

Inactivation of Spores of *Bacillus anthracis* Sterne, *Bacillus cereus*, and *Bacillus thuringiensis* subsp. *israelensis* by Chlorination

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Three species of *Bacillus* were evaluated as potential surrogates for *Bacillus anthracis* for determining the sporidial activity of chlorination as commonly used in drinking water treatment. Spores of *Bacillus thuringiensis* subsp. *israelensis* were found to be an appropriate surrogate for spores of *B. anthracis* for use in chlorine inactivation studies.

The use of spores of *Bacillus anthracis* as a bioterrorist weapon has prompted renewed interest in the study of the inactivation of *Bacillus* spores by chemical disinfectants. Information regarding the resistance of *B. anthracis* spores to chlorination is of particular interest in reference to drinking water treatment. There has been a growing awareness, due to restrictions of working with select agents, of the need to evaluate the resistance to disinfectants of spores of attenuated strains of *B. anthracis* as well as other closely related species of *Bacillus* which might serve as surrogates for the overt pathogenic agent. The three species evaluated as surrogate organisms in this study were chosen based upon their genetic homogeneity (9). Indeed, some investigators have suggested that the three organisms comprise a single species (5).

The sporidial effectiveness of free available chlorine was determined at two pH levels and two temperatures. The inactivation experiments were conducted under chlorine-demand-free (CDF) conditions. *CT* values (*C* is the concentration of chlorine in mg/liter, and *T* is the exposure time in minutes) were determined for each *Bacillus* species for the various experimental conditions. The sporulation and purification procedures were performed in the same manner for each species.

Three species of *Bacillus* were used in this study: *B. anthracis* Sterne (34F2; Colorado Serum Co., Denver, CO), *Bacillus cereus* (ATCC 7039), and *Bacillus thuringiensis* subsp. *israelensis* (ATCC 35646). *B. anthracis* Sterne is an attenuated strain of *B. anthracis*. Endospores were produced in a broth sporulation medium (3). The cultures were grown at 35°C with agitation on a rotary shaker for 5 days. Spores were purified by gradient separation using RenoCal-76 (Bracco Diagnostics, Princeton, NJ) and washed three times by centrifugation with distilled water, as previously described (7). Purified spore preparations (approximately 1×10^7 CFU/ml) were examined using phase-contrast microscopy and stored in 40% (vol/vol) ethanol at 5°C until the time of use. Microscopic examination of the purified spore preparations exhibited <0.1% vegetative cells.

Sterile CDF buffer (0.05 M KH_2PO_4) was used in the experiments. The buffer was made chlorine demand free by add-

ing reagent-grade sodium hypochlorite (4 to 6%) to the buffer to achieve a free-chlorine residual of approximately 3.0 mg/liter. The pH of the buffer was adjusted by the addition of 10 M sodium hydroxide. The buffer was boiled for 5 minutes, transferred to 4-liter beakers, and exposed to short-wave UV irradiation in a biological-safety cabinet for 48 hours to remove the chlorine. The buffer was then sterilized by autoclaving and stored in a sealed container for no longer than 3 weeks. An appropriate volume of a 1:200 (vol/vol) aqueous solution of reagent-grade sodium hypochlorite was added to the CDF buffer to obtain the desired free-chlorine level.

Inactivation experiments were conducted at 5°C in a recirculating, refrigerated water bath and at ambient room temperature (22 to 23°C) at pH 7.0 and 8.0. These parameters were chosen as representative of conditions which might normally be encountered in drinking water treatment facilities. All temperatures in the reaction vessels were verified using a mercury-filled glass thermometer. Borosilicate glass beakers (1,000 ml) containing 500 ml of buffer served as the reaction vessels. Reaction vessels were continuously stirred using a magnetic stirring apparatus. The vessels were inoculated with the various spore preparations to yield an initial level of approximately 1×10^4 CFU/ml. Chlorine concentrations were determined at each exposure time using the *N,N*-diethyl-*p*-phenylenediamine colorimetric method (1). Controls for these experiments consisted of CDF buffer without a chlorine residual. Samples were withdrawn from the reaction vessels at the various exposure times, and any residual chlorine was immediately neutralized by the addition of 0.1 ml of a 10% (wt/vol) sodium thiosulfate solution. Control and test samples were treated in the same manner throughout the experiments. Triplicate experiments were performed for each *Bacillus* species for each experimental condition. The numbers of spores present in the control and chlorine-exposed samples were determined by culture count using a membrane filtration procedure, as previously described (10).

