MICROBIOLOGICAL METHODS

Use of Alternative Carrier Materials in AOAC *Official Method*℠ 2008.05, Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard, Nonporous Surface, Quantitative Three-Step Method

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The quantitative Three-Step Method (TSM) for testing the efficacy of liquid sporicides against spores of *Bacillus subtilis* on a hard, nonporous surface (glass) was adopted as *AOAC Official Method*℠ 2008.05 in May 2008. The TSM uses 5 5 1 mm coupons (carriers) upon which spores have been inoculated and which are introduced into liquid sporicidal agent contained in a microcentrifuge tube. Following exposure of inoculated carriers and neutralization, spores are removed from carriers in three fractions (gentle washing, *fraction* A; sonication, *fraction* B; and gentle agitation, *fraction* C). Liquid from each fraction is serially diluted and plated on a recovery medium for spore enumeration. The counts are summed over the three *fractions* to provide the density (viable spores per carrier), which is $\log_{10}$-transformed to arrive at the log density. The log reduction is calculated by subtracting the mean log density for treated carriers from the mean log density for control carriers. This paper presents a single-laboratory investigation conducted to evaluate the applicability of using two porous carrier materials (ceramic tile and untreated pine wood) and one alternative nonporous material (stainless steel). Glass carriers were included in the study as the reference material. Inoculated carriers were evaluated against three commercially available liquid sporicides (sodium hypochlorite, a combination of peracetic acid and hydrogen peroxide, and glutaraldehyde), each at two levels of presumed efficacy (medium and high) to provide data for assessing the responsiveness of the TSM. Three coupons of each material were evaluated across three replications at each level; three replications of a control were required. Even though all carriers were inoculated with approximately the same number of spores, the observed counts of recovered spores were consistently higher for the nonporous carriers. For control carriers, the mean log densities for the four materials ranged from 6.63 for wood to 7.14 for steel. The pairwise differences between mean log densities, except for glass minus steel, were statistically significant ($P < 0.001$). The repeatability standard deviations (S_r) for the mean control log density per test were similar for the four materials, ranging from 0.08 for wood to 0.13 for tile. Spore recovery from the carrier materials ranged from approximately 20 to 70%: 20% (pine wood), 40% (ceramic tile), 55% (glass), and 70% (steel). Although the percent spore recovery from pine wood was significantly lower than that from other materials, the performance data indicate that the TSM provides a repeatable and responsive test for determining the efficacy of liquid sporicides on both porous and nonporous materials.

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The U.S. Environmental Protection Agency (EPA) is working to improve current laboratory methodology for determining the efficacy of sporicidal chemicals. The methods are critical to the registration of products designed for use in decontamination of personal protective gear, equipment, and facilities in the event of biological weapons (BW) attacks involving spores of *Bacillus anthracis*. The development of quantitative efficacy tests is a significant component of this effort. In 2008, the Quantitative Three-Step Method (TSM), a procedure for determining the efficacy of liquid sporicides on hard, nonporous surfaces, was granted First Action status by AOAC INTERNATIONAL. The method was assigned *Official Method* **2008.05** (1). The outcome of the collaborative study has been published (2). The TSM validation study included testing liquid formulations against spores of *B. subtilis* (a surrogate for virulent strains of *B. anthracis*) on a hard, nonporous surface (glass). In brief, the TSM uses 5 5 1 mm glass coupons to deliver spores into the sporicidal agent (400 L) contained in microcentrifuge tubes, three coupons per chemical treatment. Following exposure to the test chemical and neutralization, spores are removed from the carriers in three *fractions* by loosely washing (*fraction* A), sonication (*fraction* B), and gentle agitation (*fraction* C). Liquid from each *fraction* is plated on recovery medium for viable spore enumeration. Control (water) counts are compared to the treated counts and the level of efficacy is determined by calculating the $\log_{10}$ reduction (LR) of spores; LR = $\log_{10}$ (mean spores/control carrier) – $\log_{10}$ (mean spores/treated carrier).

The purpose of this study was to demonstrate the suitability of three alternative TSM coupon materials (stainless steel, wood, and ceramic tile) for use in the method. The current material, glass, is a smooth inert surface from which spores are relatively easy to recover and does not interact with the disinfectant chemical. In the context of decontamination of building materials, which includes a variety of porous surfaces, it is of interest to determine if relevant porous materials can provide a sensitive procedure with reproducible results for measuring the efficacy of liquid sporicides on porous surfaces. The selection of the materials was based on the following attributes: (*1*) the ability to cut the material into 5 5 mm coupons; (*2*) the uniformity of the cut coupons; (*3*) the relevance of the materials to building interiors; (*4*) the availability of the material at retail suppliers; and (*5*) the ease of sterilization without compromising the material. Based on these attributes, ceramic tile and untreated pine wood were down-selected and advanced for further evaluation. Stainless steel was included in this study as an additional hard, nonporous surface carrier material. In order to support a procedural modification of the TSM to include the use of additional coupon materials, a series of laboratory studies were conducted to elucidate the TSM performance with the new materials. Method performance indicators used in the original multilaboratory TSM validation study (2) were also examined in this study. In addition, mean percent spore recovery per carrier (10%) and mean carrier counts (6 logs) were used to determine if the coupon material is appropriate for use with the TSM.

Study Design

Laboratory testing was conducted at the U.S. Army's Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, MD, under an Interagency Agreement with the EPA. Pine wood, ceramic tile, and stainless steel were purchased from Home Depot, Edgewood, MD; U.S. Tile Co., Canton, OH; and EJ Enterprise, Glen Burnie, MD, respectively. Per AOAC Method **2008.05**, glass coupons were procured from Erie Scientific, Portsmouth, NH. The other materials were cut into 5 5 mm coupons by Advanced Design Materials at ECBC. The thickness of wall tile coupons varied between 2 and 4 mm; the thickness of the wood coupons was approximately 2 mm. Stainless steel coupons were approximately 1 mm thick. Glass, steel, and ceramic tile coupons were rinsed with distilled water and ethanol. Pine wood was vacuumed while cutting to remove sawdust, but no rinsing was performed. Cleaned coupons were dried overnight, and then all coupons were sterilized by autoclaving for 45 min using a dry cycle (30 min drying time) before use. No deformity of the coupons was visibly noted after autoclaving. Sterilized coupons (carriers) were inoculated with spores of *B. subtilis* (ATCC No. 19659) prepared according to AOAC Method **2008.05**. The target spore load for each carrier was 1 10^7 spores/carrier (7.0 logs/carrier), a level suitable for measuring an LR of 6. Mean carrier counts within a range of 5 10^6 to 5 10^7 spores/carrier are considered acceptable. Using a working stock spore suspension with a titer of 1 10^9 to 5 10^9 spores/mL, a 10 L aliquot was inoculated in the center of each coupon using a calibrated positive displacement pipet. The inoculated carriers were dried in a biological safety cabinet for a minimum of 1 h before being placed in a desiccator at room temperature. Inoculated carriers were used within 30 days after inoculation.

Each inoculated material was evaluated against three test chemicals (liquid sporicides) at two presumed levels of efficacy. Each replication included three carriers per treatment and three independent analyses of each test chemical for a total of nine carriers of each type of material per treatment. Water was used as a control to determine the control carrier counts. Glass carriers were included in all treatments as a reference material, one coupon per treatment. The carrier materials were used in efficacy tests (side-by-side comparisons) on each of the nine test days, which correspond to three consecutive days per week for three consecutive weeks. The density (CFU/carrier) was recorded for a total of 270 carriers: 27 glass carriers, 81 stainless steel carriers,

Table 1. Test chemicals and treatments

Test chemicals	Treatment level and test conditions	
	High (LR 6)	Medium (LR = 2–6)
Sodium hypochlorite (NaOCl)	6000 ± 300 ppm pH adjusted (7 ± 0.5) 30 ± 1 min contact	6000 ± 300 ppm unadjusted pH (~10) 10 min ± 10 s contact
0.08% Peracetic acid and 1.0% hydrogen peroxide (PA/HP)	30 ± 1 min contact	10 min ± 10 s contact
2.6% Glutaraldehyde	180 ± 3 min contact	60 ± 1 min contact

81 ceramic tile carriers, and 81 wood carriers. There were 90 total control carriers: one glass carrier and three carriers for each of steel, tile, and wood on each test day and 180 treated carriers: one glass carrier and three carriers for each of steel, tile, and wood for each of the two efficacy levels on each test day.

