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June 2016

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U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Richland, Washington 99352

Summary

The performance of a macrofoam-swab sampling method was evaluated using *Bacillus anthracis* Sterne (BAS) and *Bacillus atrophaeus* Nakamura (BG) spores applied at nine low target amounts (2–500 spores) to positive-control plates and test coupons (2 in. × 2 in.) of four surface materials (glass, stainless steel, vinyl tile, and plastic). Test results from cultured samples were used to evaluate the effects of surrogate, surface concentration, and surface material on recovery efficiency (RE), false negative rate (FNR), and limit of detection. For RE, surrogate and surface material had statistically significant effects, but concentration did not. Mean REs were the lowest for vinyl tile (50.8% with BAS, 40.2% with BG) and the highest for glass (92.8% with BAS, 71.4% with BG). FNR values ranged from 0 to 0.833 for BAS and 0 to 0.806 for BG, with values increasing as concentration decreased in the range tested (0.078 to 19.375 CFU/cm^2, where CFU denotes "colony forming unit"). Surface material also had a statistically significant effect. A FNR-concentration curve was fit for each combination of surrogate and surface material. For both surrogates, the FNR curves tended to be the lowest for glass and highest for vinyl title. The FNR curves for BG tended to be higher than for BAS at lower concentrations, especially for glass. Results using a modified Rapid Viability-Polymerase Chain Reaction (mRV-PCR) analysis method were also obtained. The mRV-PCR results and comparisons to the culture results will be discussed in a subsequent report.

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This report was initially written as a manuscript for submission to a journal, and hence uses the organizational structure required by the journal. The PNNL report formatting was applied for this version of the manuscript. This report contains appendices not contained in the journal manuscript.

Acronyms and Abbreviations

%RSD	percent relative standard deviation
BAS	*Bacillus anthracis* Sterne
BG	*Bacillus atrophaeus* Nakamura
BSL	Biosafety Level
CDC	Centers for Disease Control and Prevention
CFU	colony-forming units
FNR	false negative rate [the probability of a positive (contaminated) sample incorrectly being identified as a negative (not contaminated) sample]
GAO	Government Accountability Office
LOD	limit of detection
LRN	Laboratory Response Network
mRV-PCR	modified Rapid Viability-Polymerase Chain Reaction
PBS-T	phosphate buffered saline containing 0.02% Tween® 80
PCD	probability of correct detection
RE	recovery efficiency
SD	standard deviation
SSP	split-split-plot
TGA	thermogravimetric analysis
TSA	tryptic soy agar
WP	whole plot

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1.0 Introduction

After the intentional contamination of several U.S. facilities in 2001 with *Bacillus anthracis* sent in letters, questions were raised concerning the performance of surface-sampling techniques for detecting indoor *B. anthracis* contamination. The Centers for Disease Control and Prevention (CDC) noted in CDC (2006) that over 125,000 samples were taken from the contaminated buildings during the 2001 incident and processed by the Laboratory Response Network (LRN). The LRN is an integrated network of state and local public health, federal, military, and international laboratories that can respond to biological and chemical terrorism, as well as other public health emergencies. However, results from surface samples in the 2001 contaminated U.S. facilities were inconsistent. In some cases, contamination at a given location in a facility was not detected with initial samples but was detected in subsequent samples (GAO 2003). A Government Accountability Office (GAO) investigation of the 2001 incident concluded that validated sampling methods and statistical sampling plans are needed to provide confidence that contamination is absent when all sample results are negative (GAO 2005a, 2005b). This conclusion strongly reinforces the need to adequately characterize the performance of sampling and analysis methods used to respond to biothreats in order to protect the public.

After the 2001 anthrax incident, several research groups developed sampling and analysis methods and investigated (in laboratory studies) the performance of those methods using swab, wipe, and vacuum sampling devices for *B. anthracis* surrogates on various surfaces (Almeida et al. 2008; Brown et al. 2007a, 2007b, 2007c; Buttner et al. 2001; Buttner et al. 2004a, 2004b; Calfee et al. 2013; Edmonds 2009; Edmonds et al. 2009; Estill et al. 2009; Frawley et al. 2008; Hodges et al. 2006; Krauter et al. 2012; Lewandowski et al. 2010; Nellen et al. 2006; Perry et al. 2013; Quizon et al. 2007; Rose et al. 2004; Sanderson et al. 2004; Valentine et al. 2008; Valiante et al. 2003). In addition, the CDC performed validation studies on macrofoam-swab (Hodges et al. 2010) and cellulose-sponge-wipe (Rose et al. 2011) sampling methods.

A review of laboratory studies (Piepel et al. 2012) identified several gaps in the data on performance of swab, wipe, and vacuum sampling methods. A key gap was that only one study investigated low concentrations of *B. anthracis* surrogates and quantified the false negative rate (FNR) for the sampling and analysis methods investigated. A "false negative" occurs when contamination is not detected from a sample collected at a contaminated location. A false negative may be obtained because of inefficiencies (i.e., biases) and uncertainties (i.e., imprecision) at any step of the sampling and analysis process (sample collection, storage/transportation, processing/extraction, and analytical). False negatives may occur during preliminary screening or characterization sampling at low contamination levels, as well as during clearance sampling following a decontamination process. To address the GAO's concerns about method validation and increasing the confidence in negative results, it is critical to have a better understanding of FNRs and how they are influenced by different *B. anthracis* surrogates, concentrations, and surface materials. Ultimately, environmental sample collection and analysis for *B. anthracis* contamination requires methods with sufficiently low FNRs and limits of detection (LODs) to provide reasonable assurance of public safety.

Although the macrofoam-swab sampling method addressed in this study was extensively tested by the CDC (Hodges et al. 2006, 2010), it has not been tested at low concentrations of *B. anthracis* surrogates that yield false negatives. The study described in this report addressed this gap.

The macrofoam-swab sampling method was evaluated by testing low concentrations of *Bacillus anthracis* Sterne (BAS) and *Bacillus atrophaeus* Nakamura (BG) deposited on coupons of four relatively nonporous surface materials, followed by surface sampling, extraction, and analysis. Both culture and modified Rapid Viability-Polymerase Chain Reaction (mRV-PCR) analysis methods were used to detect and quantify the recovered spores. This report focuses on the culture results, while a subsequent report will focus on the mRV-PCR results and comparison of the culture and mRV-PCR results.

The culture results were used to evaluate the effects of *B. anthracis* surrogates, concentrations, and surface materials on FNR, recovery efficiency (RE), and LOD. The results of this study provide new insights for interpreting negative results from surface samples collected using macrofoam swabs. Also, the results from evaluating the dependence of RE, FNR, and LOD on different surface materials and concentrations of *B. anthracis* surrogates will be of high interest in the public-health field.

2.0 Materials and Methods

2.1 Study Overview

This study was performed to investigate the performance of a macrofoam-swab method (Hodges et al. 2006, 2010) for a range of low surface concentrations of two *Bacillus anthracis* surrogates on coupons of four surface materials using one of two analytical methods. The two surrogates were BAS and BG, and the surface concentrations tested are discussed subsequently. The surface materials were glass, stainless steel, vinyl tile, and plastic (acrylic ceiling light panel). The analytical methods were culturing and a mRV-PCR method.

As described by Piepel and Hutchison (2014), this study was conducted as a split-split-split-plot experiment (Jones and Nachtsheim 2009, Kowalski et al. 2010) in which surrogate was the whole-plot factor, concentration was the sub-plot factor, and analytical method was the sub-sub-plot factor. The fourth factor tested in the experiment was surface material, which was varied within the sub-sub-sub plots. The three splits are associated with three restrictions on randomization, such that the variance-covariance matrix of the resulting experimental data has diagonal and off-diagonal entries that are different functions of four variance components (Jones and Nachtsheim 2009, Kowalski et al. 2010).

This report discusses only the culture portion of the study, for which the primary response variables were RE and FNR. A subsequent report will (i) discuss the mRV-PCR portion of the study and (ii) compare the FNR results from the culture and mRV-PCR portions of the study. The culture and mRV-PCR portions of the study each have a split-split-plot (SSP) experimental structure involving the remaining three factors (surrogate, concentration, and surface material). For each portion, the variance-covariance matrix has diagonal and off-diagonal entries that are different functions of three variance components. For this report, appropriate statistical analysis methods (Jones and Nachtsheim 2009, Kowalski et al. 2010) were used to account for the SSP structure of the culture portion of the study.

There were 34 tests with culture analysis, which are listed in Table 6.1 of Piepel and Hutchison (2014). The portion of Table 6.1 with culture analysis tests is given in Table A.1 of Appendix A. For simplicity, the Test Run numbers rather than the Test numbers in Table A.1 are referred to in this report. Test Runs 1–8 and Test Runs 17–25 used the BAS surrogate, while Test Runs 9–16 and Test Runs 26–34 used the BG surrogate. The testing was conducted in two blocks, Test Runs 1-16 and Test Runs 17-34. One surface concentration of BG or BAS was investigated for each test run. In Test Runs 1–16, target concentrations of 0.078, 0.194, 0.388, 0.581, 0.775, 0.969, 3.875, and 19.375 colony-forming units (CFUs) per cm^2 were used on coupons of the four surface materials. These target concentrations were calculated starting with the target number of spores per test coupon or positive-control plate (2, 5, 10, 15, 20, 25, 100, and 500) and dividing by the surface area of the test coupons (25.8064 cm^2). The target numbers of spores per coupon were selected based on preliminary test results according to a process described in Section 5.1 of Piepel and Hutchison (2014).

In Test Runs 17–34, the same target concentrations were used as in Test Runs 1–16, with one exception. The highest target concentration (500 CFU/coupon, 19.375 CFU/cm^2) was replaced with a

new, second-lowest target concentration (4 CFU/coupon, 0.155 CFU/cm^2). That replacement was made because the tests (among Test Runs 1–16) at the two highest target concentrations (100 and 500 CFU/coupon) did not yield false negatives for any of the surface materials. Hence, it was not necessary to perform Test Runs 17–34 with 500 CFU/coupon. Further, the tests with target concentrations up to 10 CFU/coupon (0.388 CFU/cm^2) exhibited the highest variability in FNR (as expected). Therefore, the target concentration of 500 CFU/coupon in Test Runs 1–16 was replaced with a target concentration of 4 CFU/coupon in Test Runs 17–34 to reduce the uncertainty in the data at that concentration.

Within each of the four whole plots (WPs), corresponding to Test Runs 1–8, 9–16, 17–25, and 26–34, the concentrations were tested in the same randomized order. For each of Test Runs 1–34, six replicate coupons of each of the four surface materials were used. The six coupons of each surface material were assigned in a balanced way to the 24 locations for test coupons in a biosafety cabinet so that materials would appear (i) three times in each of the two rows and (ii) two times together in a column (Piepel and Hutchison 2014). Figure 2.1 displays the layout of the 24 test coupons within a biosafety cabinet for a given test.

