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Formaldehyde gas inactivation of *Bacillus anthracis*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surface materials

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Abstract

Aims: To evaluate the decontamination of *Bacillus anthracis*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surface materials using formaldehyde gas.
Methods and Results: *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores were dried on seven types of indoor surfaces and exposed to approx. 1100 ppm formaldehyde gas for 10 h. Formaldehyde exposure significantly decreased viable *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores on all test materials. Significant differences were observed when comparing the reduction in viable spores of *B. anthracis* with *B. subtilis* (galvanized metal and painted wallboard paper) and *G. stearothermophilus* (industrial carpet and painted wallboard paper). Formaldehyde gas inactivated ≥50% of the biological indicators and spore strips (approx. 1×10^6 CFU) when analyzed after 1 and 7 days.
Conclusions: Formaldehyde gas significantly reduced the number of viable spores on both porous and nonporous materials in which the two surrogates exhibited similar log reductions to that of *B. anthracis* on most test materials.
Significance and Impact of the Study: These results provide new comparative information for the decontamination of *B. anthracis* spores with surrogates on indoor surfaces using formaldehyde gas.

Introduction

Bacterial endospores are resistant to a wide-variety of treatments such as heat, desiccation, radiation, pressure, and chemicals (Nicholson *et al.* 2000). This spore resistance is because of factors such as the spore coat, low water content in the spore core, and the α/β-type small, acid soluble spore proteins that protect spore DNA (Setlow and Setlow 1993; Nicholson *et al.* 2000; Setlow *et al.* 2000). Various gaseous decontaminants (chlorine dioxide, hydrogen peroxide, formaldehyde, ethylene oxide) have been used for the inactivation of *Bacillus* spores (Spotts Whitney *et al.* 2003). Fumigants are advantageous for decontaminating large rooms or buildings because of the ease of dissemination and ability to contact large surface areas; however, toxicity, material compatibility, decontamination contact time and concentration, as well as ventilation requirements should be considered for each type of fumigant. Formaldehyde is a known carcinogen, which has limited its use as a decontaminant; however, formaldehyde gas can be neutralized with ammonium carbonate to reduce the toxic potential of formaldehyde. This neutralization process leads to the production of the white powder, hexamethylene tetramine, which becomes deposited on the surfaces within the decontaminated area. However, if the formaldehyde is not completely neutralized at the time of administering the ammonium carbonate then there is the potential for formaldehyde gas to leach out of porous materials, thereby posing an additional hazard.

Decontamination with aqueous and gaseous formaldehyde has been used for many years in laboratory, medical, and industrial settings (Sweet 1971; Fink *et al.* 1988; Cross and Lach 1990; Lach 1990). On a large scale, formaldehyde was used to decontaminate Gruinard Island, Scotland, after the British military conducted explosives testing with *Bacillus anthracis* spores during World War II (Inglesby *et al.* 2002). More recently, formaldehyde vapor was used in decontaminating a mail sorting machine and stamping device from the US Department of Justice mail facility in Landover, Maryland (Canter *et al.* 2005). Formaldehyde has been shown to be effective in killing *Bacillus* spores, *Mycobacterium bovis*, and poliovirus (Sagripanti and Bonifacino 1996; Loshon *et al.* 1999; Munro *et al.* 1999). The mechanism by which formaldehyde kills *Bacillus subtilis* spores is in part by DNA damage, and increases in DNA mutagenesis and DNA-protein cross-linking have also been observed (Loshon *et al.* 1999).

Temperature, relative humidity, formaldehyde concentration, and formaldehyde adsorption onto surfaces are crucial factors contributing to the sporicidal activity of formaldehyde gas (vapor phase equilibrium concentration of formaldehyde; Munro *et al.* 1999). Formaldehyde gas sporicidal efficacy is directly proportional to its concentration at a relative humidity greater than 50% (Spiner and Hoffman 1971). Munro *et al.* (1999) showed that the optimal decontamination conditions for *Bacillus* spores, *M. bovis*, and poliovirus were 66% relative humidity, a minimum temperature of 28°C, and 10·5 g paraformaldehyde/m^3 (theoretical concentration of 8500 ppm). Surface porosity affects formaldehyde decontamination of *Bacillus* spores in which porous materials are more readily decontaminated at a lower relative humidity than nonporous materials, which require a high relative humidity (Spiner and Hoffman 1971). Such differences in the relationship of relative humidity, surface porosity, and formaldehyde sporicidal efficacy may be because of formaldehyde adsorption, which increases with increasing relative humidity (Braswell *et al.* 1970). The formaldehyde adsorption onto surfaces is affected by the physical properties of the surface material, relative humidity, and time, which are factors that should be considered when conducting a decontamination procedure with formaldehyde gas (Braswell *et al.* 1970).