The test chemicals used were (*1*) NaOCl, sodium hypochlorite (reagent grade, Fisher Scientific Cat. No. SS2901, sodium hypochlorite solution, 4–6% available chlorine); (*2*) PA/HP, a combination of peracetic acid and hydrogen peroxide (Spor-Klenz Ready to Use, EPA Registration No. 1043-119); and (*3*) 2.6% glutaraldehyde (Metricide 14-Day, a commercially available sterilant). Luria-Bertani (LB) broth was used to neutralize the peracetic acid/hydrogen peroxide and glutaraldehyde-based products. Sodium thiosulfate (0.1%, w/v) was added to LB broth to neutralize the sodium hypochlorite. Previously, neutralization confirmation was performed for each neutralizer according to AOAC Method **2008.05, C(g)**. Two exposure times for each test chemical were chosen to produce the medium (partial kill) and high efficacy (complete kill or near complete kill) levels for each test chemical, thereby providing a range of efficacy for assessing the responsiveness of the TSM (Table 1). The presumed efficacy levels were selected based on the results of the previous TSM validation study. An antimicrobial test is considered responsive if a larger LR value is produced for the more efficacious treatment. For the purpose of this study, the test chemicals were experimental components only and were not evaluated to verify product label claims.

Statistical Analysis

The total number of spores recovered in three *fractions* from each carrier was $\log_{10}$-transformed to form the log density value. For the main goals of the study, all statistical calculations were performed on the log density values. The untransformed densities were used only when the proportionate contributions of *fractions* A, B, and C were calculated. Statistical calculations were performed using the Minitab statistical computer package (Minitab Inc. 2006; Minitab Statistical Software; Release 15 for Windows, State College, PA; http://minitab.com), which uses statistical methods that are compatible with AOAC guidelines (3).

Control Carriers (Mean, Variances Between- and Within-Test Days, and Repeatability of Log Densities)

The control carrier log densities were analyzed separately for each carrier material using a one-factor, random effects, analysis of variance (ANOVA) model. Let Y_{ij} denote the log density for control carrier j on test day i. Then

$$Y_{ij} = \quad + T_i + e_{ij}$$

where denotes the true overall mean log density, T_i is the random effect due to test day i, and e_{ij} is the random effect due to control carrier j in test day i. The T_i and e_{ij} effects are assumed independent and have true means of zero. The variance of T_i, denoted by V_T, is the variance among test days and the variance of e_{ij}, denoted by V, is the variance among carriers within a test day.

Let S_r denote the repeatability standard deviation. For stainless steel, ceramic tile, and wood carriers, each S_r value pertains to the mean of three carriers in a test (*see* Section **C(f)** of the method), $S_r = [V_T + V/3]^{1/2}$, and the standard error of the mean (SEM) was calculated by $SEM = [V_T/9 + V/27]^{1/2}$, with 8 degrees of freedom. Because there was only one glass carrier per test day, the variance among test days and the variance among carriers within a test cannot be separated. For glass control carriers, S_r was the standard deviation of the 9 log densities, and the SEM for the mean of the 9 log densities was $SEM = S_r/\sqrt{9}$, with 8 degrees of freedom. For glass carriers, the S_r value pertains to a single control carrier. For any two carrier materials, a 95% confidence interval was calculated for the true mean difference between control carrier log densities. Also, the two-sided *P*-value was calculated for the null hypothesis that the true mean difference equals zero. The two materials were paired by test date, and the calculations were based on a paired-*t* statistic having 8 degrees of freedom.

Treated Carriers (Mean and Repeatability of the LR)

The LR was calculated for each combination of carrier material, treatment, and replicate for a total of 72 LR values in all. The LR values were partitioned into subsets, one subset for each sporicide treatment. Within each subset, the mean, S_r, and SEM were calculated separately for each carrier material;

SEM = $S_r/\sqrt{3}$. The standard deviation across the three replications is the repeatability S_r for the LR when the specified carrier material is used to test the specified sporicide treatment. The LR values for stainless steel, ceramic tile, and wood carriers were each based on three control carriers and three disinfected carriers, but the LR for glass was based on only one control carrier and one disinfected carrier. The LR values for each treatment subset were also subjected to an ANOVA using a two-factor, mixed model; LR was the response variable, surface material was the fixed effect factor, and replicate was the random effects factor. For each pair of carrier materials, the ANOVA output provided the two-sided *P*-value for a *t*-test of the null hypothesis that the two carrier materials produced the same true mean LR. The *t*-tests were based on 6 degrees of freedom.

Responsiveness of the TSM

For each carrier material, there were three replications of each test chemical and each replication included TSM tests of both the medium and high efficacy levels. Therefore, the LR values for the two efficacy levels were paired by replication. For each combination of carrier material and test chemical, an upper one-sided, paired *t*-test was conducted to test the null hypothesis that the true mean difference of LR values, high efficacy minus medium efficacy, was zero. The *t*-tests were based on 2 degrees of freedom. If the TSM is a responsive method, it will produce a significantly larger LR for the higher efficacy treatment compared to the medium treatment.

Contribution of Fractions A, B, and C

For each carrier material and each treatment or control, the counts within each *fraction* were aggregated across carriers and expressed as a percentage of the total count. For example, for the steel control carrier densities, the viable counts per carrier were summed across the 27 carriers to produce the total count of 393 230 791. For each *fraction*, the counts in that *fraction* were summed across the 27 carriers, producing the count of 379 700 000 for *fraction* A, 13 468 182 for *fraction* B, and 62 609 for *fraction* C. The corresponding percentages are 96.6, 3.4, and 0.0 for *fractions* A, B, and C, respectively.

Statistical Diagnostics

Conventional statistical diagnostics, such as normal probability plots and homogeneous variance tests, were routinely conducted during the statistical analysis. The diagnostics indicated that the data conformed to the assumptions required by the statistical methods.

AOAC Official Method 2008.05
Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on Selected Hard Nonporous and Porous Surfaces

Quantitative Three-Step Method
First Action 2008
Revised 2009

(Applicable for determination of sporicidal activity of liquid formulations against spores of the genus *Bacillus* on selected hard, nonporous and porous surfaces: glass, stainless steel, ceramic tile, and pine wood. The method is suitable for testing strains of *B. anthracis*.)

Caution: All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated by each institution. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms, refer to the CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) manual (4). *B. anthracis* is a select agent and therefore appropriate regulations must be followed (*see* BMBL Appendix F). Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Also, hydrochloric acid (HCl) is a highly corrosive liquid and considered hazardous. Personal protective equipment (PPE) or devices are recommended during the handling of these items for purposes of activation, dilution, or efficacy testing. PPE and a chemical fume hood should be used when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine best course of action. References to water mean reagent-grade water, except where otherwise specified (5). Exact adherence to the method, good laboratory practices, and quality control (QC) are required for proficiency and validity of the results.

A. Media and Reagents

(**a**) *Media.*—(*1*) *Nutrient broth (NB).*—Dehydrated NB. For use in rehydrating test organism and preparing nutrient agar (NA). (*2*) *NA.*—For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized NB. Boil mixture until agar is dissolved. If necessary, adjust pH to 7.2 ± 0.2. Dispense 5 mL portions into 16 × 100 mm screw-cap tubes. Larger tubes may be used as well. Autoclave for 20 min at 121 C. Remove from autoclave and slant tubes to form agar slopes. Dehydrated NA may be substituted: suspend 23 g NA/L water and dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121 C. (*3*) *NA with 5 g/mL $MnSO_4$:H_2O (amended NA).*—For spore production. Suspend 11.5 g NA in 495 mL water and add 5 mL 500 ppm $MnSO_4$:H_2O. Dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121 C. Pour agar into plates. (*4*) *Trypticase soy agar*

(TSA).—Poured in plates for microbe isolation and spread plating. (*5*) *Luria-Bertani (LB) broth*.—Dehydrated LB broth (e.g., Difco, Franklin Lakes, NJ); suspend 25 g LB broth in 1 L water, mix well, if necessary adjust pH to 7.0 ± 0.2, dispense in bottles, and autoclave for 15 min at 121 C; use as neutralizer. (*6*) *Modified LB broth.*—Neutralizer in HCl resistance test, add 20 mL 1 M NaOH to 1 L LB broth, mix well, dispense in bottles, and autoclave for 15 min at 121 C. (*7*) *LB broth with 0.1% (w/v) sodium thiosulfate.*—Neutralizer for sodium hypochlorite treatments. Add 1.0 g sodium thiosulfate to 1 L LB broth, mix well, dispense in bottles, and autoclave for 15 min at 121 C.

