure was confirmed to show the target inactivation level of 1 log. Protein concentrations were determined based on a modified version (Zor and Selinger, 1996) of the Bradford assay (Bradford, 1976), a widely used method to quantify the amount of non-specific proteins. An aliquot of 3 ml 1/5 diluted Bio-Rad protein assay dye reagent (BMS 3471-6500, BMS Co., USA) was mixed with 0.06 ml aliquot of the sample supernatant. After a 20 min reaction time, the dye absorbance was measured at 595 nm, and the protein concentration was calculated according to Zor and Selinger (1996). A standard calibration was performed using a Bio-Rad Standard Protein II (BMS Co., USA).

All experiments were repeated three times, which was represented as the standard deviation in figures, and were within a 95% confidence interval.

3. Results and discussion

Figs. 1–4 show a decrease of viability of *B. subtilis* spores versus $\bar{C}T$ (i.e., the product of time-averaged disinfectant concentration and contact time) or IT (i.e., the product of constant incident UV intensity at 254 nm and contact time) during single-step inactivation with free chlorine (Fig. 1), ozone (Fig. 2), chlorine dioxide (Fig. 3) or UV (Fig. 4). Fig. 1 also shows inactivation kinetics of *B. subtilis* spores during sequential application of free chlorine followed by ozone, chlorine dioxide or UV. Figs. 2–4 show experimental results for the sequential disinfection performed in the reverse order, i.e., free chlorine primary treatment followed by secondary treatment with ozone, chlorine dioxide and UV, respectively. All single step and sequential disinfections were performed at pHs 5.6 and 8.2 and at 25 °C. All primary treatments were performed to achieve 1 log inactivation of *B. subtilis* spores by applying each primary disinfectant with $\bar{C}T$ or IT corresponding to the single step inactivation kinetics determined in Figs. 1–4. Note that the experimental data obtained from the secondary treatments were normalized such that each first data point (i.e., 1 log inactivation after primary treatment) was placed at the origin.

Most of inactivation curves presented in Figs. 1–4 were characterized by the presence of an initial lag phase during which little inactivation occurred and a subsequent pseudo-first order decrease in viability. Others followed pseudo-first order kinetics in the absence of the initial lag phase. Previous studies reported that these inactivation kinetics were well fitted using the following delayed Chick–Watson model semi-batch system (Rennecker et al., 1999; Larson and Mariñas, 2003) consider the disinfectant decomposition chemistry and the inactivation curve, the Delayed Chick–Watson model, which is modified with a $\bar{C} = \int_0^t C/t\,dt$ (time averaged ozone concentration) instead of C (constant ozone concentration), was chosen in this study (Cho et al., 2003a, b).

$$\frac{N}{N_0} = \begin{cases} 0 & \text{if} \quad \bar{C}T \leqslant \bar{C}T_{lag}, \\ \exp\{-k(\bar{C}T - \bar{C}T_{lag})\} & \text{if} \quad \bar{C}T > \bar{C}T_{lag}, \end{cases} \tag{1}$$

where N = concentration of viable *B. subtilis* spores at time t ($cfu\,ml^{-1}$), N_0 = initial concentration of viable *B. subtilis* spores ($cfu\,ml^{-1}$), $\bar{C} = \int_0^t C/t\,dt$ = time averaged disinfectant concentration ($mg\,l^{-1}$), C = concentration of disinfectant at time t; k = the inactivation rate constant ($l\,(mg\,min)^{-1}$), $\bar{C}T_{lag}$ = x-axis intercept of the inactivation curve. For disinfection with UV, $\bar{C}$ was replaced by I (i.e., constant UV light intensity), k in $l\,(mg\,min)^{-1}$ by k in $cm^2\,mJ^{-1}$, and $\bar{C}T_{lag}$ by IT_{lag}. Correlation coefficients for the linear regressions were relatively high ($R^2 > 0.97$) for the pseudo-first order phases of all the curves regardless of lag phase presence or absence. The inactivation