The intentional release of *B.anthracis* spores in the mail led to contamination of the Hart Senate Building, mail handling and distribution facilities in Washington, D.C., Trenton, NJ, and other mail processing facilities. This contamination prompted extensive clean-up efforts and increased public awareness as well as a growing interest in *B. anthracis* (and other biological agents) detection methods, sampling, and decontamination of indoor surfaces, rooms, and buildings (Canter 2005; Canter *et al.* 2005). To date, most of the decontamination studies in the scientific literature have utilized surrogates for *B. anthracis*; therefore, more research is needed to evaluate appropriate decontamination technologies for the remediation of environments contaminated with *B. anthracis* spores, as well as generating correlative data between *B. anthracis* and surrogates (Spotts Whitney *et al.* 2003).

A focus of the US Environmental Protection Agency that addresses the growing concerns of homeland security is performance verification testing of commercially available technologies intended to decontaminate buildings contaminated with biological and chemical agents. One such technology, a hydrogen peroxide gas generator, was previously evaluated for decontamination efficacy against biological agents (Rogers *et al.* 2005). The results demonstrated that hydrogen peroxide gas significantly reduced viable *B. anthracis*, B. *subtilis*, and *Geobacillus stearothermophilus* spores on various indoor surfaces materials in which efficacy appeared to be affected by material porosity. To date, there is currently no study evaluating the decontamination efficacy of formaldehyde gas against *B. anthracis* and surrogate spores deposited on indoor building surfaces. Therefore, the purpose of this study was to utilize a laboratory-scale approach for comparing the decontamination of *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores on porous and nonporous materials using a formaldehyde gas generator.

Materials and methods

Test organisms

Spores of the virulent *B. anthracis* Ames strain were prepared using a BioFlo 3000 Fermentor and Bioreactor (New Brunswick Scientific Co., Inc., Edison, NJ, USA) as previously described (Rogers *et al.* 2005). Cultures were grown in Leighton-Doi Broth (BD Diagnostic Systems, Sparks, MD, USA) in the fermentor for approx. 24 h at 37°C. Spores were purified as previously described (Rogers *et al.* 2005). Preparations having >95% refractile spores with <5% cellular debris were enumerated, diluted in sterile water to approx. $1{\cdot}0 \times 10^9$ CFU ml^{-1}, and stored at 2–8°C. *B. subtilis* and *G. stearothermophilus* are commonly used *B. anthracis* surrogates for decontamination testing (Klapes and Vesley 1990; Sagripanti and Bonifacino 1996; Setlow and Setlow 1996; Rutala *et al.* 1998; Khadre and Yousef 2001; Melly *et al.* 2002a; Sigwarth and Stark 2003). Stock suspensions of *B. subtilis* (ATCC 19659) and *G. stearothermophilus* (ATCC 12980) spores were purchased from Apex Laboratories, Inc. (Apex, NC, USA) for this study. Prior to use, these spore preparations were evaluated microscopically for refractility and debris; these preparations possessed >95% refractile spores with

<5% cellular debris. Samples from these stock cultures were enumerated, diluted to approx. $1{\cdot}0 \times 10^9$ CFU ml^{-1} in sterile water, and stored at 2–8°C.

Test materials

Seven materials representing porous and nonporous indoor surfaces commonly found in buildings were used for testing (Rogers *et al.* 2005). These included ShawTek EcoTek 6 industrial carpet (Shaw Industries, Cartersville, GA, USA), bare pine wood, painted (latex, semi-gloss) concrete cinder block ASTM C90, glass ASTM C1036, white formica laminate with matte finish, galvanized metal ductwork, and painted (latex, flat) wallboard paper. With respect to the inoculated surface, the industrial carpet, bare pine wood, and painted concrete can be considered porous, while the glass, decorative laminate, galvanized metal ductwork, and painted wallboard paper can be considered nonporous. Samples of each test material were cut from a larger piece of the representative materials to form $1{\cdot}9 \times 7{\cdot}5$ cm coupons. Visual inspection of the physical integrity and appearance of the test material coupons was performed before and after decontamination to detect any damage to the test materials.

