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SPECIAL TOPIC: DISINFECTANTS AND MICROBIAL CONTROL

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Lethality of chlorine, chlorine dioxide, and a commercial fruit and vegetable sanitizer to vegetative cells and spores of *Bacillus cereus* and spores of *Bacillus thuringiensis*

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Abstract Chlorine, chlorine dioxide (ClO_2), and a commercial raw fruit and vegetable sanitizer (Fit powder) were evaluated for their effectiveness in killing vegetative cells and spores of *Bacillus cereus* and spores of *Bacillus thuringiensis*. The ultimate goal was to use one or both species as a potential surrogate(s) for *Bacillus anthracis* in studies that focus on determining the efficacy of sanitizers in killing the pathogen on food contact surfaces and foods. Treatment with alkaline (pH 10.5–11.0) ClO_2 (200 mg/mL) produced by electrochemical technologies reduced populations of a five-strain mixture of vegetative cells and a five-strain mixture of spores of *B. cereus* by more than 5.4 and more than 6.4 log cfu/mL, respectively, within 5 min. This finding compares with respective reductions of 4.5 and 1.8 log cfu/mL resulting from treatment with 200 mg/mL chlorine. Treatment with a 1.5% acidified (pH 3.0) solution of Fit powder product was less effective, causing 2.5-log and 0.4-log cfu/mL reductions in the number of *B. cereus* cells and spores, respectively. Treatment with alkaline ClO_2 (85 mg/mL), acidified (pH 3.4) ClO_2 (85 mg/mL), and a mixture of ClO_2 (85 mg/mL) and Fit powder product (0.5%) (pH 3.5) caused reductions in vegetative cell/spore populations of more than 5.3/5.6, 5.3/5.7, and 5.3/6.0 log cfu/mL, respectively. Treatment of *B. cereus* and *B. thuringiensis* spores in a medium (3.4 mg/mL organic and inorganic solids) in which cells had grown and produced spores with an equal volume of alkaline (pH 12.1) ClO_2 (400 mg/mL) for 30 min reduced populations by 4.6 and 5.2 log cfu/mL, respectively, indicating high lethality in the presence of materials other than spores that would potentially react with and neutralize the sporicidal activity of ClO_2.

Keywords Disinfectants · ClO_2 · *Bacillus cereus* · *Bacillus thuringiensis*

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In section 'Preparation of treatment solutions' the phrase '22-28°C' was replaced by '22 ± 2°C'.

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Introduction

Concerns about international bioterrorism have kindled a renewed interest in developing and refining technologies to kill *Bacillus anthracis* spores in urban environments and foods [8]. Although spores of several *Bacillus* species known to cause spoilage of foods and foodborne disease have been studied extensively to determine conditions that affect growth and sporulation, as well as their sensitivity to physical treatments and sanitizers [3, 9, 10, 19, 23, 26], comparatively little is known about conditions that affect survival and growth of *B. anthracis* in foods and the effectiveness of sanitizers in killing spores of the organism on food contact surfaces and in foods.

B. anthracis is closely related to *B. cereus* and *B. thuringiensis*, the principal distinguishing difference being the presence of virulence genes on plasmids in *B. anthracis* [13]. Direct comparisons of the sensitivity of spores of *B. anthracis* and spores of other *Bacillus* species to sanitizers used to decontaminate food contact surfaces and foods have not been described. Information

on the sporicidal activity of chemical treatments using *B. cereus*, *B. thuringiensis*, and perhaps other *Bacillus* species as potential surrogates for *B. anthracis* would provide insights to the relative sensitivity of *B. anthracis* spores to the same treatments.

Chlorine dioxide (ClO_2) in gaseous or aqueous forms is among the sanitizers with demonstrated efficacy in killing vegetative cells and spores of food borne pathogens and spoilage microorganisms [2, 11, 12, 16, 20, 21, 27]. Young and Setlow [29] showed that although *B. subtilis* spores treated with ClO_2 can undergo initial steps of germination, outgrowth does not occur, probably because of some type of membrane damage. Compared with chlorine, ClO_2 has the ability to break down phenolic compounds and remove phenolic tastes and odors in water, does not form trihalomethanes, is capable of eliminating cyanides, sulfides, and mercaptans from wastewater, and is nonreactive with ammonia [2, 6]. The oxidizing power of ClO_2 is approximately 2.5 times that of chlorine [1], and its antimicrobial activity is less affected by pH. Like chlorine, however, the oxidizing power of ClO_2 is diminished on contact with organic matter, thus reducing its lethality [16, 27, 30].

