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Biological Sampling Variability Study

November 2016

BG Amidan
JR Hutchison

U.S. DEPARTMENT OF ENERGY

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

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Pacific Northwest National Laboratory
Richland, Washington 99352

Executive Summary

There are many sources of variability that exist in the sample collection and analysis process of surface samples for biological agents. This paper addresses some, but not all, of these sources of variability. The main focus of this study was to better understand and estimate variability due to differences between human samplers (individuals performing the surface sampling). Variability between collection and analysis days was also studied, as well as random variability within each human sampler. Experiments were performed using two surface coupons (ceramic and stainless steel), two *Bacillus atrophaeus* spore concentrations (10 CFU/coupon and 100 CFU/coupon), and with and without the presence of interfering material.

All testing was done using the cellulose sponge procedure (Centers for Disease Control and Prevention) and using 25.4 by 25.4 cm stainless steel or ceramic coupons. *B. atrophaeus* spores were used as the surrogate for *Bacillus anthracis*, the causative agent of anthrax. Spores were deposited using wet deposition onto clean or grime coated coupons prior to collection and analysis using the CDC procedure for sampling smooth, non-porous surfaces for *Bacillus* spores. A total of five sampling staff collected spores from eight coupons per day. There were four coupons with 10 spores (as measured by CFU) deposited and four coupons with 100 CFU/coupon deposited. To reduce variability, one trained sample processing personnel was used for extraction, sample processing, and analysis. The order of samplers (1st to last), sample type, and sample location within the laboratory were randomized. Each day consisted of one material being tested. The clean samples (no interfering materials) were run first, followed by the dirty samples (coated with interfering material).

There was a significant difference in recovery efficiency (number of CFU recovered divided by total number of CFU deposited) between the coupons with 10 CFU deposited (mean of 48.9%) and those with 100 CFU deposited (mean of 59.8%). There was no significant difference between the clean and dirty (containing interfering material) coupons or between the two surface materials; however, there was a significant interaction between spore surface concentration amount and presence of interfering material. The recovery efficiency was nearly equal across for all coupon types receiving 10 CFU, however, for the coupons with 100 CFU deposited, the recovery efficiency for the dirty samples was significantly larger (65.9% - dirty vs. 53.6% - clean) (see Figure 4.1).

Variance component analysis was used to estimate the amount of variability for each source of variability. Variability was small between dirty and clean samples, as well as between materials, so these results were pooled together. There was a significant difference in spore concentration achieved, so results were separated for the 10 spore and 100 spore deposited tests. In each case the within human sampler variability was the largest with variances of 426.2 for the 10 CFU/coupon condition and 173.1 for the 100 CFU/coupon condition. The within human sampler variability constitutes the variability between the four samples of similar material, interfering material, and concentration taken by each sampler. The between human sampler variance was estimated to be 0 for the 10 CFU/coupon condition and 1.2 for the 100 CFU/coupon condition. The between day variance was estimated to be 42.1 and 78.9

for the 10 CFU/coupon and 100 CFU/coupon conditions, respectively. Standard deviations (in the units of percent recovery efficiency) can be calculated in each case by taking the square root of the variance.

Acknowledgments

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Contents

1.0 Introduction

During a biological event, such as the 2001 anthrax attacks, numerous individuals and agencies respond to assess, identify, and remediate the threat (Centers for Disease Control and Prevention 2006). It is pertinent to collect and analyze samples in a timely manner so that response decisions can be made quickly and with confidence. In order to have confidence to make proper decisions, it is imperative to understand the variability that exists during the sampling and analysis process.

There are many sources of variability that exist in the process of collecting and analyzing surface samples. This report will address some, but not all, of these sources of variability. The main focus of this study was to better understand and estimate variability due to differences between sample collection personnel (human samplers). This was investigated using multiple surface materials, multiple contaminant concentrations, and with and without the presence of interfering material. Random variability between days was also investigated, as well as random variability between samples collected by the same sampler on a given day.

This report will not address other sources of variability, like analytical, sampling method, and sampling media. It will also not address variability due to sample storage and sample transportation. Variation between laboratories has been assessed previously (Rose et al. 2011) so it is not included in this study.

The remainder of this report is organized as follows:

- Section 2 – Presents the experimental design used to test the factors that might influence sampler variability and to estimate the amount of variability due to differences in sampler, differences between days, and between similar samples.

- Section 3 – Discusses the experimental procedures used during the testing. This includes details concerning the surrogate, surface materials, grime coating (interfering material), and sampling collection, processing, and analysis. Also, the statistical methods used to determine significant differences and estimate variability are discussed.

- Section 4 – Contains the results from the statistical analyses. Factors that most affected recovery efficiency are presented, as well as any significant statistical interactions. Measure of variability estimates are given for each source of variability that was tested in this study.

- Section 5 – Summarizes the conclusions that were drawn from the analytical results.

2.0 Experimental Design

An experimental design was constructed to help better understand certain aspects of variability that occurs when sampling. This experiment focuses on the following variability factors: day to day variability (referred to as between day variability), sampler-to-sampler variability (referred to as between human sampler variability), and within human sampler variability (variability between samples collected by the same sampler under similar conditions).

To provide for estimating the sources of variability, important test factors were varied and other factors remained constant. Table 2.1 gives a list of the factors that were held constant throughout the entirety of the sampling. These factors were held constant to minimize their effect on sampling variability.