Decontamination procedure

All testing was performed under Biosafety Level 3 conditions. The test coupons were cleaned by wiping with 70% isopropanol; autoclaving the materials prior to inoculation was not used in this study because of various damaging effects to the materials. Autoclaving resulted in the weave of the industrial carpet becoming unglued and falling apart, the painted wallboard paper falling apart, the concrete becoming brittle and often crumbling, the laminate curling, and the grain of the bare pine wood swelling and changing the surface texture. These damaging effects prompted the use of the 70% isopropanol wipe to maintain consistency in preparing all of the materials prior to use. Each coupon was laid flat in a Biological Safety Cabinet (BSC) Class III, and contaminated with approx. $1{\cdot}0 \times 10^8$ *B. anthracis*, *B. subtilis*, or *G. stearothermophilus* spores. For each 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). As described previously (Rogers *et al.* 2005), a micropipette was used to deliver spore suspensions (100 μl) to the surface of each coupon as small droplets and the coupons were allowed to dry overnight, undisturbed. The next day, the inoculated coupons intended for decontamination (and one blank) were transferred to a Plas-Labs Model 830-ABC Compact Glove Box (Plas-Labs, Inc., Lansing, MI, USA; volume of approx. 317 L) and the coupons were placed lying flat, inoculated surface side up on a wire rack lined with Pet-D-Fence Screening (New York Wire Co., Mt. Wolf, NY, USA) for support (Rogers *et al.* 2005).

Biological indicators (BI) containing *B. subtilis* (ATCC 19659) and *G. stearothermophilus* (ATCC 12980) and spore strips (SS) containing *Bacillus atrophaeus* (ATCC 9372) were also used to evaluate decontamination. The *B. subtilis* and *G. stearothermophilus* BI consisted of approx. $1{\cdot}8 \times 10^6$ and $2{\cdot}6 \times 10^6$ spores, respectively, on stainless steel disks sealed in Tyvek pouches (Apex Laboratories, Inc.), and the SS consisted of approx. $1{\cdot}8 \times 10^6$ spores on filter paper strips sealed in glassine envelopes (Raven Biological Laboratories, Omaha, NE, USA). For *B. anthracis* decontamination, three of each BI and SS were placed inside of the glove box during each decontamination test day. For *B. subtilis* and *G. stearothermophilus* decontamination, three of each respective BI and SS were placed inside of the glove box during each decontamination test day. Three of each BI and SS not subjected to formaldehyde gas were used as positive controls.

The CERTEK Model #1414RH formaldehyde gas generator/neutralizer (CERTEK, Inc., Raleigh, NC, USA) was used for the decontamination testing. The 1414RH unit generated formaldehyde gas by heating and depolymerizing paraformaldehyde prills (91–93% purity; Hoechst Celanese Corporation, Dallas, TX, USA). A series of six nebulizers was mounted in the sidewall of the glove box to increase relative humidity. A port for the sampling of formaldehyde gas was added to the glove box as an additional modification. Prior to the initiation of each experimental decontamination run, a leak test was performed on the glove box in which a negative pressure equivalent to two inches of a water column was generated by a vacuum pump in the glove box and maintained for a minimum of 2 min. Following this leak test, the decontamination cycle of the 1414RH was initiated. For the purposes of this testing, the operational parameters were provided by CERTEK, and included temperature (16–32°C), relative humidity (50–90%), paraformaldehyde concentration (10·5 g paraformaldehyde per cubic meter of treated volume; approx. 8500 ppm theoretical value), decontamination contact time (10 h), and neutralization with ammonium carbonate.

For each decontamination run, the formaldehyde concentration was measured inside the glove box in real-time using a fluorimetric method as previously described (Kelly and Fortune 1994). This monitor was developed to measure formaldehyde concentrations within a range of approx. 1 ppb to 1 ppm. For the present study, the concentration of formaldehyde within the glove box was much higher than 1 ppm; therefore, this monitor had to be modified to dilute the gas sample from the glove box

