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False Negative Rate and Other Performance Measures of a Sponge-Wipe Surface Sampling Method for Low Contaminant Concentrations

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ABSTRACT

Recovery of spores from environmental surfaces is known to vary due to sampling methodology, techniques, spore size and characteristics, surface materials, and environmental conditions. A series of tests were performed to evaluate a new, validated sponge-wipe method. Specific factors evaluated were the effects of contaminant concentrations and surface materials on recovery

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efficiency (RE), false negative rate (FNR), limit of detection (LOD)—and the uncertainties of these quantities. Ceramic tile and stainless steel had the highest mean RE values (48.9 and 48.1%, respectively). Faux leather, vinyl tile, and painted wood had mean RE values of 30.3, 25.6, and 25.5, respectively, while plastic had the lowest mean RE (9.8%). Results show a roughly linear dependence of surface roughness on RE, where the smoothest surfaces have the highest mean RE values. REs were not influenced by the low spore concentrations tested (3×10^{-3} to 1.86 CFU/cm^2). The FNR data were consistent with RE data, showing a trend of smoother surfaces resulting in higher REs and lower FNRs. Stainless steel generally had the lowest mean FNR (0.123) and plastic had the highest mean FNR (0.479). The LOD_{90} varied with surface material, from 0.015 CFU/cm^2 on stainless steel up to 0.039 on plastic. Selecting sampling locations on the basis of surface roughness and using roughness to interpret spore recovery data can improve sampling. Further, FNR values, calculated as a function of concentration and surface material, can be used pre-sampling to calculate the numbers of samples for statistical sampling plans with desired performance, and post-sampling to calculate the confidence in characterization and clearance decisions.

INTRODUCTION

Despite the rapid evolution of surface sampling technologies following the intentional anthrax contamination of several buildings in 2001, questions remain concerning the reliability of sampling techniques. According to the Centers for Disease Control and Prevention (8), over 125,000 samples from the contaminated buildings were taken during the 2001 incident and processed by the Laboratory Response Network (LRN)—an integrated network of state and local public health, federal, military, and international laboratories that can respond to bioterrorism, chemical terrorism and other public health emergencies. However, results from surface samples did not agree, particularly during assessment of clearance samples (19, 36). In some cases, contamination at a given location within a facility was not detected with initial samples, and subsequent samples were required to detect the contamination.

A Government Accountability Office (GAO) investigation following the 2001 anthrax incident concluded that validated sampling methods and statistical sampling designs were needed to provide confidence that there is no contamination when all sample results are negative (17, 18). This conclusion strongly reinforces the need for characterized, validated sampling methods to effectively respond to biothreats and ensure public safety. In addition, surface sampling is critical in two phases of recovery from a biological contamination incident:

- Locating contamination during the characterization phase
- Verifying areas are uncontaminated or sufficiently decontaminated during the clearance phase of the restoration process.

Following the 2001 anthrax incident, several teams developed and studied the performance of sampling methods using swab, wipe, and vacuum collection devices for *Bacillus anthracis* or surrogate contaminants on different surfaces (5; 46; 6, 7; 39; 41; 22; 28; 2,3,4, 34; 1; 16; 45;

70 13,14; 15; 25, and 23). In addition, the CDC has conducted formal validation studies on two
71 methods for sampling nonporous surfaces: macrofoam swabs (23) and cellulose sponge-wipes
72 (40).

73 A review of the laboratory studies in the literature cited identified numerous gaps in the data
74 on method performance (30). For example, none of the studies quantified the false negative rate
75 (FNR, i.e., probability of a false negative) for the sampling and analysis methods investigated.
76 False negatives could occur during characterization sampling at low contamination levels and
77 during clearance sampling. A better understanding of FNRs and how they are influenced by
78 surface materials and contamination concentration levels is critical in addressing the GAO
79 concerns about method validation and increasing the confidence in negative results.

80 Another gap was lack of wide testing of sponge-wipe sampling methods. The food industry
81 has used sponge-wipe methods for decades (12), and a newly modified and validated sponge-
82 wipe sampling and analysis method (40) is expected to find extensive use in environmental
83 sampling. However, the sponge-wipe method has not been tested at the lower contaminant
84 concentrations that may yield false negatives.

85 The study described in this article aimed at filling these two gaps. First, the study evaluated
86 the sponge-wipe sampling method by testing very low concentrations of spore (*Bacillus*
87 *atrophaeus*) deposited on coupons of a variety of non-porous surface materials, followed by
88 surface sampling, extraction, and analysis. Test results were used to evaluate the effects of
89 contaminant concentrations and surface materials on FNR, as well as recovery efficiency (RE)
90 and limit of detection (LOD) (values examined in other studies), as well as the uncertainties of
91 these values. Findings of this study will provide new insight for interpreting negative results

92 from sponge-wipe surface samples. Also, the variation in RE and FNR for different surface
93 materials will be of high interest in the field of public health.
94
95 ## MATERIALS AND METHODS
96 **Test overview**. The study investigated the performance of the cellulose sponge-wipe method
97 for a range of low surface concentrations on coupons of six surface materials. The surface
98 sampling procedure provides the following:
99 - A standardized method of processing cellulose sponge-wipes of environmental
100 surfaces to culture *Bacillus* spores
101 - A semi-quantitative estimate of the amount of contamination in a building or public
102 place.
103 The sponge-wipe method uses traditional culture methods because organism viability is
104 important in an environmental investigation. Spores are removed from the wipe by mechanical
105 extraction in phosphate buffered saline with Tween-80 (PBST) as a surfactant. The eluted
106 suspension is diluted in series and aliquots are inoculated onto aerobic growth plates, as well as
107 onto membrane filters that are placed on tryptic soy agar (TSA) growth plates. The membrane
108 filters maximize the detection of low numbers of spores. After 48-hr incubation, colony forming
109 units (CFU) are counted. The procedure was modified to reflect a Biohazard Level (BL) 1 for the
110 test microorganism, *B. atrophaeus,* rather than a BL2, as is necessary for *B. anthracis* (44).
111 The experimental work was conducted in 16 test runs, with one concentration of *B.*
112 *atrophaeus* investigated in each test run. In Test Runs 1 – 8, target spore concentrations of $3.10\times$
113 10^{-3}, 7.70×10^{-3}, 1.55×10^{-2}, 2.33×10^{-2}, 3.10×10^{-2}, 1.55×10^{-1}, and 1.86 CFU/cm^2 were used
114 on coupons of three materials (stainless steel, vinyl tile, and ceramic tile). In Test Runs 9 – 16,

target concentrations of 7.70×10^{-3}, 1.55×10^{-2}, 2.33×10^{-2}, 3.10×10^{-2}, 3.88×10^{-2}, 5.43×10^{-2}, 7.75×10^{-2}, and 1.55×10^{-1} CFU/cm^2 were used on coupons of three different materials (primed wood paneling, faux leather, and plastic (acrylic) lighting panels). Within each set of eight test runs, the concentrations were tested in a randomized order. For each test run, 10 coupons of each of the appropriate three materials were assigned in a balanced way to the bench test locations (so that materials would appear roughly the same number of times in the possible bench locations over the course of the study). Three technicians were assigned to three steps of the sampling and analysis process (collecting, processing, enumerating) in a balanced way. These balanced assignments protected against confounding any effects of test locations and technicians with the primary test variables (i.e., contaminant concentration and surface material). (29).

