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Decontamination Options for Drinking Water Contaminated with *Bacillus anthracis* Spores

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Abstract

Five parameters were evaluated with surrogates of *Bacillus anthracis* spores to determine effective decontamination options for use in a contaminated drinking water supply. The parameters were: (1) type of *Bacillus* spore surrogate (*B. thuringiensis* or *B. atrophaeus*); (2) spore concentration in suspension (10^2 to 10^6 spores/ml); (3) chemical characteristics of decontaminant [sodium dicholor-s-triazinetrione dihydrate (Dichlor), hydrogen peroxide, potassium peroxymonosulfate (Oxone), sodium hypochlorite, and VirkonS®]; (4) decontaminant concentration (0.01% to 5%); and (5) decontaminant exposure time (10 min to 24 hr). Results from 162 suspension tests with appropriate controls are reported. Hydrogen peroxide at a concentration of 5%, and Dichlor and sodium hypochlorite at a concentration of 2%, were effective at spore inactivation regardless of spore type tested, spore exposure time, or spore concentration evaluated. This is the first reported study of Dichlor as an effective decontaminant for *B. anthracis* spore surrogates. Dichlor's desirable characteristics of high oxidation potential, high level of free chlorine, and more neutral pH than that of other oxidizers evaluated appear to make it an excellent alternative. All three oxidizers were effective against *B. atrophaeus* spores in meeting EPA's biocide standard of greater than a 6 log kill after a 10-minute exposure time and at lower concentrations than typically reported for biocide use. Solutions of 5% VirkonS® and Oxone were less effective decontaminants than other options evaluated in this study and did not meet the EPA's efficacy standard for biocides. Differences in methods and procedures reported by other investigators make quantitative comparisons among studies difficult.

Introduction

Developing a decontamination approach that can be safely and effectively applied to civilian water resources and facilities following a terrorist or catastrophic release of *Bacillus anthracis* (*B. anthracis*) spores poses many challenges. For example, if a municipal drinking water system were contaminated directly or indirectly during or after such an incident, it would be essential to assess the potential health risks posed by water consumption or other water uses (e.g., recreational and bathing) and then to apply one or more proven technologies, if deemed necessary, to decontaminate the water supply quickly and cost-effectively. Treatment of drinking water supplies implies the use of a decontamination approach that would not pose adverse heath risks to humans or result in unacceptable damage to the environment. A major obstacle in killing spores of the *Bacillus* species (spp.) on or in virtually any matrix is their high level of resistance to treatments such as harsh chemicals, heat, desiccation, and ultraviolet light (8, 16). Because of the substantial and widely reported resistance of *Bacillus* spores to inactivation, a decontaminant proven to be efficacious in killing such spores for site- specific applications is likely be effective against all other biological warfare agents as well.

Whereas nearly all biological warfare agents are intended for aerosol application, many have strong potential as waterborne threats and could inflict heavy casualties when ingested (1). *B. anthracis* in particular has been identified as a “probable” (9) or an actual (19) water threat. Even though the principal risk associated with the consumption of water containing *B. anthracis* spores would likely arise from the ingestion hazard, water used for bathing, showering, or recreational purposes might also pose cutaneous exposure hazards. There is controversy regarding the long-term viability of *B. anthracis* in water, and experimental evidence is limited. However, according to a review of nonkinetic studies on survival of virulent strains in the

environment (17, Table 7), *B. anthracis* spores can survive from 2 to 18 years in pond water and 20 months in seawater or distilled water. *B. anthracis* spores have been reported by others to be stable in water for 2 years (19).

Various decontamination approaches have been evaluated for efficacy against biological warfare agents, including *Bacillus* spp. spores, on hard, nonporous surfaces. Recommendations by the U.S. Environmental Protection Agency (EPA) include the use of sodium hypochlorite [1:9 dilution of bleach to 5,250 to 6,000 ppm, corrected to pH 7, with a 60-minute contact time at 20°C (see http://www.epa.gov/pesticides/factsheets/chemicals/bleachfactsheet.htm, as well as 13)], and liquid chlorine dioxide with a 30-minute wet contact time at 20°C (http://www.epa.gov/pesticides/factsheets/chemicals/chlorinedioxidefactsheet.htm). Liquid hydrogen peroxide/peroxyacetic acid (known as peroxy compounds and marketed as ready-to-use solutions), generally with a 15- to 20-min wet contact time and concentration as specified by the manufacturer, has also been recommended (10). Other products such as hydrogen peroxide solution (3 to 25%) and potassium peroxymonosulfate have been evaluated for efficacy against *Bacillus spores* as well (22). Although disinfectants at various concentrations have been tested previously against the spores of *B. anthracis* and their surrogates, wide variations in test protocols make meaningful comparisons among studies virtually impossible (5, 7, 13).

In contrast to surface cleanup of spores, fewer assessments of efficacy have been reported for the decontamination of *Bacillus* spp. spores in water utilizing suspension tests by the aforementioned chemicals or other methods, and much of the published work has only assessed relatively high concentrations of spores in water. For example, one previous investigation commenced evaluations with 0.2-ml suspensions of approx 10^9 spores/ml of various *Bacillus* spp. to which 20 ml of aqueous ozone or 20 ml of hydrogen peroxide solution was added to assess sporicidal

action (6), and others have reported mechanisms of deactivating *B. subtilis* spores prepared in concentrations of up to approx 10^8 spores/ml (22) and approx 10^9 spores/ml (13). Inactivation by chlorination of various *Bacillus* spp. with initial concentrations of approx 1×10^4 CFU/ml has also been tested (12). However, relatively low spore concentrations would be expected to result from dilutions following contamination of a large public water system. Therefore, it is reasonable to evaluate the effectiveness of decontaminants or other methods against even lower spore concentrations in water than have been previously assessed. In addition to assessing the parameter of *Bacillus* spore concentration in water, it is essential to identify the most effective, commercially available chemical that will kill all the spores or minimize population growth, while considering the effects of the chemical on the environment and in humans.

Several objectives served to focus our investigation. First, five potential candidate decontaminants were selected because of their relative safety and ultimate degradation in the environment without substantive adverse consequences. The five chemicals were also chosen as a way of comparing the effectiveness of available free chlorine content, pH, and oxidation potential on spore inactivation. From an evaluation of those chemical parameters, we sought to determine the most effective option for inactivating *Bacillus* spore surrogates suspended in water. As a second objective, we attempted to identify the lowest concentration of the selected chemicals necessary to achieve the EPA's biocide standard of greater than a six-log kill (also known as the 1×10^6 kill rate, which means reducing the number of live organisms by 6 orders of magnitude). As a third objective, we wanted to assess the effect of reduced spore concentration on chemical biocide efficacy. As an important step in ascertaining an efficient, safe, and cost-effective water-treatment method that could potentially provide safe water to the general population in the event of *B. anthracis* contamination—and limit the potential risk of

contracting gastrointestinal or cutaneous anthrax as well—the following parameters were evaluated: chemical decontaminant type; chemical decontaminant concentration (0.01% to 5%); contact time of spores with chemical decontaminant (10 min to 24 hr); spore type (*B. atrophaeus* or *B. thuringiensis*); and low versus relatively high spore concentrations (approx 10^2 and 10^6 spores/ml).