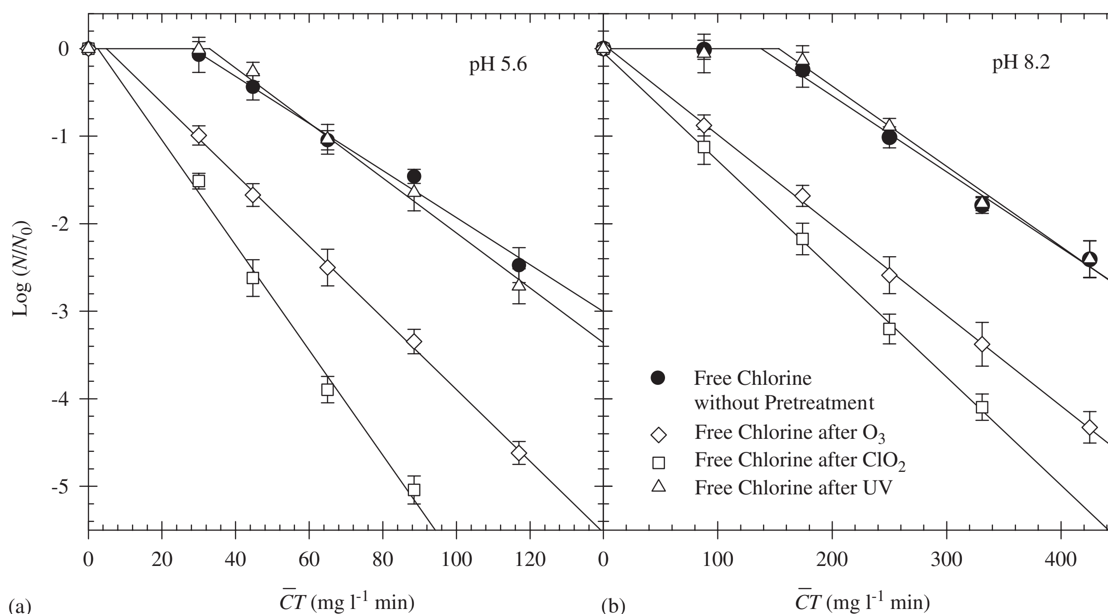

Fig. 1 – Inactivation kinetics of *B. subtilis* spores during single step application of free chlorine and sequential application of free chlorine after ozone, chlorine dioxide and UV in 20 mM phosphate buffer at pH (a) 5.6 and (b) 8.2 and at 25 °C.

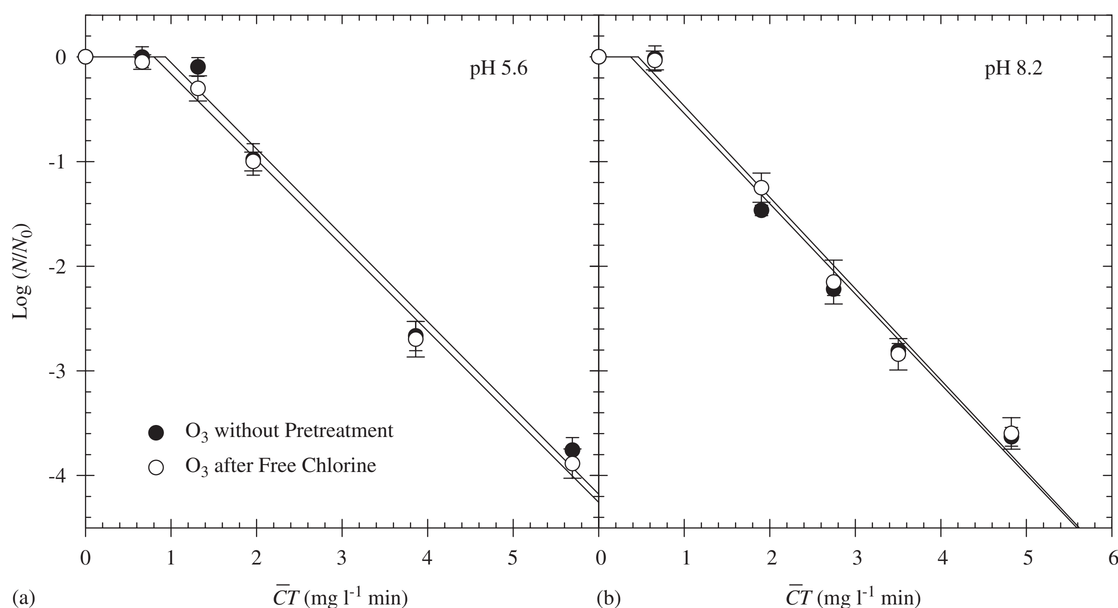

Fig. 2 – Inactivation kinetics of *B. subtilis* spores during single step application of ozone and sequential application of ozone after free chlorine in 20 mM phosphate buffer at pH (a) 5.6 and (b) 8.2 and at 25 °C.

rate constants, the extent of lag phase (i.e., $\overline{C}T_{lag}$ or IT_{lag}), and the $\overline{C}T$ and IT required for 1 log or 2 log inactivation of *B. subtilis* spores for all experimental matrices are summarized in Table 1.

3.1. Single step disinfection

The inactivation kinetics of *B. subtilis* spores during single step application of disinfectants have already been reported in literature (ozone: Driedger et al., 2001a, b; Cho et al., 2003a, b; chlorine dioxide: Radziminski et al., 2002; free chlorine: Cho et al., 2003a; monochloramine: Larson and Mariñas, 2003; UV: Nicholson and Galeano, 2003). However, these kinetics were obtained using more than one strain of *B. subtilis* and under different experimental conditions. In addition, even with the same strain and the same disinfection conditions, it has been shown that the kinetics might vary depending on how *B. subtilis* spores were prepared (Aronson and Fitz-james, 1976; Larson and Mariñas, 2003). These variations would make it difficult to accurately compare the efficiencies of different disinfectants on *B. subtilis* spore inactivation. Therefore, all the inactivation kinetics presented