The effectiveness of sanitizers in killing *Bacillus* spores is enhanced by simultaneous and sequential application of a second stress. Acidification of frankfurter emulsions, for example, increases the thermal sensitivity of *Bacillus coagulans* and *Bacillus stearothermophilus* spores [17]. Inactivation of *B. subtilis* spores by glutaraldehyde, formaldehyde, hydrogen peroxide, peracetic acid, cupric ascorbate, and sodium hypochlorite is affected by pH [24]. Inactivation of *B. subtilis* spores is not markedly affected by simultaneous exposure to pressure at low pH [28]; however, higher levels of inactivation were obtained when spores were first pressure-treated at neutral pH and then exposed to low pH. Roberts and Hoover [22] reported that *B. coagulans* spores are more sensitive to pressure, both at low pH and at high temperature.

We conducted a series of experiments to determine the effectiveness of chlorine, ClO_2, and a commercial raw fruit and vegetable sanitizer in killing vegetative cells and spores of *B. cereus* and *B. thuringiensis*. The goal is to eventually test the sensitivity of vegetative cells and spores of *B. anthracis* to treatments that cause the highest reductions in populations of these potential surrogates. Insights into the sensitivity of *B. cereus* and *B. thuringiensis* to these sanitizers will be valuable in achieving that goal.

Materials and methods

Test strains

Vegetative cells and spores of five strains of *B. cereus* isolated from foods were tested for their sensitivity to ClO_2 and other sanitizers: strain F4616A/90 (from pasteurized milk, enterotoxin producer but not emetic toxin producer), F3812/84 (pasteurized milk, atoxigenic), F4810/72 (cooked rice, emetic toxin producer), O38-2 (infant formula, diarrheal and emetic toxin producer), and C1 (pasta, toxin production unknown). Strains F4616A/90 and F3812/84 were obtained from J. Kramer (Institute for Hygiene and Technology of Milk, Munich, Germany), strain F4810/72 was obtained from S. Palumbo (United States Department of Agriculture, Wyndmoor, Pa.), strain O38-2 was obtained from S. Doores (Pennsylvania State University), and strain C1 was obtained from D. Collins-Thompson (Westreco, New Milford, Conn.). Spores of *B. thuringiensis* serovar *kurstaki* ATCC 33679 (strain HD1), isolated from diseased insect larvae, were also tested.

Production of vegetative cells and spores

The efficacy of chlorine, alkaline and acidified ClO_2, an acidified fruit and vegetable sanitizer, and ClO_2 in combination with a fruit and vegetable sanitizer in killing vegetative cells and spores of *B. cereus* was determined. The efficacy of alkaline ClO_2 in killing spores of *B. thuringiensis* was investigated.

To produce vegetative cells, five strains of *B. cereus* were grown in brain heart infusion (BHI) broth (Difco, BBL, Becton Dickinson, Sparks, Md.). Cultures were transferred by loop inocula three times at 24 h intervals to 10 mL BHI broth and incubated for 24 h at 30°C. Cultures were centrifuged (6,000 *g*, 10 min, 21°C), and cells were resuspended in sterile deionized water. Predetermined volumes of suspensions were combined to give a five-strain mixture containing approximately equal populations (10^8 cfu/mL) of each strain. This suspension (0.1 mL) was combined with 4.9 mL chemical treatment solution or water (control) to give reaction mixtures containing approximately 5.3×10^6 cfu/mL in experiments 1–3 (see procedure for treatment of vegetative cells and spores).

To produce spores, vegetative cell suspensions (0.1 mL) of each strain of *B. cereus* and *B. thuringiensis* grown in BHI broth for 24 h at 30°C were surface plated on nutrient agar (Difco, BBL, Becton Dickinson) supplemented with $MnSO_4{\cdot}7H_2O$ (50 mg/mL) (NAMS). Spores produced on plates incubated at 30°C for 72–76 h were harvested. Sterile deionized water (5 mL) was deposited on the surface of each plate, followed by rubbing with a sterile bent glass rod to suspend spores and cells that had not sporulated.

Suspensions of each strain collected from plates were coarse filtered through sterile glass wool, pooled, and centrifuged (2,600 *g*, 20 min, 21°C). Spores were resuspended in 100 mL sterile deionized water and centrifuged (6,000 *g*, 10 min, 21°C). The washing procedure was repeated until spores were free of most of the vegetative cell and spore debris originating from the culture. Suspensions (50–100 mL) of each strain were stored at

28°C until used to prepare inocula for chemical treatment solutions and deionized water (control).