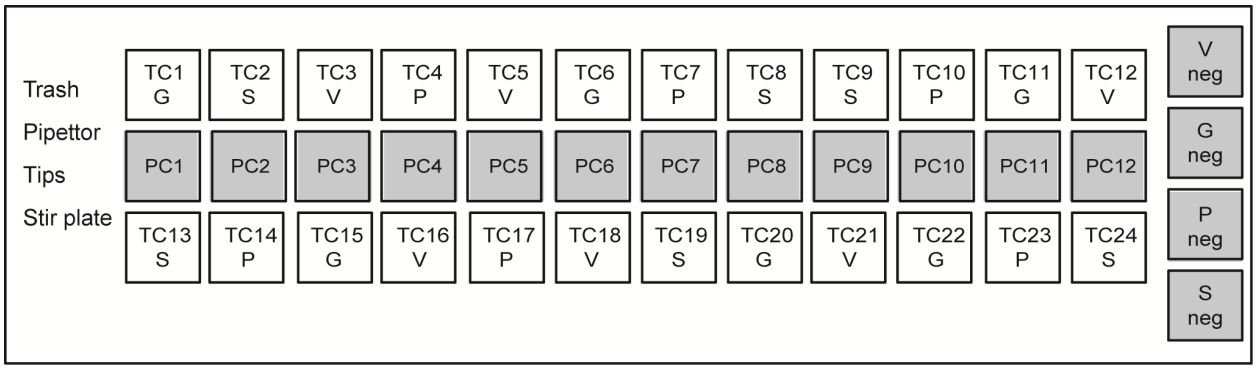

Figure 2.1. Testing Configuration in a Biosafety Cabinet Showing the Locations of the 24 Test Coupons (TC1 – TC24; white fill), the 12 Positive Controls (PC1 – PC12; gray fill), and the Four Negative Coupon Controls (neg; gray fill) for Each Test. The surface materials are denoted by G = glass, S = stainless steel, V = vinyl tile, and P = plastic light cover panel. Additionally, the needed consumables and equipment (trash, pipettor, tips, and stir plate) were located to the left of the test coupons. The working surfaces of the biosafety cabinets and coupon locations are not drawn to scale and are a pictorial representation only.

Because each combination of surrogate and concentration was tested in two different WPs, a total of 12 coupons were tested for each surface material with each combination of surrogate and concentration. Test Runs 17–25 and Test Runs 26–34 contain nine tests rather than the eight tests contained in Test Runs 1–8 and Test Runs 9–16. This resulted from testing the new target concentration (4 CFU/coupon, 0.155 CFU/cm^2) twice in Test Runs 17–25 and Test Runs 26–34 to provide results for 12 replicate coupons of each surface material.

As noted previously, the coupons for each test were assigned in a balanced way to the 24 test-coupon locations in each biosafety cabinet. Two persons were assigned to the three steps of the sampling and analysis process (surrogate deposition, sample collection and extraction, and sample analysis) in a

balanced way. These random and balanced assignments (Piepel and Hutchison 2014) protected against confounding any effects of test locations and people performing the tests with the primary test variables (i.e., surrogate, surrogate concentration, and surface material).

In this study, the CDC macrofoam-swab surface sampling procedure (CDC 2012) and the LRN swab processing procedure (Hodges et al. 2010) were used. The macrofoam-swab procedures for sampling surfaces and processing samples provide a (i) standardized method of collecting and processing swab samples of environmental surfaces to detect *Bacillus* spores, and (ii) culture-based detection and quantification method to estimate the amount of contamination for a sampled location.

Spores were removed from the swab by mechanical extraction in phosphate buffered saline (Invitrogen, Waltham, MA) containing 0.02% Tween® 80 (Sigma-Aldrich, St. Louis, MO), which is denoted PBS-T. Tween® 80 is a surfactant that supports the extraction process. The eluted suspension was then subject to traditional culture methods that included a dilution series when required (for higher numbers of CFUs). Samples were plated on tryptic soy agar (TSA) obtained from BD Bioscience (Franklin Lakes, NJ), or onto membrane filters that were placed on TSA, thus allowing the spores to germinate and form countable colonies. After 18- to 48-hour incubation (see subsequent discussion), CFUs were counted.

The environmental sampling and swab processing procedures used in this study were modified slightly to reflect a Biosafety Level (BSL) 2 for BAS rather than BSL3 that is necessary for virulent *B. anthracis* (HHS 1999). Even though BG is BSL1, the BSL2-modified procedures were used for BG as well as BAS.

2.2 Bacterial Strains and Culture Methods

Two *B. anthracis* surrogates were used in these tests: BAS and BG. The BAS ($pX01^+$ $pX02^-$) was kindly provided by Dr. David Wunschel (Pacific Northwest National Laboratory) and BG ATCC 9372 was purchased from American Type Culture Collection (Manassas, VA). The BAS was grown at 35°C and BG was grown at 30°C. Spores were prepared as previously described by Buhr et al. (2008). Briefly, overnight cultures for each strain were grown in tryptic soy broth at the appropriate temperature. Overnight cultures were diluted 1:100 in 1.6% nutrient broth with CCY salts (Buhr et al. 2008) and grown at the appropriate temperature with shaking at 200 rpm. BAS spores were harvested (after 72 hours and 7 days of sporulation, respectively) by centrifugation for 10 minutes at 10,000 × *g* at 4°C. Spore pellets were resuspended in sterile water and stored at 4°C for 7 days to enhance vegetative cell lysis. Spores were then washed three times in sterile water prior to use. BG spores were filtered through a 41-µm filter to remove cellular debris. Spore purity was evaluated using phase contrast microscopy and all preparations were >95% phase bright. Three biological spore preparations were made for each strain and pooled prior to use to reduce preparation variability. Enumeration of spore stock solutions was done periodically throughout the duration of the study by diluting 1:10 in PBS-T. Prior to each test, spores were diluted in PBS-T to the target spore number.

2.3 Sample Surface Materials

Coupons were cut to the recommended 2 in. × 2 in. (25.8064 cm^2) size for macrofoam-swab samples (Hodges et al. 2010). The surface materials selected for testing (stainless steel, glass, vinyl tile, and plastic light panel) are commonly found inside buildings. While these materials may not all typically be sampled by the macrofoam-swab method, the materials were chosen to provide a range of surface roughness values for relatively nonporous materials. Krauter et al. (2012) found that surface roughness significantly affected the FNR results for sponge-wipe samples.

Stainless steel was chosen as one of the surface materials because it has been used in many previous sampling studies (Piepel et al. 2012) and is a universally recognized material. The stainless steel coupons were cut from 18 ga (0.0480 in) 316L Stainless Steel Sheets with 2B Finish (Stainless Supply Architectural Metal Solutions, Monroe, NC). Daltile® Circa Glass Spring Green Tiles (2 in. × 2 in.) were purchased from Home Depot (Model #CG0222HD1P, Store SKU #354111). The vinyl-tile coupons were cut from Armstrong® Excelon® Vinyl Composition Tile #51830 (Armstrong World Industries Inc., Lancaster, PA). The plastic coupons were cut from an acrylic, clear cracked-ice, ceiling light panel (Professional Plastics, Fullerton, CA).

The coupons of the four surface materials were washed in a 1% solution of Liqui-nox® (Alconox Inc., New York, NY), rinsed three times in deionized water, and air dried. All coupons were placed into Chex-All® Sterilization Pouches (Propper Manufacturing Company Inc., Long Island City, NY) and sterilized by autoclaving for 60 minutes on a gravity cycle at 121°C. For each test, clean and sterile coupons were placed on petri dishes within a biosafety cabinet in a predesignated order as illustrated in Figure 2.1.

The surface roughness of stainless steel, vinyl tile, and glass coupon materials was measured using a NT1000 Optical Profiler (Veeco Instruments Inc., Plainview, NY) with a 5× magnification. For the plastic ceiling tile, a DekTak® Contact Profilometer (Veeco Instruments Inc., Plainview, NY) was used because this material was incompatible with the Veeco NT1000.

Coupon porosity was measured using classical adsorption/desorption nitrogen isotherms with a Quadrasorb SI analyzer (Quantachrome Instruments, Boynton Beach, FL). The specific surface area and pore size of each sample were determined by the Brunauer–Emmett–Teller method and the Barrett–Joyner–Halenda method, respectively.

Thermogravimetric analysis (TGA) was performed to identify any form of subsurface porosity in each of the four surface materials. TGA measures the change in mass of a material with respect to increasing/decreasing temperature. A microgram mass balance is at the core of the instrument, housed within a furnace. The high level of mass sensitivity necessitates a small sample size, so a small section of each surface material was cut to have approximately equal surface area (average of 6.6 mm^2). The samples are pictured in Figure B.1 of Appendix B. Samples were stored overnight in a vacuum oven set to 40°C, so that any residual water was removed before testing. Following this, 2 µL of PBS-T was pipetted onto the surface of each sample. Exposed surface area was closely matched for each material, given the need to access only the surface absorption of a tile. This provided an equal area of material-

droplet interaction (see Figure B.1 in Appendix B). Droplet interaction was allowed to proceed for 15 minutes, at which point all excess liquid was removed with pressurized air. Each sample was then weighed and placed in the TGA instrument for analysis. Change in mass was monitored over a period of 4 hours as the sample was slowly heated to 200°C. To protect against any form of thermal decomposition, helium shielding gas was continually flowed over the sample. Hence, any observation of mass loss can be directly related to thermal desorption.

2.4 Spore Deposition

BAS and BG spores were deposited onto TSA plates (as positive controls) and test coupons by liquid deposition. Previous studies that assessed sampling method performance primarily used spores suspended in aqueous buffers to inoculate test surfaces (Piepel et al. 2012). Liquid deposition allows relatively easy control of the number of spores and the contaminated area. Liquid deposition was used in this study because work by Krauter et al. (2012) suggested (i) some of the low concentrations to be tested would be well below the sample method's LOD and (ii) such concentrations could not be accurately achieved using dry-aerosol-deposition methods.

Spore stocks were prepared with PBS-T in a 25 mL Erlenmeyer flask and were mixed by constant stirring using a stir plate. From the spore stock solution, a 200 µL electronic repeat pipettor (Rainin E4 XLS; Rainin Instrument LLC, Oakland, CA) was used to deposit 10 droplets (10 µL each) on a clean and sterile coupon or a TSA plate. Work progressed from top to bottom and right to left within the biosafety cabinet. The inoculated coupons were dried for approximately 2 hours with the biosafety cabinet sash closed and the airflow turned off.