Materials and Methods

All work was performed in BSL-1 level laboratory facilities. The 81 suspension tests performed were run in duplicate, resulting in a total of 162 tests, excluding controls. Numerous initial control tests were also run in duplicate to ensure that there was no contamination, the starting spore concentration was accurate, and the overall methods used (including quenchers) did not affect spore growth. A total of 12 control tests were conducted.

Spores. All *B. atrophaeus* spores, formerly known as *B. globigii*, (strain ATCC: 9372) were prepared and acquired from Apex Laboratories (Apex, North Carolina) with a reported mean population of 3.0×10^3 and 1.0×10^7 spores/ml (nonheat shock value). *B. thuringiensis* spores, sp. Kurstaki, serving as a conservative surrogate, was also prepared and acquired from Apex Laboratories (Apex, North Carolina) and had a mean reported population of 3.6×10^3 spores/ml (nonheat shock value). Spore preparations were subjected to quality-acceptance criteria by the supplier to ensure good-quality spores and lack of vegetative cells and cell debris. Spores were received by investigators in a 20% ethanol solution and were refrigerated until use. The spores were not extracted from the ethanol, as ethanol poses no potential for variability in the test results. Upon receipt, spore preparations were further evaluated by microscopy to ensure that spores had not germinated prior to testing. To obtain the desired concentration of 1.0×10^6

spores/ml of *B. atrophaeus,* 0.1 ml of the starting solution (1.0×10^7 spores/ml in ethanol) was added to 0.9 ml of each decontaminant. The lower concentrations of approx 3.0×10^2 for *B. atrophaeus* and *B. thuringiensis* spores, required that 1 ml of each starting solution (3.0×10^3 *B. atrophaeus* spores/ml, and 3.6×10^3 *B. thuringiensis* spores, both in ethanol) be added to 9 ml of decontaminant to ensure adequate sample size.

Culture. 80-mm Tryptic Soy Agar Petri dishes (VWR International, West Chester, PA) were prepared from a 125-ml plastic bottle of solid agar (Wards Natural Science, Rochester, NY) by heating the agar in a microwave oven on low for a total of 5 min, stopping every 1 min to ensure mixing by swirling the bottle in large, slow rotations. Using a 25-ml graduated cylinder, 20 ml of melted agar was dispensed in each Petri dish. Petri dishes were left undisturbed at approx 24°C for a minimum of 2 hr before plating the spores.

Disinfectant Chemicals. The five chemicals assessed for sporicidal efficacy were VirkonS® (Dupont Chemical Corporation, Wilmington, DE), which is a triple salt of potassium peroxymonosulfate, potassium hydrogen sulfate, and potassium sulfate; potassium peroxymonosulfate (Oxone; Dupont Chemicals, Wilmington, DE); 27% hydrogen peroxide (Bacqucil, Winona, MN); 6% sodium hypochlorite (commercial Clorox, The Clorox Company, Oakland, CA) diluted with distilled water and without adjusting pH, as recommended by (3); and Dichlor (sodium dicholor-s-triazinetrione dihydrate) (Chlor-Brite Roswell, GA). Standard stock solutions of each chemical were prepared at concentrations of 0.01 to 5% using distilled water in 250-ml volumetric flasks. Distilled water was chosen to minimize the amount of organics present in the water. Control tests showed distilled water to be void of measurable contaminants. The solutions were maintained at approx 24°C, with temperature fluctuating a maximum of ±2°C. All mass measurements were made using a digital, centigram balance. Volume dilution

measurements were performed using either an automated pipette (small measurements) or graduated cylinder (large measurements). The pH of all chemical solutions was measured using litmus paper and then recorded. All solutions were used within 30 days.

Reaction-Quenching Chemicals. Preliminary tests were carried out to validate the methods and prove the efficacy of the neutralizing solutions. To quench hydrogen peroxide, a solution of 800 units/ml catalase powder (Wards Natural Science, Rochester, NY) was prepared using distilled water. All other chemicals were quenched using 1 molar (M) sodium thiosulfate (Wards Natural Science) in amounts depending on the concentration of chemical decontaminant used. To stop the reaction at the specified time, the indicated quencher was added to the disinfectant chemical in aliquots of 0.025 ml until pH was measured to be in the range of 6.5 to 7.5. Amounts of quencher dispensed varied as a function of the concentration and quantity of chemical present in the test tube. Pretesting was done to determine the proper ratio of quencher to chemical. Test tubes were filled with a specific quantity of chemical, and quencher was slowly added until a neutral pH was reached. These tests were done for each chemical and corresponding concentration. Specific amounts of quencher varied from 0.1 to 0.005 ml for sodium thiosulfate and from 0.005 to 0.75 ml for catalase. The use of sodium thiosulfate as well as catalase as a quencher for these types of experiments is well documented in the literature (2, 6, 22).

Exposure of Spores to Disinfectant Chemicals

Time-Dependent Studies for *B. atrophaeus* at 3.0×10^2 /ml.

A 9-ml aliquot of chemical decontaminant at a specified concentration was added to a test tube using an automated pipette. Stock solution of *B. atrophaeus* spores (3.0×10^3 /ml) was vortexed for approx 5 min to ensure consistency, followed by the addition of 1 ml of spore solution to the

test tube containing the 9 ml chemical decontaminant through the use of an automated pipette. The test tube was then vortexed for 30 sec.

After the specified spore-exposure time elapsed, the test tube was vortexed for 20 to 30 seconds, and 0.5 ml of solution was placed in a test tube containing the appropriate amount of sodium thiosulfate or catalase to quench the reaction. To account for reaction time, the solution was allowed to stand for 10 min before removing all liquid (0.5-ml spore plus quencher) and plating on a culture dish. Plating was performed using a Petri dish spinner and hovering the pipette tip approx 1.5 cm from the center of the Petri dish. While spinning, a spreader was applied to evenly spread the liquid over the agar surface. The Petri dish was covered and incubated undisturbed for 3 to 5 days at a temperature of 23° to 25°C. The procedure was repeated for all predetermined spore-exposure times (10 min, 30 min, 1 hr, 4 or 6 hr, and 24 hr) and for the five different chemicals assessed at each of three predetermined concentrations.

Time-Dependent Studies for *Bacillus atrophaeus* at 1×10^6 /ml

To evaluate the effectiveness of a decontaminant at a greater spore concentration, a series of time-dependent studies were run as seen in Figure 4. A 0.9-ml aliquot of chemical decontaminant was dispensed by automated pipette into a test tube. After vortexing and thoroughly shaking the *B. atrophaeus* for approx 5 min, 0.1 ml of starting spore solution (1×10^7/ml) was added to the test tube containing chemical decontaminant, and vortexed again for 30 seconds. After the specified, spore-exposure times of 10, 30, and 60 min, the reaction was quenched with the predetermined amount of catalase or sodium thiosulfate, and the solution was vortexed for 10 sec. After standing for 10 min, 0.2 ml of the solution was extracted and dispensed into a test tube containing 9.8 ml of distilled water. After this solution was vortexed for 20 to 30 sec, 0.5 ml was

extracted and plated on an agar dish. The result was approx 10,000 spores per plate for controls. All tests were duplicated with controls as described above.