(**b**) *Manganese sulfate monohydrate, 500 ppm*.—Add 0.25 g manganese sulfate to 500 mL water. Filter-sterilize for use.

(**c**) *Sodium thiosulfate.*

(**d**) *Sterile water.*—Use sterile reagent grade water. Reagent grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement.

(**e**) *Test organism.—Bacillus subtilis* [American Type Culture Collection (ATCC) No. 19659] obtained directly from a commercial supplier (e.g., ATCC).

B. Apparatus

(**a**) *Certified biosafety cabinet (Class II)*.—Recommended to maintain an aseptic work environment.

(**b**) *Glass coupon*.—Hard surface carrier, 5 5 1 mm, Erie Scientific Co., Portsmouth, NH; custom order Part No. EPA-1101 (minimum order of 1000 pieces), single use.

(**c**) *Stainless steel coupon*.—Hard surface carrier, EJ Enterprise, Glen Burnie, MD; type 316, cut to 5 5 1 mm, single use.

(**d**) *Ceramic tile*.—Porous surface carrier, United States Ceramic Tile Co., North Canton, OH; No. 078-66, 12.5 sq. in. tile, cut to 5 5 1 mm, use unglazed side for inoculation, single use.

(**e**) *Untreated pine wood*.—Porous surface carrier, Home Depot, nontreated select pine, No. 1, cut to 5 5 1 mm, single use.

(**f**) *Sterile 1.5–2 mL microcentrifuge tubes*.—Fisher Scientific (Pittsburgh, PA), Cat. No. 05-408-129.

(**g**) *Sterile centrifuge tubes*.—Polypropylene, 15 mL conical tubes with conical bottoms, Fisher Scientific, Cat. No. 05-538-53D.

(**h**) *Dissecting forceps*.—VWR (Westchester, PA), Cat. No. 25607-195 or Fisher Scientific, Cat. No. 13-812-42.

(**i**) *Micropipet*.—Calibrated.

(**j**) *Positive displacement pipet*.

(**k**) *Desiccator*.

(**l**) *Water bath/chiller unit*.—Constant temperature, capable of maintaining 20 ± 1 C or specified temperature; e.g., Neslab (Pittsburgh, PA) RTE-221 or Nalgene Labtop Cooler.

(**m**) *Orbital shaker.*

(**n**) *Microcentrifuge.*

(**o**) *Microcentrifuge tube lid openers*.—USA Scientific (Ocala, FL), No. 1400-1508.

(**p**) *Sonicator.*—Ultrasonic cleaner (Branson, Danbury, CT, Model 1510 Bath Sonicator, or equivalent).

(**q**) *Floating microcentrifuge tube holder*.—For sonication, VWR, No. 60986-099.

(**r**) *Hematology rotator*.—Hematology Chemistry Mixer 346 (Fisher Scientific); or suitable mixer/shaker to provide gentle agitation during incubation.

(**s**) *Vortex mixer.*

(**t**) *Vortex adapters*.—Fisher Scientific, Cat. Nos. 1281161 and 1281211.

(**u**) *Certified timer*.—Any certified timer that can display time in seconds.

(**v**) *Test tubes*.—25 150 mm.

(**w**) *Ethyl alcohol*.—40 and 95%.

C. Operating Technique

(**a**) *Culture initiation*.—Initiate *B. subtilis* culture (e.g., use NB to rehydrate a lyophilized culture, and incubate the broth culture for 24 ± 2 h at 36 ± 1 C before streak inoculation). Streak inoculate a set (e.g., six) NA slopes and incubate 24 ± 2 h at 36 ± 1 C. Perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or other identification systems). Following incubation, store at 2–5 C. Maintain stock culture on NA slants by monthly (30 ± 2 days) transfers.

(**b**) *Production of B. subtilis spore suspension*.—Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., two tubes, depending on the amount of spore suspension desired) of NB and incubate tubes 24 ± 2 h on an orbital shaker at approximately 150 rpm at 36 ± 1 C. Use this culture to inoculate amended NA plates. Inoculate each plate with 500 L broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12–14 days at 36 ± 1 C. Following incubation, harvest the spores by adding 10 mL cold (2–5 C) sterile water to each plate. Using a spreader (e.g., bent glass rod), remove growth from plates and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, approximately 10 mL each). Centrifuge tubes at 5000 rpm for approximately 10 min at room temperature. Remove and discard supernatant. Resuspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5000 rpm for 10 ± 1 min. Remove and discard supernatant. Repeat twice. Resuspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2–5 C. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of five fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores versus vegetative cells should be at least 95. Spore suspension harvested from multiple plates can be combined and realiquoted into tubes for uniformity. Prior to inoculation of carriers, determine spore titer of the concentrated spore

Table 2. Summary of log densities observed for control carriers

Carrier material	Mean	95% CI[a]	Variance components		S_r
			V_T	V	
Glass	7.13	(7.07, 7.20)	NC[b]	NC	0.09
Steel	7.14	(7.06, 7.23)	0.0093	0.0094	0.11
Tile	6.85	(6.76, 6.95)	0.0059	0.0310	0.13
Wood	6.63	(6.57, 6.69)	0.0000	0.0200	0.08

[a] CI = Confidence interval for true mean.
[b] NC = Not calculable.

suspension by plating serial dilutions (e.g., 1.0 10^{-6} through 1.0 10^{-8}) onto TSA. Incubate plates for 24 ± 2 h at 36 ± 1 C and determine titer. *Note*: When harvested and processed, 10 plates of amended NA should provide 80–100 mL concentrated spore suspension. Diluting the suspension prior to carrier inoculation will be necessary; a spore titer of approximately 1.0 10^9 CFU/mL in the suspension should be adequate to achieve the target carrier count.

(**c**) *Carrier preparation*.—Visually screen coupons (carriers) for scratches, chips, or cracks. Discard those that are damaged or defective. Rinse glass, stainless steel, and ceramic tile carriers once with water, three times with 95% ethyl alcohol, and finally three times with water. Allow carriers to dry. Ensure pine wood carriers are free of sawdust after cutting. No rinsing is required. Place carriers in glass tubes (25 150 mm), 40 carriers per tube. Steam-sterilize all carrier types 45 min at 121 C with a 30 min dry cycle or sterilize for 2 h in hot air oven at 180 C. Cool. Transfer carriers to sterile plastic Petri dishes for inoculation (approximately 40 carriers per dish).

(**d**) *Carrier inoculation*.—Transfer 10 L spore suspension with a micropipet using aerosol barrier tips or positive displacement pipet onto a 5 5 1 mm sterile, dry coupon. Apply to one central spot on each carrier. Allow carriers to dry for minimum of 1 h or until visibly dry in an open Petri dish in a biosafety cabinet, and then for a minimum of 12 ± 2 h in a desiccator. Store inoculated carriers under desiccation for up to 30 days. Carriers must be discarded after use. *Note*: During carrier inoculation, mix inoculum frequently in a vortex mixer to ensure uniform distribution of spores. Verify carrier counts (per the method for control carriers) prior to test day; mean counts must be 5.0 10^6 to 5.0 10^7 spores/carrier. *Note*: Due to the occurrence of statistical variability in the LR data, it is recommended that the analyst target carrier counts of 7–7.5 logs to ensure confidence in a 6 LR.

(**e**) *Test chemical (e.g., sporicide, disinfectant) sample preparation*.—Aseptically prepare test chemical samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use 1.0 mL or 1.0 g sample test chemical to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Place approximately 1.5 mL of each test chemical or control (sterile water) in microcentrifuge tubes. Allow to equilibrate to appropriate temperature for 15–30 min.