2.5 Swab Sampling Method

This study used the CDC swab sampling procedure to collect samples on hard, relatively nonporous surfaces (CDC 2012, Hodges et al. 2010). Macrofoam swabs (Puritan® PurCollect Environmental Sampling Collection Swab, Sterile; supplier #25-1607 1PF SC) manufactured by Puritan Medical Products (Guilford, ME) were premoistened in PBS-T buffer and excess liquid was pressed out of the swab in a sterile tube. Using a sterile technique, each test coupon was swabbed using an overlapping "S" pattern with horizontal strokes, vertical "S" strokes, and diagonal "S" strokes. Between the three different "S" strokes, the swab was rotated to expose all surfaces of the swab to the coupon.

2.6 Spore Extraction and Analysis

Spores were extracted from a macrofoam swab (the stick end was cut off and discarded) by transferring it into a screw-cap conical tube containing 5 mL PBS-T. To dislodge spores from the swabs, the swabs were vortexed at the highest level (a setting of 10) for 2 minutes using a Vortex Genie 2 (Scientific Industries, Inc. Bohemia, New York), with 10-second pulses. Excess liquid was pressed out of the swab using aseptic technique. A P1000 pipette was used to dispense 1 mL of the spore solution onto a TSA plate, which was done in triplicate and the same tip was used to avoid spore loss. If all three

experimental plates for a given test coupon were negative for growth, then a 1 mL aliquot of the remaining spore elution was filtered onto a membrane (MicroFunnel™ Filter Funnels 0.45-µm mixed cellulose esters membrane, VWR International, catalog #28143-544; MicroFunnel Manifold, Pall Corporation, Port Washington, NY) that was placed on a TSA plate. All plates were incubated for 48 hours to confirm the lack of viable bacteria. If plates were negative for growth, they were incubated for an additional 24 hours to verify no viable bacteria were present.

2.7 Negative and Positive Controls

Negative and positive controls were included in each of the 34 tests. There were two types of negative controls: process negatives and coupon negatives. For each test (within a biosafety cabinet), there were four process negative controls, each of which consisted of a swab that was simply removed from the packaging and transferred into the collection tube. For each coupon type there was one coupon negative control that was processed in the same manner as the test coupons. These negative controls were conducted for each biosafety-cabinet run. All negative control samples for all tests were negative for spore growth.

For each test, 12 TSA plates were used as positive controls. These plates were located in the middle row of samples in a biosafety cabinet (see Figure 2.1). Ten 10-µL droplets from the spore stock were placed directly onto each TSA plate. For the 500 CFU target, a 1:10 dilution was made of the original stock so that countable plates would be obtained. Plates were incubated overnight at 30°C or 35°C (BG or BAS, respectively). If plates were negative for growth, they were incubated for an additional 24 hours to verify no viable bacteria were present.

2.8 Positive-Control Concentrations and Uncertainties

The counts of the 12 positive-control plates for each test were averaged, where $\overline{C}_{hij}$ denotes the mean CFUs for the j^{th} target concentration of the i^{th} surrogate in the h^{th} block. The $\overline{C}_{hij}$ values were used as the reference values for RE calculations. The $\overline{C}_{hij}$ values were converted to mean concentrations (CFU/cm^2) via

$$\overline{c}_{hij} = \overline{C}_{hij} / 25.8064 \quad (1)$$

and used to assess the relationships of RE and FNR (for test coupons) with actual concentrations of the surrogates.

The mean and standard deviation of the actual concentrations for the j^{th} target concentration of the i^{th} surrogate were calculated using different formulas depending on whether the target concentration was investigated only in Test Runs 1–16 (500 CFU/coupon), Test Runs 17–34 (4 CFU/coupon), or in both Test Runs 1–16 and 17–34 (the remaining concentrations). The formulas for the mean are

$$\bar{c}_{ij} = \begin{cases} \bar{c}_{hij} & \text{4 and 500 CFU/coupon} \\ (\sum_{h=1}^{2} \bar{c}_{hij}) / 2 & \text{Remaining target concentrations} \end{cases} \quad (2)$$

while the formulas for the standard deviation (SD) are

$$SD(c_{ij}) = \begin{cases} SD(c_{ij}^{Plate}) & \text{4 and 500 CFU/coupon} \\ \{[SD(c_{ij}^{WP})]^2 + [SD(c_{ij}^{Plate})]^2\}^{0.5} & \text{Remaining target concentrations.} \end{cases} \quad (3)$$

In the second formula of Eq. (3), the squared quantities are the two variance components affecting the positive-control concentration data for a given "*ij*" combination associated with WP and plates. The percent relative standard deviation (%RSD) of c_{ij} was calculated as

$$\%RSD(c_{ij}) = 100\ SD(c_{ij}) / \bar{c}_{ij} \quad (4)$$

using the appropriate formulas in Eqs. (2) and (3) for each target concentration.

2.9 Recovery Efficiency and Uncertainties

The notation R_{hijkm} denotes the number of CFUs recovered from the m^{th} coupon of the k^{th} material with the j^{th} concentration of the i^{th} surrogate in the h^{th} block. An R_{hijkm} is calculated as

$$\begin{aligned} R_{hijkm} = &\text{(Mean CFU from 3 or 4 plates)} \times \text{5 ml (total volume)} \\ &\times \text{final dilution factor on plate} \end{aligned} \quad (5)$$

where "3 or 4 plates" is explained in Section 2.6. The multiplication by 5 mL is because 1 mL samples are plated from the total 5 mL volume. Tests with a target of 500 CFU/plate were diluted (1:10) for the positive controls only, and required using a 10 dilution factor. Appendix C contains formulas for calculating recovery concentrations (r_{hijkm}), as well as their means ($\bar{r}_{ijk}$) and %RSDs [%RSD(r_{ijk})].

A RE was calculated for each test coupon as

$$RE_{hijkm} = R_{hijkm} / \bar{C}_{hij}, \quad (6)$$

where RE_{hijkm} is the RE of the macrofoam-swab method with culture analysis for the m^{th} coupon of the k^{th} material with the j^{th} concentration of the i^{th} surrogate in the h^{th} block of tests, and RE_{hijkm} and $\bar{C}_{hij}$ are as defined previously. The mean and SD of REs over replicate coupons of the k^{th} material with the j^{th} concentration of the i^{th} surrogate are denoted as $\bar{\bar{R}E}_{ijk}$ and $SD(RE_{ijk})$. These quantities were calculated

using different formulas depending on the target concentration, as discussed previously. The formulas for the mean are

$$\overline{RE}_{ijk} = \begin{cases} \sum_{m=1}^{6} RE_{hijkm} / 6 & 500 \text{ CFU/coupon } (h = 1) \\ \sum_{m=1}^{12} RE_{hijkm} / 12 & 4 \text{ CFU/coupon } (h = 2) \\ \sum_{h=1}^{2} (\sum_{m=1}^{6} RE_{hijkm} / 6) / 2 & \text{Remaining target concentrations } (h = 1, 2) \end{cases} \quad (7)$$

and the formulas for the SD are

$$SD(RE_{ijk}) = \begin{cases} SD(RE_{ijk}^{Coupon}) & 4 \text{ and } 500 \text{ CFU/coupon} \\ \{[SD(RE_{ijk}^{WP})]^2 + [SD(RE_{ijk}^{Coupon})]^2\}^{0.5} & \text{Remaining target concentrations.} \end{cases} \quad (8)$$

In the second formula of Eq. (8), the squared quantities are the two variance components affecting the RE data for a given "*ijk*" combination. The %RSD of RE_{ijk} was calculated as

$$\%RSD(RE_{ijk}) = 100\ SD(RE_{ijk}) / \overline{RE}_{ijk} \quad (9)$$

using the appropriate formulas in Eqs. (7) and (8) for each target concentration. The standard error of $\overline{RE}_{ijk}$ was calculated as

$$SE(\overline{RE}_{ijk}) = \begin{cases} SD(RE_{ijk}) / 6 & 500 \text{ CFU/coupon } (h = 1) \\ SD(RE_{ijk}) / 12 & 4 \text{ CFU/coupon } (h = 2) \\ \left(\frac{[SD(RE_{ijk}^{WP})]^2}{2} + \frac{[SD(RE_{ijk}^{Coupon})]^2}{12} \right)^{0.5} & \text{Remaining target concentrations} \end{cases} \quad (10)$$

where the WP variance component is reduced by a factor of 2 because of the two WPs while the coupon variance component is reduced by a factor of 12 because there are 12 coupons for each "*ijk*" combination.

2.10 False Negative Rate and Uncertainties

The notation FNR_{hijkm} represents the FNR of the macrofoam-swab method with culture analysis for the m^{th} coupon of the k^{th} material with the j^{th} concentration of the i^{th} surrogate in the h^{th} block. As discussed previously, three 1-mL samples of the extraction fluid from a given test coupon were analyzed

using standard microbiology culturing techniques. Each of these was identified as a "detect" or "non-detect" of the surrogate for that test coupon. A FNR estimate of 0, 1/3, 2/3, or 1 for each test coupon was obtained based on the proportion of non-detects obtained for the three samples. As discussed previously, when the three samples were all non-detects, a fourth sample was taken. In such cases, the FNR estimate was set to 3/4 or 4/4 = 1, depending on whether the fourth sample yielded a "detect" or "non-detect," respectively.

The mean and SD of FNRs over replicate coupons of the k^{th} material with the j^{th} concentration of the i^{th} surrogate are denoted as $\overline{FNR}_{ijk}$ and $SD(FNR_{ijk})$. These quantities were calculated using different formulas depending on the target concentration, as discussed previously. The formulas for the mean are

$$\overline{FNR}_{ijk} = \begin{cases} \sum_{m=1}^{6} FNR_{hijkm} / 6 & 500 \text{ CFU/coupon } (h = 1) \\ \sum_{m=1}^{12} FNR_{hijkm} / 12 & 4 \text{ CFU/coupon } (h = 2) \\ \sum_{h=1}^{2} (\sum_{m=1}^{6} FNR_{hijkm} / 6) / 2 & \text{Remaining target concentrations } (h = 1, 2) \end{cases} \quad (11)$$

and the formulas for the SD are

$$SD(FNR_{ijk}) = \begin{cases} SD(FNR_{ijk}^{Coupon}) & 4 \text{ and } 500 \text{ CFU/coupon} \\ \{[SD(FNR_{ijk}^{WP})]^2 + [SD(FNR_{ijk}^{Coupon})]^2\}^{0.5} & \text{Remaining target concentrations.} \end{cases} \quad (12)$$

In the second formula of Eq. (12), the squared quantities are the two variance components affecting the FNR data for a given "*ijk*" combination. The standard error of $\overline{FNR}_{ijk}$ was calculated as

$$SE(\overline{FNR}_{ijk}) = \begin{cases} SD(FNR_{ijk}) / 6 & 500 \text{ CFU/coupon } (h = 1) \\ SD(FNR_{ijk}) / 12 & 4 \text{ CFU/coupon } (h = 2) \\ \left(\frac{[SD(FNR_{ijk}^{WP})]^2}{2} + \frac{[SD(FNR_{ijk}^{Coupon})]^2}{12} \right)^{0.5} & \text{Remaining target concentrations} \end{cases} \quad (13)$$

with explanations similar to those following Eq. (10).