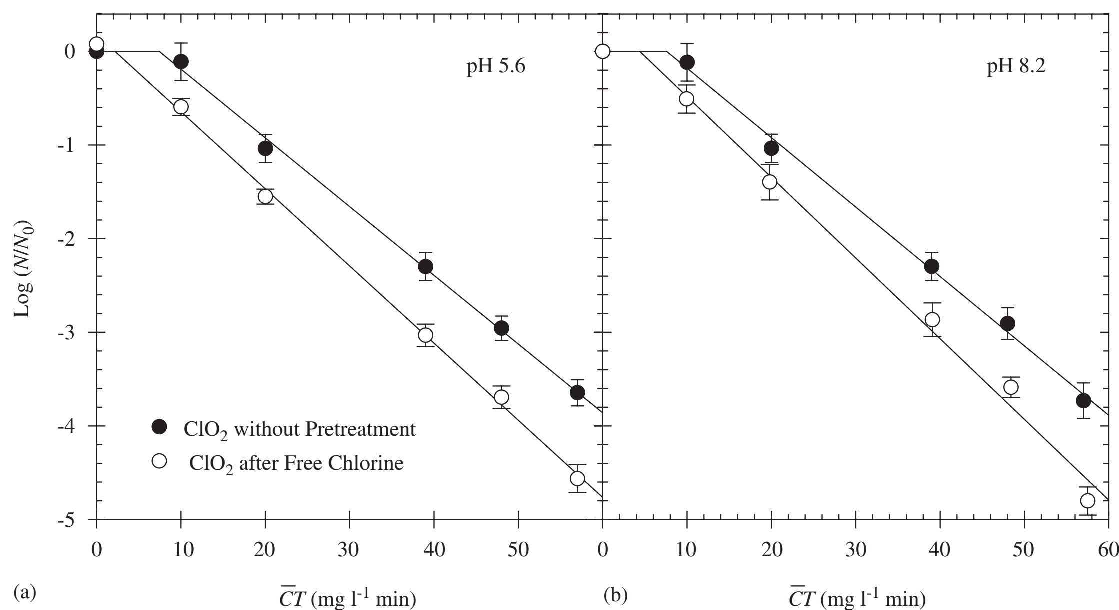

Fig. 3 – Inactivation kinetics of *B. subtilis* spores during single step application of chlorine dioxide and sequential application of chlorine dioxide after free chlorine in 20 mM phosphate buffer at pH (a) 5.6 and (b) 8.2 and at 25 °C.

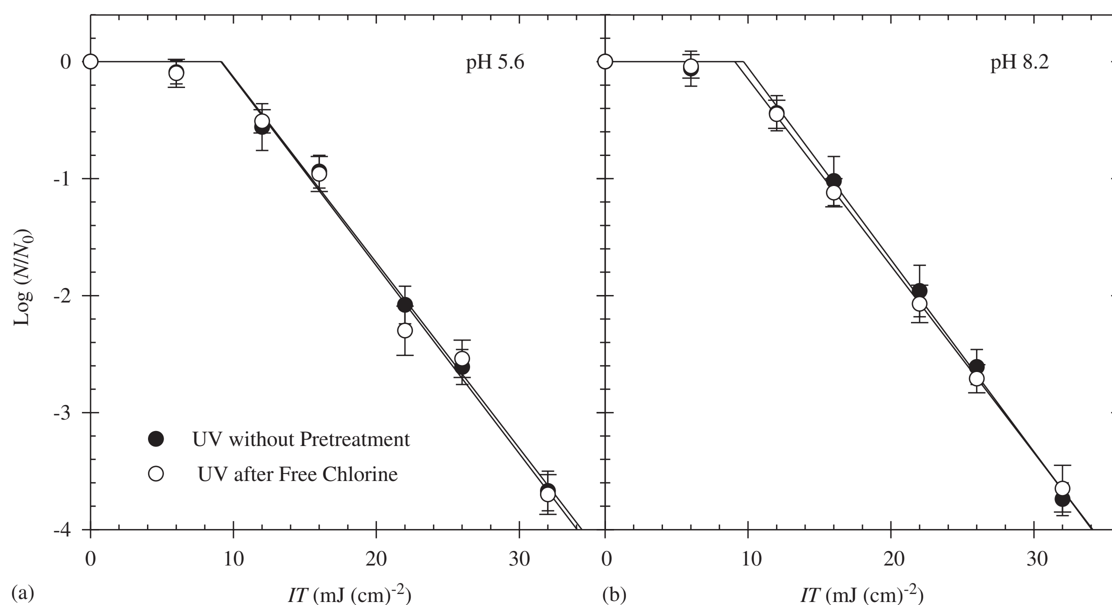

Fig. 4 – Inactivation kinetics of *B. subtilis* spores during single step application of UV and sequential application of UV after free chlorine in 20 mM phosphate buffer at pH (a) 5.6 and (b) 8.2 and at 25 °C.

in Figs. 1–4 for single step and sequential disinfection processes were obtained using the same lot of *B. subtilis* spores (i.e., prepared at the same time).