Time-Independent Studies for *B. atrophaeus* at 3×10^2/ml and *B. thuringiensis* at 3.6×10^2/ml

Procedures were identical to those described above, except that all spore-exposure times were 10 min, a specified amount of quencher was added to the test tube, and the test tube was vortexed for 10 sec. The test tube was then allowed to stand for 10 min before being vortexed again for 20 to 30 sec, after which 0.5 ml of the solution was plated on an agar dish.

Controls

A total of 12 control groups, run in duplicate, were treated in an identical manner as experimental groups, discussed above, except for the following. Some control groups consisted of spores only in solution with neither chemical decontaminant nor quencher added to the spore preparation. Such controls were assessed to verify the starting concentrations of spores on plates and to ensure that no contamination was introduced during any step of the process. Blank controls were prepared with no spores added, but were otherwise subject to the same process as experimental groups. Additional controls were evaluated with the necessary amounts of sodium thiosulfate or catalase added to spore preparations, but with no chemical decontaminant added. Such controls were evaluated to exclude the possibility that either of the two quenchers alone influenced spore inactivation.

Results

Results are summarized as a function of spore type, spore concentration, exposure time, and decontaminant concentration. All results are reported as average counts derived from duplicate

tests, for which the variability can be seen in Table 1. Results are also reported as percent kill for each of the five decontaminants tested.

Controls groups were assessed to determine starting concentrations of spores on culture plates, by counting colony forming units (CFUs) for all initial suspensions. Following serial dilutions, an average of 141 ±7 CFUs per plate were counted for *B. atrophaeus* spores (derived from initial preparations of 3.0×10^3 spores/ml); an average of 140 ±3 CFUs per plate were counted for *B. thuringiensis* spores (derived from initial preparations of 3.6×10^3 spores/ml); and an average of 11,059 ±1006 CFUs per plate were counted for *B. atrophaeus* spores (derived initial from preparations of 1.0×10^7 spores/ml). The number of counted *B. atrophaeus* spores per plate, derived from a starting concentration of 1.0×10^7 spores/ml and following serial dilutions, was greater than anticipated as a result of evaporation of ethanol during storage. For this control group, CFUs in three representative 1-cm^2 areas within the culture dish were counted, the results averaged, and then multiplied by the area of the dish, expressed in cm^2, to estimate spore concentrations. The distribution of spores was judged as uniform across the surface of agar.

For controls evaluated following the addition of sodium thiosulfate or catalase, but with no chemical decontaminant added, it was found that neither quencher had an effect on spore inactivation at the concentrations used.

Table 1 summarizes the results of 82 initial test results for all cases in which *B. atrophaeus* spore-exposure times to a given concentration of decontaminant were varied (i.e., from 10 minutes to as long as 24 hours). From the counts of *B. atrophaeus* CFUs following spore incubation, only slight improvements in killing were observed following greater than 10 minutes of exposure to a given concentration of decontaminant in most cases. Stated another way, a

decontaminant at a given concentration either worked effectively in killing all spores within 10 minutes, or it did not work well at all. Because an exposure duration of 10 min appeared to be sufficient time for effective decontamination in most cases, further experiments focused on 10-min exposure times only. In no test cases were *B. thuringiensis* spores exposed to a decontaminant for longer than 10 min. Table 2 summarizes 162 test results, expressed as percent kill, for all cases in which *B. atrophaeus* and *B. thuringiensis* spores were exposed to decontaminants for 10 min.

Figures 1 through 3 show the results following 10 min of spore exposure, expressed as percent kill as a function of each of three decontaminant concentrations ranges, identified as low (0.01 to 0.05%), medium (0.1 to 0.5%), and high (1 to 5%). Figure 1 shows the results for *B. atrophaeus* spores at a concentration of 1×10^6/ml; Figure 2 shows the results *B. atrophaeus* spores at a concentration of 3×10^2/ml, and Figure 3 shows the results of *B. thuringiensis* spores at a concentration of 3.6×10^2/ml. Figure 4 shows the time-dependent results for three different exposure times (10, 30, and 60 minutes) for *B. atrophaeus* spores at a concentration of 1×10^6/ml and for all five decontaminants.

The following results are noteworthy. The percent kill ranged from 14.5% to 100% over the 162 experimental tests performed, and, as expected, percent kill increased as the concentration of a decontaminant increased. In general, *B. thuringiensis* spores were somewhat more difficult to kill than *B. atrophaeus* spores after exposure to the five decontaminants tested. Tests conducted with the higher concentration of *B. atrophaeus* spores (1×10^6 spores/ml) generally required greater concentrations of chemical to achieve the same percent kill as that achieved using the lower concentration of spores (3×10^2 spores/ml). However, the use of lower concentrations of chemical decontaminant often resulted in a greater percent kill than might be expected from

suggestions in the literature to the effect that only higher concentrations for many of these oxidants have been effective (6,13). However, most of the reported tests were done with higher concentrations of spores than those used in the present study, and they were not aimed at potential water treatment, which would be expected to have lower concentrations of spores because of dilution. Most of the previous studies were also aimed at surface treatment where higher levels of spores were considered more realistic.

In terms of the five decontaminants evaluated in this study, sodium hypochlorite, hydrogen peroxide, and Dichlor were effective for use as biocides. A solution of 2% sodium hypochlorite achieved 100% kill at both spore concentrations tested. A solution of 0.5% hydrogen peroxide was no less than 99.9% effective at both the high and low concentrations of spores tested. Although the 0.5% hydrogen peroxide was not entirely effective in all cases, a solution of 5% hydrogen peroxide was completely effective in killing 100% of the spores at both spore concentrations. A solution of 2% Dichlor also achieved 100% kill at both spore concentrations tested.

Earlier publications are somewhat contradictory regarding reported results using the peroxy-based compounds VirkonS® (4, 5, 20) and Oxone (11, 22). The results reported here show these compounds are not as effective as the other oxidizers tested for use as biocides for the types of spores and at both spore concentrations evaluated for drinking water treatment as compared to the other oxidizers tested. A 1% solution of VirkonS® was effective in killing *B. atrophaeu*s spores at a concentration of 3.0×10^2 spores/ml and *B. thuringiensis* spores at a concentration of 3.6×10^2 spores/ml, as reported by other investigators (5), specifically for such lower spore concentrations. However, a 1% VirkonS® solution was not effective against *B. atrophaeus* spores at our higher concentration of approx 1×10^6 spores/ml, producing in that case only a

39.3% kill, a finding similar to that reported by (5) against spores of *B. Cereus* in accordance with Association Francaise de Normalisation (AFNOR) Guidelines. Solutions of Oxone at the tested chemical concentrations of up to 5% were also not completely effective in killing all spores at the spore concentrations we tested. For example, a 1% solution of Oxone resulted in only a percent kill of 98.6% using *B. atrophaeus* spores at a concentration of 3×10^2/ml; a percent kill of 98.4% using *B. atrophaeus* spores at a concentration of 3×10^6/ml; and a percent kill of only 72.0% using *B. thuringiensis* at a concentration of 3.6×10^2/ml. However, a 5% Oxone concentration resulted in a 99.9% kill using *B. atrophaeus* spores at a concentration of 3×10^6/ml.