The variance components in Eqs. (3), (8), (10), (12) and (13) were estimated from the experimental data using the restricted maximum likelihood method for variance component estimation (Harville 1977).

2.11 False Negative Rates as Functions of Surrogate Concentrations

For a given surrogate and surface material, the FNR will increase as the surrogate concentration decreases below the level at which false negatives begin to occur. The three-coefficient, cumulative-distribution form of the Johnson SB model (Hahn and Shapiro 1968, Mathwave 2011)

$$FNR_{hijkm} = 1 - \Phi\left(\gamma_{ik} + \delta_{ik}\, ln\left(\frac{\bar{c}_{hij}}{\lambda_{ik} - \bar{c}_{hij}}\right)\right) \tag{14}$$

was used to relate FNR to the surrogate concentration for each combination of the two surrogates (i = 1, 2) and the four surface materials (k = 1, 2, 3, 4). The notations FNR_{hijkm} and $\bar{c}_{hij}$ were defined previously, where $0 \le \bar{c}_{hij} \le \lambda_{ik}$; Φ is the standard normal (Gaussian) cumulative distribution function. The coefficients γ_{ik}, δ_{ik} (>0), and λ_{ik} (>0) depend on the surrogate and surface material. The three coefficients were estimated from the experimental data for each combination of surrogate and surface material using nonlinear weighted-least-squares regression (Seber and Wild 2003). The high correlations among some coefficient estimates made it necessary to manually select the value of the λ_{ik} coefficients (for each combination of surrogate and surface material) to be slightly higher than the lowest actual concentration (CFU/cm^2) for which all FNR_{hijkm} values were zero. The weights for the FNR_{hijkm} values were reciprocals of the estimated variances of the FNR values for the six coupons at a given "*hijk*" combination.

The models of the form in Eq. (14) allow predicting the FNR at any concentration within the range where false negatives occur. Also, statistical methods enable calculating the uncertainty in the predicted FNR at a given concentration (Seber and Wild 2003).

2.12 Limits of Detection and Uncertainties

In this report, the LOD of the macrofoam-swab method for each combination of a surrogate and surface material is defined as the surrogate concentration for which there is a 95% probability of correct detection (PCD), denoted LOD_{95}. An estimate of the LOD_{95} is calculated for a given combination of surrogate and surface material using the corresponding FNR-concentration equation of the form in Eq. (14). Specifically, the LOD_{95} is the concentration at which the equation predicts FNR = 0.05 (i.e., the PCD = 0.95). A method for nonlinear models was used to calculate the SD of the LOD_{95} for each combination of surrogate and surface material (Seber and Wild 2003).

2.13 Split-Split-Plot Data Analyses

Statistical data analyses accounting for the SSP structure of the RE and FNR data (RE_{hijkm} and FNR_{hijkm}) for the culture portion of the study were conducted using PROC MIXED in the SAS software (SAS Institute Inc. 2014). The restricted-maximum-likelihood and Kenward-Rogers methods were used.

Because the uncertainties of the RE_{hijkm} values were larger for lower concentrations (discussed subsequently), a weighted analysis was performed using reciprocals of the estimated variances for the six coupons at a given "*hijk*" combination. Because FNR_{hijkm} values are between 0 and 1, a logit transformation [$\ln(FNR_{hijkm} /(1 - FNR_{hijkm}))$] was employed to meet the assumptions of the statistical analyses performed. The effects of the test factors (surrogate, concentration, and surface material) and two-factor interactions were declared statistically significant if the confidence level was 95% or greater.

3.0 Results

3.1 Actual Concentrations

For each target concentration, Tables 3.1 and 3.2 display for BAS and BG, respectively, the means and %RSDs of actual concentrations [$\bar{c}_{ij}$ and $\%RSD(c_{ij})$] calculated from positive-control data using Eqs. (2) to (4).

3.2 Surface Property Analyses

The roughness indices for the four surface materials are listed in Table 3.3. They range from 0.019 μm for glass to 139.7 μm for plastic.

TGA measurements were conducted to further characterize the materials for liquid absorption potential. Figure B.2 of Appendix B shows the mass (percentage of initial) as a function of increasing temperature for each of the four surface-material samples. The vinyl tile was the only material to lose any mass over the period of heating. In contrast, the other three materials showed a slight increase in mass. The onset of mass loss in the vinyl material occurred at around 100°C, from which we can infer that liquid was being desorbed. This suggests that liquid was absorbed into the vinyl material during contact with the droplet of PBS-T. From this finding it can be concluded that vinyl tile has some form of porosity, which absorbs and retains liquid due to capillary action. Spores placed onto a surface will become attached to the material by hydrophobic and other forces during drying.

3.3 Recovery Efficiencies and Uncertainties

For each combination of target concentration and surface material, Tables 3.1 and 3.2 summarize for BAS and BG, respectively, the (i) means and %RSDs of recovery concentrations [$\bar{r}_{ijk}$ and $\%RSD(r_{ijk})$] and (ii) means and %RSDs of recovery efficiency [$\overline{RE}_{ijk}$ and $\%RSD(RE_{ijk})$] for the macrofoam-swab method. Because a positive control was associated with a pair of coupons for each of the 12 "columns" of coupons on a biosafety cabinet surface for a test (Figure 2.1), the option existed to calculate the RE for each coupon using the result from the associated positive control. However, statistical analysis of the positive-control data revealed no significant differences in results by column in a biosafety cabinet, which confirmed a similar conclusion from preliminary tests. Hence, REs were calculated using Eq. (6), the denominator of which is the mean count of the surrogate over the 12 positive-control samples in a given test.

Table 3.1. Performance Measures of the Macrofoam-Swab Method with Culture Analysis and Liquid-Deposited *B. anthracis* Sterne Spores on Coupons of Four Surface Materials

Target Deposition, CFU/coupon (CFU/cm^2)[a]	Test #s[b]	Positive-Control CFU/sample (CFU/cm^2)		Surface Material	# Test Coupons	Test Coupons					
						Recovery Conc. (CFU/cm^2)		Recovery Efficiency (%)		FNR	
		Mean[c]	%RSD			Mean[f]	%RSD[f]	Mean[g]	%RSD[g]	Mean[h]	SD[h]
2 (0.078)	6, 40	2.08 (0.0807)	84.8	S	12	0.0592	113.6	69.8	101.6	0.750	0.255
				G	12	0.1238	64.7	154.1	71.8	0.500	0.309
				V	12	0.0323	134.8	41.1	128.8	0.833	0.225
				P	12	0.0431	136.9	50.4	126.8	0.833	0.176
4 (0.155)	36, 50	3.25 (0.1259)	70.4	S	12	0.1063	51.1	85.2	60.0	0.535	0.269
				G	12	0.1224	102.1	98.6	103.5	0.590	0.255
				V	12	0.0579	53.4	47.2	113.1	0.771	0.201
				P	12	0.0996	36.9	80.1	46.1	0.535	0.179
5 (0.194)	9, 43	5.04 (0.1954)	62.2	S	12	0.1346	60.4	71.3	63.0	0.417	0.289
				G	12	0.1978	69.6	106.8	81.7	0.368	0.300
				V	12	0.0538	86.1	32.2	99.5	0.722	0.239
				P	12	0.1453	88.0	73.7	75.2	0.500	0.225
10 (0.388)	8, 41	9.54 (0.3697)	28.3	S	12	0.2691	36.7	72.9	36.2	0.194	0.264
				G	12	0.2960	50.3	79.7	48.4	0.250	0.289
				V	12	0.1978	68.2	53.3	68.6	0.451	0.334
				P	12	0.2260	56.5	60.9	54.6	0.333	0.246
15 (0.581)	14, 47	14.50 (0.5619)	17.7	S	12	0.4037	39.3	71.8	38.9	0.111	0.164
				G	12	0.4521	40.9	80.7	42.2	0.139	0.223
				V	12	0.3283	62.5	58.4	62.2	0.194	0.264
				P	12	0.4521	48.7	80.3	47.8	0.139	0.172
20 (0.775)	1, 34	22.54 (0.8735)	25.6	S	12	0.4736	21.6	54.2	19.2	0.111	0.164
				G	12	0.6297	43.6	71.1	34.3	0.083	0.151
				V	12	0.4413	41.1	49.9	34.3	0.167	0.176
				P	12	0.6674	34.9	76.7	36.2	0.083	0.207
25 (0.969)	3, 38	23.04 (0.8929)	24.6	S	12	0.6135	39.0	69.4	42.1	0.028	0.096
				G	12	0.7050	27.9	78.6	19.8	0.056	0.130
				V	12	0.5597	41.3	62.3	39.0	0.083	0.167
				P	12	0.5759	36.5	64.1	32.0	0.056	0.136
100 (3.875)	12, 45	86.99 (3.371)	10.8	S	12	2.3196	31.1	68.3	24.4	0	0
				G	12	2.6425	23.9	78.2	20.8	0	0
				V	12	1.5446	35.6	45.9	36.2	0	0
				P	12	2.3519	20.2	69.9	20.8	0	0
500 (19.375)	16	580.85 (22.508)	24.8	S	6	19.3212	5.8	82.2	7.0	0	0
				G	6	20.5375	7.5	87.4	8.5	0	0
				V	6	15.7261	10.1	66.9	15.1	0	0
				P	6	18.6000	6.1	79.1	7.7	0	0

[a] Target number and concentration of spores (CFUs) deposited per 2 in. × 2 in. coupon (25.8064 cm^2).

[b] Test numbers are from Piepel and Hutchison (2014) and include tests analyzed by mRV-PCR as well as culture; thus not all of the test numbers are included in Tables 3.1 and 3.2.

[c] The means and %RSDs of actual CFU/cm^2 were calculated using Eqs. (2) and (4) for each target concentration. The CFU/sample mean values were obtained via multiplying the CFU/cm^2 values by 25.8064. The %RSD values are the same for both CFU/sample and CFU/cm^2.

[d] S = stainless steel, G = glass, V = vinyl tile, P = plastic light cover panel

[e] The number of test coupons for each combination of surrogate concentration and surface material.