Determination of number of spores in suspensions harvested from NAMS

The number of spores (cfu per milliliter) in stock suspensions of each strain of *B. cereus* and *B. thuringiensis* was determined. Deionized water (4 mL) in glass test tubes (16×100 mm) was adjusted to 80°C by immersing in a water bath. Stock spore suspensions were diluted 10-fold in deionized water, and 1 mL was added to the hot water. After heating for 10 min, 1 mL was withdrawn and added to 9 mL sterile 0.1% peptone (21°C). Suspensions serially diluted in 0.1% peptone were surface plated (0.1 mL) on duplicate plates of BHI agar. Plates were incubated at 30°C for 24 h before colonies were counted. Populations of spores in stock suspensions were then calculated. Differences in populations among the five strains of *B. cereus* necessitated centrifugation (6,000 *g*, 10 min) of some suspensions followed by resuspending spores in different volumes of sterile deionized water before combining to form a five-strain suspension containing approximately equal numbers of each strain of *B. cereus*. The five-strain mixture of *B. cereus* spores and the single-strain suspension of *B. thuringiensis* spores served as inocula for chemical treatment solutions and deionized water (control). Preparation of inoculum containing approximately equal populations of heat-shocked spores of each of the five strains of *B. cereus* and *B. thuringiensis* was carried out immediately before determining the lethality of chemical treatments.

The influence of organic materials other than *B. cereus* or *B. thuringiensis* in the reaction mixture on inactivation by test chemicals was determined. In this experiment, *B. cereus* strain C1 was not used. Stock suspensions of spores of *B. cereus* strains F3812/84, F4616A/90, F4810/72, and 038-2 harvested from NAMS were centrifuged (5,000 *g*, 15 min, 21°C). Spores in pellets of each strain of *B. cereus* and *B. thuringiensis* were suspended in spent modified G medium (MGM; see description of preparation) to give approximately 10^8 cfu/mL. Predetermined volumes of suspensions of *B. cereus* were combined to give a four-strain suspension containing approximately equal populations of each strain. Suspensions (90 mL) of *B. cereus* spores and *B. thuringiensis* spores were separately deposited in 250-mL Erlenmeyer flasks and immersed in a water bath at 80°C for 10 min during which the surface of the suspensions was below the surface of the water in the bath. Suspensions were constantly agitated throughout the 10-min heat treatment. On removal from the water bath, suspensions were cooled to 22–28°C by immersing in tap water. These spore suspensions were combined with a spent culture medium (see description of preparation) and treated with alkaline ClO_2 to determine lethality to spores in an environment with high organic content.

Preparation of organic medium in which spores were suspended before treatment

In experiment 4, spores of *B. cereus* and *B. thuringiensis* were suspended before chemical treatment in a spent culture medium containing a high organic load instead of water. MGM, a sporulation broth formulated by Kim and Goepfert [14] for *B. anthracis* and widely used to culture the organism, was used to culture the two surrogate *Bacillus* species. Spent MGM was chosen to represent a high-organic environment simulating conditions under which *B. cereus* and *B. thuringiensis* can grow, and, thus, environmental conditions mimicking those presented for decontamination. The medium consists of the following (in grams per liter of deionized water): yeast extract (Difco, BBL, Becton Dickinson), 2.0; $CaCl_2{\cdot}2H_2O$, 0.025; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}4H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 0.005; $CuSO_4{\cdot}5H_2O$, 0.005; $FeSO_4{\cdot}7H_2O$, 0.0005; and $(NH_4)_2SO_4$, 2.0 (pH 7.1 after autoclaving). *B. cereus* and *B. thuringiensis* were grown in BHI broth at 30°C for 24 h. Each culture (1.0 mL) was inoculated into 100 mL MGM in a 500-mL Erlenmeyer flask and incubated on a rotary shaker (90 rpm) at 30°C for 72 h. Cells and spores were collected by centrifugation (5000 *g*, 15 min, 21°C). The supernatants (spent MGM) from cultures of each strain of *B. cereus* were combined. Spent MGM from *B. cereus* and *B. thuringiensis* cultures was stored at 28°C until used in tests to determine sporicidal activity of alkaline ClO_2.

Determination of solids content in spent MGM

Triplicate 10-mL samples of spent MGM were deposited in tared aluminum pans and dried at 95°C for 18 h. The weight of solids in each sample was calculated. The solids content is presented on the basis of total weight (milligrams) of organic and inorganic materials per milliliter of spent MGM.