(**f**) *Test procedure overview*.—Use three carriers per test chemical and three carriers for the water control (control carriers) per product test. Use one pair of sterile forceps per *fraction* for each test chemical. *Fractions* may be refrigerated briefly to allow for processing of other *fractions*. It is recommended that two analysts perform this method so that dilution and plating of the multiple *fractions* may be conducted as soon as possible.

Using sterile forceps, carefully transfer one inoculated carrier into each microcentrifuge tube labeled *fraction* A. Avoid touching inoculated area of carrier and sides of microcentrifuge tube. Discard carrier and tube if carrier touches sides of tube. Place *fraction* A tubes containing carriers and tubes containing test chemical(s) and sterile water (control) into chiller water bath at 20 ± 1 C, or use a labtop cooler to maintain temperature of the tubes. Equilibrate approximately 10 min. Add 400 L test chemical (test

Table 3. Mean difference between control carrier log densities, row material minus column material, followed by the associated 95% confidence interval

Material	Wood	Tile	Steel
Glass	0.50[a] (0.45, 0.55)	0.28[a] (0.19, 0.37)	–0.01 (–0.07, 0.05)
Steel	0.51[a] (0.45, 0.58)	0.29[a] (0.20, 0.38)	
Tile	0.23[a] (0.14, 0.32)		

[a] Statistically significantly different from 0 ($P < 0.001$).

Table 4. Mean LR values and S_r for each of the 24 combinations of treatment and carrier material

		Medium efficacy level		High efficacy level	
Test chemical	Carrier material	Mean	S_r	Mean	S_r
Glutaraldehyde	Glass	4.30	0.53	6.42	0.12
	Steel	4.12	0.25	6.46	0.09
	Tile	4.68	0.37	5.86	0.40
	Wood	0.66	0.06	1.81	0.29
NaOCl	Glass	2.42	0.35	5.08	1.04
	Steel	2.31	0.18	6.43	0.14
	Tile	2.12	0.21	5.98	0.22
	Wood	1.19	0.06	1.63	0.12
PA/HP	Glass	6.40	0.09	6.30	0.23
	Steel	6.35	0.12	6.41	0.17
	Tile	5.80	0.61	6.15	0.26
	Wood	4.32	0.64	5.80	0.23

carriers) or 400 L sterile water (control carriers) at 15 or 30 ± 5 s intervals to appropriate microcentrifuge tube (in triplicate). Allow contact of the carriers to the test chemical or water in *fraction* A tubes for the appropriate exposure period.

Following the exposure period, add 600 L of appropriate ice-cold neutralizer (e.g., LB broth) to each test chemical *fraction* A tube. Add 600 L LB broth as neutralizer for water control *fraction* A tubes. Slightly agitate tubes to thoroughly mix liquid components. Transfer each carrier using one pair of sterile forceps per carrier set (i.e., three carriers) from *fraction* A tube to corresponding *fraction* B tube. *Fraction* B tubes contain 400 L ice-cold (0–5 C) sterile water.

Place *fraction* A tubes in microcentrifuge, and centrifuge for 6 min ± 30 s at 13 000 rpm (15 500 × *g*). Remove 900 L from each tube without disturbing pellet. Discard supernatant. Carefully add 900 L ice-cold LB broth to each tube. Repeat two additional times. After third centrifugation, remove 900 L from each tube. Carefully add 100 L ice-cold LB broth to each *fraction* A tube and resuspend pellet by mixing in a vortex mixer 5 min ± 30 s (use the vortex adapter) at midrange speed. Add 800 L ice-cold LB broth to each *fraction* A tube. Proceed to dilution and plating if another analyst is available, or store *fraction* A tubes in refrigerator. *Note*: Fluid remaining in the *fraction* A tubes contains spores dislodged from carrier by exposure to the test chemical or water control. Consistent orientation of the microcentrifuge tubes in the microcentrifuge is important in locating the pellet. The pellet may range in size and be difficult to visualize depending on the treatment. *Fraction* B and *fraction* C tubes can be evaluated while *fraction* A tubes are being centrifuged.

Sonicate *fraction* B tubes 5 min ± 30 s using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner. After sonication is complete, add 600 L ice-cold LB broth to *fraction* B tubes. Mix on a vortex approximately 1 min. Transfer each carrier using one pair of sterile forceps per carrier set from *fraction* B tube to corresponding *fraction* C tube (*fraction* C tubes contain 400 L ice-cold LB broth). Proceed to dilution and plating if another analyst is available, or store *fraction* B tubes at 2–5 C; storage should be limited to 2 h. *Note*: Fluid remaining in the *fraction* B tubes contains spores dislodged from the carrier by sonication.

Place *fraction* C tubes in a hematology rotator inside incubator for 30 ± 2 min at 36 ± 1 C. Remove *fraction* C tubes after 30 ± 2 min rotation/incubation from incubator. Add 600 L ice-cold LB broth to each tube. The carriers remain in the *fraction* C tubes. Proceed to dilution and plating if another analyst is available, or store *fraction* C tubes at 2–5 C; storage should be limited to 2 h. *Note*: Fluid remaining in *fraction* C tubes contains spores dislodged from the carrier by gentle agitation for 30 min.

Mix each microcentrifuge tube thoroughly in a Vortex mixer before making dilutions. For each *fraction* and control tube, remove 100 L and serially dilute 10-fold in 900 L ice-cold LB broth. For each carrier, direct plate 100 L of the appropriate dilutions onto TSA to ensure obtaining counts within the target range of 30–300 CFU/plate. Incubate plates a minimum of 24 ± 2 h at 36 ± 1 C. Record control counts at 24 ± 2 h. Record treated carrier counts at 24 ± 2 h and at 48 ± 2 h. Confirm the identity of a minimum of one representative colony taken from at least one plate per treatment level (if available) using Gram staining, general growth media (e.g., TSA), or other confirmation procedure. *B. subtilis* is a large Gram-positive rod. On general growth media, *B. subtilis* colonies are opaque, rough, round, low, convex colonies with irregular margins. *Note*: After plating, dilution tubes may be stored at 2–5 C until the results are recorded; the tubes may be used for additional plating if initial plate counts are beyond the recommended target range.

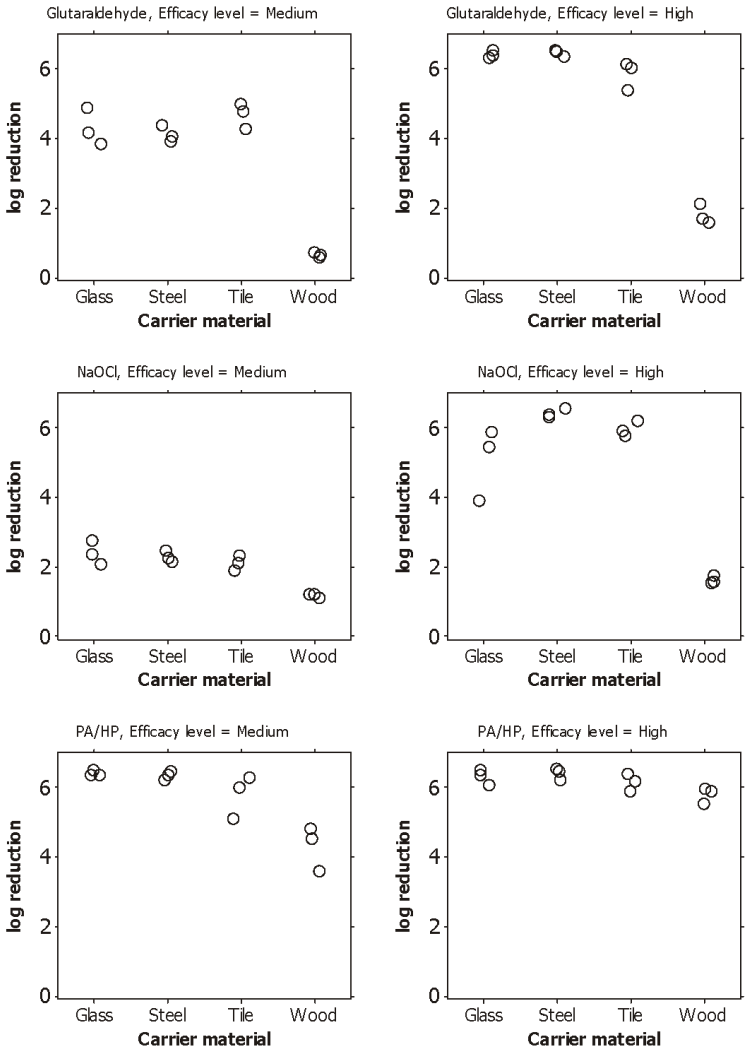

Figure 1. The triplicate log reduction values for each carrier material, with a separate panel for each combination of test chemical and efficacy level and the same axes in each panel.