[f] Mean and %RSD of recovery concentrations for each combination of concentration and surface material, calculated using Eqs. (C.1) to (C.4) of Appendix C.

[g] Mean and %RSD of RE for each combination of concentration and surface material, calculated using Eqs. (7) and (9).

[h] Mean and SD of FNR for each combination of concentration and surface material, calculated using Eqs. (11) and (12).

Table 3.2. Performance Measures of the Macrofoam-Swab Method with Culture Analysis and Liquid-Deposited *B. atrophaeus* Nakamura Spores on Coupons of Four Surface Materials

Target Deposition, CFU/coupon (CFU/cm^2)[a]	Test #s[b]	Positive-Control CFU/Sample (CFU/cm^2)		Surface Material	# Test Coupons	Test Coupons					
						Recovery Conc. (CFU/cm^2)		Recovery Efficiency (%)		FNR	
		Mean[c]	%RSD			Mean[f]	%RSD[f]	Mean[g]	%RSD[g]	Mean[h]	SD[h]
2 (0.078)	22, 58	2.33 (0.0904)	62.8	S	12	0.0525	126.6	57.4	124.0	0.785	0.257
				G	12	0.0592	108.7	65.4	105.5	0.750	0.251
				V	12	0.0538	123.3	62.4	127.7	0.806	0.184
				P	12	0.0552	100.2	60.6	96.7	0.736	0.277
4 (0.155)	54, 68	4.75 (0.1841)	38.9	S	12	0.1346	65.4	75.8	86.2	0.472	0.300
				G	12	0.1668	44.6	92.9	48.1	0.389	0.239
				V	12	0.0807	41.8	42.6	98.0	0.611	0.372
				P	12	0.0969	40.6	52.0	78.0	0.639	0.300
5 (0.194)	26, 61	4.83 (0.1873)	45.1	S	12	0.1238	98.1	66.2	97.4	0.556	0.385
				G	12	0.1157	81.5	61.5	81.0	0.569	0.339
				V	12	0.0780	88.6	41.8	89.7	0.653	0.270
				P	12	0.0982	82.6	52.2	81.8	0.597	0.295
10 (0.388)	23, 60	11.67 (0.4521)	26.5	S	12	0.2960	47.9	65.9	50.0	0.306	0.289
				G	12	0.2691	54.0	59.8	55.5	0.194	0.223
				V	12	0.1453	73.7	32.1	74.5	0.472	0.332
				P	12	0.1830	74.9	41.1	78.4	0.389	0.283
15 (0.581)	29, 66	14.87 (0.5764)	27.6	S	12	0.3875	35.5	67.3	35.2	0.083	0.151
				G	12	0.4252	53.2	73.7	54.0	0.194	0.223
				V	12	0.2032	56.9	35.5	58.4	0.368	0.186
				P	12	0.3337	56.1	57.5	54.2	0.194	0.229
20 (0.775)	17, 51	23.83 (0.9235)	22.8	S	12	0.5113	52.5	55.6	54.0	0.056	0.130
				G	12	0.6512	34.0	70.6	33.6	0.028	0.096
				V	12	0.4090	67.8	44.6	69.4	0.194	0.373
				P	12	0.5920	51.4	64.5	53.7	0.056	0.136
25 (0.969)	19, 55	29.01 (1.124)	22.9	S	12	0.6889	20.9	61.5	22.3	0.056	0.136
				G	12	0.8181	41.7	72.0	36.0	0.028	0.096
				V	12	0.3445	37.8	30.8	37.2	0.167	0.242
				P	12	0.6405	40.2	56.6	35.3	0.056	0.130
100 (3.875)	28, 63	108.93 (4.221)	11.1	S	12	3.1215	17.6	74.0	16.9	0	0
				G	12	3.4229	12.2	81.5	16.5	0	0
				V	12	1.8514	37.8	44.4	42.3	0	0
				P	12	2.3842	30.2	57.3	37.2	0	0
500 (19.375)	31	566.66 (21.958)	25.8	S	6	13.9716	5.3	63.6	8.3	0	0
				G	6	14.2191	3.9	64.8	6.0	0	0
				V	6	6.1354	12.9	27.9	46.0	0	0
				P	6	10.2472	6.1	46.7	13.2	0	0

[a] Target number and concentration of spores (CFUs) deposited per 2 in. × 2 in. coupon (25.8064 cm^2).

[b] Test numbers and are from Piepel and Hutchison (2014) and include tests analyzed by mRV-PCR as well as culture, thus not all of the test numbers are included in Tables 3.1 and 3.2.

[c] The means and %RSDs of actual CFU/cm^2 were calculated using Eqs. (2) and (4) for each target concentration. The CFU/sample mean values were obtained via multiplying the CFU/cm^2 values by 25.8064. The %RSD values are the same for both CFU/sample and CFU/cm^2.

[d] S = stainless steel, G = glass, V = vinyl tile, P = plastic light cover panel

[e] The number of test coupons for each combination of surrogate concentration and surface material.

[f] Man and %RSD of recovery concentrations for each combination of concentration and surface material, calculated using Eqs. (C.1) to (C.4) of Appendix C.

[g] Mean and %RSD of RE for each combination of concentration and surface material, calculated using Eqs. (7) and (9).

[h] Mean and SD of FNR for each combination of concentration and surface material, calculated using Eqs. (11) and (12).

Table 3.3. Recovery Efficiency (RE) and False Negative Rate (FNR), Averaged Over All Spore Concentrations, for Each Combination of Surrogate and Surface Material with the Corresponding Roughness Index Measurement

Surrogate	Surface Material	RE Mean (%)	RE Least-Squares Mean (%)[a]	Comparison of RE Least-Squares Means[b]	FNR, Mean[c]	Roughness Index[d] (µm)
Bacillus anthracis Sterne (BAS)	Glass	92.8	82.1		0.2207	0.019
	Stainless Steel	71.7	68.5	A	0.2384	0.118
	Vinyl Tile	50.8	50.4		0.3579	2.55
	Plastic Panel	70.6	69.3	A	0.2754	139.7
Bacillus atrophaeus Nakamura (BG)	Glass	71.4	74.9	B	0.2391	0.019
	Stainless Steel	65.3	65.5	B	0.2571	0.118
	Vinyl Tile	40.2	34.7		0.3634	2.55
	Plastic Panel	54.3	52.0		0.2963	139.7

[a] Least-squares means account for the unequal variances of the data and the SSP structure of the data.

[b] The Tukey's multiple-comparison procedure (Miller 1981) was used in the SSP data analyses to statistically compare the least-squares means for all pairs of surface materials (separately for BAS and BG). The least-squares means for all pairs were statistically different (> 95% confidence) except those with the same letters (A or B), namely (i) stainless steel and plastic for BAS and (ii) glass and stainless steel for BG.

[c] The FNR means are averaged over all concentrations of a given surrogate despite FNR being a strong function of surrogate concentration for each surface material. Hence, these means are solely for assessing whether there is a macro relationship between FNR and surface roughness.

[d] The roughness index (Ra) is the average from 6 to 11 locations on a representative test coupon.

Over the range of mean actual concentrations tested (0.0807 to 22.508 CFU/cm^2 for BAS and 0.0904 to 21.958 CFU/cm^2 for BG), the $\%RSD(RE_{ijk})$ values tend to increase as concentration decreases, as shown in Tables 3.1 and 3.2. Hence, a weighted SSP statistical analysis was performed, as discussed previously. The conclusions were that surrogate and surface material had statistically significant effects on RE, but concentration did not. The effects of surrogate and surface material on RE are discussed in the following paragraph. The nondependence of RE on concentration is illustrated for stainless steel coupons in Figure 3.1, which displays for BAS and BG the $\overline{RE}_{ijk}$ values with ± 1 $SE(\overline{RE}_{ijk})$ error bars. The $\overline{RE}_{ijk}$ values for stainless steel range from 54.2 to 85.2% (average of 71.7%) for BAS and (ii) 55.6 to 75.8% (average of 65.3%) for BG. The $SE(\overline{RE}_{ijk})$ values for stainless steel (not listed in Tables 3.1 and 3.2) range from 0.96 to 22.2% for BAS and from 0.85 to 20.5% for BG.

As part of the SSP data analysis, Tukey's multiple-comparison procedure (Miller 1981) was used for each of the surrogates to statistically compare the RE results for the four surface materials. Table 3.3 lists, for each surrogate, the RE means and least-squares means (see footnote a) of the surface materials. Glass and stainless steel had the highest RE means for both BAS (92.8 and 71.7%, respectively) and BG (71.4 and 65.3%, respectively). Vinyl tile and plastic panel had the lowest RE means for both BAS (50.8 and 70.6%, respectively) and BG (40.2 and 54.3%, respectively). The data analysis results in Table 3.3 also show that all pairs of surface materials have statistically different RE least-squares means except (i) stainless steel and plastic for BAS, and (ii) glass and stainless steel for BG.

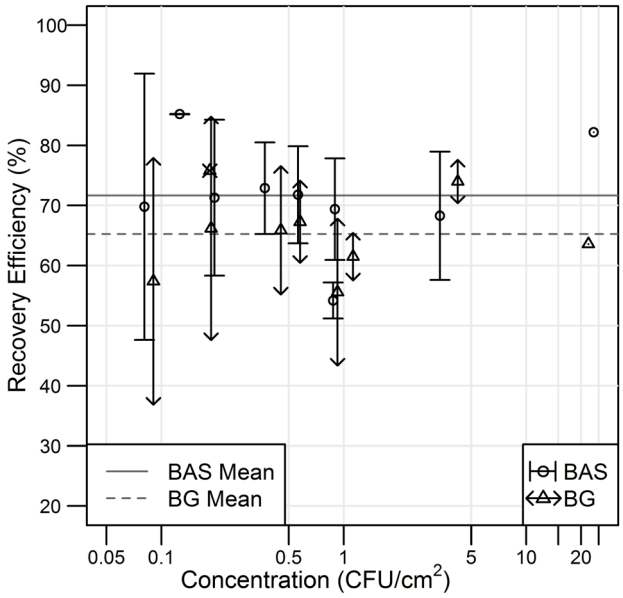

Figure 3.1. Mean Values of Recovery Efficiency (%) for the Macrofoam-Swab Method Applied to Stainless Steel Coupons with *B. anthracis* Sterne (BAS) and *B. atrophaeus* Nakamura (BG), Plotted versus Mean Concentrations of the Two Surrogates from Positive-Control Samples. The error bars are ±1 SE, as calculated by Eq. (10). The horizontal lines across the figure show the average recovery efficiency for all concentrations of BAS (71.7%) and BG (65.3%).