Preparation of treatment solutions

Solutions (pH 8.1–12.1) containing ClO_2 at concentrations up to 500 mg/mL were prepared via an electrogenerator using patent-pending electrochemical technologies (Procter and Gamble, Cincinnati, Ohio). A solution acidified to pH 3.4 with citric acid was used in experiment 3. An acidic powdered sanitizer [Fit Powder Product (FPP), Procter and Gamble], developed for commercial treatment of raw fruits and vegetables, was tested at concentrations of 0.5 and 1.5% (pH 3.0) in deionized water; FPP (0.5%) in alkaline (pH 10.5) ClO_2 (85 mg/mL) was also evaluated for lethality. FPP consists of anhydrous citric acid, sodium lauryl sulfate, $NaHCO_3$, $MgCO_3$, polypropylene glycol (PPG 2000), antifoam, and grapefruit oil. All ingredients are generally recognized as safe (GRAS) by the United States Food and Drug

Administration. Equal volumes of ClO_2 (150 mg/mL) solution and a 1.0% solution of FPP in deionized water were combined to give a solution (pH 3.5) containing 85 mg/mL ClO_2 and 0.5% FPP. A chlorine solution (200 mg/mL) was prepared by combining NaOCl (Aldrich, Milwaukee, Wis.) with 0.05 M potassium phosphate buffer (pH 6.8). The concentration of free chlorine was determined using an amperometric titrator (Hach, Loveland, CO.).

Procedure for treatment of vegetative cells and spores

Alkaline ClO_2 was tested for its lethality to vegetative cells and spores of *B. cereus* in experiments 1–3 and to spores of *B. cereus* and *B. thuringiensis* in experiment 4. Chlorine, acidified ClO_2, FPP, and FPP in combination with ClO_2 were tested in selected experiments. All treatments were performed at 22 ± 2°C.

Experiment 1 was performed to compare the efficacy of chlorine (100, 200, and 1,000 mg/mL, pH 6.8), ClO_2 (200 and 500 mg/mL, pH 10.5–11.0), and FPP (0.5 and 1.5% solutions, pH 3.0) in killing a five-strain mixture of vegetative cells and a five-strain mixture of spores of *B. cereus* in deionized water. Chemical solutions or water (control) (4.9 mL, 21°C) were deposited in test tubes measuring 16×150 mm. A vegetative cell suspension (0.1 mL) or spore suspension (0.1 mL), prepared as described above, was added and thoroughly mixed. After treatment for 5 min, 5.0 mL 23 Dey-Engley (DE) neutralizing broth (Difco, BBL, Becton Dickinson) was added to achieve neutralization of potentially lethal residual chemicals and adjust the pH to a range not lethal to vegetative cells or spores.

Experiment 2 focused on determining the lethality of lower concentrations of alkaline (pH 8.1–11.5) ClO_2 (5, 10, 50, 100, and 200 mg/mL) to five-strain suspensions of vegetative cells and spores of *B. cereus* in deionized water. Chlorine (200 and 1,000 mg/mL) was also tested. The treatment protocol was the same as that used in experiment 1.

In experiment 3, a five-strain suspension of vegetative cells and spores of *B. cereus* was treated with chlorine (200 mg/mL), alkaline (pH 10.5) ClO_2 (85 mg/mL), ClO_2 (85 mg/mL) acidified to pH 3.4 with citric acid, and a mixture of ClO_2 (85 mg/mL) and FPP (0.5%) (pH 3.5). The protocol for treating cells and spores was the same as that used in experiment 1.

Experiment 4 investigated the influence of the presence of organic material other than spores on the lethality of alkaline ClO_2 to *B. cereus* and *B. thuringiensis* spores. Heat-shocked suspensions (2.8 mL) of *B. cereus* and *B. thuringiensis* spores in spent MGM were separately deposited in plastic Petri dishes (60-mm diameter by 15-mm rim height). Deionized water (2.8 mL) or solutions (2.8 mL) containing ClO_2 (200, 400, and 800 mg/mL) were combined with the spore suspension and thoroughly mixed to give initial ClO_2 concentrations of 100, 200, and 400 mg/mL, respectively. The pH of reaction mixtures containing ClO_2 ranged from 10.9 to 12.1. After treatment for 10 s and 30 min (control), or 10 s and 5, 10, and 30 min (ClO_2 solution), 2.8 mL 33 DE neutralizing broth was added to the 5.6 mL reaction mixture and thoroughly mixed.

Microbiological analysis

Immediately after neutralizing reaction mixtures with DE broth, populations of vegetative cells or spores were determined. Treated and control suspensions were surface plated in quadruplicate (0.25 mL) and duplicate (0.1 mL) on BHI agar. Suspensions serially diluted in sterile 0.1% peptone water were also surface plated (0.1 mL, in duplicate) on BHI agar. Plates were inoculated at 30°C for 24 h before colonies were counted.

Statistical analysis

Experiments 1–3 were replicated three times. Experiment 4 was performed twice. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests to determine significant differences ($P \leq 0.05$) between mean values in each experiment.

