

# Large-scale Inactivation of *Bacillus anthracis* Ames, Vollum, and Sterne Spores Using Vaporous Hydrogen Peroxide

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## Abstract

*This study evaluated the inactivation of Bacillus anthracis Ames, Vollum, and Sterne spores on various materials (glass, Hypalon® rubber glove, and stainless steel) using vaporous hydrogen peroxide fumigation of a ~15 m<sup>3</sup> aerosol research and component assessment (ARCA) chamber. Suspensions of each spore type (~1 x 10<sup>8</sup> CFU) were dried on coupons made from each type of test surface and exposed to vaporous hydrogen peroxide fumigation for a decontamination time of 5.5 hours. For these three materials, the log reductions ranged from 7.7 to 7.9, 8.2 to 8.5, and 7.6 to 7.8 for B. anthracis Ames, Vollum, or Sterne spores, respectively. The effectiveness of vaporous hydrogen peroxide fumigation on the growth of Geobacillus stearothermophilus biological indicators (BI) was evaluated in parallel as a qualitative assessment of decontamination. At 1 and 7 days post-exposure, all decontaminated BI exhibited no growth. This study provides information for using vaporous hydrogen peroxide fumigation as an approach for the surface decontamination of B. anthracis spores within a large-scale chamber.*

## Introduction

Fumigants (e.g., gases or vapors) for decontaminating *Bacillus anthracis* spores have been utilized for decades to treat animal hides and skins and to mitigate the health problems associated with the handling of contaminated wool and hair (Rogers et al., 2008a). Many of these fumigants have included gaseous or vaporous forms of ammonia, bromine, carbon tetrachloride, chlorine, chlorine dioxide, ethylene oxide, formaldehyde, hydrochloric acid, hydrogen peroxide, iodine, methyl bromide, nitrogen dioxide, ozone, phosgene, sulfur dioxide, and trichloroethylene (Rogers et al., 2008a). Fumigants offer advantages over liquids, sprays, and wipes as they can be easily dispersed within an extensive volume (e.g., rooms or buildings) and provide coverage over the corresponding large surface areas. More recently, some of these fumigants have been tested and implemented following the release of *B. anthracis* spores in the mail in 2001. Specifically, hydrogen peroxide, chlorine dioxide, and formaldehyde were used to decontaminate five mail facilities and two office buildings (Canter, 2005; Canter et al., 2005).

The use of vaporous hydrogen peroxide is a common approach for decontamination and disinfection in the scientific, medical, and pharmaceutical fields. Hydrogen peroxide is considered less toxic to humans than other fumigants (e.g., formaldehyde, chlorine dioxide); therefore, it has been widely used to treat laboratory and medical equipment, pharmaceutical facilities, hospital rooms, ambulances, animal holding rooms, and air ducts (Anderson et al., 2006; Dryden et al., 2008; Fichet et al., 2004; French et al., 2004; Heckert et al., 1997; Hillman, 2004; Johnston et al., 2005; Klapes & Vesley, 1990; Krause et al., 2001; ; Krishna et al., 2000; Verce et al., 2008; Wagenaar & Snijders, 2004). The use of hydrogen peroxide as a fumigant, dry mist, or an aqueous solution promotes decontamination efficacy against a wide range of microorganisms, including bacterial spores, vegetative bacteria, fungi, viruses, and bacteriophages (French et al., 2004; Grare et al., 2008; Hall et al., 2007; Hall et al., 2008; Heckert et al., 1997; Hillman, 2004; Johnston et al., 2005; Klapes & Vesley, 1990; Melley et al., 2002; Otter & Dudde-Niekie, 2009; Rastogi et al., 2009; Rogers et al., 2005; Rogers et al., 2008b; Rogers & Choi, 2008).