Change in the solution pH had the greatest effect on single step treatment with free chlorine (Fig. 1). Since HOCl has a higher germicidal effect than OCl^-, a decrease in pH resulted in an increase in inactivation efficiency. In single step treatment with ozone (Fig. 2), slightly faster inactivation kinetics was observed at higher pH, consistent with previous findings by Cho et al. (2003a) and Larson and Mariñas (2003). Cho et al. (2003a) attributed this effect to an increased hydroxyl radical concentration at higher pHs based on the observation that differences in inactivation kinetics at different pHs were negligible in the presence of hydroxyl radical scavengers. Inactivation of *B. subtilis* spores by chlorine dioxide was not affected by pH. This observation is consistent with the absence of a pH effect for *B. subtilis* spore inactivation in treated river water over a pH range from 6.0 to 8.0 reported by Radziminski et al. (2002) and in a phosphate-buffered natural water studied by Driedger et al. (2001a, b). However, Radziminski et al. (2002) also reported that inactivation rate increased as pH increased from 6.0 to 8.0 when *B. subtilis* spores were suspended in a phosphate-buffered organic-free solution. These authors proposed a possible

Table 1 – Rate constants and lag phase factors for *B. subtilis* spore inactivation by single step and sequential disinfection processes and $\overline{C}T$ or IT required for 1 log and 2 log inactivation

Disinfectant	Pretreatment	K[a]	$\overline{C}T_{lag}$ or IT_{lag} [b]	$\overline{C}T$ or IT [b]	
				1 log	2 log
pH 5.6					
O_3	None	8.3×10^{-1}	9.3×10^{-1}	2.1	3.4
	Free chlorine	8.2×10^{-1}	8.0×10^{-1}	2.0	3.2
ClO_2	None	7.3×10^{-2}	7.4×10^{0}	21	35
	Free chlorine	8.2×10^{-2}	2.2×10^{0}	14	26
Free chlorine	None	2.7×10^{-2}	2.8×10^{1}	65	103
	O_3	4.1×10^{-2}	4.9×10^{0}	29	54
	ClO_2	6.0×10^{-2}	2.6×10^{0}	19	36
	UV	3.1×10^{-2}	3.3×10^{1}	65	97
UV	None	1.6×10^{-1}	1.6×10^{-1}	15	22
	Free chlorine	9.2×10^{0}	9.1×10^{0}	15	22
pH 8.2					
O_3	None	8.6×10^{-1}	3.8×10^{-1}	1.5	2.7
	Free chlorine	8.7×10^{-1}	4.6×10^{-1}	1.6	2.8
ClO_2	None	7.4×10^{-2}	7.6×10^{0}	21	35
	Free chlorine	8.6×10^{-2}	4.4×10^{0}	16	28
Free chlorine	None	8.7×10^{-3}	1.4×10^{2}	253	368
	O_3	1.0×10^{-2}	4.9×10^{0}	102	198
	ClO_2	1.2×10^{-2}	-3.6×10^{0}	77	158
	UV	9.2×10^{-3}	1.5×10^{2}	262	371
UV	None	1.6×10^{-1}	9.7×10^{0}	16	22
	Free chlorine	1.5×10^{-1}	9.1×10^{0}	15	22

[a] $l\,(mg\,min)^{-1}$ for chemical disinfectants and $cm^2\ mJ^{-1}$ for UV.
[b] $mg\,l^{-1}$ min for chemical disinfectants and $mJ\,(cm^2)^{-1}$ for UV.

direct and indirect protection of spores by the phosphate buffer. Our results as well as those by Driedger et al. (2001) were contrary to their findings, and therefore the proposed effect of phosphate buffer might warrant further research. Finally, the inactivation with UV was also unaffected by pH.

Results shown in Figs. 1–4 suggest that single step disinfection using free chlorine would be most challenging for treating *B. subtilis* spores due to its relatively high $\overline{C}T$ values required. For example, $\overline{C}T$ required to achieve 2 log inactivation with free chlorine treatment at pH 5.6 and 8.2 was 103 and 386 $mg\,l^{-1}$ min at 25 °C, respectively, which was approximately 30–136 times larger than what would be required with ozone treatment. This $\overline{C}T$ requirement for *B. subtilis* inactivation and possibly for other spore forming bacteria such as *B. anthracis*, for which *B. subtilis* is often used as an indicator, might be difficult to achieve, if not impossible, in many drinking water treatment processes especially at higher pHs.

3.2. Sequential disinfection

Fig. 1 shows that both ozone and chlorine dioxide primary treatment greatly enhanced the efficiency of secondary treatment with free chlorine. Dependency of inactivation kinetics on pH during secondary treatment with free chlorine was similar to that of single step treatment with free chlorine, i.e., higher inactivation at lower pH. On the contrary, there was no synergistic effect exerted by primary treatment with UV. Figs. 2–4 show the experimental results for the sequential application of disinfectants in the reverse order. Figs. 2 and 4 show that there were little or no synergistic effects when ozone or UV was applied after free chlorine. Only the secondary treatment with chlorine dioxide showed a relatively small synergistic effect. For example, experimental results in Fig. 3 and Table 1 show that the $\overline{C}T$ required for 2 log inactivation of *B. subtilis* spores by chlorine dioxide treatment after free chlorine pretreatment was 26 $mg\,l^{-1}$ min at pH 5.6, which was 26% less than that required when chlorine dioxide was applied in the single step.