Results and discussion

Experiment 1

Populations of five-strain mixtures of vegetative cells or spores of *B. cereus* surviving treatment with chlorine, alkaline ClO_2, and FPP are shown in Table 1. Chlorine dioxide was the most lethal to vegetative cells and spores, killing more than 5.4 log cfu/mL and more than 6.4 log cfu/mL, respectively, at a treatment concentration of 200 mg/mL. At the concentrations tested, treatment with chlorine was less effective than ClO_2 and treatment with FPP was least effective in killing *B. cereus*. Although treatment with FPP caused significant reductions ($P \leq 0.05$) of 2.5–2.6 log cfu/mL of vegetative cells, it was largely ineffective in killing spores.

Experiment 2

The lethality of concentrations of alkaline ClO_2 lower than those tested in experiment 1 was determined. Reductions in populations of a five-strain mixture of vegetative cells of *B. cereus* treated with ClO_2 or chlorine at 200 mg/mL (Table 2) were similar to those observed using the same concentration in experiment 1. In both experiments, treatment with 200 mg/mL ClO_2 caused a significantly ($P \leq 0.05$) higher number of cells to die compared with treatment with 200 mg/mL chlorine. The number of spores killed by treatment with 1,000 mg/mL

Table 1 Populations of vegetative cells and spores of *Bacillus cereus* recovered from water (control) and sanitizers after treatment for 5 min. Mean values (log cfu/mL sanitizer solution or water) in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$). Lower limit of detection is 2 cfu/mL (0.3 log cfu/mL) of treatment suspension

Treatment	pH	Concentration	Population (log cfu/mL)			
			Vegetative cells		Spores	
			cfu/mL	R^f	cfu/mL	R
Water (control)			5.7 a		6.7 a	
Chlorine	6.8	100 μg/mL	1.7 c	4.0		
		200 μg/mL	1.2 d	4.5	4.9 b	1.8
		1,000 μg/mL			3.8 c	2.0
ClO_2	10–5-11.0	200 μg/mL	< 0.3 e	> 5.4	< 0.3 d	> 6.4
		500 μg/mL	< 0.3 e	> 5.4	< 0.3 d	> 6.4
FPP	3.0	0.5%	3.2 b	2.5	6.3 a	0.4
		1.5%	3.2 b	2.5	6.3 a	0.4

[f]Reduction (log cfu/mL) in population in sanitizer solutions after treatment for 5 min compared with the population in water (control) after treatment for 5 min

chlorine or 200 mg/mL ClO_2 exceeded 5.8 log cfu/mL. The spore population was reduced by 5.9 log cfu/mL when treated with 100 mg/mL ClO_2.

Experiment 3

This experiment focused on determining the effects of alkaline ClO_2, ClO_2 combined with FPP, and ClO_2 acidified with citric acid on inactivation of five-strain mixtures of vegetative cells and spores of *B. cereus*. The results are shown in Table 3. Treatment of vegetative cells with 200 mg/mL chlorine reduced the number of viable vegetative cells by 4.2 log cfu/mL compared with treatment with water. This agrees with reductions of 4.5 log and 4.9 log cfu/mL in experiments 1 and 2, respectively. Treatment of spores with 200 mg/mL chlorine resulted in a 2.0-log cfu/mL reduction compared with treatment with water; this compares with a 1.8-log cfu/mL reduction in experiment 1. Reductions in the number of viable vegetative cells and spores caused by treatment with alkaline ClO_2 (85 mg/mL) are consistent with reductions achieved using 100 mg/mL ClO_2 in experiment 2. All treatments containing ClO_2 caused significant ($P \leq 0.05$) reductions in populations of vegetative cells and spores compared with respective reductions resulting from treatment with 200 mg/mL chlorine. Reductions in numbers of viable vegetative cells and spores caused by treatment with alkaline ClO_2 (85 mg/mL), a mixture of ClO_2 (85 mg/mL) and FPP (0.5%), and acidified ClO_2 (85 mg/mL) were not significantly different from each other.

Experiment 4

The influence of organic and inorganic materials (1.7 mg/mL reaction mixture) on inactivation of *B. cereus* and *B. thuringiensis* spores by alkaline ClO_2 was investigated. Spores suspended in spent MGM instead of deionized water were subjected to treatment with ClO_2 at concentrations up to 400 mg/mL.