For laboratory testing of fumigant decontamination efficacy against virulent *B. anthracis* spores, studies have been done at the bench scale using small-sized chambers of approximately 300 L (Rogers et al., 2005; Rogers et al., 2007; Rogers et al., 2008a). However, as research keeps moving forward into the testing of fumigants against *B. anthracis* spores and other biological select agents, the need to move towards larger-scale testing is growing. Recently, our facility has acquired a custom-made biosafety level 3 (BSL-3)-capable aerosol research and component assessment (ARCA) chamber that is designed to generate aerosols of biological agents to enable testing of sensors, detectors, and decontaminants, or to enable the ability to perform other large-scale biological agent studies. The ARCA chamber has a total internal working volume of approximately 520 ft<sup>3</sup> (~14.7 m<sup>3</sup>) and working surface area of approximately 58.6 m<sup>2</sup>. During operation, the airflow through the ARCA is unidirectional with complete exhaust from the chamber. As the ARCA chamber is large enough for multiple people to enter into the main chamber, it is crucial that successful decontamination is performed and demonstrated to maximize safety, prevent the risk of any potential secondary aerosolization and exposure, and enable safe removal of any test equipment or devices.

Therefore, the purpose of this study was to demonstrate the ability to decontaminate multiple strains of *B. anthracis* spores on surfaces within the ARCA chamber.

## Materials and Methods

### Test Organisms

All portions of testing were performed under BSL-3 conditions. Spores of the virulent *B. anthracis* Ames strain were prepared by fermentation as previously described (Rogers et al., 2005). Virulent *B. anthracis* Vollum and avirulent *B. anthracis* Sterne spores were prepared using a shaker flask method as previously described (Rogers et al., 2006). The purified spore preparations were resuspended in sterile water and evaluated by phase-contrast microscopy. Preparations having >95% refractile spores with <5% cellular debris were enumerated, diluted to approximately  $1 \times 10^9$  colony-forming units (CFU)/ml, and stored at 2°-8°C.

### Test Materials

Three materials commonly found in the ARCA chamber (The Baker Company, Sanford, ME) were used as the test surfaces and included glass, Hypalon® glove, and stainless steel. The glass coupons were plain microscope slides (Fisher HealthCare, Houston, TX), while Hypalon® coupons were cut to approximately 2.0 cm x 2.0 cm from a Hypalon® glove. The stainless steel coupons were approximately 1.5 cm x 6.5 cm. All coupons were sterilized by autoclaving at 121°C for 20 minutes prior to testing. Visual assessment of the coupons used for the study was performed to ensure that no physical changes or defects such as warping or discoloration occurred as a result of the autoclaving. A visual inspection of the physical integrity and appearance of the test material coupons was also performed before and after decontamination to detect any damage to the test materials.

### Decontamination Procedure

Each test coupon was laid flat in a Biological Safety Cabinet (BSC) Class III and inoculated with approximately  $1.0 \times 10^8$  CFU by dispensing ten, 10  $\mu$ L droplets across each coupon surface as previously described (Rogers et al., 2005). For each type of test material, three coupons were used for decontamination, three coupons were used as controls (inoculated; not decontaminated), and two coupons were used as blanks (not inoculated). Following inoculation, the coupons were allowed to dry overnight, undisturbed. The next day, the inoculated coupons intended for decontamination (and one blank) were transferred into the ARCA chamber. Due to the size and complexity of the ARCA chamber, one of each coupon type (and blanks) were placed in the upstream, middle, and downstream sections (Figure 1) to demonstrate decontamination efficacy throughout the chamber. Four 12-inch fans were placed inside the ARCA chamber at various locations to provide turbulence that