Discussion

Spore Type. Both *B. atrophaeus* and *B. thuringiensis* spores are close relatives to, and are frequently reported to behave similarly to, *B. anthracis* spores in tests of chemical decontamination. For example, the sensitivity of the two nonpathogenic simulants used in the present investigation—*B. atrophaeus* and *B. thuringiensis*—to chemical decontaminants when deposited on painted metal, polymeric rubber, or glass surfaces and exposed to peroxide, chlorine, or other oxidants has been reported to be similar to that of *B. anthracis* (15). Although *B. atrophaeus* and *B. thuringiensis* spores have been documented in the literature as behaving quite similarly to *B. anthracis* spores, some differences between them are evident in response to the decontaminants we evaluated. Our results suggest that these two strains may vary to some small extent following exposure in solution to liquid VirkonS®, Oxone, and hydrogen peroxide (calculated as percent kill), with *B. thuringiensis* somewhat more difficult to kill than *B. atrophaeus* spores.

In addition, *B. thuringiensis* spores were more difficult to count, potentially introducing some uncertainty in the assessment of viable CFUs following culture. The literature is somewhat contradictory as to the certainty with which data obtained with simulant nonpathogenic *Bacillus* spp. spores can be extrapolated to virulent spores of *B. anthracis*, with some researchers suggesting that extrapolation of decontamination data may be done safely (15). Given the slight differences we observed between the two spore surrogates evaluated, it is recommended that the same tests as those described here be replicated using *B. anthracis* or *B. sterne* spores to more definitively evaluate the results. Another suggestion in the literature is that spore preparations, including the temperature of the preparations, may account for differences in susceptibility when trying to evaluate decontamination effectiveness with surrogates, and even when *B. anthracis* or *B. sterne* spores are used (21, 22). Differences in resistance to various oxidants have been observed between alpha/beta type spores, wild-type spores, and spores lacking both the alpha/beta-type SASP and the *recA* gene (22). It is therefore possible that the spore preparation or specifics relating to gene expression, or both, influenced the overall results of the present study as well as other results reported in the literature.

Chemical Characteristics. Table 3 summarizes the characteristics of the five chemical decontaminants evaluated. Of the five chemicals tested, 5% hydrogen peroxide, 2% Dichlor, and 2% sodium hypochlorite were found to be 100% effective in all the tests we conducted, regardless of spore exposure time to a chemical decontaminant over the range tested, spore concentration evaluated, or spore species used. From this finding, it is clear that effectiveness of a decontaminant appears to be independent of pH and relies more on other factors, as discussed below. However, the effectiveness of individual chemicals as a function of varying pH was not evaluated as part of this study. Other researchers have shown that pH can be an important

parameter for certain disinfectants (13). By comparison, Oxone and VirkonS® were generally less effective than the other three decontaminants evaluated. Whereas a 1% solution of Oxone achieved a maximum percent kill of 98.4% following exposure to *B. atrophaeus* spores at a concentration of 10^6/ml, a 1% solution of VirkonS® achieved a maximum percent kill of only 39.3%. Such findings support the results published by some researchers and are in disagreement with other reports, reinforcing the ongoing controversy on the topic of decontamination efficacy of those two chemicals, although differences in procedures may also explain some of the discrepancies (4, 5, 11, 20, 22). Regarding the overall effectiveness of hydrogen peroxide, its high oxidation potential (1.78 V) may underlie its success over that of the peroxy compounds, Oxone and VirkonS®. Because the application of hydrogen peroxide results in the formation of water as a byproduct, it is a nearly ideal biocide that would have essentially no adverse effects on humans or the environment from its byproducts. Other researchers have concluded that oxidizing agents, such as hydrogen peroxide and ozone, cause major spore injury to proteins in the inner membrane resulting in the killing or damaging of spores (2).

Sodium hypochlorite and Dichlor at concentrations of 2% both contain free chlorine in abundance, which could provide at least some of the basis for their success in achieving 100% kill for all spore concentrations we evaluated (12). Dichlor, which is commonly used to treat swimming pools, with its relatively neutral pH, should be carefully considered as a relatively new decontaminant chemical for *B. anthracis* spore inactivation in water. This compound has the highest value of available free chlorine (56%) of all the decontaminants we tested, as well as a high oxidation potential (1.34 V), second only to hydrogen peroxide, the combination of which may also contribute to its efficacy. To evaluate the specific relationship of free chlorine to spore inactivation and the potential for use in a water treatment system, further testing would be

necessary. Ours is the first apparent evaluation of this chemical as a potential decontaminant for *B. anthracis* contamination, and the results reported here clearly demonstrate its effectiveness.

Chemical Concentration. It appears that the concentration of chemical reagent is a key parameter in its ability to inactivate spores. More importantly, lower concentrations of some chemical decontaminants than have been previously evaluated in the literature were found to be effective in the current work. In addition, during preliminary experiments conducted to test *B. anthracis* surrogates prepared at a concentration of 3,000 spores/ml, then diluted and treated at the level of approx 300 spores/ml, we observed no spore growth with either hydrogen peroxide or sodium hypochlorite at concentrations of 0.05% and 0.2%, respectively (see Table 2 for further details on experimental results). Previous evaluations of commercial liquid disinfectant products also suggest that they would be more effective in solution, rather than on contaminated surfaces, than is generally acknowledged (14), and that the use of suspension test protocols for this purpose also makes it difficult to extrapolate findings to the inactivation of the spores on environmental surfaces. As stated previously, wide variations in test protocols make meaningful comparisons between other studies virtually impossible (5, 7, 13).

The procedures and materials used in our experiments differed in several ways compared with many reported in previous studies of spore inactivation. Some of those differences may account for the unexpected success reported here of the relatively low concentrations for at least three of the chemical decontaminants we tested. The most significant of the differences appears to be the practice of multiple washings with water and/or phosphate-buffered saline solutions and, in some cases, additional centrifugation of spores before plating and germination (6, 8, 22).

It is difficult to understand the nature of this effect, in that there is no consensus in the literature regarding the actual mechanism of spore killing with respect to the use of various oxidizers.