Table 2 Populations of vegetative cells and spores of *B. cereus* recovered from water (control) and sanitizers after treatment for 5 min. Mean values (log cfu/mL of sanitizers or water) in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$). Lower limit of detection is 2 cfu/mL (0.3 log cfu/mL) of treatment suspension

Treatment	Concentration (μg/mL)	pH	Population (log cfu/mL)			
			Vegetative cells		Spores	
			cfu/mL	R^f	cfu/mL	R
Water (control)			5.4 a		6.1 a	
Chlorine	200	6.8	0.5 d	4.9		
	1,000	6.8			0.1 d	6.0
ClO_2	5	8.1	3.9 b	1.5	5.9 ab	0.2
	10	8.8	3.9 b	1.5	5.7 a	0.4
	50	10.4	3.2 c	2.2	4.7 c	1.4
	100	10.9	0.2 e	5.2	0.2 d	5.9
	200	11.5	< 0.3 e	> 5.1	< 0.3 d	> 5.8

[f]Reduction (log cfu/mL) in population in sanitizer solutions after treatment for 5 min compared with the population in water (control) after treatment for 5 min

Table 3 Populations of vegetative cells and spores of *Bacillus cereus* recovered from water (control) and sanitizers after treatment for 5 min. Mean values (log cfu/mL sanitizers or water) in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$). Lower limit of detection is 2 cfu/mL (0.3 log cfu/mL) of treatment suspension

Treatment	pH	Concentration (μg/mL)	Population (log cfu/mL)			
			Vegetative cells		Spores	
			cfu/mL	R^d	cfu/mL	*R*
Water (control)			5.6 a		6.3 a	
Chlorine	6.8	200	1.4 b	4.2	4.2 b	2.1
ClO_2	10.5	85	< 0.3 c	> 5.3	< 0.7 c	5.6
ClO2 + FPP (0.5%)	3.5	85	< 0.3 c	> 5.3	< 0.3 c	> 6.0
Acidified ClO_2	3.4	85	< 0.3 c	> 5.3	0.6 c	5.7

[d]Reduction (log cfu/mL) in population in sanitizer solutions after treatment for 5 min compared with the population in water (control) after treatment for 5 min

Table 4 lists the mean values of the number of viable spores of *B. cereus* (log cfu/mL spent MGM plus water or ClO_2 solution) after treatment for up to 30 min. The number of spores suspended in water did not change significantly ($P > 0.05$) during the 30-min exposure period. Treatment with 100, 200, or 400 mg/mL ClO_2 resulted in significant ($P \leq 0.05$) reductions in populations between 10 s and 5 min, but not between 5 and 30 min. Within 10 s of exposure to 100 mg/mL ClO_2, the number of spores was significantly reduced. An additional significant reduction in the number of viable *B. cereus* spores resulted from treatment with 400 vs. 200 mg/mL ClO_2. Exposure to ClO_2 for 5, 10, or 30 min resulted in a similar trend (i.e., increased reductions resulted from treatment with increased concentrations of ClO_2). Treatment with 400 mg/mL $ClO_2$for 30 min resulted in the highest reduction (4.6 log cfu/mL) caused by various combinations of ClO_2 concentration and exposure time.

Also shown in Table 4 are mean values of the number of *B. thuringiensis* spores detected after treatment in water or alkaline ClO_2 solution. The number of viable spores in water did not change significantly ($P > 0.05$) during the 30-min treatment period. Significant reductions ($P \leq 0.05$) occurred between 10 s and 5 min, regardless of the concentration of ClO_2 in treatment solutions. Further significant reductions did not occur between 5 and 30 min in treatment solutions containing 100 or 400 mg/mL ClO_2; however, a significant reduction resulted from treatment with 200 mg/mL ClO_2 for 10 min compared with 5 min. This may be an aberrant value, because the number of spores recovered from the 200-mg/mL treatment after 30 min was not significantly different than the number recovered after 5 min. Treatment with 100 mg/mL ClO_2 for 10 s or 30 min significantly reduced populations compared with the control. An increase in concentration of ClO_2 in the treatment solution resulted in further significant reductions after 10 s, 5 min, and 10 min of exposure.

Even though large differences in numbers of spores were detected within treatment time as the concentration of alkaline ClO_2 was increased, some of these differences were not significant. This is attributed in part to large deviations in values for the two replicate experiments. Nevertheless, alkaline ClO_2 has a high lethal activity against *B. cereus* and *B. thuringiensis* spores suspended in a medium containing a high organic load.