by 10 000-fold. To accomplish this, two identical systems, consisting of a calibrated mass flow controller (MFC; Sierra Instruments, Monterey, CA, USA) and a valveless rotating and reciprocating piston metering pump (Fluid Metering, Inc., Syosset, NY, USA), were connected to each other in series. Each dilution system was set to a 10 ml min^{-1} flow rate, yielding a 100-fold dilution. The first system pulled 10 ml min^{-1} from the glove box, which was mixed with 990 ml min^{-1} air gas stream from a gas cylinder controlled by the MFC. From the exhaust stream of the first dilution system, the second system pulled 10 ml min^{-1}, which was also mixed with 990 ml min^{-1} air that was controlled by the second MFC; the sample from the glove box was then diluted 10 000-fold. For approx. 15 min prior to operating the 1414RH unit, the glove box was monitored for background formaldehyde concentration in ppm. Once a background baseline had been established, the 1414RH unit was operated according to the operation manual for the CERTEK Model #1414RH. The formaldehyde concentration in the glove box was monitored in real-time throughout the complete operational cycle of the technology. Using a formaldehyde standard (Sigma, St. Louis, MO, USA) and the known dilution factor, the formaldehyde concentration in ppm in the glove box was determined.

Sample processing and data collection

The processing and data collection procedure was performed as previously described (Rogers *et al.* 2005) with slight modification. Briefly, formaldehyde gas-exposed, control unexposed, and blank coupons were placed in a 50 ml tube containing 10 ml of 0·1% Triton X-100 (Sigma) in sterile phosphate-buffered saline (PBS). The inoculated control (not decontaminated) and blank coupons were also placed in a 50 ml tube containing 10 ml of sterile PBS with 0·1% Triton X-100. Spores were extracted by agitating tubes at 200 rev min^{-1} on an orbital shaker for 15 min at room temperature. Each tube was then heat-shocked at 65°C for 1 h to kill vegetative bacteria, and 1·0 ml of each extract was removed and serially diluted from 10^{-1} through 10^{-7} in sterile water.

Spore viability was determined by dilution plating in which 100 μl of the undiluted extract and each serial dilution were plated onto tryptic soy agar plates (Remel, Lexena, KS, USA) in triplicate, allowed to dry, and incubated overnight at 37°C for *B. anthracis*, 35°C for *B. subtilis*, and 55–60°C for *G. stearothermophilus*. Following 18–24 h incubation, plates were enumerated and colony-forming units (CFU) ml^{-1} was determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data were expressed as the mean ± standard deviation (SD) of observed CFU. For all samples tested, the micro-organisms observed growing on TSA plates (*B. anthracis*, *B. subtilis*, or *G. stearothermophilus*) had the appearance of a homogenous mixture. Moreover, the identification of each organism was confirmed by comparing the colony morphology from spores in the coupon sample extracts to that of the spore stock suspension when grown on TSA plates.

Efficacy calculations and statistical analysis

To calculate the efficacy of the decontamination treatment, the number of viable spores extracted from the decontaminated test coupons was compared with the number of viable spores extracted from the control coupons. Efficacy for biological agents was expressed in terms of a log reduction using the following equation:

$$\text{Log Reduction} = \log(N/N')$$

where N is the mean number of viable organisms recovered from the control coupons (i.e., those not subjected to decontamination), and N' is the number of viable organisms recovered from each test coupon after decontamination. For decontaminated coupons where viable organisms were not detected, the efficacy was calculated as the log of the mean number of viable organisms recovered from the control coupons. Using the calculated log reduction for each test coupon, the mean (±SD) log reduction was calculated. Mean (±SD) percent recovery was calculated for each type of test material inoculated with each biological agent or surrogate by dividing the number of viable organisms extracted from the test sample (decontaminated or nondecontaminated control) by the spore inoculum.

For statistical comparisons, the two-way ANOVA and *t*-tests (SAS version 8·2, SAS Institute, Inc., Cary, NC, USA) were used for data analysis. For each material and species combination, log reduction was calculated as described above. The two-way ANOVA was used to assess main effects for each organism and test material and interactions were fitted to the log reduction data. This model was used to compare the mean log reduction for each bacterial species tested, and compare the log reduction in *B. subtilis* and *G. stearothermophilus* spores to *B. anthracis* spores for each test material. The *t*-tests or statistical contrasts were used for the comparisons, with no adjustment for multiple comparisons. The ANOVA model was fitted using the SAS GLM procedure. $P \leq 0{\cdot}05$ was used as the level for significance.

Results

In all tests, the formaldehyde concentration (as measured by the formaldehyde monitor) was maintained at approx.