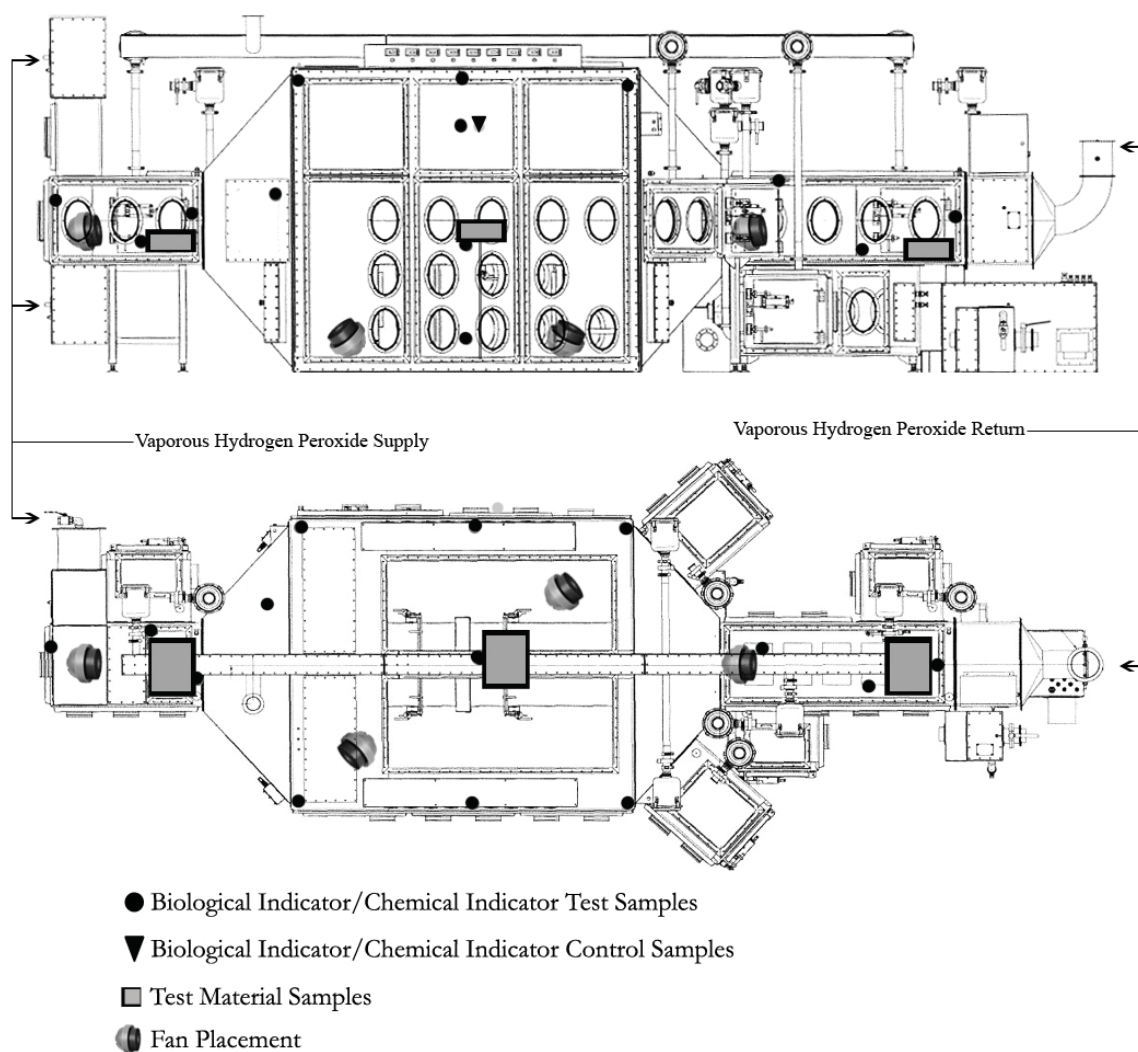
would help maximize vaporous hydrogen peroxide distribution (Figure 1). Control coupons (and blanks) were transferred to a Plas Labs Model 830-ABC Compact Glove Box (Plas Labs, Inc., Lansing, MI), and the coupons were placed lying flat, inoculated surface side up on a wire rack.

In parallel, Steris Sporedex® single species biological indicators (BI) containing  $4 \times 10^5$  *Geobacillus stearothermophilus* (ATCC 7593) spores (STERIS Corporation, Mentor, OH) were used to evaluate decontamination. Sixteen BI were placed at separate locations within the ARCA chamber and subjected to decontamination; a single BI affixed to the outside of the ARCA was not decontaminated and used as a positive control (Figure 1). Additionally, VHP Chemical Indicator NB305 strips (STERIS Corporation) were used to provide a visual indication (color change) that fumigation of the ARCA chamber had occurred. Sixteen indicator strips were placed at separate locations within the ARCA chamber during the decontamination run, while a single indicator strip was placed with the non-decontaminated control BI (Figure 1). Following the decontamination run, all indicator strips were visually inspected for a color change.