Spores of *Bacillus* and *Clostridium* species are known to be resistant to killing by acid and alkali, but these compounds are generally used as components of disin-

Table 4 Populations of *B. cereus* and *Bacillus thuringiensis* spores recovered from water (control) and MGM after treatment with ClO_2 for up to 30 min. Mean values (log cfu/mL spent MGM suspension plus water or ClO_2 solution) in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$). Within each *Bacillus* species, mean values in the same column that are not preceded by the same letter are significantly different ($P \leq 0.05$)

Species	Control/treatment	pH	Concentration (μg/mL)	Population (log cfu/mL)					
				10 s	R^d	5 min	10 min	30 min	*R*
B. cereus	Water ClO_2			a 8.0 a				a 8.0 a	
	ClO_2	10.9	100	b 7.7 a	0.3	a 7.0 b	a 7.2 b	b 7.0 b	1.0
		11.5	200	b 7.7 a	0.3	b 5.9 b	b 6.0 b	C 6.1 b	1.9
		12.1	400	c 7.4 a	0.6	b 6.3 b	c 3.9 b	C 3.4 b	4.6
B. thuringiensis	Water			a 8 .0 a				a 8.1 a	
	ClO_2	10.9	100	b 7.7 a	0.3	a 7.0 b	a 7.0 b	b 6.8 b	1.3
		11.5	200	c 7.3 a	0.7	a 6.9 b	b 5.1 c	b 6.3 bc	1.8
		12.1	400	c 7.2 a	0.5	b 5.0 b	b 3.7 b	b 2.9 b	5.2

[d]Reduction (log cfu/mL) in population in ClO_2 solution compared with the population in water after the same treatment time 10 s or 30 min)

fectants rather than alone [18, 20]. However, the mechanisms of spore resistance to acid and alkali have not been fully described and neither have the precise mechanisms of lethality attributable to these agents [25]. This latter study reported that dipicolinic acid (DPA) released from *B. subtilis* spores treated with acid parallels the death of ungerminated spores. Spores killed by alkali did not release DPA until initiation of germination, but did not initiate metabolism and degraded their cortex poorly, suggesting that lethality caused by acid involves disruption of a spore permeability barrier, whereas killing by alkali is due to inactivation of cortex lytic enzymes. A previous report [15] concluded that death of *B. cereus* spores treated with 5% NaOH was caused by modification of the outer spore coats, leading to disruption of permeability barriers.

Young and Setlow [29] reported that treatment of *B. subtilis* spores with hypochlorite at 2,500 mg/mL (pH 11) or 50 mg/mL (pH 7) or ClO_2 at 10–14 mg/mL (pH 3.5) gave 3 logs of killing within approximately 45, 8, and 9 min, respectively. We observed that ClO_2 at 85 mg/mL (pH 3.4 or 10.5), also in the absence of a high organic load, killed 5.6 to 5.7 log cfu/mL *B. cereus* spores within 5 min (Table 3). Reductions caused by treatment with chlorine (200 mg/mL, pH 6.8) were 2.0 log cfu/mL. These observations support those of Young and Setlow [29], showing greater lethality of ClO_2 compared with chlorine in killing *Bacillus* spores.

The addition of hypochlorite (200 mg/mL) to NaOH has been reported to enhance sporicidal activity [7]. This may be due to extraction of alkali-soluble spore coat protein, thus increasing sensitivity to chlorine. Bloomfield and Arthur [4] showed that lethality of chlorine-releasing agents is affected by NaOH. Hypochlorite in the presence or absence of NaOH and sodium dichloroisocyanurate in the presence of NaOH degraded *B. subtilis* spore coat and cortex materials, which may be related to their sporicidal activity. Similar mechanisms of action may be occurring in spores of *B. cereus* and *B. thuringiensis* treated with alkaline ClO_2.

Our study clearly confirms the well-known fact that lethality of disinfectants that owe their activity to oxidizing reactions is diminished when spores are in an environment that contains organic materials. Although more than 4.6-log reductions in spores of *B. cereus* and *B. thuringiensis* in MGM were achieved by treating with ClO_2 at 400 mg/mL, higher reductions of *Bacillus* spores in foods may be desirable in some situations. Coates and Death [5] showed significant potentiation of the action of hypochlorite against *B. subtilis* spores could be achieved by the addition of methanol, ethanol, or propanol to treatment solutions. Setlow et al. [25] attributed death of *B. subtilis* spores treated with ethanol to a release of DPA. The use of GRAS alcohols and perhaps other solvents in combination with ClO_2 to enhance lethality to *Bacillus* spores on the surface of, or enmeshed in, organic matrices and on hard inert surfaces deserves further research attention.