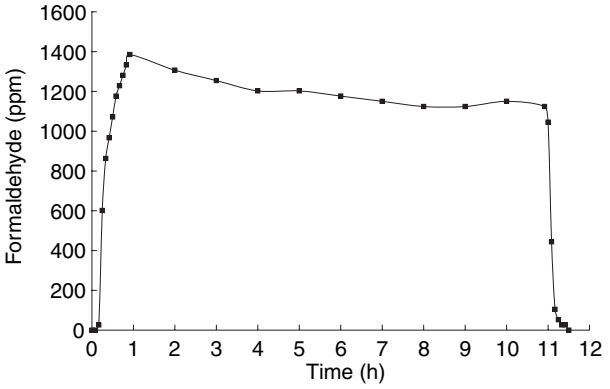

Figure 1 Formaldehyde concentration measured inside the glove box. This figure represents the data collected for a single decontamination run from the start of gassing through neutralization. During the decontamination runs, the temperature ranged from 22–23°C and the relative humidity ranged from 70–75%.

1100 ppm (Fig. 1) with a relative humidity range of 70–75% and a temperature range of 22–23°C during the 10-h contact time. Prior to introduction of formaldehyde gas, a 5 min humidification of the decontamination chamber using a nebulizing system was performed. Once the relative humidity inside the glove box was greater than 70%, the formaldehyde gassing commenced for approx. 1 h. Following the 10-h contact time, neutralization was accomplished in approx. 30–60 min. Following all experimental decontamination runs, the test coupons were evaluated qualitatively for visible surface damage and no changes to any of the test materials were observed. However, a white powder film of hexamethylene tetramine was formed and deposited on all surfaces and test materials inside the glove box following the neutralization step with ammonium carbonate.

Exposure of test coupons contaminated with *B. anthracis* Ames, *B. subtilis*, or *G. stearothermophilus* spores to formaldehyde gas resulted in a reduction of viable spores that varied according to the type of the test material (Tables 1–3). The mean log reduction of detectable viable *B. anthracis* Ames spores ranged from 5·2 to 7·9 for all seven test materials (Table 1). For all seven test materials, the log reduction of detectable viable *B. subtilis* and *G. stearothermophilus* spores ranged from 6·0 to 8·0 and 5·7 to 7·6, respectively (Tables 2 and 3). No viable organisms were detected in any of the blank samples.

Statistical analysis of the data revealed that all mean log reductions were significantly different from zero (Tables 1–3), indicating that exposure to formaldehyde gas significantly reduced ($P \leq 0{\cdot}05$) the mean number of all three species of spores. In general, comparisons within each material indicated that the two selected surrogates had similar mean log reductions to *B. anthracis*. However,

Table 1 Decontamination efficacy of *Bacillus anthracis* Ames spores following formaldehyde exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
Control	$1{\cdot}0 \pm 0{\cdot}37 \times 10^7$	11 ± 4·0	NA
Formaldehyde	0	0	≥7·0 ± 0†
Bare pine wood			
Control	$4{\cdot}0 \pm 0{\cdot}24 \times 10^7$	40 ± 2·4	NA
Formaldehyde	0	0	≥7·6 ± 0†
Painted concrete			
Control	$5{\cdot}8 \pm 0{\cdot}25 \times 10^7$	56 ± 2·5	NA
Formaldehyde	$2{\cdot}2 \pm 3{\cdot}9 \times 10$	<0·0001	7·2 ± 1·1†
Glass			
Control	$5{\cdot}1 \pm 1{\cdot}4 \times 10^7$	55 ± 15	NA
Formaldehyde	0	0	≥7·7 ± 0†
Decorative laminate			
Control	$4{\cdot}6 \pm 0{\cdot}60 \times 10^7$	45 ± 5·9	NA
Formaldehyde	$4{\cdot}9 \pm 5{\cdot}8 \times 10$	<0·0001	6·5 ± 1·1†
Galvanized metal ductwork			
Control	$7{\cdot}2 \pm 1{\cdot}5 \times 10^7$	71 ± 15	NA
Formaldehyde	0	0	≥7·9 ± 0†
Painted wallboard paper			
Control	$1{\cdot}5 \pm 0{\cdot}43 \times 10^5$	0·2 ± 0·05	NA
Formaldehyde	0	0	≥5·2 ± 0†

NA, not applicable.

**Bacillus anthracis* Ames spores were subjected to formaldehyde gas exposure and assessed for viability as described in the materials and methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and formaldehyde-treated samples. Values are expressed as mean ± SD from triplicate samples of each test material.

†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

the mean log reduction in *B. anthracis* spores was significantly lower ($P \leq 0{\cdot}05$) than the two surrogates on painted wallboard paper (Tables 2 and 3). The mean reduction in *B. subtilis* spores was significantly lower than *B. anthracis* on galvanized metal ductwork, while the mean log reduction in *G. stearothermophilus* spores was significantly lower ($P \leq 0{\cdot}05$) than *B. anthracis* for industrial carpet.