Levels of inactivation were determined by plotting the \log_{10} ratio of survivors against the exposure time (minutes) for each experimental condition. Covariance analysis was performed to compare the inactivation rates. The analysis was performed separately for each of the four experimental conditions of pH and temperature. The Chick-Watson model was used for determining *CT* values (6). *CT* values were calculated based upon

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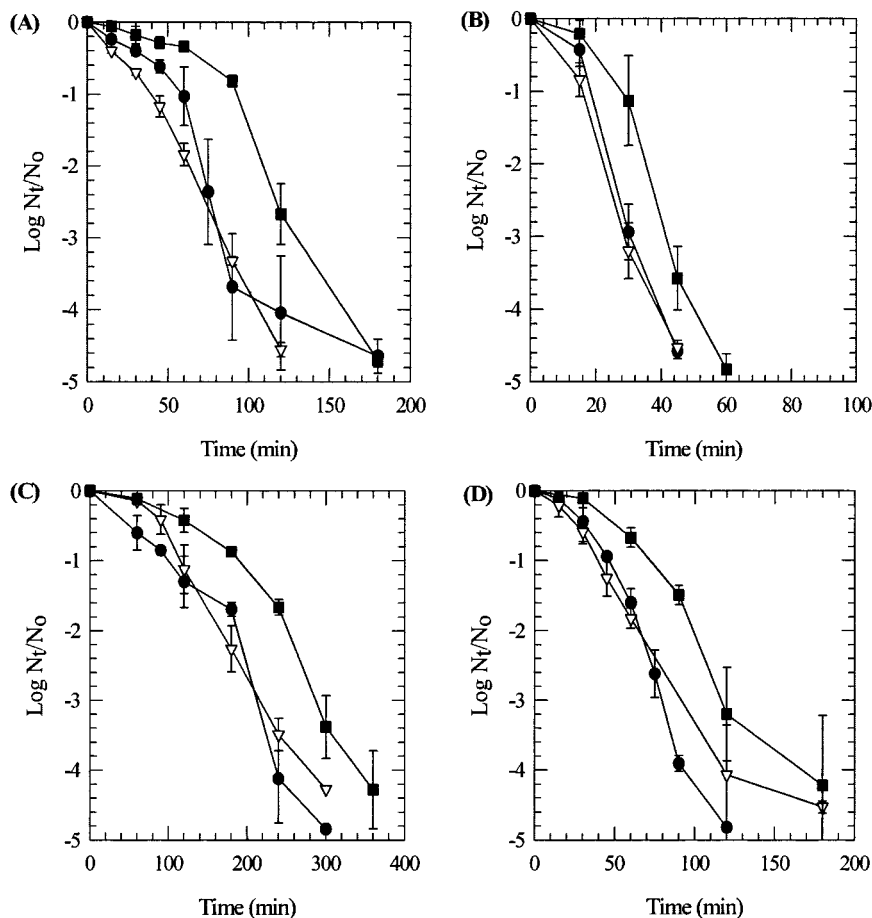


FIG. 1. Inactivation of *Bacillus* species spores exposed to 2.0 mg/liter free chlorine under different conditions. (A) pH 7, 5°C; (B) pH 7, 23°C; (C) pH 8, 5°C; (D) pH 8, 23°C. Symbols: ●, *B. anthracis* Sterne; ▽, *B. cereus*; ■, *B. thuringiensis* subsp. *israelensis*.

a first-order exponential relationship for chlorine decay. The chlorine decay rate was calculated from the slope of the line plotted on the basis of the ratio of chlorine concentrations at each exposure time and at time zero (C_t/C_0) against the exposure time. The mean chlorine decay rate was 0.0012 min^{-1} (range, 0.00023 to 0.00259 min^{-1}).