Spore Matrix. The surrogate organism for *B. anthracis* in these tests was *B. atrophaeus* spores, American Type Culture Collection ATCC# 9372 (formerly *B. subtilis* var. Niger) from Apex Laboratories (Apex, NC). The material is Lot# 718701-E9 with a mean population of 3.5×10^9 CFU/mL in water, post-heat shocked at (80 − 85ºC) for 10 min. These spores were not surface-enhanced and ranged in size from about 0.9 to 1.1 μm. Spore stock solutions were made in PBS with 0.01% Tween buffer, autoclaved, and then sterile-filtered. Each spore concentration was verified periodically (throughout the duration of the corresponding test run) by plating eight replicate samples per concentration.

Coupon characteristics. Coupons were cut to match the recommended area for surface wipe samples, 25.4 by 25.4 cm (645.16 cm^2). Sample surface materials selected for testing **(**stainless steel, vinyl tile, ceramic tile, faux leather, plastic light panel, and primed wood) were relatively nonporous and chosen to represent materials commonly found in buildings. Stainless steel served as the standard test surface, as the majority of previous sampling studies were performed on

stainless steel. It represents a universally recognized carrier and also serves as a conservative proxy for building material roughness. The stainless steel coupons were cut from 1.2-mm-thick 316-L stainless steel (Neeley Plastic Fabrication Inc., Albuquerque, NM). The vinyl tiles were Armstrong Excelon Vinyl Composition Tile #51830 (Armstrong World Industries Inc., Lancaster, PA). The glazed ceramic tiles were from Dal-Tile Corp. (Dallas, TX). The faux leather is a fabric made of polyester yarn with a vinyl surface finished with a urethane topcoat (Spradling International, Inc., Pelham, AL). The plastic light cover was an acrylic, cracked-ice, ceiling light panel (Plaskolite, Inc., Columbus, OH). The wood panel was primed with acrylic paint (DPI Decorative Panels Intl., Toledo, OH).

Prior to spore deposition, the stainless steel, ceramic tile, vinyl tile, and plastic light cover coupons were washed with Alcojet powdered detergent (Alconox Inc., New York, NY), rinsed, treated with 10% bleach (30-min contact time) and rinsed in deionized water and air dried. The faux leather and primed wood coupons were treated with 10% bleach (30-min contact time), wiped with sodium thiosulfate to neutralize the bleach, then air-dried. This modification to the coupon cleaning treatment was necessary to avoid damaging these two materials. Pre-cleaned coupons were placed on clean laboratory tabletops in pre-determined locations. Each was wiped with 95% ethanol using a sterile gauze wipe and the right corner of each coupon (outside the sampling area) was labeled with a unique number linked to the sample's type, number, and placement.

The surface roughness of coupon materials was measured using a surface roughness tester (Phase II Machine & Tool Inc., SRG-1000). Roughness is a measure of vertical deviations of the surface material. The tester uses a piezoelectric pick-up stylus with diamond tip to measure a range between 0.05 – 10.0µm.

Spore deposition. Spores were deposited onto coupons using a liquid inoculation technique. Sampling method performance studies have primarily employed protocols that use spores suspended in aqueous buffers to prepare test surfaces (35, 37, 43). Liquid inoculation methods allow relatively easy control of the number of spores and the contaminated area. Liquid-deposition was used in this study because the concentrations to be tested were well below the sample method's LOD and thus needed to be accurately controlled.

The spores in buffer were dropped onto the cleaned and sterilized 25.4 by 25.4 cm coupons. Each spore stock solution was stirred constantly using a stir plate (Scienceware, F37017-0000 Battery-Powered Magnetic Stirrer). From the spore stock solution, a 1-mL pipettor (Thermo Electron 4500120, calibrated following manufacture's recommendations) was used to deposit 20 droplets (0.05 mL each) on each coupon in a pattern from left to right. During the tests, the temperature was maintained at 25 $\pm$ 2°C and the relative humidity was maintained between 30 − 45%. The inoculated coupons were dried for approximately 2 hr.

Positive and negative controls. A positive control (reference) sample was co-located with each test coupon (i.e., 30 test coupons and 30 co-located positive control samples per test). A 1-mL sample of the spore titer solution was directly inoculated onto Petrifilm™ aerobic count plates (3M, St. Paul, MN). The reference plates were incubated for 48 hr; the results were counted and recorded on an automatic plate count reader (3M Petrifilm™ Plate Reader Model 6499, St. Paul, MN); and the counts were manually verified. The counts of reference plates were used to verify the concentrations of each spore stock solution and served as the reference value for recovery efficiency calculations. Extraction and processing was the same for the positive control and test samples.

For each of the 16 tests, 16% of the samples (5 samples per 30 test samples) were laboratory process controls and 25% of the samples (8 media samples per 30 test samples) were analyzed as material and media (culture and buffer) control samples. All laboratory material and media control samples were negative for spore growth.

Wipe sampling and analysis method. This study used the sampling procedure for collection of samples on hard non-porous surfaces in both indoor and outdoor environments (9). Using a sterile technique and a sterile, pre-moistened sponge-wipe, each test coupon was wiped using an overlapping 'S' pattern with horizontal strokes. The wipe was then rotated, and the coupon was wiped using vertical 'S' strokes. The sample area was then wiped using a diagonal "S' strokes, and the process was concluded by wiping the edges of the coupon.