The synergism consisted of a decrease in the lag phase and an increase in the pseudo-first order rate constant, and the magnitude of one was in general not correlated to that of the other. Since comparison based on a single quantitative parameter would be more convenient, a parameter Percent Synergistic Effect was defined as how much additional log inactivation could be achieved due to the synergistic effect compared to the target log inactivation level which would have been achieved if there were no synergism (i.e., note that this term was defined based on log inactivation). For each primary and secondary disinfectant, $\overline{C}T$ or IT to achieve 1 log inactivation (i.e., overall 2 log inactivation) was first calculated. Then, the actual log inactivation that would be achieved at target $\overline{C}T$ or IT by sequential application of the same primary and secondary disinfectants was calculated. This actual log inactivation was compared to the target inactivation level of 2 log. Percent Synergistic Effects for sequential application of free chlorine, ozone, chlorine

Table 2 – Percent synergistic effect in sequential inactivation of *B. subtilis* spores

Disinfection method	Primary	O_3	UV	ClO_2	HOCl	HOCl	HOCl
	Secondary	HOCl	HOCl	HOCl	O_3	UV	ClO_2
pH 5.6	Target[a]	2.0 log	2.0 log	2.0 log	2.0 log	2.0 log	2.0 log
	Observed	3.5 log	2.0 log	4.9 log	2.0 log	2.0 log	2.5 log
	Percent synergistic effect	75%	0%	145%	0%	0%	25%
pH 8.2	Target[a]	2.0 log	2.0 log	2.0 log	2.0 log	2.0 log	2.0 log
	Observed	3.6 log	2.0 log	4.2 log	2.0 log	2.0 log	2.4 log
	Percent synergistic effect	80%	0%	110%	0%	0%	20%

[a] Predicted if synergistic effect is absent.

dioxide, and UV are summarized in Table 2. For example, chlorine dioxide pre-treatment resulted in 145% and 110% synergistic effect and ozone pre-treatment resulted in 75% and 80% synergistic Effect at pH 5.6 and 8.2, respectively, when free chlorine was used as a secondary disinfectant. Therefore, chlorine dioxide would provide additional 70% and 30% synergism compared to ozone at each pH. This term may provide a more convenient way of comparing synergistic effect when several sequential disinfection schemes are compared.

It is interesting to note that the overall synergistic effect was more evident with the removal of lag phase when compared to increase in the rate constant for sequential inactivation of *B. subtilis* spores. This observation is in contrast with a relatively large increase in inactivation rate in addition to decrease of lag phase observed in the sequential application of ozone and free chlorine (Driedger et al., 2000; Rennecker et al., 2000) for *C. parvum* Oocyst inactivation. This difference might originate from the functional difference between the spore coat of *B. subtilis* and the Oocyst wall of *C. parvum*. According to Driks (1999), the major function of the coat of *B. subtilis* spores was protection and therefore deletion of many of *B. subtilis* coat proteins, and the resulting elimination of spore coats had little or no effect on viability of spores in the absence of disinfectants. Foegeding (1985) also suggested that the spore coat was the primary protective barrier against ozone inactivation based on the observation that *Bacillus* and *Clostridium* species were rapidly inactivated when the spore coats were removed. In similar experiments, Young and Setlow (2003) observed that *B. subtilis* deficient of spore coats were inactivated readily by chlorine and chlorine dioxide.

In the sequential disinfection process, the secondary disinfectant transfers through the spore coat that has been partially damaged by the primary disinfectant and then into the inner spore, where it reacts with vital components. The extent of resistance that the secondary disinfectant experiences as it transfers through the partially damaged spore coat is therefore expressed as a lag phase. It is apparent that this resistance is effectively reduced by primary treatment with the strong disinfectant for the case of *B. subtilis* spores. Assuming that the reactions of the primary disinfectant with vital component inside the spore coat are not rate limiting (i.e., the spore population with partial damage of inner components is relatively small after primary disinfection), the secondary disinfectant should exhibit similar inactivation rates with and without primary disinfection. On the contrary, the Oocyst wall of *C. parvum*, while its major function is to serve as a protective barrier, also plays a critical functional role in excystation of infective sporozoites and, therefore, capability to infect the host. When the Oocyst walls are partially damaged by the primary disinfectant, further damage by the secondary disinfectant could have a greater probability of additional inactivation at a faster rate, which is exhibited by the greater inactivation rate in the post-shoulder phase.