The results of the inactivation experiments are shown in Fig. 1. Each data point represents the mean of the results of three experiments. Under the chlorine-demand-free conditions, the chlorine concentrations averaged 2.0 ± 0.2 mg/liter during the course of the experiments. The standard error of the mean ranged from 0 to 0.58, with the average standard error being 0.14 for the \log_{10} microbial counts for the individual data points (Fig 1). As expected, in all cases the rates of inactivation were greater at the higher temperature (23°C). Inactivation also occurred more rapidly, as anticipated, at the lower pH level (pH 7.0). These results concur with established observations regarding the role of temperature and pH in chlorine inactivation studies (6).

Differences were observed for the inactivation of the different *Bacillus* species. *B. thuringiensis* subsp. *israelensis* spores were consistently more resistant than spores of either *B. anthracis* Sterne or *B. cereus*. Spores of *B. anthracis* Sterne and *B. cereus* behaved similarly but did not exhibit consistent patterns

of resistance under the various conditions. Spores of *B. anthracis* Sterne were more resistant than spores of *B. cereus* at pH 7.0, but spores of *B. cereus* were more resistant than spores of *B. anthracis* Sterne at pH 8.0 at both temperatures (Fig. 1). The covariance analysis indicated that the rate of inactivation for spores of *B. thuringiensis* was significantly lower ($P < 0.05$) than the rate of inactivation for spores of *B. anthracis* or *B. cereus*. There was no significant difference ($P < 0.05$) between the inactivation rates for spores of *B. anthracis* Sterne or *B. cereus*.

The calculated CT values ($\text{mg} \cdot \text{min}/\text{liter}$) for inactivation of spores of the three *Bacillus* spp. at 2, 3, and 4 orders of magnitude are given in Table 1. The CT values derived from the model illustrate the differences in inactivation observed for the three species of *Bacillus*. Also shown in Table 1 are CT values which have recently been reported for 2 and 3 \log_{10} levels of inactivation at pH 7.0 for spores of a virulent strain of anthrax, *B. anthracis* Ames (11).

Chlorine is the most widely used disinfectant for water treatment in the United States, but there is a limited amount of data on the effectiveness of chlorination in inactivating spores of *B. anthracis* under conditions used in drinking water treatment (2,4). The CT values for two of the surrogate organisms used in this study (*B. anthracis* Sterne and *B. cereus*) were substantially

TABLE 1. *CT* values for inactivation of spores of *Bacillus* spp. exposed to 2.0 mg/liter free chlorine

Temp (°C)	pH	Log ₁₀ inactivation	<i>CT</i> (mg · min/liter)			
			<i>B. anthracis</i> Ames ^a	<i>B. anthracis</i> Sterne	<i>B. cereus</i>	<i>B. thuringiensis</i>
23	7	2	79	45	41	66
		3	102	68	62	99
		4	ND	90	82	132
	8	2	ND	127	132	246
		3	ND	191	199	369
		4	ND	254	264	492
5	7	2	220	140	117	229
		3	339	210	175	344
		4	ND	280	233	458
	8	2	ND	319	340	481
		3	ND	478	510	721
		4	ND	637	680	961

^a Data from reference 11. ND, not determined.

lower than the *CT* values for spores of *B. thuringiensis* subsp. *israelensis* and for the virulent *B. anthracis* Ames strain. The *CT* values which most closely approximated those for the virulent strain were seen for spores of *B. thuringiensis* subsp. *israelensis*. Based upon these findings, spores of *B. thuringiensis* subsp. *israelensis* would be an appropriate surrogate to use in place of *B. anthracis* in chlorine inactivation studies.

Methodological variations can substantially alter the measured activities of sporicidal agents (12). In a recent report on UV inactivation of *Bacillus* species spores (8), it was noted that the most reliable method for testing intrinsic differences between strains requires parallel testing, using identical conditions for sporulation, purification, and survival determinations. The present study meets these criteria. Future studies directly

comparing the inactivation of spores of other surrogate species and other strains of the proposed surrogate organisms with that of other virulent strains will be beneficial in evaluating the use of surrogate *Bacillus* spp. as an alternative to *B. anthracis* in disinfection studies.

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