For decontamination, a VHP® 1000ED Biodecontamination System (STERIS Corporation) was connected to the ARCA chamber in which all airflow through the ARCA chamber had been shut off. The vaporous hydrogen peroxide supply was connected to one end of the ARCA chamber and the vaporous hydrogen peroxide return was connected to the opposite end of the ARCA chamber (Figure 1). The decontamination process consisted of dehumidification, conditioning, decontamination, and aeration phases, which were controlled by the hydrogen peroxide generator. Due to the size and complexity of the ARCA chamber, the established cycle parameters were based on the maximum amount of hydrogen peroxide (1,950 g) that could be utilized in a single run using the VHP® 1000ED. The specifics of each phase are provided in Table 1. During the decontamination run, the hydrogen peroxide concentration, relative humidity, and temperature were monitored in real-time using data loggers set to a data point capture frequency of 1 minute. The hydrogen peroxide was monitored using an ATI Series B12 two-wire gas transmitter connected to a 0-1000 ppm remote hydrogen peroxide sensor (Analytical Technology, Inc., Collegeville, PA). The sensor was suspended approximately 1 m from the middle of the ARCA main chamber ceiling. The temperature and relative humidity were monitored in real-time using a HOBO U12-006 data logger (Onset Computer Corporation, Bourne, MA) that was placed in the middle of the ARCA main chamber. A Yokogawa DX2010 (Yokogawa Electric Corporation, Tokyo) connected to an Omega HX93AC temperature/relative humidity probe (Omega Engineering Inc., Stamford, CT) was used to monitor temperature and relative humidity in real-time in the control chamber.

**Figure 1**

Experimental set-up of the ARCA chamber. (A) Side view. (B) Top view.

**Table 1**

Decontamination Parameters for the ARCA Chamber

Phase	Parameters
Dehumidification	Duration: 10 min Flow Rate: 20 CFM <sup>a</sup> Relative Humidity: ≤6.9 mg/L
Conditioning	Duration: 20 min Flow Rate: 20 CFM Injection Rate: 6.5 g/min
Decontamination	Duration: 5.5 hours Flow Rate: 20 CFM Injection Rate: 5.0 g/min
Aeration	Duration: 30 min Flow Rate: 20 CFM
Additional Aeration	Duration: 4 hours Flow Rate: 20 CFM

<sup>a</sup>CFM = cubic feet per minute

## Sample Processing and Analysis

Decontamination efficacy of *B. anthracis* spores was quantified by measuring the viable spores extracted from both decontaminated and control coupons as previously described (Rogers et al., 2005; Rogers et al., 2007). Briefly, after the decontamination run, test and control coupons were placed in a 50 ml conical tube containing 10 ml of sterile phosphate-buffered saline and agitated at 200 rpm on an orbital shaker for 15 minutes at room temperature. A 1.0 ml aliquot of each sample was removed, serially diluted 10-fold in sterile water, plated onto tryptic soy agar plates (Hardy Diagnostics, Santa Maria, CA) in triplicate, and incubated overnight at 37°C. Following an 18-24 hour incubation, plates were enumerated and CFU/ml determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data were expressed as the mean  $\pm$  standard deviation (SD) of observed CFU. Decontamination efficacy was determined by calculating the log reduction in viable spores.

Following the decontamination run, BIs were placed into 15 ml conical tubes containing 10 ml of tryptic soy broth (Remel, Lenexa, KS), gently agitated, and incubated at 55°-60°C. As the extraction process does not typically yield 100% recovery of spores, all material coupons were also placed in tryptic soy broth (TSB) and cultured to determine whether any viable spores remained on the coupons. All cultures were visually inspected for growth (cloudy culture) or no growth (clear culture) at 1 and 7 days post-decontamination as previously described (Rogers et al., 2005; Rogers et al., 2007). Streak plates were made of all positive cultures to verify the growing organism.

## Results

### Decontamination Run

During the conditioning phase, the hydrogen peroxide concentration peaked to approximately 460 ppm and decreased to a level of approximately 270 ppm during the decontamination phase. Figure 2 provides a representative hydrogen peroxide curve during an entire decontamination run. The relative humidity profile followed a curve similar to the hydrogen peroxide, peaking at approximately 95% and decreasing to a level of approximately 86% during the decontamination phase (Figure 3). The mean ( $\pm$ SD) temperature in the ARCA chamber during the decontamination run was 21.7°  $\pm$  0.3°C (Figure 3). In the control chamber, the mean ( $\pm$ SD) temperature and relative humidity were 24.2°  $\pm$  0.4°C and 46.2°  $\pm$  1.4%, respectively.