Use counts that fall within 0–300 CFU/plate for calculations. Obtain the total number of spores per *fraction* by dividing the number of colonies counted in each *fraction* by its dilution, and account for volume plated. Obtain the total number of spores per carrier by adding the total number of viable spores per *fraction* for *fractions* A, B, and C. Determine log density (LD) of total number of viable spores per carrier by taking $\log_{10}$ (total number of spores per carrier). Determine the LR by subtracting the mean LD of test carriers from the mean LD of control carriers. Determine average LD and LR for each test chemical.

(**g**) *Neutralization confirmation.*—Use 12 microcentrifuge tubes. Add 400 L sterile water to tubes 1–6 and 400 L of test chemical to tubes 7–12. Allow tubes to equilibrate approximately 10 min at 20 ± 1 C (or other specified temperature). Add 600 L neutralizer in ice-cold LB broth (or only LB broth depending on the product) to tubes 4–6 (neutralizer controls). Add 600 L neutralizer in ice-cold LB broth to tubes 7–9 (ability of neutralizer to inactivate the test chemical). Gently mix. Add 10 L *B. subtilis* spore suspension (approximately 10^9 spores/mL) to each tube and mix on a vortex mixer for approximately 15 s. Incubate tubes for 30 ± 2 min at 20 ± 1 C (or temperature specified by test chemical manufacturer). After incubation, add 600 L ice-cold LB broth to tubes 1–3 (survival controls). Add 600 L ice-cold LB broth to tubes 10–12 (test chemical controls). Serially dilute each tube (e.g., 10 L into 990 L ice-cold LB broth or 100 L into 900 L ice-cold LB broth) to achieve plate counts of 30–300 CFU/plate. Plate 100 L of each dilution onto TSA. Incubate 24 ± 2 h at 36 ± 1 C. Count colonies on each plate. LD (CFU/mL) in tubes 1–3 and 4–6 should reflect the original spore suspension titer and should be within 1 log of each other. If the difference in LD between tubes 1–3 and 4–6 is greater than 1 log, then the neutralizer has a sporicidal effect. If the test

Table 5. Mean difference between LR values, row material minus column material, followed by the associated 95% confidence interval, calculated separately for each combination of test chemical and efficacy level

Test chemical	Material	Wood	Tile	Steel
		Medium efficacy level		
Glutaraldehyde	Glass	3.64[a] (3.03, 4.25)	–0.39 (–0.99, 0.22)	0.18 (–0.43, 0.79)
	Steel	3.46[a] (2.85, 4.07)	–0.57 (–1.17, 0.04)	
	Tile	4.03[a] (3.42, 4.63)		
		High efficacy level		
	Glass	4.62[a] (4.05, 5.18)	0.56 (–0.01, 1.13)	–0.04 (–0.61, 0.53)
	Steel	4.65[a] (4.08, 5.22)	0.60[b] (0.03, 1.17)	
	Tile	4.05[a] (3.48, 4.62)		
		Medium efficacy level		
NaOCl	Glass	1.22[a] (0.77, 1.68)	0.29 (–0.16, 0.75)	0.11 (–0.35, 0.57)
	Steel	1.12[a] (0.66, 1.57)	0.18 (–0.27, 0.64)	
	Tile	0.93[a] (0.48, 1.39)		
		High efficacy level		
	Glass	3.45[a] (2.35, 4.55)	–0.90 (–2.00, 0.20)	–1.35[b] (–2.45, –0.25)
	Steel	4.80[a] (3.69, 5.90)	0.45 (–0.65, 1.55)	
	Tile	4.35[a] (3.25, 5.45)		
		Medium efficacy level		
PA/HP	Glass	2.08[a] (1.08, 3.08)	0.60 (–0.40, 1.60)	0.06 (–0.95, 1.06)
	Steel	2.02[a] (1.02, 3.02)	0.55 (–0.45, 1.55)	
	Tile	1.48[b] (0.47, 2.48)		
		High efficacy level		
	Glass	0.50[b] (0.09, 0.91)	0.15 (–0.26, 0.56)	–0.11 (–0.52,0.30)
	Steel	0.61[b] (0.20, 1.02)	0.26 (–0.15, 0.67)	
	Tile	0.35 (–0.06, 0.76)		

[a] Statistically significantly different from 0 ($P < 0.01$).
[b] Statistically significantly different from 0 ($0.01 < P < 0.05$).

chemical is highly effective, LD in tubes 10–12 should be approximately 5–6 logs lower than LD in tubes 1–6. To be an effective neutralizer, LD in tubes 7–9 should be within 1 log of the LD in tubes 1–6. For this assay, produce a spore preparation according to the procedure for amended NA. Harvest growth from plates (e.g., five plates) per the method, except resuspend pellet after final centrifugation step in approximately 100 mL aqueous (40%) ethanol.

(**h**) *HCl resistance*.—Perform on each preparation of inoculated carriers. Conduct TSM procedure on 2.5 M HCl. Follow procedure as specified in part (**f**) with 2 and 5 min exposure periods with three inoculated carriers per time period. Include three control (sterile water) carriers to determine control carrier counts. Use LB broth modified with NaOH as the neutralizer instead of LB broth for HCl treatments. Perform test at 20 ± 1 C. Calculate LR. Spores should resist HCl for 2 min (i.e., based on presence of viable spores after 2 min) to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described. *Note*: Compared to the water control, anticipate LR of 0–3 at 2 min exposure and LR of 2–6 after the 5 min exposure.

Reference: *J. AOAC Int.* **93**, 259(2010).

Results and Discussion

The counts by fraction and the LD for each control carrier are listed in *Appendix A*. The control carrier LD, the variance among test days (V_T), the variance among carriers within a test day (V), and the repeatability standard deviation (S_r) are reported in Table 2. The variance among carriers within a test was always larger than the variance among test days, a result similar to the initial TSM validation study using glass carriers (2). The S_r values were similar for the four materials, ranging from 0.08 for wood to 0.13 for tile. The mean control LD values were also similar, varying from 6.63 for wood to

7.14 for steel. The range of 0.51 corresponds to a geometric mean LD for steel that is 3.2 times larger than for wood. The target range of the control counts (5.0 10^6 to 5.0 10^7 spores/carrier) was always successfully obtained for stainless steel and glass; however, the mean LD on the porous carriers was sometimes slightly below the specified range. Among the 9 test days, the mean of control carrier LD was between 6.53 and 6.70 on 1 day for tile carriers and on 8 days for wood carriers. The pairwise differences between mean control LD, except for glass minus steel, were highly statistically significant ($P < 0.001$). The glass and steel means were not statistically significantly different ($P = 0.65$). Even though the differences between means of LD for the carrier materials were statistically significant (Table 3), those differences are probably too small to be of practical importance. The titer of the spore suspension (10 L volume) ranged between 6.98 and 7.09 log CFU. Mean percent recovery of spores from all coupon types exceeded 10; 72, 62, 38, and 19% was obtained from stainless steel, glass, tile, and wood, respectively.

Counts by *fraction* and the LD for each disinfected carrier, including the associated LR values, are listed in *Appendix B*. The mean and S_r of LR values for each combination of carrier material and treatment are presented in Table 4. The LR is based on three control and three disinfected carriers per TSM test, except for the tests with glass that used one control and one disinfected carrier per test. The LR means ranged from 0.66 to 6.46 and the S_r ranged from 0.06 to 1.04. The LR values for each carrier material chemical treatment combination are plotted in Figure 1. Chemicals tested against wood carriers typically produced a smaller LR than the same test using glass, steel, or tile carriers. The vertical scatter of the three LR values was typically wider for an LR value between 2 and 6 than for an LR value <2 or >6. For each test disinfectant, the LR value for the high efficacy level was usually greater than the LR for the medium efficacy level. For each treatment, the mean LR values were compared for every pair of materials (Table 5). The results show that, except for testing the high efficacy level of PA/HP, the mean LR for wood carriers was always statistically significantly smaller than the means of the LR values for glass, steel, and tile. Of the 18 differences between the mean LR for wood carriers and the mean LR for each of the other materials, 14 were >1.0. Therefore, there was a difference of practical importance between the wood carrier LR and the corresponding LR for each of the other materials. Wood consists of lignin, celluloses, and hemicelluloses. It is likely that these components either inactivate the active components of these test chemicals or simply interact adversely, resulting in significant lowering of active component. Spore deposition within the pores and cavities of wood surface might also account for reduced ineffectiveness of the test chemicals.