Previous studies have shown that the killing of spores by oxidants is not through damage to the spore DNA, nor through oxidation of unsaturated fatty acids. Also, the role of specific spore coat and inner membrane proteins in resisting various decontaminants or oxidizers is not known but speculated to be important (2, 22). We hypothesize that if an oxidation reaction were occurring before washing, and a specific amount of chemical reagent were adsorbed or bound to the spore coat proteins, a reaction could continue throughout the experimental procedure, finally reaching and permeating the inner membrane barrier even though the solution may be neutralized. Multiple washings however could remove more, or perhaps all, of the chemical from the exterior of spores, possibly requiring a greater starting concentration of chemical for inactivation than would be otherwise necessary. Because spore washing would not be part of an actual cleanup process, experiments conducted in the absence of washing may be more representative of actual conditions expected during cleanup.

Spore Concentration. Our results show that spore concentration greatly affects the amount of chemical reagent necessary to ensure 100% kill. All decontaminants at the highest concentrations evaluated, with the exception of Oxone (98.6% kill at 1% concentration), resulted in a 100% kill at the lower of two spore concentrations (3×10^2/ml) evaluated against *B. atrophaeus*. In contrast, our results for the greater spore concentration of *B. atrophaeu*s (1×10^6/ml) showed a 100% kill only for three of the five decontaminants tested, namely hydrogen peroxide (5%), sodium hypochlorite (2%), and Dichlor (2%).

The EPA requires that a biocide demonstrate a 6 log kill to meet Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) environmental regulations and to be registered as a pesticide. However, if a *B. anthracis* release resulted in contamination of a water system, the resulting concentration would probably not be as great as 1 million spores/ml, especially after dilution

from the point of discharge. Furthermore, it is not clear from the literature exactly how many spores are necessary to cause gastrointestinal anthrax. The success of the sporicides we tested at the relatively low chemical reagent concentrations and low spore concentrations evaluated suggests that the current standard of a 6 log kill may be unrealistically high for drinking water. If it were deemed unlikely that a contaminated water system would have such a large number of spores, owing principally to the effects of dilution, and the EPA agreed to lower its standard for biocide application in water, then many more chemical treatment options might be considered as effective biocides for water treatment.

Spore Exposure Time. In general, our data show that exposure times greater than 10 min have little effect on the success of a chemical decontaminant in killing spores. However, as shown in Figure 4, increased exposure time did provide some improved effect for hydrogen peroxide at 0.05% and for sodium hypochlorite at 0.2% when applied to the higher concentration (1×10^6/ml) of *B. atrophaeus* spores. It is possible that there is a marginal, time-dependent relationship when more spores are present, compared to lower concentrations of spores. Most tests in the literature report results after 30 min of exposure, although one study (7) showed that bleach was effective to achieve a 6-log kill at 10 minutes, which is similar to our results. In addition, a time-dependent effect for Oxone and other peroxy compounds has been reported (22).

Our test results for 0.05% hydrogen peroxide for up to 1 hr of exposure suggest that such exposure time was not sufficient to kill all of the spores at this concentration. We suggest that it is likely, from our limited results for this reagent, that hydrogen peroxide at a concentration of 0.05% might attain a 100% kill if given 24-hr exposure to a 1×10^6/ml concentration of spores. Additional tests would need to be done to support any such hypothesis.

Conclusions

In terms of designing an effective method for water treatment following contamination by *B. anthracis* spores, our data, together with that of others (12) suggest that a successful, efficient, and relatively safe biocide would have the following properties: relatively neutral pH, high oxidation potential, an excess of free chlorine, and the ability to form nontoxic byproducts, perhaps aided by UV radiation. Such a product or approach could be used in municipal water treatment systems and has potential for point-of-use application in homes.

There is currently no standard approach for decontaminating drinking water supplies contaminated with *B. anthracis* spores. Given that typical water treatment systems already chlorinate drinking water, it may be possible to simply increase the amount of free chlorine to some concentration deemed appropriate in the event of a contamination incident. Results of this study and others suggest that increasing the amount of chlorine (and available free chlorine), perhaps by a factor of 10, might be an effective treatment method (12, 18).

We found that VirkonS® and Oxone were not as effective as other options evaluated. In general, both were successful at the higher chemical concentrations (5%) tested when used to treat lower concentrations of spores. However, VirkonS® and Oxone were not as effective when used with the *B. atrophaeus* preparations at the higher spore concentration of 1×10^6/ml. Therefore, these two decontaminants are not recommended for use as biocides at the higher spore concentrations, which were assessed in our research.

Our data suggests that both 5% hydrogen peroxide and 2% Dichlor would be effective and efficient decontaminants for use in a water treatment system. The effectiveness of hydrogen peroxide is likely a result of its high oxidation potential relative to that of the other chemicals

evaluated. In a water-treatment application, hydrogen peroxide would first oxidize the spore coating and then form water as a byproduct, making its use a nearly ideal biocide in that its byproduct would have no adverse effects on the environment or human health. In addition, with a pH in the range 4.0 to 5.0, hydrogen peroxide is less corrosive than NaClO, although bleach at 2% was also effective.

The most promising, newly identified option for decontamination of water would be the use of Dichlor, which has a high oxidation potential and contains large amounts of free chlorine. Our data suggest that Dichlor should be evaluated as an excellent, new option. This is an important conclusion because no other evaluations of this chemical were found in the current literature. Additional advantages of Dichlor derive from its availability and relatively common use in swimming pool decontamination and its relatively neutral pH (in the range of 4.5 to 5.3).

Future studies should repeat and verify the work presented here, taking note of the lower chemical concentrations assessed and their ability to effectively oxidize surrogates of *B. anthracis* spores in water. In addition, the amount of time in excess of 10 minutes that spores were exposed to a given chemical did not, in most cases, affect our spore viability results. Such a finding has important implications when applying viability data to a water treatment method because it suggests that treatment can occur on a relatively fast timescale and have minimal impacts on operations. Additional studies should include a more detailed evaluation of actual water treatment design parameters to optimize an overall decontamination process.

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Figure legends

Figure 1. Percent kill for cultured *B. atrophaeus* spores derived from a starting suspension of 1×10^6 spores/ml after 10 minutes of exposure to each of the five decontaminants evaluated at three different concentrations.

Figure 2. Percent kill for cultured *B. atrophaeus* spores derived from a starting suspension of 3×10^2 spores/ml after 10 minutes of exposure to each of the five decontaminants evaluated at three different concentrations.

Figure 3. Percent kill for cultured *B. thuringiensis* spores derived from a starting suspension of 3.6×10^2 spores/ml after 10 minutes of exposure to each of the five decontaminants evaluated at three different concentrations.

Figure 4. CFUs remaining for cultured *B. atrophaeus* spores derived from a starting suspension of 1×10^6 spores/ml after 10, 30, or 60 minutes of exposure to each of the five decontaminants evaluated and at the one concentration specified. All data represent the average of duplicate tests.

Table 1 footnote

[a]The data represented in the table is based off of an average control group count of 11059 ±1006 CFUs.

[b]The data represented in Table 1 is based off of an average control group count of 141 ± 7 CFUs

Table 2 footnote

[a]The value for NaClO at 0.02% concentration is approximate because of potential experimental error.