The BI and SS evaluated in parallel as a qualitative decontamination assessment were partially inactivated by formaldehyde gas where no growth was observed for ≥50% of all exposed BI and SS as determined by the lack of visibly cloudy liquid cultures at 1 and 7 days postexposure. For all BI and SS, the percent number of positive samples increased from day one to day seven. The *G. stearothermophilus* BI displayed the most resistance to decontamination in which approx. 17% and 50% of the samples were positive for growth at 1 and 7 days, respectively.

Table 2 Decontamination efficacy of *Bacillus subtilis* (ATCC 19659) spores following formaldehyde exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
Control	$1{\cdot}1 \pm 0{\cdot}08 \times 10^8$	88 ± 6·3	NA
Formaldehyde	0	0	≥8·0 ± 0†
Bare pine wood			
Control	$1{\cdot}2 \pm 0{\cdot}41 \times 10^7$	12·0 ± 3·9	NA
Formaldehyde	$1{\cdot}1 \pm 1{\cdot}9 \times 10$	<0·0001	6·6 ± 0·88†
Painted concrete			
Control	$5{\cdot}5 \pm 0{\cdot}43 \times 10^7$	52 ± 4·1	NA
Formaldehyde	$6{\cdot}6 \pm 5{\cdot}8 \times 10$	<0·0001	6·0 ± 0·35†
Glass			
Control	$6{\cdot}2 \pm 2{\cdot}2 \times 10^7$	50 ± 18	NA
Formaldehyde	0	0	≥7·8 ± 0†
Decorative laminate			
Control	$5{\cdot}5 \pm 1{\cdot}3 \times 10^7$	53 ± 12	NA
Formaldehyde	$2{\cdot}2 \pm 3{\cdot}9 \times 10$	<0·0001	7·3 ± 0·78†
Galvanized metal ductwork			
Control	$7{\cdot}4 \pm 1{\cdot}9 \times 10^7$	71 ± 18	NA
Formaldehyde	$1{\cdot}9 \pm 1{\cdot}7 \times 10^2$	<0·001	6·2 ± 1·4†,‡
Painted wallboard paper			
Control	$4{\cdot}8 \pm 1{\cdot}2 \times 10^7$	39 ± 10	NA
Formaldehyde	0	0	≥7·7 ± 0†,‡

NA, not applicable.

**Bacillus subtilis* spores were subjected to formaldehyde gas exposure and assessed for viability as described in the materials and methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and formaldehyde-treated samples. Values are expressed as mean ± SD from triplicate samples of each test material.

†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

‡Mean log reduction is significantly different ($P \leq 0{\cdot}05$) than the corresponding mean log reduction for *Bacillus anthracis* presented in Table 1.

Table 3 Decontamination efficacy of *Geobacillus stearothermophilus* (ATCC 12980) spores following formaldehyde exposure*

Test material/ treatment	Total spores recovered (CFU)	% Recovery	Log reduction
Industrial carpet			
Control	$1{\cdot}5 \pm 0{\cdot}19 \times 10^7$	17 ± 2·2	NA
Formaldehyde	$1{\cdot}2 \pm 1{\cdot}2 \times 10^2$	<0·001	5·7 ± 1·3†,‡
Bare pine wood			
Control	$6{\cdot}6 \pm 1{\cdot}6 \times 10^6$	9·0 ± 2·1	NA
Formaldehyde	0	0	≥6·8 ± 0†
Painted concrete			
Control	$1{\cdot}9 \pm 0{\cdot}60 \times 10^7$	27 ± 8·2	NA
Formaldehyde	$0{\cdot}61 \pm 1{\cdot}1 \times 10^3$	<0·001	6·2 ± 1·9†
Glass			
Control	$1{\cdot}7 \pm 0{\cdot}05 \times 10^7$	20 ± 0·51	NA
Formaldehyde	0	0	≥7·2 ± 0†
Decorative laminate			
Control	$1{\cdot}3 \pm 0{\cdot}59 \times 10^7$	18 ± 8·1	NA
Formaldehyde	0	0	≥7·1 ± 0†
Galvanized metal ductwork			
Control	$4{\cdot}3 \pm 4{\cdot}8 \times 10^7$	59 ± 66	NA
Formaldehyde	0	0	≥7·6 ± 0†
Painted wallboard paper			
Control	$1{\cdot}5 \pm 0{\cdot}20 \times 10^7$	17 ± 2·3	NA
Formaldehyde	0	0	≥7·2 ± 0†,‡

NA, not applicable.