Lab processing methods. A modified LRN procedure for recovering spores from wipes (40) was used. Spore extraction from the sponge-wipe was accomplished by transferring each sponge-wipe to the stomacher bag (Seward Stomacher® 400 Circulator, Seward; catalog #0400/001/AJ; closure bags, Seward, catalog # BA6141/CLR) using sterile forceps. PBST (90 mL) was added to the bag and the stomacher was set to 260 revolutions per minute (RPM) for 1 min. The wipe was squeezed and moved to the top of the bag, then removed with sterile forceps. The elution suspension was mixed and then centrifuged at 3500 × g for 15 min. The 9-mL elution suspension was concentrated to 6 mL. The concentrated suspension was vortexed for 30 sec and sonicated at 40 kHz in a sonicator bath (Branson Ultrasonic Cleaner Model 1510, Process Equipment and Supply, Inc.; #952-116) for 30 sec; sonication and vortexing steps were repeated twice. The final volume of suspension was measured. Aliquots of the spore elution were serially diluted in Butterfield buffer using standard methods. One mL of the dilution was plated on growth media, in triplicate.

If there was no growth on a plate, then an aliquot of the remaining spore suspension was filtered (vacuum filtration manifold and MicroFunnel Filter Funnels, Fisher Scientific) using micro-funnel membranes (0.45 μm MCE membrane, VWR; catalog #28143-544) and the membrane was cultured on TSA plates.

Recovery efficiency, false negative rate, and uncertainties. Recovery efficiencies (RE) were calculated using the formula

$$RE_{hijk} = R_{hijk} / \overline{C}_{hi}, \quad (1)$$

where RE_{hijk} is RE for the k^{th} coupon of the j^{th} material with the i^{th} concentration in the h^{th} block; R_{hijk} is the CFUs recovered from the k^{th} coupon of the j^{th} material with the i^{th} concentration in the h^{th} block; and $\overline{C}_{hi}$ is the mean CFUs from the positive controls for the i^{th} concentration in the h^{th} block. The mean and standard deviation (SD) of RE over the n_{hij} (usually 10) coupons of the j^{th} material with the i^{th} concentration in the h^{th} block were calculated (using standard formulas) and are denoted as $\overline{RE}_{hij}$ and $SD(RE_{hij})$. The standard error of $\overline{RE}_{hij}$ was calculated as

$$SE(\overline{RE}_{hij}) = SD(RE_{hij}) / \sqrt{n_{hij}} \quad (2)$$

and the percent relative standard deviation (%RSD) was calculated as

$$\%RSD(RE_{hij}) = 100\, SD(RE_{hij}) / \overline{RE}_{hij}. \quad (3)$$

False negative rate values are denoted by FNR_{hijk}, which is the FNR of the k^{th} coupon of the j^{th} material with the i^{th} concentration in the h^{th} block. The mean and standard deviation of these FNR_{hijk} values for each "hij" combination were calculated (using the standard formulas) over the n_{hij} (typically 10) test-coupon samples, and are denoted as $\overline{FNR}_{hij}$ and $SD(FNR_{hijk})$. The standard error of $\overline{FNR}_{hij}$ was calculated as

$$SE(\overline{FNR}_{hij}) = SD(FNR_{hij}) / \sqrt{n_{hij}}. \quad (4)$$

Below the contaminant concentration at which false negatives first begin to occur for the sponge-wipe method, the FNR generally increases as concentration decreases. Using statistical methods to develop equations that relate experimentally determined FNRs to the concentration of the contaminant was therefore of interest. Such equations would allow prediction of the FNR at any concentration within the range that false negatives occur. Further, statistical methods enable calculation of the uncertainty in the predicted FNR at a given concentration.

The three-coefficient cumulative-distribution form of the Johnson SB equation (20, Section 6.1, 26)

$$FNR_{hijk} = 1 - \Phi\left(\gamma_j + \delta_j \ln\left(\frac{\overline{C}_{hi}}{\lambda_j - \overline{C}_{hi}}\right)\right) \quad (5)$$

was used to relate FNR to contaminant concentration for each of the six surface materials (j = 1, 2, …, 6). FNR_{hijk} and $\overline{C}_{hi}$ were defined previously, where $0 \le \overline{C}_{hi} \le \lambda$; Φ is the standard normal (Gaussian) cumulative distribution function; and γ, δ (>0), and λ (>0) are three coefficients estimated from the experimental data by nonlinear weighted-least-squares regression (42).

Limits of detection and uncertainties. The limit of detection of the sponge-wipe method for a given surface material is defined and estimated in two ways. The first method (denoted LOD_{90}) is the lowest concentration reliably detected (i.e., at least one CFU >90% of the time; AOAC 1999). The second (denoted LOD_{95}) is the contaminant concentration for which there is a 95% probability of correct detection (PCD). An estimate of the LOD_{95} is calculated for a given surface material using the corresponding FNR-concentration equation of the form in Eq. (5). Specifically, the LOD_{95} is the concentration at which the equation predicts FNR = 0.05 (i.e., the PCD = 0.95). The statistical bootstrap method (11) was used to calculate a 95% confidence interval for the estimated LOD_{95} value of each surface material.

250

251

RESULTS

Recovery efficiencies and uncertainties. Table 1 summarizes the mean recovery efficiencies ($\overline{RE}_{hij}$) and uncertainties ($\%RSD(RE_{hij})$) for the sponge-wipe method for each combination of concentration and surface material. Because a positive control was paired with each of the 30 test coupons in each test run, the option existed to calculate RE using the results from each pair of test coupon and corresponding positive control. However, analysis of the positive control data revealed no significant differences in results by bench location or technician. Hence, REs were calculated using Eq. (1), the denominator of which is the mean contaminant concentration of the positive-control samples (generally 30) in a given test run.

Over the range of *B. atrophaeus* concentrations tested (2.48×10^{-3} to 1.85 CFU/cm^2, based on average concentrations of positive control samples) there was no dependence of RE on spore concentration for any surface material. However, uncertainties in RE values tend to increase as the concentration decreases, as shown in Table 1. To illustrate these conclusions for stainless steel coupons, Figure 1 displays the $\overline{RE}_{hij}$ values with ± 1 $SE(RE_{hij})$ error bars. The $\overline{RE}_{hij}$ values for stainless steel range from 36 to 63% (average of 52%), while the $SE(RE_{hij})$ values ranged from 2 to 21%.

A weighted analysis of variance with Tukey's multiple comparison procedure showed that the different surface materials tested have different mean RE values. Table 2 lists the mean RE (across all concentrations) for each surface material, noting the pairs of surface materials with statistically different RE mean values ($p < 0.0001$). Ceramic tile and stainless steel had the largest mean RE values (48.1 and 48.9%, respectively); these mean values were not statistically different. Faux leather, vinyl tile, and painted wood had mean RE values of 30.3, 25.6, and 25.5, respectively. Some, but not all, pairs of these materials had statistically different mean RE values

(see Table 2). Plastic had the lowest mean RE (9.8%), which is significantly lower than the mean REs of all other materials.