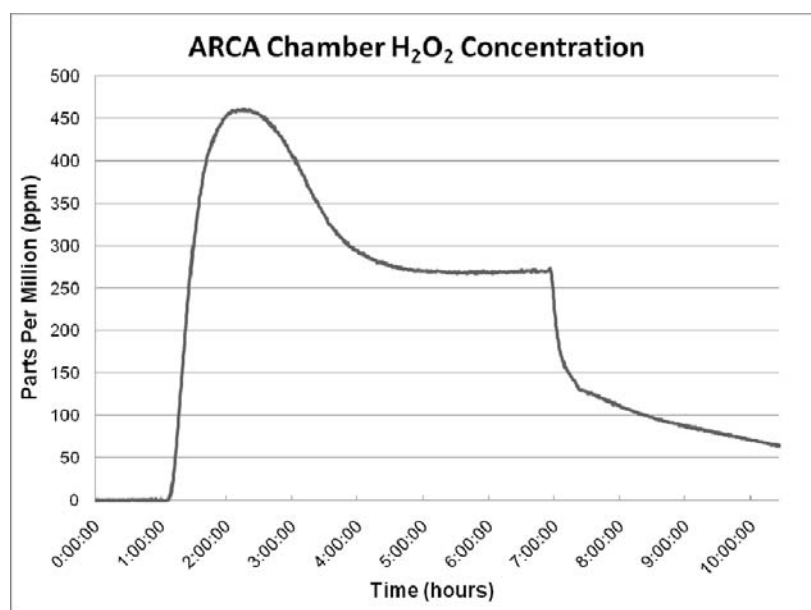
Following decontamination, all chemical indicator strips present in the ARCA chamber during the decontamination run changed color, indicating exposure to the vaporous hydrogen peroxide. The control chemical indicator strips did not change color. Upon visual inspection, no physical damage was observed for the hydrogen peroxide-exposed coupons.

### Decontamination Efficacy

Exposure of test coupons contaminated with *B. anthracis* Ames, Vollum, or Sterne spores to vaporous hydrogen peroxide resulted in the inactivation of observable viable spores on all glass, Hypalon®, and stainless steel materials. For these three materials, the log reductions calculated from the number of viable spores recovered from each coupon inoculated with approximately

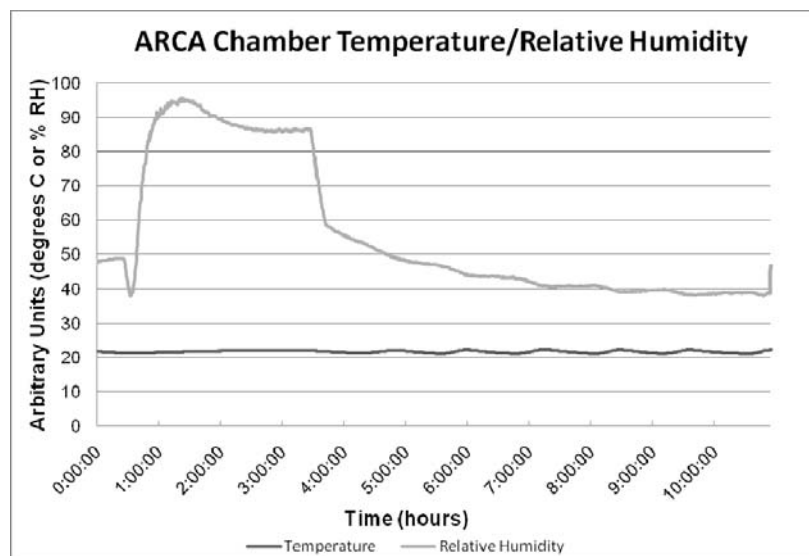
**Figure 2**

Real-time profile of hydrogen peroxide concentration in the ARCA chamber.



**Figure 3**

Real-time profiles of temperature and relative humidity in the ARCA chamber.