The dependence of RE on surface materials was further investigated by comparing the RE means to the roughness indices of the test materials, which was done separately for the two surrogates. For each combination of surrogate and surface material, the RE mean (across all spore concentrations) and the corresponding roughness index measurement are listed in Table 3.3. The roughness indices range from 0.019 μm (glass) to 139.7 μm (plastic). As shown in Table 3.3, for both surrogates the RE means for glass, stainless steel, and plastic increase with decreasing surface roughness indices. The vinyl tile has the lowest RE means for BAS and BG, but not the highest roughness value. These data suggest that the RE for a sample collected with a macrofoam swab is not completely dependent on the surface roughness. Surface material porosity was investigated as an additional possible factor affecting RE for two reasons. First, during testing the liquid drops on vinyl tile coupons appeared to dry the fastest, suggesting increased porosity or wicking action compared to other surface materials. Second, previous swab studies found lower REs for porous surface materials compared to nonporous materials (Buttner et al. 2001, Frawley et al. 2008, Valentine et al. 2008). We determined that (i) stainless steel, glass, and plastic were nonporous and (ii) vinyl tile was porous with pore sizes from 4–6 nm. Porosity was measured by classical adsorption/desorption nitrogen isotherms.

While the vinyl tile used in this study is not typically considered a porous or absorbent surface (such as brick, cotton cloth, and synthetic carpet), it is possible that the combined factors of surface roughness, porosity, and surface chemistry contributed to vinyl tile having the lowest RE for both BAS and BG.

3.4 FNRs and Uncertainties

Negative results occurred for a small number of positive-control samples at the lowest test concentrations. For BAS there were 3, 2, and 2 negatives out of the 24 positive controls at the target concentrations of 2, 4, and 5 CFU/coupon, respectively. For BG, there were 3 and 1 negatives at target concentrations of 2 and 4 CFU/coupon, respectively. Because there might not have been any spores deposited on some positive control plates (or test coupons) at these very low concentrations, it is possible that negatives are "true negatives" rather than "false negatives". However, the numbers of negatives for the positive control samples is very small, and for conservatism in quantifying FNRs in this work the negative results are assumed to be false negatives.

FNRs were estimated for each test coupon based on the data (with possible values of 0, 1/3, 2/3, 3/4, and 1), as described previously. Tables 3.1 and 3.2 list for BAS and BG, respectively, the mean ($\overline{FNR}_{ijk}$) and SD [$SD(FNR_{ijk})$] values of FNR for each combination of surrogate, target concentration, and surface material (*ijk*). The $\overline{FNR}_{ijk}$ values range from 0 to 0.833 for BAS and 0 to 0.806 for BG. The $SD(FNR_{ijk})$ values are relatively high (ranging up to 0.334 for BAS and 0.385 for BG) because they are the uncertainties in FNR values for a single test coupon. The $SE(\overline{FNR}_{ijk})$ values calculated using Eq. (13) are notably lower, ranging up to 0.146 for BAS and 0.195 for BG (not shown in Tables 3.1 and 3.2).

The SSP statistical analysis showed that concentration and surface material had statistically significant effects on FNR. The effect of surrogate was not statistically significant over all combinations of concentration and surface material. This latter topic is discussed in more detail subsequently.

Table 3.4 shows the coefficients and R^2 statistics from fitting Eq. (14) to the FNR (FNR_{hijkm}) versus concentration ($\bar{c}_{hij}$) data for each combination of surrogate and surface material. The R^2 statistics are in the range 0.591 to 0.768 because of the relatively large uncertainty in FNR values for the individual test coupons used to fit Eq. (14). The 12 test coupons used for each combination of surrogate, concentration, and surface material provide for reducing the uncertainties in FNR values predicted using the fitted equations. The FNR for a given surrogate, concentration, and surface material can be predicted by substituting the coefficients from Table 3.4 into Eq. (14). For example, the FNR of the macrofoam-swab method (with culture analysis) for a 0.50 CFU/cm^2 concentration of BAS on vinyl tile is predicted by substituting the coefficients from Table 3.4 into Eq. (14), which yields FNR = 0.383.

Table 3.4. Coefficients and R^2 Values for the Johnson SB Equations in Eq. (14), which Relate False Negative Rate to Concentration for Each Combination of Surrogate and Surface Material

	***Bacillus anthracis* Sterne**				***Bacillus atrophaeus* Nakamura**			
	Coefficient[a]				**Coefficient[a]**			
Surface Material	γ	δ	λ	$R^{2\,b}$	γ	δ	λ	$R^{2\,b}$
Glass	1.316	0.497	1.550	0.591	1.109	0.656	1.182	0.698
Stainless steel	1.087	0.677	0.975	0.673	2.666	0.917	3.969	0.689
Vinyl tile	0.272	0.490	0.975	0.764	2.072	0.837	3.969	0.760
Plastic	0.761	0.582	0.975	0.768	2.402	0.888	3.969	0.703

[a] The coefficients γ and δ are two shape parameters, while λ is a scale parameter. For this application, λ essentially represents the concentration (CFU/cm^2) at which FNR reaches the zero asymptote. The regression estimates of γ and δ were highly correlated with the estimates of λ for all surface materials. Hence, λ was set equal to a value slightly higher than the lowest mean actual concentration for which the FNR was 0 for all test coupons at a given combination of surrogate and surface material.

[b] R^2 values were calculated using a formula that accounts for the model fits being performed using weighted-least-squares regression. The values are lower than typically considered desirable because of the limited number of FNR values for a given test coupon and hence relatively large uncertainty in those values. However, this is compensated by having 12 test coupons for each combination of surrogate, concentration, and surface material. The data for the target concentration of 500 CFU/coupon were not used in the model fits.

The left and right panels of Figure 3.2 show, for BAS and BG respectively, the mean FNR results from test coupons for each of the four surface materials ($\overline{FNR}_{ijk}$) plotted against mean concentrations of the surrogates ($\bar{c}_{ij}$) from the positive-control data. Figure 3.2 also shows the curves corresponding to the fitted equations in Table 3.4. The relatively high uncertainty in the test data, and hence in the fitted curves, leads to some differences between the curves and certain mean FNR values (as plotted in Figure 3.2). The curves in the figure illustrate the strong dependence of FNR on concentration, with smaller but still notable differences in FNRs between some surface materials (discussed in the next paragraph). Figure 3.2 also shows that FNRs tend to be higher for BG than BAS at lower concentrations, which is most noticeable with glass as the surface material. We hypothesize that the differences between FNRs for BG and BAS are a result of the phenotypic properties of the bacterial spore. Phenotypic differences include (but are not limited to) BG spores lacking an exosporium and tending to be smaller in size compared to BAS spores (for a review, see Greenburg et al. 2010).

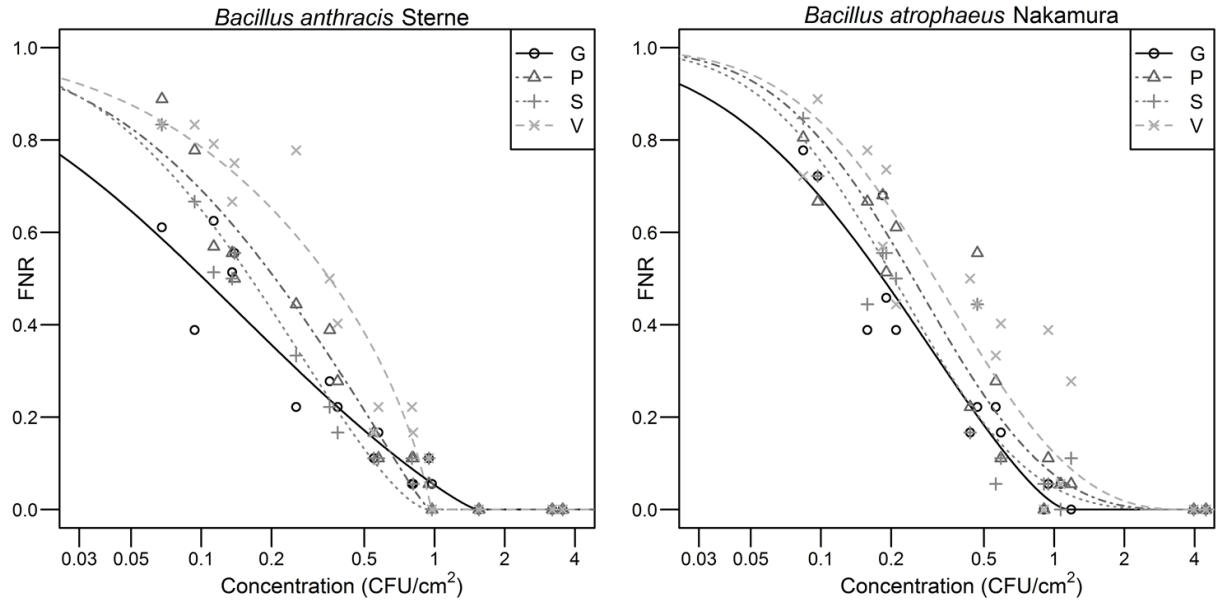

Figure 3.2. Average False Negative Rate Data from Culture Analyses and Fitted Equations as Functions of *B. anthracis* Sterne and *B. atrophaeus* Nakamura Concentrations (from positive controls) for Each of Four Surface Materials. The surface materials are denoted by G = glass, S = stainless steel, V = vinyl tile, and P = plastic light cover panel.

Table 3.3 summarizes the mean FNRs (across all concentrations) for each combination of surrogate and surface material. The FNR curves in Figure 3.2 and the mean FNR values for each combination of surrogate and surface material in Table 3.3 show for both surrogates that the materials with the smoothest surfaces (glass and stainless steel) tend to have the lowest FNRs, while the materials with the roughest surfaces (vinyl tile and plastic panel) tend to have the highest FNRs. The FNRs for both surrogates were the highest with the vinyl tile. Of the tested materials, the vinyl tile was the only material that had measurable pores (4–6 nm) and was the only material that displayed some form of porosity where buffer was absorbed into the material. Based on this, FNR may be dependent on porosity and surface chemistry in addition to surface roughness.

3.5 Limits of Detection

Table 3.5 lists the estimates of LOD_{95} for the macrofoam-swab method with each of the four surface materials for each of the two surrogates. The LOD_{95} values were calculated using the FNR-concentration equations of the form in Eq. (14) with coefficients listed in Table 3.4. The lowest LOD_{95} value for (i) BAS is 0.678 CFU/cm^2 with stainless steel and (ii) BG is 0.820 CFU/cm^2 with glass. The highest LOD_{95} value for (i) BAS is 1.023 CFU/cm^2 with glass and (ii) BG is 1.489 CFU/cm^2 with vinyl tile. It is unexpected that the highest LOD_{95} for BAS occurs with glass. However, this is explained by the FNR-concentration curve for BAS on glass reaching FNR = 0.05 at a higher concentration than for the other surface materials (see Figure 3.2).