To further explore the dependence of RE on surface materials, mean RE values were compared to the roughness indices of the test materials. The mean RE (across all spore concentrations) for each surface material and the corresponding roughness index measurement are listed in Table 2. The roughness indices range from 0.13 to 5.88 μm. Figure 2 shows that there is a roughly linear dependence of surface roughness on RE, with the smoothest surfaces showing the largest mean RE values. The standard errors for the mean RE values shown in Figure 2 were calculated using a bootstrap approach (11), accounting for two sources of variation (between and within concentrations) and the fact that variations in RE values depend on concentration.

FNRs and uncertainties. FNRs were calculated using the positive control data and test coupon data, as described previously. No false negatives occurred for positive control samples, except for a single test at a target concentration of 3.10×10^{-3} (CFU/cm^2 = 2 CFU/coupon). For that test, the FNR values for the 30 positive control samples had mean = 0.133 and standard error = 0.024.

For the test coupon data, Table 1 lists the mean ($\overline{FNR}_{hij}$) and standard deviation ($SD(FNR_{hijk})$) values of FNR for each combination of surface material and target concentration (ij). The $\overline{FNR}_{hij}$ values range from 0 to 1. The $SD(FNR_{hijk})$ values are relatively large (ranging up to 0.367) because they are the uncertainties in FNR values for a single test coupon (FNR_{hijk}), and FNR_{hijk} could only take values of 0, 1/3, and 1 in this work. Although not shown in Table 1, the $SE(\overline{FNR}_{hij})$ values were calculated using Eq. (4), and range up to 0.122.

297 Table 3 shows the coefficients from fitting Eq. (5) to the FNR-concentration data for each of
298 the six surface materials. Figure 3 shows the mean FNR results from test coupons of the six
299 surface materials ($\overline{FNR}_{hij}$) plotted against contaminant concentration ($\overline{C}_{hi}$) from the positive
300 control data. Figure 3 also shows the corresponding fitted equations from Table 3.
301 The FNR-concentration equations listed in Table 3 and shown in Figure 3 are subject to
302 considerable uncertainty, because the data used to fit the equations are subject to considerable
303 uncertainty. The relatively high uncertainty in the test data, and hence in the fitted curves, leads
304 to differences between the curves and certain data points in some cases. The FRN curves are
305 consistent with RE data, showing a trend of smoother surfaces resulting in higher REs and lower
306 FNRs. Stainless steel generally had the lowest FNRs and plastic had the highest FNRs. Figure 3
307 curves below FNR = 0.4 had low to high occurrence of false negatives, which also trended with
308 the surface roughness (stainless steel < ceramic < vinyl < faux leather < painted wood < plastic
309 light covers). Table 2 summarizes the mean FNR (across all concentrations) for each surface
310 material. The lowest mean FNR (0.12) occurs for the smoothest surface (stainless steel) and the
311 largest mean FNR (0.48) occurs for the roughest surface (plastic panel). The linear trend between
312 FNR and roughness index is not as strong as for RE, because FNR depends on concentration as
313 well as surface material. Hence, the mean FNR value for each surface material is affected by the
314 nature of FNR dependence on concentration for each surface material.
315 **Limit of detection.** Two methods were used to define and estimate the limit of detection for
316 the sponge-wipe method, denoted LOD_{90} and LOD_{95}, as discussed above. Table 4 lists the
317 estimates of these quantities for the sponge-wipe method with each of the six surface materials.
318 The LOD_{95} values for the six surface materials were calculated using the FNR-concentration

equations of the form in Eq. (5) with coefficients listed in Table 3. Also shown in Table 4 is the 95% confidence interval on each LOD_{95} value.

Stainless steel and ceramic tile have the smallest LOD_{90} and LOD_{95} values, while the plastic lighting panel and vinyl tile have the largest LOD_{90} and LOD_{95} values. These results are consistent with stainless steel and ceramic tile having the lowest, and the plastic lighting panel and vinyl tile having the largest, values of surface roughness index. Figure 3 curves cross midway. However, at the low FNR (e.g., below 0.4), the effects of surface materials on FNR, LOD_{90}, and LOD_{95} occur in the same order (stainless steel < ceramic < faux leather < painted wood < vinyl < plastic light cover).

DISCUSSION

The RE, FNR, and LOD results from the sponge-wipe study described in this article are discussed relative to results from previous studies. In many cases, this section attempts to explain the underlying reasons for the differences in results, which seem to largely stem from different test conditions and methods, and different contaminant concentrations. This section also notes information gaps filled by this study that can aid in future decontamination, recovery, and public health efforts.

Recovery efficiency

Comparisons of our study's RE results to those from previous sampling studies are problematic because those studies used a variety of surface materials, surface concentrations, and methods, each of which introduces specific limitations. When RE values from our study were compared to other surface sampling studies using stainless steel as a test surface, we saw greater recovery efficiency (RE in our study ranged from 36 to 63% with an average of 48%).

342 Presumably this higher RE rate resulted from our study's use of an improved sponge-wipe
343 method compared to methods tested in previous studies and different test methods, e.g., spore
344 type, deposition, or environmental controls. In our study, REs were not influenced by the low
345 spore concentrations tested (3×10^{-3} to 1.86 CFU/cm^2). Rose et al. (40) conducted a national
346 validation study with stainless steel coupons using the same sampling and analytical methods as
347 used in our study. Their study resulted in an overall average RE of 29.7 (ranging from 24.4 to
348 34.6%) when testing liquid deposited *B. anthracis* Sterne concentrations of 0.013, 0.277 and
349 17.127 CFU/cm^2. Similar to our study, Rose et al. (40) saw no effect of spore concentration on
350 RE. Our higher RE may be due in part to the difference in surrogate and the lack of a mixed
351 microbial culture.
352 Studies that used aerosol deposition have the difficult task of collecting samples with loosely
353 attached spores particularly in conditions of low relatively humidity. Spores can potentially be
354 reaerosolized during sampling. Edmunds (13) found that recovery of liquid-deposited spores
355 differs significantly from recovery of dry aerosol-deposited spores in most instances. He reported
356 89% RE from liquid-deposited spores and 61% from aerosol-deposited spores when using
357 macrofoam swabs and testing recovery from glass coupons. Estill et al. (15), using gauze wipes
358 to sample aerosol-deposited spores on stainless steel, reported REs ranging from 18%
359 (deposition concentration of 2.7 CFU/cm^2) to 31% (deposition concentration of 0.08 CFU/cm^2).
360 Brown (2007a), using polyester-rayon blend (gauze) wipes to sample aerosol-deposited spores
361 on stainless steel, reported a mean RE of 35%.
362 Because swabs made from macrofoam have material similarity to cellulose sponge-wipes,
363 whereas cotton swabs or gauze wipes are considerably different from sponge wipes, RE
364 information from macrofoam swabs is of interest. Hodges's (22) study using macrofoam swab