Table 2 footnote

[a]The value for NaClO at 0.02% concentration is approximate because of potential experimental error.

Table 3 footnote

[a]pH range measured for concentrations used.

Tables

TABLE 1. Counts of *B. atrophaeus* CFUs surviving after varying chemical exposure times. Results are the mean of duplicate tests (± population standard deviation). Control blanks showed no growth.

Spore concentration	Decontaminant tested	Decontaminant concentration (%)	Spore-exposure time to decontaminant				
			10 min	30 min	1 hr	6 hr	24 hr
1×10^6/ml[a]	VirkonS®	0.1	8545 (±503)	8219 (±679)	8269 (±227)	—	—
1×10^6/ml[a]	Oxone	0.1	6716 (±171)	7389 (±352)	5755 (±980)	—	—
1×10^6/ml[a]	Dichlor	0.2	5 (±3)	0	4 (±4)	—	—
3×10^2/ml[b]	H_2O_2	5	0	0	0	0	0
3×10^2/ml[b]	H_2O_2	10	—	0	0	0	0
3×10^2/ml[b]	H_2O_2	27	—	0	0	0	0
1×10^6/ml[a]	H_2O_2	0.05	210 (±59)	32 (±3)	7 (±1)	—	—
3×10^2/ml[b]	NaClO	2	0	0	0	0	0
3×10^2/ml[b]	NaClO	4	—	0	0	0	0
3×10^2/ml[b]	NaClO	6	—	0	0	0	0
1×10^6/ml[a]	NaClO	0.2	3 (±3)	0	0	—	—

TABLE 2. Percent kill of *B. atrophaeus* or *B. thuringiensis* spores following 10-min exposure to each of the five decontaminants tested. Results are the mean of duplicate tests.

Spore type	Spore concentration	Decontaminant concentration (%)	Percent kill for each decontaminant				
			VirkonS®	Oxone	H_2O_2	NaClO	Dichlor
B. atrophaeus	3×10^2/ml	0.01	79.4	77.3	—	—	—
		0.02	—	—	—	92.9	99.3
		0.05	—	—	100	—	—
		0.1	82.3	83.0	—	—	—
		0.2	—	—	—	100	100
		0.5	—	—	100	—	—
		1	100	98.6	—	—	—
		2	—	—	—	100	100
		5	—	—	100	—	—
B. atrophaeus	1×10^6/ml	0.01	18.0	14.50	—	—	—
		0.02	—	—	—	48.4[a]	52.0
		0.05	—	—	98.1	—	—
		0.1	22.7	39.30	—	—	—
		0.2	—	—	—	99.9	99.9
		0.5	—	—	99.9	—	—
		1	39.3	98.4	—	—	—
		2	—	—	—	100	100
		5	82.6	99.9	100	—	—
B. thuringiensis	3.6×10^2/ml	0.01	47.7	42.7	—	—	—
		0.02	—	—	—	100	99.3
		0.05	—	—	97.1	—	—
		0.1	58.1	52.3	—	—	—
		0.2	—	—	—	100	100
		0.5	—	—	100	—	—
		1	100	72.0	—	—	—
		2	—	—	—	100	100
		5	—	—	100	—	—

[a]The value for NaClO at 0.02% concentration is approximate because of potential experimental error.

1 TABLE 3. Chemical oxidizers evaluated.

Chemical	Chemical formula	Measured pH range[a]	Available chlorine (%)	Oxidation potential (V)	Environmental parameters
Hydrogen peroxide	H_2O_2	Acidic 4.0 to 5.0	0	1.78	Acidic (depends on concentration used); degradable to nontoxic byproducts
Sodium hypochlorite	NaClO	Basic 8.5 to 12+	12	0.90	Caustic; degradable to nontoxic byproducts
Sodium dicholor-s-triazinetrione dihydrate (Diclor)	$C_3HCl_2N_3O$	Acidic 4.5 to 5.3	56	1.34	More neutral than all other decontaminants tested; degradable to nontoxic byproducts
VirkonS® (potassium peroxymonosulfate: 40 to 60%; sodium dodecylbenzene-sulfphonate: 10 to 20%; sulfamic acid: 1 to 10%)	K_2SO_5 $C_{18}H_{29}NaO_3S$ H_2NSO_3H	Acidic 1.0 to 3.7	0	Unknown	Acidic (depends on concentration used); degradable to nontoxic byproducts
Potassium peroxymonosulfate	K_2SO_5	Acidic 1.0 to 4.0	0	Unknown	Acidic (depends on concentration used); degradable to nontoxic byproducts

2 [a]pH range measured for concentrations used.