Table 3.5. Estimates of the LOD_{95} Values and Approximate Standard Deviations when Sampling Four Surface Materials with the Macrofoam-Swab Method

	***Bacillus anthracis* Sterne**		***Bacillus atrophaeus* Nakamura**	
Surface Material	**LOD_{95}[a] (CFU/cm^2)**	**Approximate $SD(LOD_{95})$ (CFU/cm^2)**	**LOD_{95}[a] (CFU/cm^2)**	**Approximate $SD(LOD_{95})$ (CFU/cm^2)**
Glass	1.023	0.139	0.820	0.081
Stainless steel	0.678	0.072	0.981	0.138
Vinyl tile	0.920	0.025	1.489	0.181
Plastic	0.800	0.051	1.186	0.156

[a] LOD_{95} is the concentration at which the contamination would be correctly detected 95% of the time, calculated as the concentration corresponding to the 5th percentile of the FNR-versus-concentration equation for each combination of surrogate and surface material (in Table 3.4).

4.0 Discussion

This section discusses the results of our macrofoam-swab study relative to the results of previous studies.

4.1 Recovery Efficiency

Comparing the RE results from our study to those from previous swab sampling studies is complicated by several differences. Previous studies used a variety of swab sampling methods and materials (collection, extraction, analytical), surface materials, spore sizes and characteristics, surface concentrations, and were subject to different environmental conditions, each of which may affect REs (Piepel et al. 2012). We focus on comparing the results of our study to those from previous studies using a macrofoam swab.

Hodges et al. (2006) used macrofoam swabs to sample liquid-deposited BAS spores on stainless steel coupons, with RE means ranging from 32 to 49% (with an average of 39%) for six concentrations from 0.4 to 6000 CFU/cm^2. Hodges et al. (2010) conducted a national validation study of the macrofoam-swab sampling method with liquid-deposited BAS spores on stainless steel coupons, and used the same sampling and analytical methods used in our study. Their study resulted in RE means ranging from 15.8 to 31% (with an average of 24.2%) for tests without background dust or other organisms at three concentrations from 1.88 to 1607.2 CFU/cm^2. With dust and other organisms, the RE means ranged from 27.9 to 55% (with an overall average of 41.6%) at BAS concentrations from 1.38 to 1188.5 CFU/cm^2. With stainless steel as the surface material, the RE means from our study ranged from (i) 54.2–85.2% (with an average of 71.7%) over nine BAS concentrations from 0.0807–22.508 CFU/cm^2, and (ii) 55.6–75.8% (with an average of 65.3%) over nine BG concentrations from 0.0904–21.958 CFU/cm^2. It is not clear why noticeably higher RE means were obtained in our study compared to the studies by Hodges et al. (2006) and Hodges et al. (2010). A major difference between this study and the Hodges et al. studies is the method used to prepare spores, including the storage buffer and diluent. Hodges et al. stored and diluted BAS spores in 95% ethanol, while spores for this work were stored in water and diluted in PBS-T. We hypothesize that the presence of Tween 80 could account for the higher RE in this study. DaSilva et al. (2011) investigated parameters affecting spore recovery from wipes. Their study concluded that RE was greater when Tween 80 was present, and that the hydrophobicity or hydrophilicity of the cell surface can be altered when a surfactant is present.

In our study, the magnitudes of REs were not a function of the spore concentrations tested for BAS (0.0807–22.508 CFU/cm^2) or BG (0.0904–21.958 CFU/cm^2). A similar conclusion holds for the Hodges et al. (2010) study, despite the higher range of concentrations investigated in that study (1.38–1607.2 CFU/cm^2). In contrast, the RE values for the Hodges et al. (2006) study tended to increase with increasing concentration over the range 0.4–6000 CFU/cm^2. The results for macrofoam swabs in the Edmonds et al. (2009) study also showed the RE means increasing from 42.1 to 92.7% as the concentration of liquid-deposited BG spores increased (10^3 to 10^6 CFU/cm^2). It is not clear why the Hodges et al. (2006) and Edmonds et al. (2009) studies show RE tending to increase with surface

concentration, which is counter to the results of our study and the study of Hodges et al. (2010). The Hodges et al. (2006) and Edmonds et al. (2009) studies tested far higher spore concentrations than were tested in our study. High spore concentrations can result in layers and clumps of spores rather than a single layer of evenly dispersed spores. This layering could result in greater recovery of the top layers of spores because they are not subject to van der Waals', Coulombic, and other surface forces. Additional variables that may contribute to the differences in RE include the potential purity of the spore preparations, the method used to generate spores, and the presence of a surfactant.

Our study used liquid deposition because dry-aerosol deposition does not reliably achieve the low concentrations at which false negatives occur. However, it is of interest to consider how the RE results of our study might have differed if dry-aerosol deposition had been possible. Edmonds et al. (2009) compared RE means of liquid-deposited spores and dry-aerosol-deposited spores (at high concentrations) when using four different types of swabs to sample each of four surface materials. For some combinations of surface material and swab type, the RE means were statistically significantly higher for liquid-deposited spores than dry-aerosol-deposited spores. For other combinations the opposite was the case, while still other combinations showed no statistically significant difference in RE means from the two deposition methods. Focusing on the macrofoam-swab results of Edmonds et al. (2009), the RE means were statistically significantly higher for liquid deposition than dry-aerosol deposition for two surface materials (89% vs. 61% for glass, 88% vs. 76% for polycarbonate). However, the differences in RE means for liquid deposition versus dry-aerosol deposition were not statistically significant for the other two surface materials (56% vs. 52% for painted steel, 72% vs. 67% for vinyl tile). Hence, we conclude that our study is likely to have at most only slightly to moderately higher RE mean values than if dry-aerosol deposition had been used.

4.2 False Negative Rate and Limit of Detection

Piepel et al. (2012) noted that the FNR performance of sampling methods has been investigated in only one previous study by Krauter et al. (2012). They investigated the cellulose-sponge-wipe method and reported FNRs ranging from 0 to 1 depending on the surface material (six tested) and the concentration of BG (ranging from 0.00248–1.854 CFU/cm^2). These values are comparable to the FNR results in this study, which range from 0 to 0.833 for BAS and 0 to 0.806 depending on the concentration (0.0807–22.508 CFU/cm^2 for BAS and 0.0904–21.958 CFU/cm^2 for BG) and the surface material (four tested). In both our study and that of Krauter et al. (2012), FNR is strongly dependent on surrogate concentration, with smaller but still significant differences in FNR between some surface materials.

Values for LOD (defined and estimated in various ways) have been reported in previous studies with macrofoam swabs. Hodges et al. (2006) reported LOD = 1.2 CFU/cm^2 based on inoculating BAS in a liquid suspension onto growth plates. Estill et al. (2009) reported LOD = 0.4 and 1.9 CFU/cm^2 for BAS aerosolized onto stainless steel and carpet samples, respectively. In our study, LOD_{95} values ranged from 0.678–1.489 depending on the surrogate and surface material. The performance of macrofoam-swab sampling and processing procedures will vary with the bacterial species, soil load, surface material and its characteristics, size of the area sampled, and many other factors. Hence, any LOD is a reflection of the environmental conditions in which the test was conducted.

The FNR of a sampling and analysis method (such as the macrofoam-swab method addressed in this report) can have a significant impact on health-risk decisions. For concentrations below the level at which false negatives begin to occur, but above the level determined to have a health risk, a negative sample result cannot be wholly trusted to indicate the absence of contamination or health risk. One solution that compensates for FNR > 0 is to collect more samples, which increases the probability of detecting the contamination. The FNR-concentration equations developed in this study can be used to estimate the FNRs of the macrofoam-swab method for various *B. anthracis* concentrations and surface materials. The FNR estimates can in turn be used in responding to a future contamination event. For example, FNR estimates are required as inputs to formulas for calculating the number of samples required (with a given statistical sampling approach) to obtain the desired confidence in characterization and clearance decisions (Piepel et al. 2013). Also, after samples are collected and analyzed following a contamination event, FNR values are needed as inputs to formulas for calculating the statistical confidence in characterization and clearance decisions (Piepel et al. 2013).

4.3 Effects of Surface Materials

Surface chemistry effects were previously shown to influence REs of spores in a study using wipes (Buttner et al. 2001, Frawley et al. 2008, Valentine et al. 2008). The four surface materials selected for our macrofoam-swab study are hard, nonporous surfaces (except for vinyl tile, which had pore sizes of 4–6 nm and displayed some liquid-absorption capability). The materials also varied in surface roughness, which ranged from 0.019 to 139.7 μm. Our study showed, for both BAS and BG, that smoother surfaces (glass and stainless steel) tend to have higher RE, lower FNR, and lower LOD_{95} values than for rougher surfaces (vinyl tile and plastic panel).

Probst et al. (2010) reported that REs for BG spores collected from several surface materials using a nylon-flocked-swab sampling method ranged from 5.9 to 62.0%, depending on the roughness of the surface analyzed. Probst et al. (2011) reported REs ranging from 1.4 to 6.6% for samples collected from a rough spacesuit fabric using several swab sampling methods. However, roughness values were not reported in either study. These results agree qualitatively with those of the macrofoam-swab study discussed in this report and the previous sponge-stick wipe study by Krauter et al. (2012) in which smoother surfaces had higher REs. However, two previous studies presented results in which RE did not appear to be correlated with the surface roughness. Estill et al. (2009) obtained lower REs for stainless steel than carpet with all BAS concentrations tested when using macrofoam swabs and sponge-stick wipes. Edmonds et al. (2009) used macrofoam swabs to sample BG deposited (liquid and dry aerosol) on glass, stainless steel, and polycarbonate surfaces. Stainless steel had the lowest REs for both deposition methods, while glass, polycarbonate, and vinyl had the highest REs, depending on the deposition method. Hence, it is unclear when using macrofoam swabs whether RE, FNR, and LOD values will necessarily be better for smoother surfaces. More work is needed to assess the effects of surface properties on RE, FNR, and LOD.

4.4 Effects of Surrogate Microorganisms

The (i) sample collection, extraction, and recovery characteristics of different *Bacillus* species, and (ii) additives or contaminants (such as background dust or other microorganisms) may lead to different results from those reported in this study. In our study, the effects of two *B. anthracis* surrogates (BAS and BG) on the results were assessed. We note that BG spores do not possess an exosporium like BAS (and *B. anthracis*) spores, which suggests that the spore exosporium (or surficial exosporium proteins) or other physical differences between the two spore types could contribute to the higher RE in this study due to physical interactions of the exosporium with the surface material (Ronner et al. 1990).