samples and *B. anthracis* Sterne spores on stainless steel coupons resulted in RE ranging from 32 to 49% (spore concentrations from 0.1 to 2.9 × 10^3 CFU/cm^2). In the Edmonds et al. (2009) study using macrofoam swabs, the RE increased as the concentration of liquid-deposited spores increased (10^3 to 10^6 CFU/cm^2). Edmonds et al. (14) results are counter to the results of our study, but the spore concentrations used by Edmonds were far greater and the test methods differed. High spore concentrations can result in layers of spores rather than a monolayer of spores. This layering can result in greater recovery of the top layers of spores because they are not subject to van der Waals', Coulombic, and other surface forces.

Surrogate selection

The extraction and recovery characteristics of a different *Bacillus* species, native spore materials, or additives may lead to different results from those reported in this study. Spore inoculum used in this study did not include any surface treatment, such as silicon dioxide coating. It is recognized that the *Bacillus* species investigated in this study, *B. atrophaeus*, does not possess an exosporium like that seen in *B. anthracis*. Nonetheless, the sample RE results reported in the literature provide valuable information for the interpretation of sponge-wipe sample analytical results. Probst et al. (32) reported that the REs for *B. atrophaeus* spores removed from stainless steel coupons were greater than those found with *B. anthracis* spores. This result suggests that different physiochemical adhesive properties, such as hydrophobicity or molecular composition of spore sheaths, may affect the release of spores from surfaces (38). In addition, our study did not attempt to evaluate method efficiency in the presence of dust, bacterial vegetative cells, fungal spores, detritus, or other native background material that might interact with extraction or RE.

False negative rate and level of detection

388 The FNR performance of sampling methods has not been investigated in previous studies
389 documented in the literature (30). Hence, there are no previous results to compare with our FNR
390 results reported above. In contrast, values for limits of detection (defined and estimated in
391 various ways) have been reported in previous studies. Rose et al. (40) reported a LOD_{90} of 0.031
392 CFU/cm^2 using the sponge-wipe method on stainless steel. Our study found a slightly lower
393 LOD_{90} value for stainless steel (0.015 CFU/cm^2). Brown (2007a), using aerosol-deposited spores
394 on stainless steel and polyester-rayon blend (gauze) wipes, reported a LOD estimated at 3.6
395 CFU/cm^2. This result may be attributed to deposition methods of spore concentrations which
396 ranged from 10^2 to 10^5 CFU/cm^2. The efficiency of the sampling and processing procedures will
397 vary with the surface material and texture, the soil load, the size of the sample area, the bacterial
398 species, and may other factors. Hence, any LOD is a reflection of the environmental conditions
399 in which the test was conducted.
400 The FNR of a sampling and analysis method (such as the sponge-wipe method addressed in
401 this article) can potentially have a major impact on health-risk decisions. For concentrations
402 below the level at which false negatives begin to occur, but above levels determined to have a
403 health risk, a negative sample result cannot be completely trusted to indicate the absence of
404 contamination or health risk. One solution for compensating for FNR is to collect more samples.
405 The FNR-concentration equations developed in this study can be used to estimate the FNR (of
406 the sponge-wipe method) for various concentrations and surface materials. This FNR estimate
407 can in turn be used in responding to a future *Bacillus anthracis* contamination event. For
408 example, estimates of the FNR are required as inputs to formulas for calculating the number of
409 samples required (with a given statistical sampling approach) to obtain the desired confidence in
410 characterization and clearance decisions. Also, after samples are collected and analyzed

411 following a contamination event, FNR values are needed as inputs to formulas for calculating the
412 statistical confidence in characterization and clearance decisions.

413 **Surface materials**

414 All of the surfaces selected for this study are hard non-porous surfaces, but varied in surface
415 roughness. The surface characteristics evaluated, including hydrophobicity, statics, contact angle
416 and porosity (unpublished data), did not show a relationship with RE, FNR, or LOD. However, a
417 roughly linear relationship was seen in mean RE (ranging from 9.8 to 48.9%), with the highest
418 REs from the smoothest surfaces.

419 Probst et al. (32, 33) reported that REs for *B. atrophaeus* spores from different surfaces
420 showed a variation from 5.9 to 62.0%, depending on the roughness of the surface analyzed.
421 However, roughness values were not provided. Estill et al. (15) tested stainless steel and carpet
422 and found higher RE on stainless steel for all spore concentrations tested. These findings,
423 combined with the similar findings of our study, may have implications for field sampling.
424 Specifically, technicians could sample locations with smooth surfaces and take a quick
425 measurement of the roughness index in order to roughly estimate RE, FNR, and LOD.

426 **Sample media**

427 Our study used a 3M™ cellulose sponge-wipe on a stick that was pre-moistened with a
428 neutralizing buffer. We found that the depths of folded sponge-wipe samples varied by up to
429 300%, a variation that may introduce variation in RE. Because the sponge-wipes came pre-
430 moistened, the difference in sponge volume could lead to a difference in buffer distribution
431 throughout the sponge, which would cause the outside of the sponge to be drier in a low-
432 humidity environment. This in turn, could affect the spore RE. However, in our study the relative
433 humidity was maintained at a consistent range (RH 30-45%) during all testing.

Sampling variation

Imperfect (< 100%) sampling recovery is common, and the variation in sampling methodology, techniques, spore size and characteristics, surface materials, and environmental conditions will cause variation in REs, FNR, and LOD. Da Silva et al. (10) stated that the overall RE is sensitive to the applied experimental conditions due to a wide range of potential variables in surface sample collection methodologies, such as differences in extraction solution, adsorptive material, surface substrate, and surrogate biomaterial. Further, spore recovery from a surface is complex due to multiple factors, including spore characteristics, environmental factors, presence of grime and/or competing microorganisms, sample media, and method (41). A way to control some of these factors is to use standardized test methods. New validated methods (such as the new sponge-wipe method) are improving the RE and LOD, as well as decreasing the FNR, of surface sampling.