**Geobacillus stearothermophilus* spores were subjected to formaldehyde gas exposure and assessed for viability as described in the materials and methods. Each test material was inoculated with approx. $1{\cdot}0 \times 10^8$ CFU and dried overnight. Spores were extracted from the test materials and enumerated. Percent recovery and log reduction calculation were based on the number of detectable viable spores in the control and formaldehyde-treated samples. Values are expressed as mean ± SD from triplicate samples of each test material.

†Mean log reduction is significantly different than zero ($P \leq 0{\cdot}05$).

‡Mean log reduction is significantly different ($P \leq 0{\cdot}05$) than the corresponding mean log reduction for *Bacillus anthracis* presented in Table 1.

Discussion

The results of this study show that a 10-h exposure to formaldehyde gas resulted in significant reduction in *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores dried on various porous and nonporous materials. The observed log reduction in viable spores for the three organisms inoculated on all test materials evaluated were >6·0, with the exception of *B. anthracis* on painted wallboard paper and *G. stearothermophilus* on industrial carpet. These results suggest that when using the decontamination parameters outlined in this study, material porosity did not appear to affect decontamination efficacy of formaldehyde gas.

As with previous studies (Braswell *et al.* 1970; Hoffman and Spiner 1970; Spiner and Hoffman 1971; Sweet 1971; Ackland *et al.* 1980; Canter *et al.* 2005), a similar starting paraformaldehyde concentration (10·5 g per cubic meter) was used in this study to yield a theoretical vapor phase concentration of approx. 8500 ppm formaldehyde gas in the decontamination chamber. Most studies in the scientific literature express formaldehyde concentrations in terms of the theoretical value without secondary confirmatory measurements of the formaldehyde in the vapor phase. Within an enclosed system, the vapor phase equilibrium concentration of formaldehyde at 20–21°C is 2·0 g per cubic meter, which coverts to 1628 ppm (Ackland *et al.* 1980). When exceeding this vapor phase equilibrium concentration, condensation can occur on the surfaces; however, the vapor phase equilibrium concentration of formaldehyde increases with increasing temperature (Ackland *et al.* 1980). In our study, we measured

an average of approx. 1100 ppm formaldehyde in the vapor phase, which is similar to a previously reported value of 1·75 g per cubic meter, or 1425 ppm (Ackland *et al.* 1980). Furthermore, Ackland *et al.* (1980) utilized a controlled fumigation approach to assess optimal vapor phase formaldehyde levels without exceeding the vapor phase equilibrium concentration, thereby resulting in condensation within the decontaminated area. This information can be advantageous for potentially implementing formaldehyde gas decontamination of large areas, such as buildings.

The majority of the data available for *Bacillus* spore decontamination are derived from using avirulent *B. anthracis* strains or surrogates, such as *B. subtilis*. Under controlled conditions, surrogates selected for decontamination studies should result in comparable performance data to that of *B. anthracis*. Previous work by Rogers *et al.* (2005) demonstrated statistically significant differences in decontamination efficacy of hydrogen peroxide gas between *B. anthracis* Ames, *B. subtilis*, and *G. stearothermophilus* spores on the same indoor surface materials evaluated in the present study. Statistical analyses were made to compare the decontamination efficacy of formaldehyde gas for *B. anthracis* and the surrogate spores on all test surfaces evaluated. In general, the two surrogates exhibited similar log reductions to that of *B. anthracis*; however significant differences were observed when comparing the reduction in viable spores of *B. anthracis* with *B. subtilis* on galvanized metal and painted wallboard paper, and *G. stearothermophilus* on industrial carpet and painted wallboard paper. These results suggest that for most of the test materials there did not appear to be a difference in spore killing, suggesting the decontamination efficacy of formaldehyde gas for both surrogates appeared to reflect that of *B. anthracis*.