3.3. *Protein extraction*

According to Driks (1999), the spore coats of *B. subtilis* are mainly composed of tyrosine- and cysteine-rich proteins with minor amounts of carbohydrates and lipids. It is therefore hypothesized that the disinfectant treatment might extract proteins from the spore coats and the amount of extracted protein might be related to the degree of damage induced by the disinfectant. Fig. 5 shows the quantity of extracted protein measured by the Bradford assay when each disinfectant was used to inactivate *B. subtilis* spores. The inset of Fig. 5 shows the degradation and consequential loss of reagent protein by each disinfectant. For these experiments, the concentration of disinfectant was adjusted such that 1 log inactivation of *B. subtilis* spores would be achieved for 60 s exposure. While the reagent protein may not accurately represent the proteins extracted from the spore coat, the results might provide a reasonable estimate of the effect of protein loss during protein extraction. The experimental results showed that protein degradation was the fastest with ozone followed by chlorine dioxide and free chlorine and the slowest with UV irradiation (Fig. 5, inset). Therefore, the actual rate of protein extraction should be much higher than those determined by residual proteins as shown in Fig. 5 and the differences should be greater in the order of ozone, chlorine dioxide, free chlorine and UV.

The total amount of extracted protein appeared to be only a small fraction of protein present in the spore coat. Aronson and Fitz-James (1976) estimated that the protein content of the spore coat of *B. subtilis* was approximately $1.6 \times 10^{-13}\,g\,spore^{-1}$. This would correspond to $1.6\,mg\,l^{-1}$ of

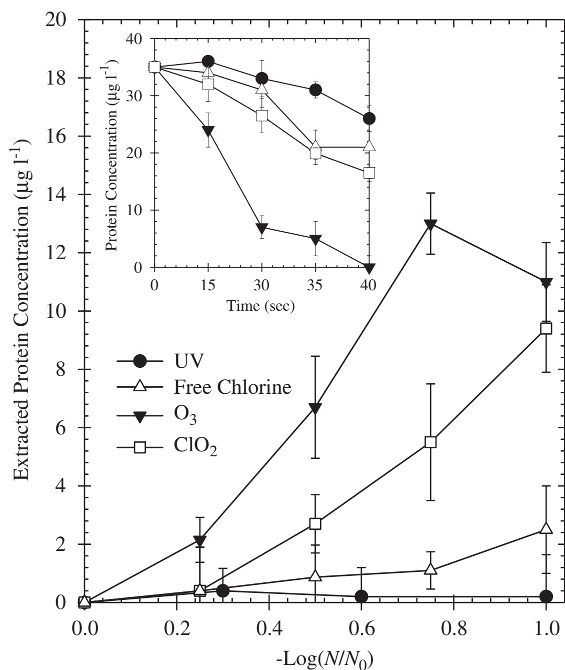

Fig. 5 – Amount of proteins extracted from 1.0×10^7 cfu ml^{-1} of *B. subtilis* spores during chemical and UV inactivation in 20 mM PBS at pH 5.6 and 25 °C. Disinfectant doses were adjusted such that 1 log inactivation was achieved by 60 s exposure.

proteins for the case of the present experimental system in which 10^7 spores ml^{-1} was used. The total extracted protein, for example, by chlorine dioxide was about 12 g l^{-1}, assuming 50% reduction of extracted protein (Fig. 5 and inset of Fig. 5). Therefore, roughly less than 1% of proteins have been detected.

As shown in Fig. 5, when 0.75 log inactivation of *B. subtilis* spores was achieved by ozone treatment, approximately 13 μg l^{-1} of residual protein was measured. As previously hypothesized, the presence of proteins in the supernatant of treated solution might indicate the physical damage to the spore coat induced by ozone treatment. The concentration of extracted protein slightly decreased to 11 μg l^{-1} at higher level of inactivation, possibly due to further degradation of the extracted protein by ozone. This experimental result is consistent with the earlier transmission electron microscopic observation by Khadre and Yousef (2001) in which ozone might inactivate spores by disintegrating and extracting spore coat material and facilitating penetration of ozone into the cortex and the protoplast. Chlorine dioxide was also able to extract proteins from spore coats, substantially more than free chlorine and UV, consistent with findings by Foegeding et al. (1986) that chlorine dioxide removed proteinaceous components from the spore coats of different *Bacillus* species. Chlorine dioxide treatment, however, yielded smaller amount of proteins compared to ozone, despite the fact that the same level of inactivation was achieved. UV irradiation produced a very small amount of proteins. Even though the primary action of UV in inactivation is known as DNA damage, this small amount of protein might have been detected since UV light denaturates some types of proteins (Kalisvaart, 2001).

3.4. Mechanism of synergism

As previously mentioned, the protective coat of *B. subtilis* spores does not have any functional role in germination. This is in marked difference with other types of pathogens of concern in water treatment such as *C. parvum*, bacteria and viruses since the damage in oocyst wall, bacterial wall, and virus coat may result in their inability to reproduce. In the case of *B. subtilis* spores, the chemical disinfectants must undergo reactive transport through the spore coat and reach the cortex and spore core to damage the vital components such as DNA and proteins. In this regard, the synergism is determined by the ability of the primary disinfectant to alter the physical and chemical structures of the spore coat and facilitate the subsequent transport of the secondary disinfectant.