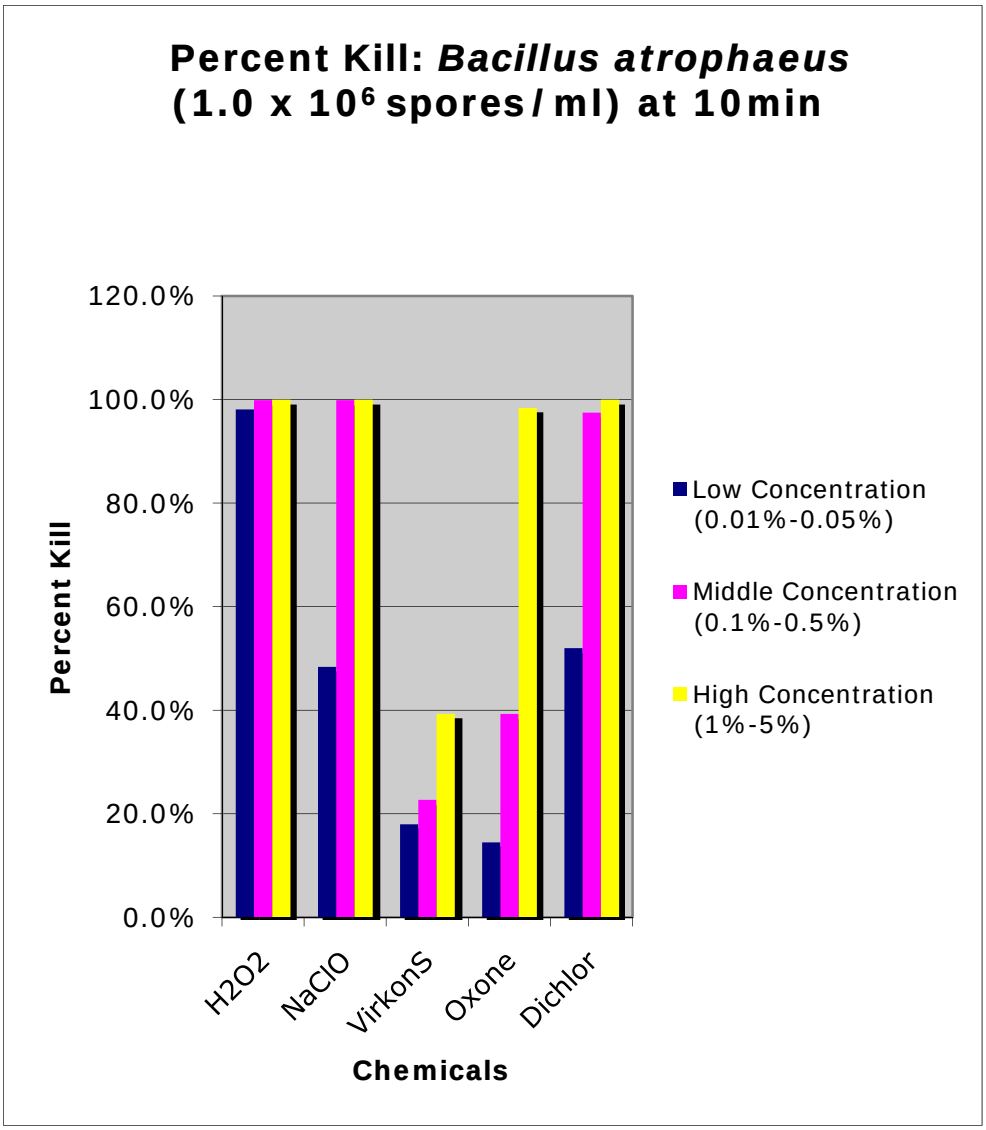

Percent Kill: Bacillus atrophaeus
(1.0 x 10⁶ spores/ml) at 10min
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Percent Kill
H2O2
NaClO
VirkonS
Oxone
Dichlor
Chemicals
Low Concentration (0.01%-0.05%)
Middle Concentration (0.1%-0.5%)
High Concentration (1%-5%)

Figure 1.

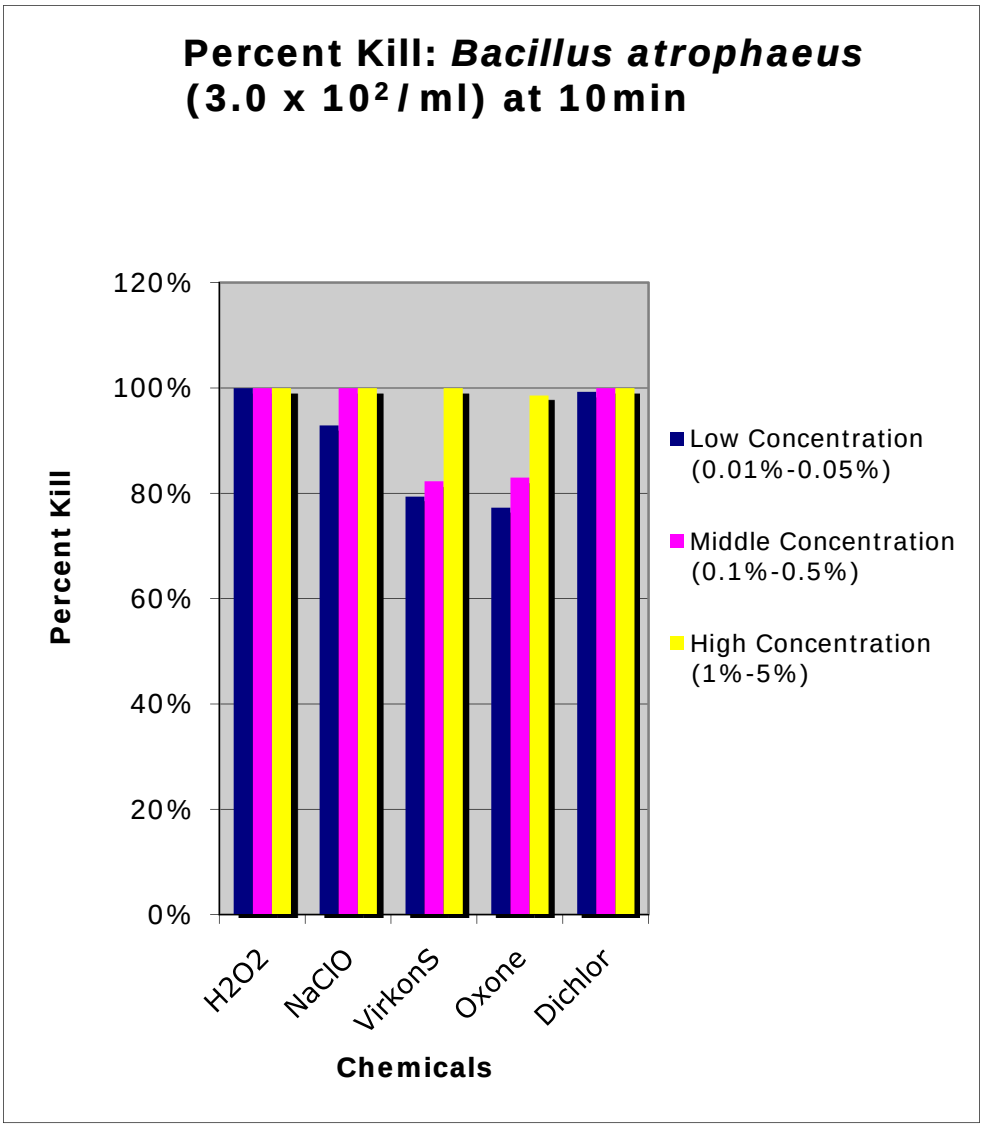

Percent Kill: Bacillus atrophaeus
(3.0 x 10²/ml) at 10min
120%
100%
80%
60%
40%
20%
0%
Percent Kill
H2O2
NaClO
VirkonS
Oxone
Dichlor
Chemicals
Low Concentration
(0.01%-0.05%)
Middle Concentration
(0.1%-0.5%)
High Concentration
(1%-5%)

Figure 2.