$1 \times 10^8$  CFU in 10 droplets ranged from 7.7 to 7.9, 8.2 to 8.5, and 7.6 to 7.8 for *B. anthracis* Ames, Vollum, or Sterne spores, respectively (Table 2). No viable organisms were detected from any of the blank samples.

All decontaminated material coupons cultured in TSB exhibited no growth at 7 days, while the control coupons were positive for growth at 1 day (Table 3). For all BI exposed to the vaporous hydrogen peroxide, no growth was observed as determined by the lack of visibly cloudy liquid cultures through 7 days post-exposure, while the unexposed control BI exhibited growth as determined by the presence of visibly cloudy cultures (Table 3). Streak plates of the positive cultures for the coupons and BI exhibited homogenous growth of *B. anthracis* and *G. stearothermophilus*, respectively. All blank coupons exhibited no growth through 7 days.

## Discussion

Virulent *B. anthracis* spores have been decontaminated on a large scale to include buildings and facilities contaminated as a result of their intentional release in the mail in 2001 and at a textile mill that processed goat hair (Canter et al., 2005; Young et al., 1970). To our knowledge, the present study is the first laboratory-controlled, large-scale decontamination test using vaporous hydrogen peroxide against *B. anthracis* spores. The results of this study show that *B. anthracis* Ames, Vollum, or Sterne spores were inactivated on glass, Hypalon® rubber glove, and stainless steel surfaces after decontamination with vaporous hydrogen peroxide. The complete inactivation of these spore types is supported by the results in which no growth was observed from any of the material coupons during the quantitative counts of

spores from the coupon extracts, and all liquid cultures of each decontaminated coupon were negative for growth. These results suggest that the decontamination parameters outlined in this study were effective in inactivating three strains of *B. anthracis* spores in a 520 ft<sup>3</sup> (~14.7 m<sup>3</sup>) ARCA chamber using vaporous hydrogen peroxide.

The relationship between the response of virulent *B. anthracis* and surrogates deposited on various surfaces and treated with different types of decontaminants is not well-characterized. Although many studies regarding the decontamination of *Bacillus* spores have been published, most of the available data has been obtained from the use of *B. anthracis* surrogates such as *B. atrophaeus*, *B. subtilis*, *B. anthracis* Sterne, *B. anthracis* NNR1Δ1, and *G. stearothermophilus* (Rastogi et al., 2009; Rogers et al., 2008a; Spotts Whitney et al., 2003). With respect to fumigants, a couple of studies have directly compared the decontamination efficacy of *B. anthracis* and surrogate spores on porous and non-porous surfaces using vaporous hydrogen peroxide and formaldehyde (Rogers et al., 2005; Rogers et al., 2007). Comparative data have also been presented in studies evaluating the use of liquid decontaminants against *B. anthracis* and surrogate spores on non-porous materials (Majcher et al., 2008; Sagripanti et al., 2007). Although these studies provide useful comparative data on many surfaces with multiple types of decontaminants, the lack of data using virulent *B. anthracis* spores has increased the attention to filling data gaps by directly comparing biological agents and their surrogates. Therefore, in this study we chose to assess the inactivation of both virulent and avirulent *B. anthracis* spores in parallel as a means to have direct comparative efficacy data obtained from identical decontamination conditions in a large-scale laboratory test.

**Table 2**

Mean ( $\pm$  SD) Total Viable Colony-forming Units (CFU) and Mean Log Reduction of *B. anthracis* Ames, Vollum, and Sterne Spores Exposed to Vaporous Hydrogen Peroxide. Each coupon was inoculated with  $1 \times 10^8$  CFU in 10 droplets.