The mean log reduction in *B. anthracis* spores on painted wallboard paper was significantly lower than both *B. subtilis* and *G. stearothermophilus* spores. The mean percent recovery of viable *B. anthracis* spores (based on the nondecontaminated controls) was 0·2%, while the mean percent recovery of viable *B. subtilis* and *G. stearothermophilus* spores on painted wallboard paper was approx. 39% and 17%, respectively. No countable *B. anthracis* CFU were observed from the dilution plating of spores extracted from painted wallboard paper exposed to formaldehyde gas. Previously, we observed a recovery rate of *B. anthracis* spores on painted wallboard paper of 7·7% (Rogers *et al.* 2005), which is higher than the value reported in the present study. Although there is a difference in these two values, a log reduction value for painted wallboard paper was calculated and statistical comparisons made between *B. anthracis* and the two surrogates. The significantly lower log reduction in *B. anthracis* spores for the painted wallboard paper results from the low spore recovery; therefore, the calculated value may not reflect the full extent of *B. anthracis* spore inactivation by formaldehyde on painted wallboard paper.

Growth assessments of various BI are often used to qualify decontamination performance (Heckert *et al.* 1997; Sigwarth and Moirandat 2000; Sigwarth and Stark 2003; French *et al.* 2004; Johnston *et al.* 2005; Rogers *et al.* 2005). In the present study, a qualitative evaluation of the performance of the 1414RH unit was accomplished using BI and SS containing spore loads of approx. $1{\cdot}0 \times 10^6$ spores. For all decontamination runs, the nondecontaminated control BI and SS displayed growth (cloudy cultures) in the liquid cultures at both 1 and 7 days. The BI and SS exposed to formaldehyde gas were partially inactivated (≥50% for all tests) where the number of samples exhibiting growth varied among the dates of experimentation in which no clear trend was observed between decontamination runs. However, it is interesting to note that the number of positive samples increased from day one to day seven. Formaldehyde has a relatively poor penetration capacity (Hoffman and Spiner 1970); therefore, the Tyvek and glassine pouches in which the BI and SS were sealed may have partially inhibited the penetration of formaldehyde through the pouches where it could reach the spores. This inhibition could play a role in preventing complete inactivation of the BI and SS, supporting our observations of incomplete kill of BI and SS at 1 and 7 days. Another factor contributing to this partial inactivation of BI and SS could be because of the difference in spore carrier materials (stainless steel disks *vs* paper), which can affect the performance of BI (Shintani and Akers 2000; Johnston *et al.* 2005). Similar results have been reported where the results of BI decontaminated with formaldehyde gas did not parallel the inactivation of *Bacillus* spores dried on stainless steel test materials (Munro *et al.* 1999).

Spore production conditions, heat shock, and the mechanism of spore deposition onto surfaces could potentially contribute to the spore inactivation observed in this study. Methods for producing spores could vary between laboratories and differences in incubation temperature and growth medium can influence spore resistance to decontamination (Palop *et al.* 1999; Cazemier *et al.* 2001; Melly *et al.* 2002b). The presence or absence of debris in spore preparations can also influence the rate at which formaldehyde gas inactivates spores, especially on nonporous surfaces (Spiner and Hoffman 1971). However, the spore preparations used in this study exhibited little to no debris and greater than 95% spore refractility under phase contrast microscopy, suggesting the effect of spore cleanliness on formaldehyde decontamination in this study was probably not a factor. The heat shock step

implemented in this study could have also contributed to spore killing following formaldehyde gas decontamination and spore extraction from the material coupons. This is possible as *B. subtilis* spores pretreated with decontaminating agents were more sensitive to killing by subsequent incubation at 84°C (Cortezzo *et al.* 2004). Another factor that could potentially influence formaldehyde decontamination efficacy is the method of spore deposition. In this study, spores were delivered to the material surfaces as droplets from an aqueous suspension that subsequently dried. In 2001 intentional release of *B. anthracis* spores in the mail, the spores were delivered as a dry, fine powder aggregate. It is possible that the spore preparation and delivery mechanism of a fine powder could affect the decontamination efficacy of formaldehyde gas compared with the spores that are delivered as an aqueous suspension.

This study demonstrates the decontamination efficacy of formaldehyde gas for spores of *B. anthracis* Ames and the two surrogates *B. subtilis* and *G. stearothermophilus* dried on indoor surface materials. The formaldehyde gas exposure demonstrated a significant reduction in spores on all materials evaluated. The current assessment for effective remediation is no growth of *B. anthracis* spores from all postremediation sampling; however, the potential exists for the establishment of risk-based clean-up levels resulting from dose-response assessments and availability of methodologies for measuring *B. anthracis* spores on surfaces (Canter 2005). Ultimately, there will need to be an acceptable level of reduction in spores (e.g., 6 logs or complete kill) prior to the safe re-entry of personnel back into a building or environment that has been decontaminated.

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