References

1. Bernarde MA, Snow WB, Olivieri P, Davidson B (1967) Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl Microbiol 15:265–267
2. Beuchat LR (1998) Surface decontamination of fruits and vegetables eaten raw: a review. Food Safety Issues, Food Safety Unit, World Health Organization, Geneva, WHO/FSF/FOS/98.2
3. Bloomfield SF (1999) Resistance of bacterial spores to chemical agents. In: Russell AD, Hugo WB, Ayliffe GAJ (eds) Principles and practices of disinfection, reservation and sterilization. Blackwell, London, pp 303–320
4. Bloomfield SF, Arthur M (1992) Inactivation of *Bacillus subtilis* spores with sodium hypochlorite, sodium dichloroisocyanurate, and chloraurine-T. J Appl Bacteriol 72:166–172
5. Coates D, Death JE (1978) Sporicidal activity of mixtures of alcohol and hypochlorite. J Clin Pathol 31:148–152
6. Cords BR, Dychadala GR (1993) Sanitizers: halogens, surface-active agents, and peroxides. In: Davidson PM, Branen AL (eds) Antimicrobials in foods, 2nd edn. Dekker, New York, pp 469–537
7. Cousins CM, Allan CD (1967) Sporicidal properties of some halogens. J Appl Bacteriol 30:168–174
8. Erickson MC, Kornacki JL (2003) *Bacillus anthracis*: current knowledge in relation to contamination of food. J Food Prot 66:691–699
9. Granum PE (2001) *Bacillus cereus*. In: Doyle MP, Beuchat LR, Montville TJ (eds) Food microbiology: fundamentals and frontiers, 2nd edn. American Society for Microbiology, Washington D.C. pp 373–381
10. Granum PE, Baird-Parker TC (2000) *Bacillus* species. In: Lund BM, Baird-Parker TC, Gould GW (eds) The microbiological safety of quality of food, vol 2. Aspen, Gaithersburg, Md., pp 1029–1039
11. Han Y, Linton RH, Nielson SS, Nelson PE (2000) Inactivation of *Escherichia coli* O157:H7 on surface-uninjured and-injured green pepper (*Capsicum annuum* L.) by chlorine dioxide gas demonstrated by confocal laser scanning microscopy. Food Microbiol 17:643–655
12. Han Y, Linton RH, Nielson SS, Nelson PE (2001) Reduction of *Listeria monocytogenes* on green peppers (*Capsicum annuum* L.) by gaseous and aqueous chlorine dioxide and water washing and its growth at 78C. J Food Prot 64:1730–1738
13. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolsto A-B (2002) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 66:2627–2630
14. Kim HU, Goepfert JM (1974) A sporulation medium for *Bacillus anthracis*. J Appl Bacteriol 37:265–267
15. Kulikovsky A, Pankratz HS, Sadoff HL (1975) Ultrastructural and chemical changes in spores of *Bacillus cereus* after action of disinfectants. J Appl Bacteriol 38:39–46
16. Lindsay D, Brozel VS, Mostert JF, von Holy A (2002) Differential efficacy of a chlorine dioxide-containing sanitizer against single species and binary biofilms of a dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. J Appl Microbiol 92:352–361
17. Lynch DJ, Potter NN (1988) Effects of organic acids on thermal inactivation of *Bacillus stearothermophilus* and *Bacillus coagulans* spores in frankfurter emulsion slurry. J Food Prot 51:475–480
18. McConnell G, Russell AD (1999) Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 2:147–179
19. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Mol Biol Microbiol Rev 64:548–572
20. Reina LD, Fleming, Humphries EG (1995). Microbiological control of cucumber hydrocooling water with chlorine dioxide. J Food Prot 58:541–546

21. Revkin AC (2001) Evicting an unwelcome tenant: anthrax. New York Times, Available at: http://www.nytimes.com/2001/11/06/science/physical/06CLEA.htmL. Accessed 6 October 2003
22. Roberts CM, Hoover DG (1996) Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity, and nisin. J Appl Bacteriol 81:363–368
23. Russell AD (1990) Bacterial spores and chemical sporicidal agents. Clin Microbiol Rev 3:99–119
24. Sagripanti J-L, Bonifacino A (1996) Comparative sporicidal effects of liquid chemical agents. Appl Environ Microbiol 62:545–551
25. Setlow B, Loshon CA, Genest PC, Cowan AE, Setlow C, Setlow P (2002) Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali, and ethanol. J Appl Microbiol 92:362–375
26. Setlow P (2000) Resistance of bacterial spores. In: Storz G, Hengge-Aronis R (eds) Bacterial stress. American Society for Microbiology, Washington, D.C., pp 217–230
27. Stampi S, De Luca G, Onorato M, Ambrogiana E, Zanetti F (2002) Peracetic acid as an alternative wastewater disinfectant to chlorine dioxide. J Appl Microbiol 93:725–731
28. Wuytack EY, Michiels CW (2001) A study on the effects of high pressure and heat on *Bacillus subtilis* spores at low pH. Int J Food Microbiol 64:333–341
29. Young SB, Setlow P (2003) Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. J Appl Microbiol 95:54–67
30. Zhang S, Farber JM (1996) The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. Food Microbiol 13:311–321