Environmental contaminant concentrations and health risk

Price et al. (31) and Hong *et al*. (24) discussed approaches for linking environmental concentrations of *B. anthracis* spores on surfaces in buildings with human health risk. For example, for a retrospective inhalation risk of 10^{-3}, Price calculates an estimated environmental concentration range of 0.36 to 3.4×10^2 spores/m^2 (3.6×10^{-5} to 0.034 CFU/cm^2). Hong *et al.* states that the ability to reliably sample is a prerequisite for the implementation of environmental standards and that sampling in this very low concentration range would require large sampling areas. Our study found an LOD for stainless steel of 0.015 CFU/cm^2 using the validated sponge-wipe method; other surface materials tested ranged from 0.015 to 0.039 CFU/cm^2. These findings suggest that the performance improvements obtained with the validated sponge-wipe method is approaching what is required to reliably detect *B. anthracis* at lower surface

concentrations. However, sampling techniques for large areas is an important topic for future efforts.

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TABLE 1. Performance measures of sponge-wipes with liquid-deposited *Bacillus atrophaeus* spores on coupons (645.16 cm^2) of six surface materials

Target Deposition, CFU/coupon[a] (CFU/cm^2)	Block[b]	Positive Control Concentration (CFU/cm^2)		Surface Material[d]	# Test Coupons	Test Coupons					
						Recovery Conc. (CFU/cm^2)		Recovery Efficiency (%)		FNR	
		Mean[c]	%RSD[c]			Mean[e]	%RSD[e]	Mean[f]	%RSD[f]	Mean[g]	SD[g]
2 (0.00310)	1	0.00248	58.3	S	10	0.00155	105.41	62.5	105.94	0.600	0.322
				V	10	0.00031	316.23	12.5	316.41	0.933	0.211
				C	10	0.00093	161.02	37.5	161.37	0.800	0.322
5 (0.00775)	1	0.00677	25.9	S	10	0.00372	76.58	55.0	76.72	0.367	0.367
				V	10	0.00217	96.42	32.1	96.54	0.600	0.344
				C	10	0.00217	96.42	32.1	96.54	0.567	0.387
	2	0.00775	15.8	L	10	0.00031	316.23	4.0	316.62	0.933	0.211
				W	10	0.00124	129.10	16.0	129.13	0.733	0.344
				P	10	0.0	0.0	0.0	0.0	1.0	0.0
10 (0.01550)	1a	0.01669	10.5	S	10	0.00961	32.08	57.6	32.14	0.033	0.105
				V	10	0.01085	43.12	65.0	43.16	0.0	0.0
				C	10	0.00899	59.62	53.9	59.65	0.100	0.161
	1b	0.01519	14.3	S	10	0.00961	28.25	63.3	28.37	0.0	0.0
				V	10	0.00713	21.00	46.9	21.16	0.0	0.0
				C	10	0.01147	42.35	75.5	42.43	0.0	0.0
	2	0.01535	15.3	L	10	0.00651	41.70	42.4	41.79	0.100	0.316
				W	10	0.00279	81.98	18.2	82.03	0.500	0.360
				P	10	0.00031	316.23	2.0	316.24	0.933	0.211
15 (0.02325)	1	0.02253	12.6	S	10	0.01116	14.34	49.5	14.53	0.0	0.0
				V	10	0.00341	67.08	15.1	67.12	0.367	0.367
				C	10	0.01085	27.77	48.2	27.86	0.0	0.0
	2a	0.02356	10.1	L	10	0.00837	30.49	35.5	30.55	0.033	0.105
				W	10	0.00713	21.00	30.3	21.08	0.0	0.0
				P	10	0.00155	105.41	6.6	105.43	0.67	0.351
	2b	0.02289	11.6	L	10	0.00682	28.75	29.8	28.83	0.033	0.105
				W	10	0.00589	29.88	25.7	29.95	0.077	0.141
				P	10	0.00031	316.23	1.4	316.23	0.933	0.211
20 (0.03100)	1	0.03064	10.4	S	10	0.01581	19.50	51.6	19.59	0.0	0.0
				V	9	0.00586	31.81	19.1	31.87	0.074	0.147
				C	10	0.01581	28.41	51.6	28.48	0.033	0.104
	2	0.03276	9.8	L	10	0.00899	25.44	27.4	25.51	0.033	0.105
				W	10	0.00341	51.60	10.4	51.64	0.333	0.272
				P	10	0.00093	161.02	2.8	161.03	0.800	0.322
25 (0.03875)	1	0.03725	9.4	S	10	0.01333	22.06	35.8	22.13	0.0	0.0
				V	10	0.00682	28.75	18.3	28.80	0.067	0.141
				C	9	0.01584	18.16	42.5	18.24	0.0	0.0
	2	0.03834	7.0	L	10	0.00930	35.14	24.3	35.16	0.033	0.105
				W	10	0.00930	31.43	24.3	31.45	0.0	0.0
				P	10	0.00527	39.70	13.7	39.72	0.200	0.322
35 (0.05425)	2	0.05430	6.6	L	10	0.01550	21.08	28.5	21.12	0.0	0.0
				W	10	0.01798	15.84	33.1	15.89	0.0	0.0
				P	10	0.00651	35.14	12.0	35.16	0.100	0.161
50 (0.07750)	2	0.07905	4.4	L	10	0.06076	27.86	76.9	27.87	0.0	0.0
				W	10	0.03906	16.39	49.4	16.41	0.0	0.0
				P	10	0.01395	24.00	17.6	24.02	0.0	0.0
100 (0.15500)	1	0.15629	3.7	S	10	0.07194	14.34	46.0	14.35	0.0	0.0
				V	9	0.03548	18.07	22.7	18.08	0.0	0.0
				C	10	0.07037	12.29	45.0	12.31	0.0	0.0
	2	0.15371	3.0	L	10	0.08339	18.99	54.3	19.00	0.0	0.0
				W	10	0.05642	18.82	36.7	18.83	0.0	0.0
				P	10	0.03968	22.04	25.8	22.04	0.0	0.0
1200 (1.86000)	1	1.85380	2.9	S	10	0.97402	8.84	52.5	8.86	0.0	0.0
				V	10	0.46934	10.55	25.3	10.56	0.0	0.0
				C	10	0.99355	6.56	53.6	6.58	0.0	0.0

[a] Target number of spores deposited per 25.4 cm x 25.4 cm coupon (645.16 cm^2)

[b] Block of testing (1, 2), where a and b denote replicate tests at a given concentration

[c] Mean and %RSD of concentrations, calculated over 30 positive controls for each target

concentration in a block

[d] S = stainless steel, V = vinyl tile, C = ceramic tile, L = faux leather, W, painted wood, P = plastic