The responsiveness of the TSM method for each carrier type and each test chemical was assessed by calculating the mean difference in LD, i.e., the LR for the test of the high efficacy level minus the LR for the test of the medium efficacy level (Table 6). Figure 2 presents, separately for each carrier material, the LR and mean LR for each test chemical at both the medium and high efficacy levels. Of the 12 mean differences, nine were statistically significant. Large, statistically significant differences indicate that the TSM was responsive to increased efficacy. The three differences that were not significant were tests involving PA/HP. Tests with glass, steel, and tile showed that the medium efficacy level of PA/HP killed almost all spores; the high efficacy level could not improve on that. A similar efficacy profile for the PA/HP was observed in the initial TSM validation study using glass. Wood treated with PA/HP was the notable exception, where the mean difference between the high and medium efficacy levels was statistically significant. Overall, the results demonstrate that the TSM with all three new carrier materials was responsive by successfully delineating between the two treatment efficacy levels.

Data from previous studies using glass carriers indicate the largest percentages of spores are contained in *fractions* A and B, thereby reducing the need for *fraction* C. For this study, the percentages of the total number of spores for each carrier material that were counted in the TSM *fractions* A, B, or C, are shown in Table 7. The coupon material impacted the distribution of the spores recovered from the control carriers. The results show that *fraction* A contributed a large percentage of the control count for nonporous carriers: 93%

Table 6. Mean difference, high efficacy LR minus medium efficacy LR, followed by the associated 95% lower, one-sided confidence limit, for each combination of test chemical and carrier material

	Test chemical		
Carrier material	Glutaraldehyde	NaOCl	PA/HP
Glass	2.13[a] (1.13)	2.67[a] (0.34)	–0.10 (–0.39)
Steel	2.35[b] (1.77)	4.12[b] (3.61)	0.07 (–0.13)
Tile	1.18[a] (0.20)	3.86[b] (3.42)	0.35 (–0.69)
Wood	1.15[b] (0.69)	0.44[a] (0.14)	1.48[a] (0.73)

[a] Statistically significantly greater than 0 ($0.01 < P < 0.05$).
[b] Statistically significantly greater than 0 ($P < 0.01$).

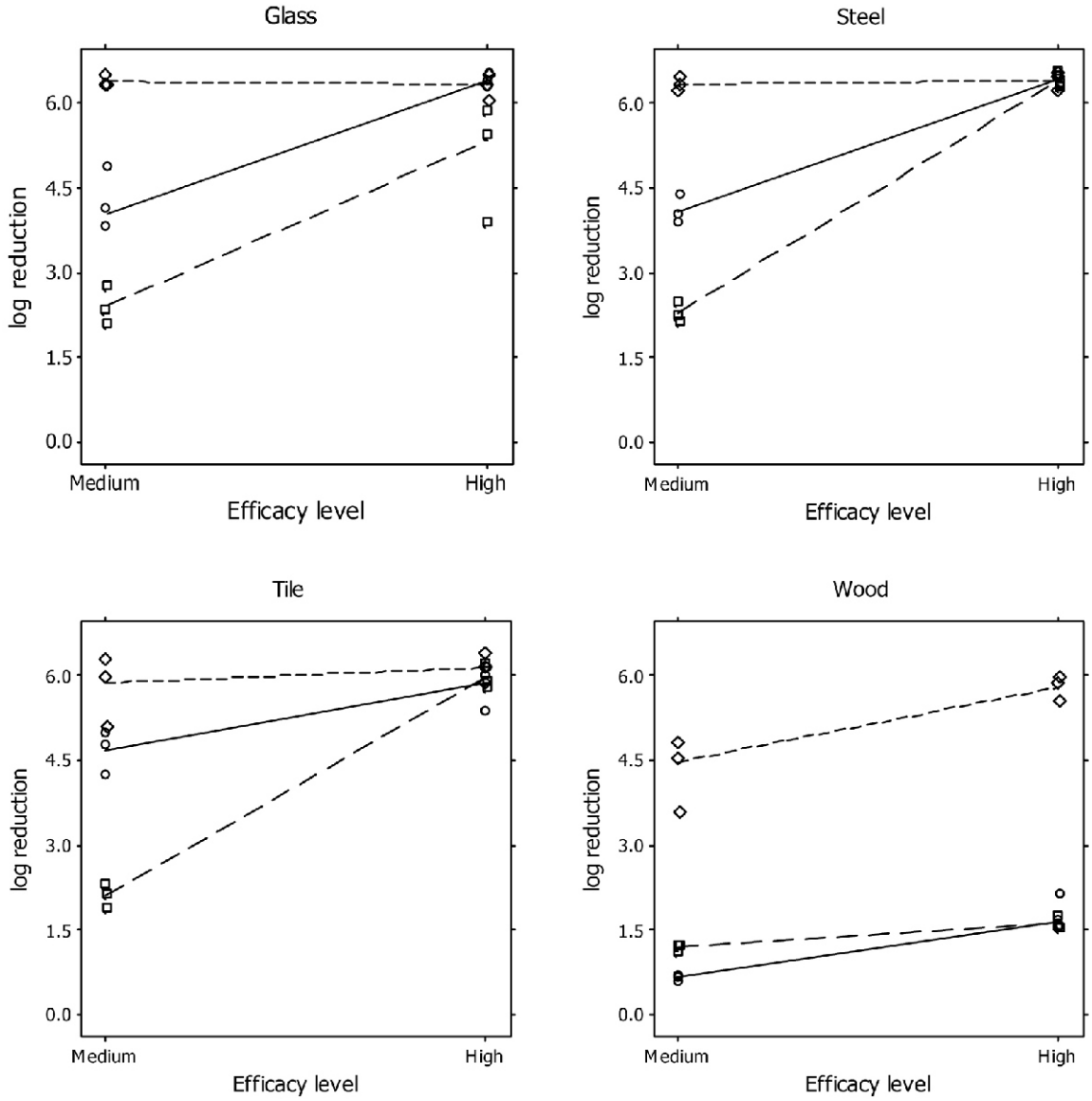

Figure 2. The log reduction (LR) for each test chemical at both efficacy levels, with a separate panel for each carrier material and the same axes in each panel. Within each panel, each symbol is the LR for a TSM test. For each test chemical, the lines connect the mean LR at the medium efficacy level and the mean LR at the high efficacy level. Glutaraldehyde is represented by circles and a solid line, NaOCl by squares and a dashed line, and PA/HP by diamonds and a dotted line.

for glass and 97% for steel; however, the percentage in *fraction* A was much smaller when the control coupons were made of porous materials: 14% for tile and 53% for wood. For the treated carriers, there appears to be an interaction between carrier materials and the test chemicals. The contribution of *fraction* B was greatest for the treated carriers for most material by test chemical combinations (*see* medium efficacy results in Table 7). The recovery of spores for the glutaraldehyde and nonporous material combinations was notably different where *fractions* B and C together accounted for 22% of the carrier counts for glass and 9% for steel for the medium treatment. The longer exposure period and the fixative properties of glutaraldehyde may in part sponsor easier removal (e.g., flaking off of spore inoculum) in *fraction* A on nonporous materials while providing an increase in spore adhesion in porous materials. When exposed to the medium treatment of NaOCl, *fractions* B and C together accounted for 90% for glass and 61% for steel. For tile and wood carriers exposed to the medium treatment of NaOCl or glutaraldehyde, *fractions* B and C together contributed at least 83% of the total carrier count. These results suggest that it is more difficult to remove spores from the porous carrier materials and that the last two steps of the method, sonication and gentle agitation, are important.