In our study, RE means were statistically higher for BAS than BG spores, while FNR was only significantly lower for BAS than BG at lower concentrations with glass as the surface material. Probst et al. (2010) reported that the REs for BG spores collected from stainless steel coupons using three swab methods were greater than REs for BAS spores. In contrast, our study of macrofoam swabs with stainless steel (and three other surface materials) found REs to be higher for BAS than BG.

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Appendix A

Tests with Culture Analyses in the Macrofoam-Swab Study

Appendix A: Tests with Culture Analyses in the Macrofoam-Swab Study

Table A.1 lists the tests from Table 6.1 of Piepel and Hutchison (2014) with culture analyses. Note that values are missing in the "Test" column because the tests performed using an mRV-PCR analysis are not included.

Table A.1. Test Matrix for the Split-Split-Plot Experimental Design of the Macrofoam-Swab Study with Culture Analyses[a]

Test Run	Test[b]	BA Surrogate[c]	Concentration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons				# Positive Controls[g]	# Negative Controls		Testing Task[j]		
						Stainless Steel	Glass	Vinyl Tile	Plastic		Coupons[h]	Swabs[i]	A	B	C
1	1	BAS	20	C	BSC1	6	6	6	6	12	4	4	2	1	2
2	3	BAS	25	C	BSC2	6	6	6	6	12	4	4	1	2	1
3	6	BAS	2	C	BSC1	6	6	6	6	12	4	4	1	2	1
4	8	BAS	10	C	BSC2	6	6	6	6	12	4	4	2	1	2
5	9	BAS	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
6	12	BAS	100	C	BSC2	6	6	6	6	12	4	4	2	1	2
7	14	BAS	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
8	16	BAS	500	C	BSC2	6	6	6	6	12	4	4	1	2	1
9	17	BG	20	C	BSC2	6	6	6	6	12	4	4	1	2	1
10	19	BG	25	C	BSC1	6	6	6	6	12	4	4	2	1	2
11	22	BG	2	C	BSC2	6	6	6	6	12	4	4	1	2	1
12	23	BG	10	C	BSC2	6	6	6	6	12	4	4	2	1	2
13	26	BG	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
14	28	BG	100	C	BSC2	6	6	6	6	12	4	4	2	1	2
15	29	BG	15	C	BSC1	6	6	6	6	12	4	4	1	2	1
16	31	BG	500	C	BSC2	6	6	6	6	12	4	4	2	1	2
17	34	BAS	20	C	BSC1	6	6	6	6	12	4	4	2	1	2
18	36	BAS	4	C	BSC1	6	6	6	6	12	4	4	1	2	1
19	38	BAS	25	C	BSC2	6	6	6	6	12	4	4	1	2	1
20	40	BAS	2	C	BSC2	6	6	6	6	12	4	4	2	1	2
21	41	BAS	10	C	BSC1	6	6	6	6	12	4	4	1	2	1
22	43	BAS	5	C	BSC1	6	6	6	6	12	4	4	1	2	1
23	45	BAS	100	C	BSC1	6	6	6	6	12	4	4	2	1	2
24	47	BAS	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
25	50	BAS	4	C	BSC1	6	6	6	6	12	4	4	1	2	1

Table A.1. Test Matrix for the Split-Split-Plot Experimental Design of the Macrofoam-Swab Study with Culture Analyses (contd)

Test Run	Test[b]	BA Surrogate[c]	Concentration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons: Stainless Steel	Glass	Vinyl Tile	Plastic	# Positive Controls[g]	# Negative Controls: Coupons[h]	Swabs[i]	Testing Task[j]: A	B	C
26	51	BG	20	C	BSC2	6	6	6	6	12	4	4	2	1	2
27	54	BG	4	C	BSC1	6	6	6	6	12	4	4	1	2	1
28	55	BG	25	C	BSC1	6	6	6	6	12	4	4	1	2	1
29	58	BG	2	C	BSC1	6	6	6	6	12	4	4	1	2	1
30	60	BG	10	C	BSC2	6	6	6	6	12	4	4	1	2	1
31	61	BG	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
32	63	BG	100	C	BSC2	6	6	6	6	12	4	4	1	2	1
33	66	BG	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
34	68	BG	4	C	BSC2	6	6	6	6	12	4	4	1	2	1

(a) Specifics of the test matrix for each test run not shown in this table are given in Figure 2.1.
(b) The Test number also represents the Run Order number of the tests. Some Test numbers are missing because mRV-PCR tests are not included in this table. The complete table including culture and mRV-PCR tests is given as Table 6.1 in Piepel and Hutchison (2014).
(c) BAS = *Bacillus anthracis* Sterne, BG = *Bacillus atrophaeus* Nakamura.
(d) The concentrations are the target numbers of spores per coupon or positive-control sample, where the coupons are 2 in. × 2 in. (25.806 cm^2). The concentrations were assigned to test runs in a pseudo-random manner.
(e) C = culture.
(f) Two biosafety cabinets (BSCs) were located adjacent to each other on one wall of a laboratory. The left one was designated BSC1 and the right one was designated BSC2. The two BSCs ran tests simultaneously, with the one started first in each pair of tests determined randomly.
(g) There was one positive-control sample associated with the two test coupons on either side of it as shown in Figure 2.1.
(h) For each test in a BSC, there was one blank (uncontaminated) coupon of each of the four surface materials, for a total of four negative coupon controls.
(i) For each test in a BSC, there were four blank swabs, removed from their packages and immediately placed in the same kind of sample containers used for swabs that sampled contaminated coupons.
(j) Two persons performed the testing tasks. These tasks were: (A) sample deposition, (B) sample collection and extraction, and (C) sample analysis. The numbers 1 and 2 represent the specific person assigned to perform each task for each test. For logistics reasons, the person who performed Task A also performed Task C. Persons 1 and 2 were randomly assigned to Tasks A and B for each test, such that each person performed Tasks A and B eight times within the sets of Tests 1–16, 17–32, 33–48, and 51–66. The extra tests (49-50 and 67-68) at a target concentration of 4 spores/coupon had the persons assigned separately.

Appendix B

Thermogravimetric Analysis to Identify Subsurface Porosity in the Four Surface Materials

Appendix B: Thermogravimetric Analysis to Identify Subsurface Porosity in the Four Surface Materials

Thermogravimetric analysis (TGA) was performed on the four surface materials to identify subsurface porosity. Figure B.1 shows the small samples of approximately equal surface area (average of 6.6 mm^2) for each of the four surface materials, after drops of phosphate buffered saline containing 0.02% Tween® 80 (PBS-T) were pipetted onto the surfaces. The TGA results for the four materials are shown in Figure B.2. Given the different densities of each material, mass (percentage of initial) is used for direct comparison.

Figure B.1. Pieces of Vinyl, Plastic, Stainless Steel, and Glass with 2 µL of PBS-T Pipetted onto to the Surface of Each Sample Fragment

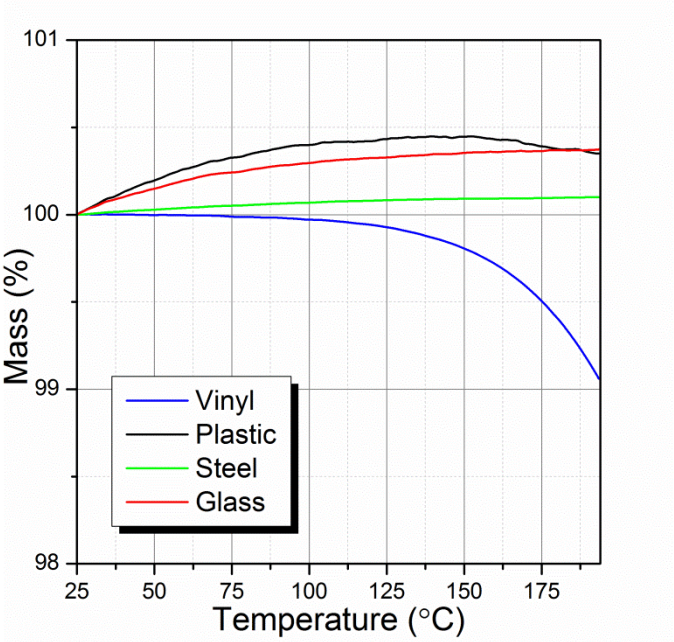

Figure B.2. TGA Profiles for Each of Four Surface Materials as a Function of Increasing Temperature

Appendix C

Summary Statistics Equations for Recovery Concentrations

Appendix C: Summary Statistics Equations for Recovery Concentrations

This appendix presents the formulas for calculating the means, standard deviations (SDs), and percent relative standard deviations (%RSDs) of recovery concentrations (CFU/cm^2) listed in Tables 3.1 and 3.2. The formulas are more complicated than traditional formulas because of the structure of the data, with culture analyses being a split-split-plot experiment.

R_{hijkm} denotes the number of CFUs recovered from the m^{th} coupon of the k^{th} material with the j^{th} concentration of the i^{th} surrogate in the h^{th} block. Recovery concentrations are calculated as

$$r_{hijkm} = R_{hijkm} / 25.8064 \tag{C.1}$$

The mean and SD of r_{hijkm} values over replicate coupons of the k^{th} material with the j^{th} concentration of the i^{th} surrogate are denoted as $\bar{r}_{ijk}$ and $SD(r_{ijk})$. These quantities were calculated using different formulas depending on the target concentration, as discussed previously. The formulas for the mean are

$$\bar{r}_{ijk} = \begin{cases} \sum_{m=1}^{6} r_{hijkm} / 6 & 500 \text{ CFU/coupon } (h = 1) \\ \sum_{m=1}^{12} r_{hijkm} / 12 & 4 \text{ CFU/coupon } (h = 2) \\ \sum_{h=1}^{2} (\sum_{m=1}^{6} r_{hijkm} / 6) / 2 & \text{Remaining target concentrations } (h = 1, 2) \end{cases} \tag{C.2}$$

and the formulas for the standard deviation are

$$SD(r_{ijk}) = \begin{cases} SD(r_{ijk}^{Coupon}) & 4 \text{ and } 500 \text{ CFU/coupon} \\ \{[SD(r_{ijk}^{WP})]^2 + [SD(r_{ijk}^{Coupon})]^2\}^{0.5} & \text{Remaining target concentrations.} \end{cases} \tag{C.3}$$

In the second formula of Eq. (C.3), the squared quantities are the two variance components affecting the r_{hijkm} data for a given "*ijk*" combination. The %RSD of r_{ijk} was calculated as

$$\%RSD(r_{ijk}) = 100\ SD(r_{ijk}) / \bar{r}_{ijk}\ . \tag{C.4}$$

using the appropriate formulas in Eqs. (C.2) and (C.3) for each target concentration.

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