[e] Mean and %RSD of recovery concentrations calculated over the # test coupons for a target concentration and surface material

[f] Mean and %RSD of recovery efficiency calculated over the # test coupons for a target concentration and surface material

[g] Mean and SD of FNRs calculated over the # test coupons for a target concentration and surface material

Table 2. Recovery efficiency and false negative rate (averaged over all spore concentrations) for each surface material with the corresponding roughness index measurement

Surface Material	Recovery Efficiency, Mean (%)	Comparison of RE Means[a]	False Negative Rate, Mean	Roughness Index[b] (□m)
Stainless Steel	48.1	A	0.1229	0.13
Ceramic Tile	48.9	A	0.1812	0.59
Vinyl Tile	25.6	B,D	0.2551	1.63
Faux Leather	30.3	B,C	0.1417	3.27
Painted Wood	25.5	C,D	0.2000	4.11
Plastic Panel	9.8		0.4792	5.88

[a] All pairs of surface materials have statistically different mean RE values ($p < 0.0001$) except those pairs with the same letters (A, B, C, D), based on a weighted analysis of variance with Tukey's multiple comparison procedure (Miller 1981).For example, stainless steel (A) and ceramic (A) are not statistically different from each other but are different from all other materials. No letter is shown for Plastic Panel because its mean RE was significantly lower than the mean REs of all other surface materials.

[b] Roughness parameter Ra (arithmetic average of absolute values) was computed to conform to ISO class 3.

Table 3. Coefficients of the Johnson SB equations in Eq. (5), which relate false negative rate to contaminant concentration for each surface material

Surface Material	Coefficient		
	γ	Δ	λ
Stainless steel	3.205	0.958	0.079
Ceramic tile	4.736	1.898	0.079
Vinyl tile	1.705	0.929	0.079
Faux leather	5.873	3.483	0.079
Painted wood	3.506	1.838	0.079
Plastic	0.506	1.900	0.079

Table 4. Estimates of the LOD_{90} and LOD_{95} values for sponge-wipe method for the six surface materials, with 95% confidence intervals for the LOD_{95} estimates

Surface Material	LOD_{90}[a] (CFU/cm^2)	LOD_{95}[b] (CFU/cm^2)	95% CI on LOD_{95} (CFU/cm^2)
Stainless steel	0.015	0.013	(0.010, 0.015)
Ceramic tile	0.015	0.013	(0.007, 0.015)
Vinyl tile	0.031	0.038	(0.029, 0.047)
Faux leather	0.015	0.018	(0.010, 0.022)
Painted wood	0.023	0.021	(0.018, 0.024)
Plastic	0.039	0.051	(0.049, 0.054)

[a] LOD_{90} is the lowest concentration tested that yielded 90% samples with >1 CFU per sample (AOAC 1999).

[b] LOD_{95} is the concentration at which the contamination would be correctly detected 95% of the time, calculated as the concentration corresponding to the 5^{th} percentile of the FNR versus concentration equation for each surface material (in Table 3).

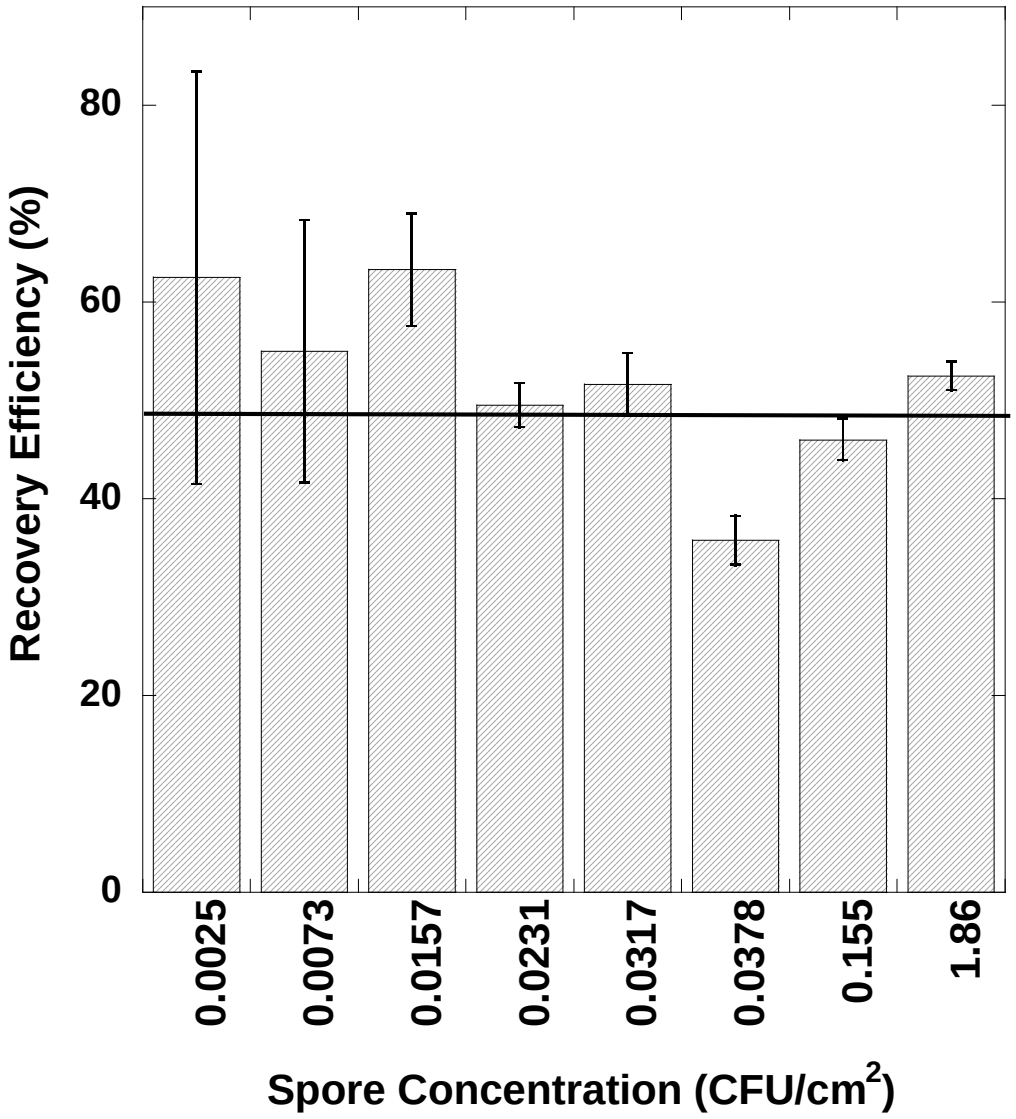

Figure 1. Mean values of recovery efficiency for the sponge-wipe method applied to stainless steel coupons plotted versus spore concentration from positive control samples. The error bars are ± 1 SE, as calculated by Eq. (2). The horizontal line across the figure shows the average recovery efficiency for all concentrations (48%).