Table 7. Percentage of total counts across all carriers that were in each *fraction* (A, B, or C)

	Glutaraldehyde		NaOCl		PA/HP		Control
Fraction	Medium efficacy	High efficacy	Medium efficacy	High efficacy	Medium efficacy	High efficacy	—
				Glass			
A	78	NC[a]	10	94	NC	100	93
B	19	NC	73	5	NC	0	7
C	3	NC	17	1	NC	0	0
Total count	3400	0	2.16×10^5	1630	0	10	1.24×10^8
No. carriers	3	3	3	3	3	3	9
				Steel			
A	91	NC	38	100	100	NC	97
B	9	NC	61	0	0	NC	3
C	0	NC	0	0	0	NC	0
Total count	12183	0	7.43×10^5	10	20	0	3.93×10^8
No. carriers	9	9	9	9	9	9	27
				Tile			
A	17	99	3	0	2	50	14
B	75	0	94	20	92	50	83
C	8	1	2	80	6	0	3
Total count	1730	770	6.16×10^5	50	530	20	2.09×10^8
No. carriers	9	9	9	9	9	9	27
				Wood			
A	6	5	3	3	0	75	53
B	89	86	66	53	87	25	41
C	5	9	31	44	13	0	6
Total count	8.95×10^6	5.57×10^5	2.45×10^6	1.01×10^6	5306	40	1.21×10^8
No. carriers	9	9	9	9	9	9	27

[a] NC = Not calculable.

Conclusions

The TSM, modified with the additional coupon materials, successfully met the statistical parameters described in this report. Satisfactory validation parameters for control and treated carriers such as repeatability and responsiveness were obtained for controls and LR values associated with the chemical treatments. Based on the results, the proposed procedural change for the inclusion of the additional coupon materials is recommended. The revised method, including information on the additional carriers, is provided in this report.

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Appendix A. Counts and log densities for control carriers

Test date	Carrier material	Counts by *fraction* A	B	C	Log density
03/05/08	Glass	11100000	1540000	1636	7.10180
03/12/08		9700000	700000	10364	7.01747
03/18/08		17000000	618182	5091	7.24609
03/06/08		9700000	1530000	5727	7.05060
03/10/08		10700000	450000	182	7.04728
03/19/08		15300000	554546	1636	7.20020
03/07/08		11300000	1390909	4000	7.10363
03/11/08		14400000	990909	2000	7.18732
03/17/08		15900000	872727	5091	7.22474
03/05/08	Stainless steel	15100000	570000	5400	7.19522
03/05/08		16100000	760000	1691	7.22690
03/05/08		15100000	590000	2364	7.19569
03/12/08		10400000	550000	1809	7.03949
03/12/08		12200000	310000	6000	7.09747
03/12/08		10100000	294546	5500	7.01704
03/18/08		17000000	204546	1409	7.23568
03/18/08		18000000	350000	2145	7.26369
03/18/08		14200000	260909	1391	7.16024
03/06/08		5700000	430000	1382	6.78756
03/06/08		8300000	191818	809	6.92904
03/06/08		11000000	350000	1364	7.05505
03/10/08		17700000	700000	1055	7.26484
03/10/08		11700000	244546	818	7.07720
03/10/08		13500000	570000	855	7.14832
03/19/08		20200000	380000	1264	7.31347
03/19/08		15500000	600000	1936	7.20688
03/19/08		16000000	510000	1882	7.21780
03/07/08		11200000	281818	1936	7.06008
03/07/08		14600000	690000	2336	7.18447
03/07/08		9800000	630000	2964	7.01841
03/11/08		9900000	910000	3400	7.03396
03/11/08		11300000	980000	2800	7.08930
03/11/08		16100000	610000	3500	7.22307
03/17/08		15300000	440000	1800	7.19705
03/17/08		30000000	510000	3300	7.48449
03/17/08		13700000	550000	1500	7.15386
03/05/08	Ceramic tile	630000	6000000	232000	6.83645
03/05/08		2010000	8700000	93000	7.03354
03/05/08		880000	3500000	237000	6.66436
03/12/08		3000000	2700000	149000	6.76708
03/12/08		1410000	6300000	254000	6.90113
03/12/08		12727	6500000	1273	6.81385
03/18/08		1654545	8600000	440000	7.02916
03/18/08		1236364	2518182	300000	6.60794

Appendix A. (*continued*)

Test date	Carrier material	Counts by *fraction* A	B	C	Log density
03/18/08		163636	6900000	210000	6.86175
03/06/08		2010000	6600000	273000	6.94856
03/06/08		680000	5300000	225000	6.79274
03/06/08		980000	2800000	300000	6.61066
03/10/08		307273	9500000	300000	7.00463
03/10/08		99091	5200000	300000	6.74812
03/10/08		233636	6600000	300000	6.85331
03/19/08		2845455	10400000	173636	7.12772
03/19/08		618182	11200000	183636	7.07925
03/19/08		5100000	7000000	98182	7.08630
03/07/08		1130000	2836364	217000	6.62153
03/07/08		230909	1454545	182000	6.27125
03/07/08		1680000	7000000	224000	6.94959
03/11/08		545455	7700000	229091	6.92812
03/11/08		436364	6500000	315455	6.86045
03/11/08		1172727	6500000	260000	6.89942
03/17/08		254546	4700000	248182	6.71623
03/17/08		600000	10300000	160000	7.04376
03/17/08		372727	9700000	231818	7.01303
03/05/08	Wood	1980000	1670000	222000	6.58794
03/05/08		1410000	2150000	215000	6.57692
03/05/08		1970000	1560000	231000	6.57530
03/12/08		2240000	2100000	165455	6.65374
03/12/08		1820000	1010000	215455	6.48365
03/12/08		1660000	1720000	149091	6.54766
03/18/08		5500000	1745455	301818	6.87779
03/18/08		1400000	1290909	225455	6.46484
03/18/08		6600000	2318182	410000	6.96980
03/06/08		1180000	2050000	191000	6.53415
03/06/08		2940000	1430000	294000	6.66876
03/06/08		1270000	1960000	172000	6.53173
03/10/08		1520000	1830000	259000	6.55739
03/10/08		4600000	2110000	600000	6.86392
03/10/08		1360000	2530000	177000	6.60927
03/19/08		2381818	1454545	450000	6.63209
03/19/08		3300000	1672727	260000	6.71873
03/19/08		1781818	1881818	224546	6.58975
03/07/08		1250000	2560000	212000	6.60444
03/07/08		1460000	2080000	186000	6.57124
03/07/08		1170000	1310000	300000	6.44405
03/11/08		2072727	2890909	176364	6.71096
03/11/08		981818	1045455	219091	6.35148
03/11/08		3800000	1972727	300000	6.78338
03/17/08		2527273	1700000	380000	6.66344
03/17/08		2472727	1618182	510000	6.66284
03/17/08		2845455	2472727	223636	6.74365

Appendix B. Counts, log densities, and log reductions for disinfected carriers

Date	Test chemical	Efficacy level	Carrier material	Counts by *fraction* A	B	C	Log density	Log reduction
03/07/08	NaOCl	Medium	Glass	2709	17909	150	4.31740	2.78623
03/11/08				6800	81000	35000	5.08920	2.09812
03/17/08				12900	58000	1640	4.86058	2.36416
03/07/08			Stainless steel	9091	51818	610	4.78901	2.26657
03/07/08				10000	43636	280	4.73172	
03/07/08				16364	70909	330	4.94252	
03/11/08				4000	16364	70	4.31035	2.49920
03/11/08				20000	39091	100	4.77226	
03/11/08				30000	28182	180	4.76613	
03/17/08				74000	84545	640	5.20190	2.15509
03/17/08				58000	60909	520	5.07711	
03/17/08				64000	59091	250	5.09111	
03/07/08			Ceramic tile	1773	27818	836	4.48326	2.33010
03/07/08				11100	20545	782	4.51091	
03/07/08				4200	2909	100	3.85788	
03/11/08				1	86000	2500	4.94695	1.90431
03/11/08				170	67000	4200	4.85352	
03/11/08				80	148000	1400	5.17458	
03/17/08				1	50000	1000	4.70758	2.13763
03/17/08				960	153000	2600	5.19468	
03/17/08				3000	25000	700	4.45788	
03/07/08			Wood	10600	262000	69000	5.53352	1.12847
03/07/08				5800	151000	50000	5.31555	
03/07/08				8800	124000	110000	5.38525	
03/11/08				22091	92727	64545	5.25373	1.21804
03/11/08				2273	180909	79091	5.41875	
03/11/08				12364	217273	100909	5.51923	
03/17/08				5727	126364	75455	5.31711	1.23061
03/17/08				4455	225455	142727	5.57129	
03/17/08				2455	231818	74545	5.48970	
03/07/08		High	Glass	1530	20	0	3.19033	3.91330
03/11/08				0	0	20	1.30103	5.88629
03/17/08				0	60	0	1.77815	5.44658
03/07/08			Stainless steel	0	0	0	0.69897	6.38869
03/07/08				0	0	0	0.69897	
03/07/08				0	0	0	0.69897	
03/11/08				0	0	0	0.69897	6.31613
03/11/08				0	0	0	0.69897	
03/11/08				10	0	0	1.00000	
03/17/08				0	0	0	0.69897	6.57950
03/17/08				0	0	0	0.69897	
03/17/08				0	0	0	0.69897	
03/07/08			Ceramic tile	0	0	0	0.69897	5.91515
03/07/08				0	0	0	0.69897	
03/07/08				0	0	0	0.69897	