It is interesting to note that different amounts of proteins were extracted by different chemical disinfectants even when the same level of inactivation was achieved. This result suggests a fundamental difference in the underlying mechanism behind how disinfectants act on *B. subtilis* spores. Ozone is very reactive toward electron rich moieties and reacts fast with the aromatic amino acid units of proteins such as tyrosine, which are abundant in the spore coats of *B. subtilis*. These reactions lead to partial oxidation and denaturation of proteins, although they are not very effective in breaking amide bonds (Catalo, 2003). Therefore, it is inferred that ozone effectively releases proteins from spore coats by partially oxidizing and denaturating proteins and possibly damaging surrounding structures composed of saturated fatty acids and carbohydrates. This process may induce structural damage to the spore coat and facilitate the transport of secondary disinfectants. Chlorine dioxide also reacts fast with amino acids such as cysteine and tyrosine, whereas it is relatively less reactive toward most other amino acids compared to free chlorine (Tan et al., 1987). In the study of inactivation of *f2* virus by chlorine dioxide, Noss et al. (1986) also observed rapid oxidation of tyrosine residues of proteins on the viral coat. Similar to ozone, chlorine dioxide may also induce structural damage to the spore coats, as evidenced by protein extraction experiments in this study but to a lesser degree. However, contrary to ozone, chlorine dioxide may further react with functional groups that favour chlorine substitution. For example, Ghanbari et al. (1982) reported that both free chlorine and chlorine dioxide are readily incorporated into lipids. While chlorine dioxide mostly oxidizes and does not incorporate chlorine, some of the same reaction sites would be consumed by primary application of chlorine dioxide. Therefore, the greater synergistic effect (i.e., compared to primary treatment with ozone) might result from the ability of chlorine dioxide to consume some common reaction sites that free chlorine may otherwise attack, in addition to the structural damage that facilitates the transport of free chlorine. This hypothesis is consistent with the observation that free chlorine enhanced secondary disinfection with chlorine dioxide but not ozone (Figs. 2 and 3). It is possible that free chlorine consumed some reactive sites that chlorine dioxide would preferably react with and, therefore, chlorine dioxide transferred through the spore coat more efficiently after free chlorine treatment.

However, in order to further support this hypothesis, additional research is needed to quantitatively analyze the reactions of different disinfectants with spore coat constituents.

Finally, the sequential disinfections of both free chlorine followed by UV and UV followed by free chlorine did not show any synergistic effect. It is relatively well known that UV light inactivates microorganisms, such as *C. parvum* and *E. coli*, by damaging DNA and RNA which are vital for the synthesis of key proteins and reproduction (2001). On the contrary, it has been suggested that the major mechanism of free chlorine and chlorine dioxide inactivation of *B. subtilis* spores is not by DNA damage (Young and Setlow, 2003). Therefore, differences in inactivation mechanisms lead to the absence of the synergistic effect when UV is combined with free chlorine.

4. Conclusion

This study reveals the presence of synergistic effect in the sequential application of ozone, chlorine dioxide followed by free chlorine. Especially, the greater synergism was observed when chlorine dioxide was used as a primary disinfectant followed by secondary disinfection with free chlorine. And no synergism was observed when UV was used as the primary disinfectant. This enhanced inactivation was successfully explained by the damaged surface components of *B. subtilis* spore during primary disinfection.

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REFERENCES

Aieta, E., Berg, J.D., 1986. The removal of chlorine dioxide in drinking water treatment. Journal American Water Works Association 78, 62–72.

Ameriican Public Health Association (APHA); American Water Works Association (AWWA); Water Environment Federation (WEF). Standard Methods for the Examination of Water and Wastewater 1992; 18th ed., American Public Health Association, Washington DC.

Armon, R., Weitch-Cohen, G., Bettane, P., 2004. Disinfection of *Bacillus* spp. Spores in drinking water by TiO_2 photocatalysis as a model for *Bacillus anthracis*. Water Sci. Technol. 4, 7–14.

Aronson, A.I., Fitz-James, P., 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. 40, 360–402.

Bader, H., Hoigné, J., 1981. Determination of ozone in water by the indigo method. Water Res. 15, 449–456.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Catalo, F., 2003. On the action of ozone on proteins. Polym. Degrad. Stabil. 82, 105–114.

Cho, M., Chung, H., Yoon, J., 2003a. Quantitative evaluation of the synergistic sequential inactivation of *bacillus subtilis* spores with ozone followed by chlorine. Environ. Sci. Eng. 37, 2134–2138.

Cho, M., Chung, H., Yoon, J., 2003b. Disinfection of water containing natural organic matter by using ozone-initiated radical reactions. Appl. Environ. Microbiol. 69, 2284–2291.

Corona-Vasques, B., Rennecker, J.L., Driedger, A.M., Mariñas, B.J., 2002. Sequential inactivation of *Cryptosporidium parvum* oocysts with chlorine dioxide followed by free chlorine or monochloramine. Water Res. 36, 178–188.