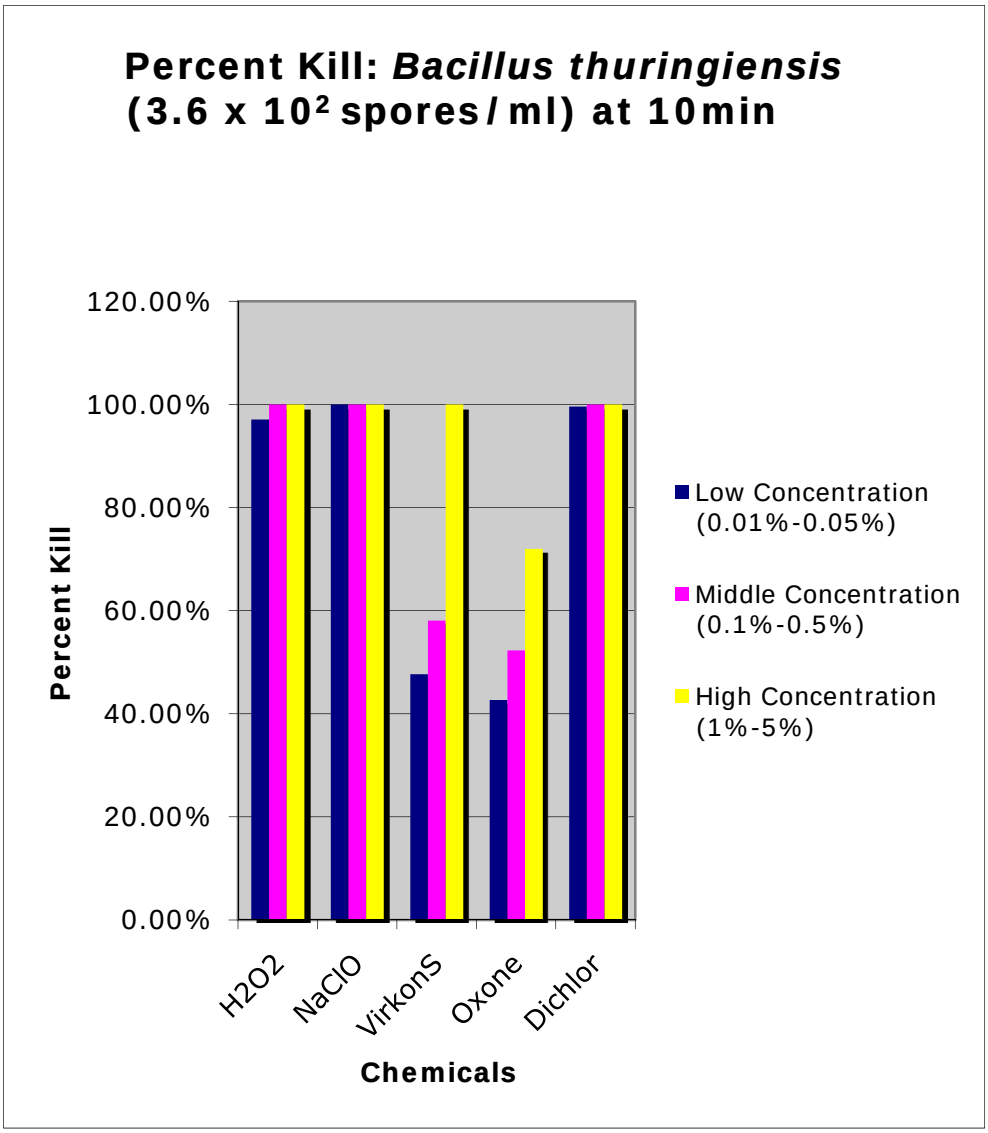
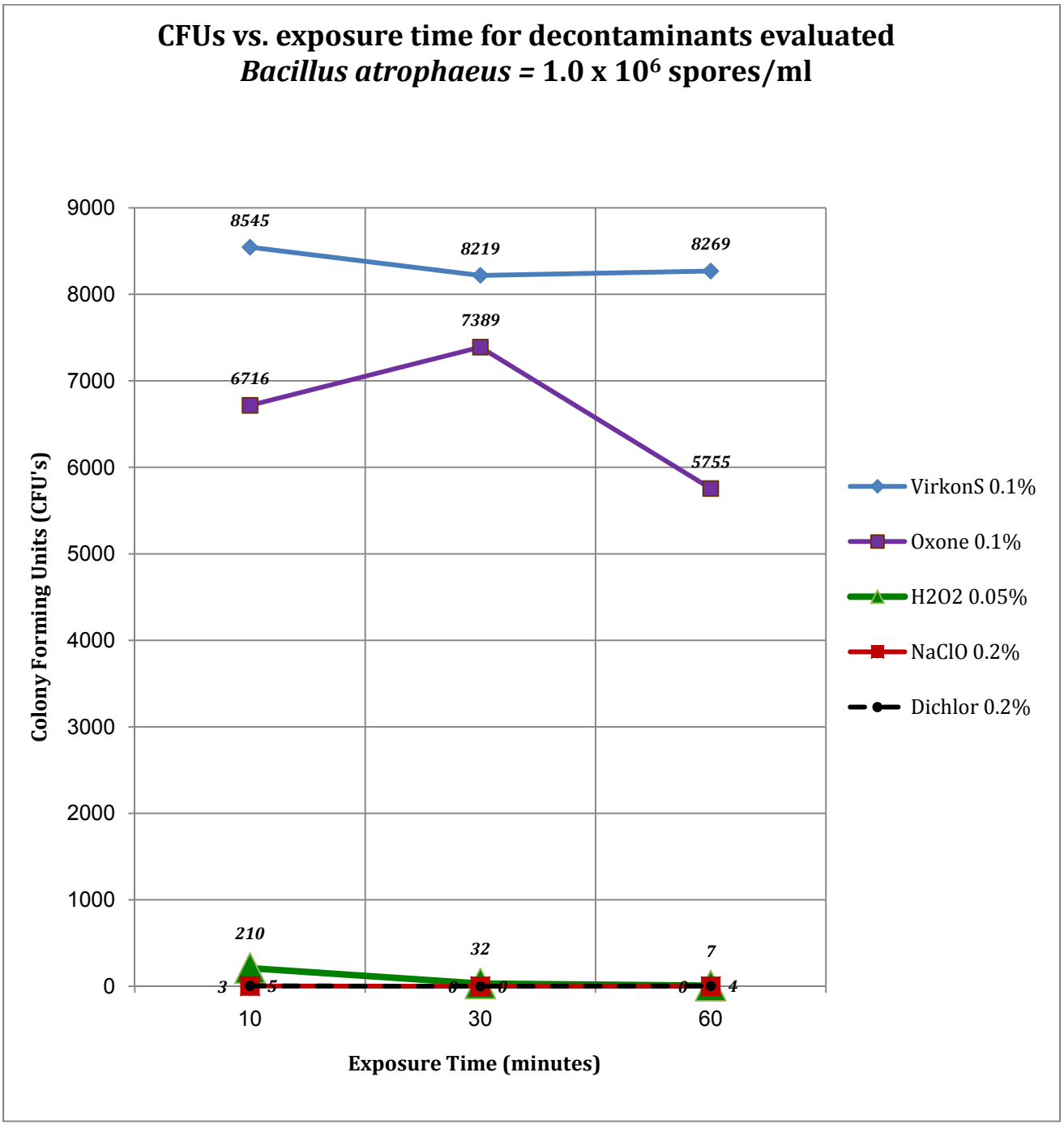

Percent Kill: Bacillus thuringiensis
(3.6 x 10² spores/ml) at 10min
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Percent Kill
H2O2
NaClO
VirkonS
Oxone
Dichlor
Chemicals
Low Concentration (0.01%-0.05%)
Middle Concentration (0.1%-0.5%)
High Concentration (1%-5%)

Figure 3.

1


CFUs vs. exposure time for decontaminants evaluated
Bacillus atrophaeus = 1.0 x 10⁶ spores/ml
Colony Forming Units (CFU's)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
8545
8219
8269
7389
6716
5755
210
32
7
3
5
0
0
4
10
30
60
Exposure Time (minutes)
VirkonS 0.1%
Oxone 0.1%
H2O2 0.05%
NaClO 0.2%
Dichlor 0.2%

2

3 Figure 4.