Appendix B. (*continued*)

Date	Test chemical	Efficacy level	Carrier material	Counts by *fraction* A	B	C	Log density	Log reduction
03/11/08				0	10	0	1.00000	5.79565
03/11/08				0	0	0	0.69897	
03/11/08				0	0	40	1.60206	
03/17/08				0	0	0	0.69897	6.22537
03/17/08				0	0	0	0.69897	
03/17/08				0	0	0	0.69897	
03/07/08			Wood	10100	30000	30000	4.84572	1.76591
03/07/08				12500	30000	30000	4.86034	
03/07/08				1000	22900	17400	4.61595	
03/11/08				10	56364	39091	4.97984	1.58630
03/11/08				545	58182	44545	5.01399	
03/11/08				273	72727	50909	5.09310	
03/17/08				1909	51818	43636	4.98840	1.53904
03/17/08				1000	56364	34545	4.96336	
03/17/08				2455	158182	156364	5.50106	
03/06/08	PA/HP	Medium	Glass	0	0	0	0.69897	6.35163
03/10/08				0	0	0	0.69897	6.34831
03/19/08				0	0	0	0.69897	6.50123
03/06/08			Stainless steel	0	0	0	0.69897	6.22491
03/06/08				0	0	0	0.69897	
03/06/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	6.46448
03/10/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	
03/19/08				0	0	0	0.69897	6.34639
03/19/08				20	0	0	1.30103	
03/19/08				0	0	0	0.69897	
03/06/08			Ceramic tile	0	10	0	1.00000	5.98467
03/06/08				0	0	0	0.69897	
03/06/08				0	0	0	0.69897	
03/10/08				0	10	0	1.00000	5.11375
03/10/08				0	20	20	1.60206	
03/10/08				0	450	10	2.66276	
03/19/08				0	0	0	0.69897	6.29844
03/19/08				10	0	0	1.00000	
03/19/08				0	0	0	0.69897	
03/06/08			Wood	0	200	20	2.34242	4.82407
03/06/08				0	0	0	0.69897	
03/06/08				0	136	30	2.22106	
03/10/08				0	250	10	2.41497	4.54294
03/10/08				0	10	0	1.00000	
03/10/08				0	860	110	2.98677	
03/19/08				10	840	70	2.96379	3.60272
03/19/08				0	1120	400	3.18184	
03/19/08				10	950	10	2.98677	
03/06/08		High	Glass	10	0	0	1.00000	6.05060

Appendix B. (*continued*)

Date	Test chemical	Efficacy level	Carrier material	Counts by *fraction* A	B	C	Log density	Log reduction
03/10/08				0	0	0	0.69897	6.34831
03/19/08				0	0	0	0.69897	6.50123
03/06/08			Stainless steel	0	0	0	0.69897	6.22491
03/06/08				0	0	0	0.69897	
03/06/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	6.46448
03/10/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	
03/19/08				0	0	0	0.69897	6.54708
03/19/08				0	0	0	0.69897	
03/19/08				0	0	0	0.69897	
03/06/08			Ceramic tile	0	0	0	0.69897	5.88433
03/06/08				0	10	0	1.00000	
03/06/08				10	0	0	1.00000	
03/10/08				0	0	0	0.69897	6.16972
03/10/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	
03/19/08				0	0	0	0.69897	6.39879
03/19/08				0	0	0	0.69897	
03/19/08				0	0	0	0.69897	
03/06/08			Wood	0	0	0	0.69897	5.87925
03/06/08				0	0	0	0.69897	
03/06/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	5.97789
03/10/08				0	0	0	0.69897	
03/10/08				0	0	0	0.69897	
03/19/08				10	10	0	1.30103	5.54651
03/19/08				10	0	0	1.00000	
03/19/08				10	0	0	1.00000	
03/05/08	Glutaraldehyde	Medium	Glass	109	55	0	2.21388	4.88792
03/12/08				445	191	100	2.86709	4.15037
03/18/08				2091	409	0	3.39794	3.84815
03/05/08			Stainless steel	1000	209	0	3.08246	4.05364
03/05/08				1591	218	0	3.25746	
03/05/08				1073	236	0	3.11697	
03/12/08				227	18	0	2.38997	4.38580
03/12/08				564	45	0	2.78468	
03/12/08				627	36	0	2.82193	
03/18/08				2109	36	0	3.33152	3.90652
03/18/08				2491	182	0	3.42696	
03/18/08				1409	100	10	3.18158	
03/05/08			Ceramic tile	0	36	10	1.66618	4.98221
03/05/08				9	82	0	1.95861	
03/05/08				0	82	10	1.96293	
03/12/08				91	118	0	2.32034	4.79333
03/12/08				18	73	20	2.04497	

Appendix B. (*continued*)

Date	Test chemical	Efficacy level	Carrier material	Counts by *fraction* A	B	C	Log density	Log reduction
03/12/08				0	55	0	1.73676	
03/18/08				145	282	40	2.66957	4.26640
03/18/08				0	327	40	2.56499	
03/18/08				36	245	10	2.46511	
03/05/08			Wood	30000	1070000	30000	6.05308	0.58939
03/05/08				30000	650000	23100	5.84702	
03/05/08				30000	1120000	30000	6.07188	
03/12/08				69000	650000	55000	5.88874	0.66821
03/12/08				67000	940000	75000	6.03423	
03/12/08				26091	500000	46000	5.75747	
03/18/08				91000	1350000	50000	6.17348	0.71077
03/18/08				71000	780000	84000	5.97081	
03/18/08				115000	910000	61000	6.03583	
03/05/08		High	Glass	0	0	0	0.69897	6.40283
03/12/08				0	0	0	0.69897	6.31850
03/18/08				0	0	0	0.69897	6.54712
03/05/08			Stainless steel	0	0	0	0.69897	6.50697
03/05/08				0	0	0	0.69897	
03/05/08				0	0	0	0.69897	
03/12/08				0	0	0	0.69897	6.35236
03/12/08				0	0	0	0.69897	
03/12/08				0	0	0	0.69897	
03/18/08				0	0	0	0.69897	6.52090
03/18/08				0	0	0	0.69897	
03/18/08				0	0	0	0.69897	
03/05/08			Ceramic tile	0	0	0	0.69897	6.14582
03/05/08				0	0	0	0.69897	
03/05/08				0	0	0	0.69897	
03/12/08				760	0	0	2.88081	5.40110
03/12/08				0	0	0	0.69897	
03/12/08				0	0	0	0.69897	
03/18/08				0	0	0	0.69897	6.03364
03/18/08				0	0	0	0.69897	
03/18/08				0	0	10	1.00000	
03/05/08			Wood	1182	17273	3000	4.33152	1.59442
03/05/08				2636	27091	3000	4.51491	
03/05/08				909	18364	2720	4.34228	
03/12/08				727	9909	2291	4.11151	2.13501
03/12/08				455	15273	8100	4.37707	
03/12/08				818	59000	2045	4.79144	
03/18/08				1091	153000	9200	5.21296	1.69257
03/18/08				19600	120000	8200	5.16967	
03/18/08				1636	59000	10500	4.85209	