Spores/Test Material	Total Viable CFU Recovered (Mean $\pm$ SD)	Mean Log Reduction
<b><i>B. anthracis</i> Ames</b>		
Glass		
Unexposed Control	$4.65 \pm 1.24 \times 10^7$	- <sup>a</sup>
Decontaminated	0	$\geq 7.7$
Hypalon® Rubber Glove		
Unexposed Control	$7.47 \pm 0.78 \times 10^7$	-
Decontaminated	0	$\geq 7.9$
Stainless Steel		
Unexposed Control	$8.64 \pm 2.64 \times 10^7$	-
Decontaminated	0	$\geq 7.9$
<b><i>B. anthracis</i> Vollum</b>		
Glass		
Unexposed Control	$2.74 \pm 1.18 \times 10^8$	-
Decontaminated	0	$\geq 8.4$
Hypalon® Rubber Glove		
Unexposed Control	$3.49 \pm 2.90 \times 10^8$	-
Decontaminated	0	$\geq 8.5$
Stainless Steel		
Unexposed Control	$1.64 \pm 0.12 \times 10^8$	-
Decontaminated	0	$\geq 8.2$
<b><i>B. anthracis</i> Sterne</b>		
Glass		
Unexposed Control	$4.32 \pm 2.38 \times 10^7$	-
Decontaminated	0	$\geq 7.6$
Hypalon® Rubber Glove		
Unexposed Control	$6.00 \pm 1.17 \times 10^7$	-
Decontaminated	0	$\geq 7.8$
Stainless Steel		
Unexposed Control	$5.37 \pm 0.35 \times 10^7$	-
Decontaminated	0	$\geq 7.7$

<sup>a</sup> "-" = Not applicable.

In this study, the material types chosen for the decontamination testing were representative of those comprising most of the surface area within the ARCA chamber, and the results show that *B. anthracis* spores on glass, Hypalon® rubber glove, and stainless steel materials can be inactivated. However, these materials are considered non-porous and may not be representative of additional materials that could be introduced into the ARCA chamber for research purposes. The decontamination efficacy of vaporous hydrogen peroxide has been shown to be affected by different factors, such as material porosity and spore density (Han et al., 2003; Rogers et al., 2005; Rastogi et al., 2009; Spiner & Hoffman, 1971; Young et al., 1970). For more complex and porous materials, the inoculated spores may penetrate and stratify within the test material and could interfere with the contact of the decontaminant with the spores, thereby potentially decreasing spore inactivation. There-

fore, when decontaminating large-scale enclosures such as the ARCA chamber, laboratory, or room where re-entry is imminent, it is important to evaluate the inactivation of *B. anthracis* spores (and other biological agents) on material types that may be present in the enclosure to provide both best- and worst-case scenarios for challenging the decontamination process.

The generation of biological agent aerosols is one of the capabilities of the ARCA chamber that would be advantageous for conducting future decontamination research. An important factor that could potentially influence the decontamination efficacy of vaporous hydrogen peroxide or other fumigants is the method of spore deposition. In this study and as done previously (Rastogi et al., 2009; Rogers et al., 2005; Rogers et al., 2007), spores were delivered to the material surfaces as droplets from an aqueous suspension that were left undisturbed to dry. However, the 2001 intentional release of

**Table 3**

Qualitative Growth Assessments of Test Materials and Biological Indicators Exposed to Vaporous Hydrogen Peroxide

Spores/Test Material <sup>a</sup>	No. Tested (No. Positive)	
	Day 1	Day 7
<b><i>B. anthracis</i> Ames</b>		
Glass		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Hypalon® Rubber Glove		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Stainless Steel		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
<b><i>B. anthracis</i> Vollum</b>		
Glass		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Hypalon® Rubber Glove		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Stainless Steel		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
<b><i>B. anthracis</i> Sterne</b>		
Glass		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Hypalon® Rubber Glove		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
Stainless Steel		
Unexposed Control	3(3)	3(3)
Decontaminated	3(0)	3(0)
<b>Biological Indicators</b>		
Unexposed Control	1(1)	1(1)
Decontaminated	16(0)	16(0)

<sup>a</sup>All blanks (not inoculated) for each material were negative for growth.

*B. anthracis* spores in the mail consisted of a fine, dry powder aggregate that formed an aerosol depositing over surfaces. It is possible that the spore preparation and delivery mechanism may have an effect on the decontamination efficacy of spores deposited as an aerosol compared to spores delivered as an aqueous suspension. Therefore, it is our intent to conduct future research in which spores aerosolized within the ARCA chamber will be allowed to settle onto the surface of material coupons and subsequently be decontaminated with a fumigant such as vaporous hydrogen peroxide. Such studies will enable a comparison that will address the question of whether differences exist in the inactivation of spores on surfaces that were deposited as an aerosol or dried from an aqueous suspension.

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