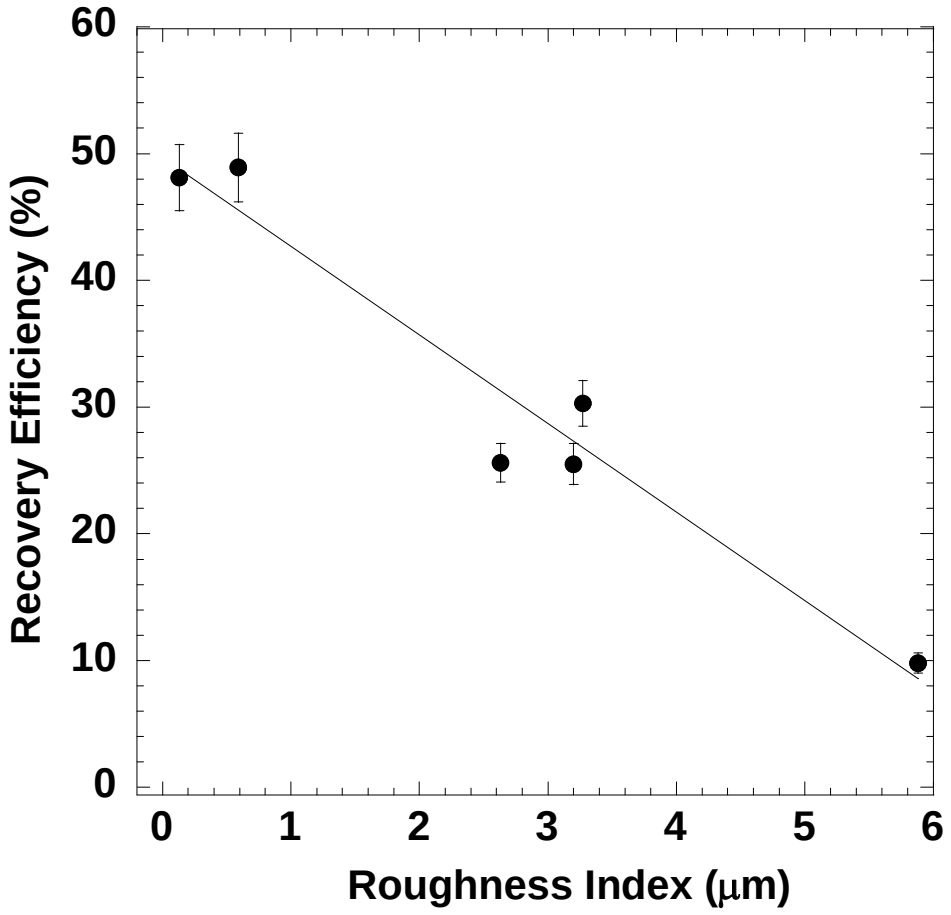

Figure 2. Mean percent recovery efficiency versus roughness index (μm) of the six material surfaces tested using the sponge-wipe method (see Table 2 for the roughness index of surface materials)

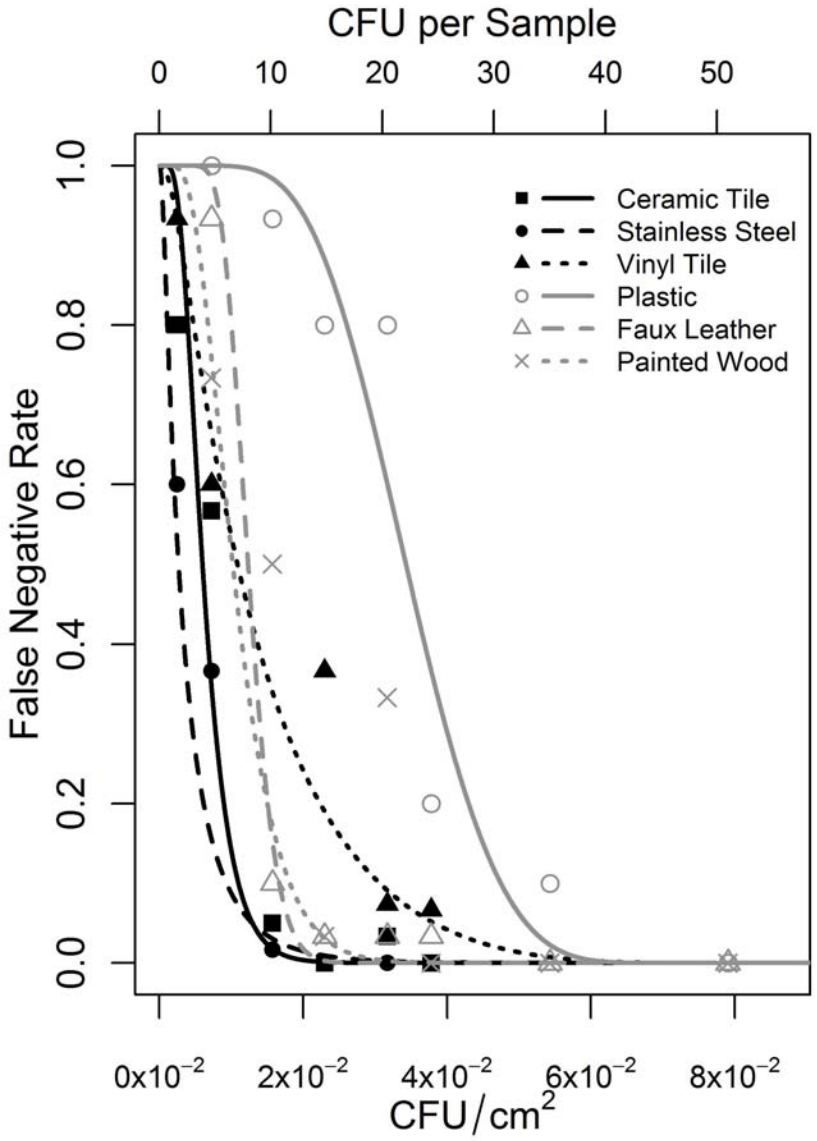

Figure 3. Average false negative rate data and fitted equations as a function of *B. atrophaeus* concentration (from positive controls) for each of six surface materials.