Driedger, A.M., Rennecker, J.L., Mariñas, B.J., 2000. Sequential inactivation of *Cryptosporidium parvum* oocysts with ozone and free chlorine. Water Res. 34, 3591–3597.

Driedger, A.M., Rennecker, J.L., Mariñas, B.J., 2001a. Inactivation of *Cryptosporidium parvum* oocysts with ozone and monochloramine at low temperature. Water Res. 25, 41–48.

Driedger, A.M., Staub, E., Pinkernell, U., Mariñas, B.J., Köster, W., von Gunten, U., 2001b. Inactivation of *Bacillus subtilis* spores and formation of bromate during ozonation. Water Res. 35, 2950–2960.

Driks, A., 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. 63, 1–20.

Foegeding, P.M., 1985. Ozone inactivation of *Bacillus* and *Clostridium* spore populations and the importance of the spore coat to resistance. Food Microbiol. 2, 123–134.

Foegeding, P.M., Hemstapat, V., Giesbrecht, F.G., 1986. Chlorine dioxide inactivation of *Bacillus* and *Clostridium* spores. J. Food Sci. 51, 197–201.

Ghanbari, H.A., Wheeler, W.B., Kirk, J.R., 1982. Reaction of aqueous chlorine and chlorine dioxide with lipids: chlorine incorporation. J. Food Sci. 47, 482–485.

Gyürék, L.L., 1997. Ozone and chlorine inactivation of *Cryptosporidium* in water. Ph.D. dissertation 1997, University of Alberta, Canada.

Huffman, D.E., Gennaccaro, A., Rose, J.B., Dussert, B.W., 2002. Low- and medium-pressure UV inactivation of microsporidia *Encephalitozoon intestinalis*. Water Res. 36, 3161–3164.

Kalisvaart, B.F., 2001. Photobiological effects of polychromatic medium pressure UV lamps. Water Sci. Technol. 43, 191–197.

Khadre, M.A., Yousef, A.E., 2001. Sporicidal action of ozone and hydrogen peroxide: a comparative study. Int. J. Food Microbiol. 71, 131–138.

Larson, M.A., Mariñas, B.J., 2003. Inactivation of *Bacillus subtilis* spores with ozone and monochloramine. Water Res. 37, 833–844.

Lewin, N., Craik, S., Li, H., Smith, D.W., Belosevic, M., 2001. Sequential inactivation of *Cryptosporidium* using ozone followed by free chlorine in natural water. Ozone Sci. Eng. 23, 411–420.

Liyanage, L.R.J., Finch, G.R., Belosevic, M., 1997. Sequential disinfection of *Cryptosporidium parvum* by ozone and chlorine dioxide. Ozone Sci. Eng. 19, 409–423.

Nakayama, A., Yano, Y., Kobayashi, S., Ishikawa, M., Sakai, K., 1996. Comparison of pressure resistances of spores of six *Bacillus* strains with their heat resistances. Appl. Environ. Microbiol. 62, 3897–3900.

Nicholson, W.L., Galeano, B., 2003. UV resistance of *Bacillus anthracis* spores revisited: validation of *bacillus subtilis* spores as UV surrogate for spores of *B. anthracis* Sterne. Appl. Environ. Microbiol. 69, 1327–1330.

Noss, C.I., Hauchman, F.S., Olivier, V.P., 1986. Chlorine dioxide reactivity with proteins. Water Res. 20, 351–356.

Radziminski, C., Ballantyne, L., Hodson, J., Creason, R., Andews, R.C., Chauret, C., 2002. Disinfection of *Bacillus subtilis* spores with chlorine dioxide: a bench-scale and pilot-scale study. Water Res. 36, 1629–1639.

Rennecker, J.L., Mariñas, B.J., Rice, E.W., Owens, J.H., 1999. Inactivation of *Cryptosporidium parvum* oocyst with ozone. Water Res. 33, 2481–2488.

Rennecker, J.L., Driedger, A.M., Rubin, S.A., Mariñas, B.J., 2000. Synergy in sequential inactivation of *Cryptosporidium parvum*

with ozone/free chlorine and ozone/monochloramine. Water Res. 34, 4121–4130.

Rennecker, J.L., Kim, J.H., Corona-Vasquez, B., Mariñas, B.J., 2001. Role of disinfectant concentration and pH in the inactivation kinetics of *Cryptosporidium parvum* oocysts with ozone and monochloramine. Environ. Sci. Technol. 35, 2752–2757.

Shin, G., Linden, K.G., Arrowood, M.J., Sobsey, M.D., 2001. Low-Pressure UV inactivation and DNA repair potential of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 67, 3029–3032.

Tan, H., Sen, A.C., Wheeler, W.B., Cornell, J.A., Wei, C.I., 1987. A kinetic study of the reaction of aqueous chlorine and chlorine dioxide with amino acids, peptides, and proteins. J. Food Sci. 52, 1706–1711.

Young, S.B., Setlow, P., 2003. Mechanism of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. J. Appl. Microbiol. 95, 54–67.

Zor, T., Selinger, Z., 1996. Linearization of the bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal. Biochem. 236, 302–308.