Table 2.2 gives a list of the factors that were varied. These factors were varied to support estimating variability for different levels of these factors. These factors included surface material (stainless steel and ceramic), concentration of contaminant (10 and 100 CFU/coupon), sampling personnel (five samplers were used), and interfering material (clean and dirty surfaces). Each combination was performed three separate days. Four test coupons were sampled for each combination of factors on each day by each sampler.

Table 2.1. Factors Held Constant in the Study

Factor	Test Level
Sample collection media	Cellulose sponge
Sample/Coupon Size	25.4 by 25.4 cm (645.16 cm^2)
Wetting agent	PBS-T (PBS with 0.02% Tween 80)
BA surrogate	*Bacillus atrophaeus* Nakamura ATCC 9372 (BG)
Deposition	Wet deposition
Storage/transportation	None
Laboratories	One (PNNL) [(a)]
Preparation/extraction	Per method
Analytical Method	Culture
Personnel for sample processing	One person

[a] Lab-to-lab variation is typically a major contributor to reproducibility uncertainty, which ideally would be estimated via tests at several laboratories.

Table 2.2. Factors Varied in the Study

Factor	Test Levels
Surface materials	Stainless steel (SS) and ceramic (CE)
Surrogate surface concentrations	10 and 100 CFU/coupon
Interfering material	Clean and dirty surfaces
Personnel for sample collection	Five personnel
Day	Three different sampling days were used for each material
Number of test coupons per surface material	4 test coupons for each material, concentration, interfering material, and sampler combination.

The experiment was conducted in two parts. The first part was done with the clean samples and is discussed in Section 2.1. The second part was conducted with the dirty samples and is discussed in Section 2.2.

2.1 Clean Sample Variability Experimental Design

Table 2.3 shows the experimental plan for the sampling from clean surfaces. For each day only one material was sampled and this order was randomly determined. For each day, the order of the samplers was randomized. Each sampler sampled eight total coupons, four with 10 CFU/coupon and four with 100 CFU/coupon. These eight coupons were randomly ordered and the sampler was unaware of which coupons contained which concentration amount. There were a total of six days of sampling, three days for each of the two materials.

Table 2.3. Experimental Plan for Sampling from Clean Surfaces

Day 1: Clean CE

Sampler5	Sampler1	Sampler4	Sampler2	Sampler3
CE-100	CE-100	CE-100	CE-10	CE-10
CE-100	CE-10	CE-100	CE-100	CE-10
CE-10	CE-10	CE-100	CE-10	CE-100
CE-100	CE-10	CE-10	CE-100	CE-100
CE-10	CE-100	CE-10	CE-100	CE-100
CE-10	CE-100	CE-10	CE-10	CE-100
CE-100	CE-10	CE-100	CE-100	CE-10
CE-10	CE-100	CE-10	CE-10	CE-10

Day 2: Clean SS

Sampler5	Sampler3	Sampler2	Sampler4	Sampler1
SS-10	SS-10	SS-100	SS-100	SS-100
SS-10	SS-100	SS-100	SS-100	SS-10
SS-100	SS-100	SS-10	SS-10	SS-100
SS-10	SS-100	SS-100	SS-10	SS-100
SS-10	SS-10	SS-10	SS-10	SS-100
SS-100	SS-10	SS-10	SS-100	SS-10
SS-100	SS-10	SS-100	SS-100	SS-10
SS-100	SS-100	SS-10	SS-10	SS-10

Day 3: Clean SS

Sampler1	Sampler5	Sampler4	Sampler2	Sampler3
SS-100	SS-100	SS-100	SS-10	SS-100
SS-10	SS-10	SS-10	SS-100	SS-10
SS-10	SS-10	SS-10	SS-10	SS-100
SS-100	SS-10	SS-10	SS-100	SS-10
SS-100	SS-10	SS-100	SS-10	SS-100
SS-10	SS-100	SS-100	SS-100	SS-100
SS-10	SS-100	SS-10	SS-10	SS-10
SS-100	SS-100	SS-100	SS-100	SS-10

Table 2.3 continued. Experimental Plan for Sampling from Clean Surfaces

Day 4: Clean CE

Sampler4	Sampler5	Sampler3	Sampler2	Sampler1
CE-10	CE-100	CE-10	CE-10	CE-100
CE-10	CE-10	CE-100	CE-100	CE-10
CE-100	CE-10	CE-100	CE-10	CE-100
CE-100	CE-10	CE-100	CE-100	CE-100
CE-100	CE-100	CE-10	CE-10	CE-10
CE-10	CE-100	CE-10	CE-100	CE-10
CE-10	CE-100	CE-100	CE-10	CE-100
CE-100	CE-10	CE-10	CE-100	CE-10

Day 5: Clean CE

Sampler1	Sampler2	Sampler3	Sampler5	Sampler4
CE-10	CE-10	CE-10	CE-100	CE-100
CE-100	CE-100	CE-10	CE-100	CE-100
CE-10	CE-100	CE-100	CE-10	CE-10
CE-100	CE-100	CE-100	CE-10	CE-10
CE-100	CE-10	CE-10	CE-100	CE-100
CE-10	CE-10	CE-100	CE-100	CE-100
CE-100	CE-10	CE-100	CE-10	CE-10
CE-10	CE-100	CE-10	CE-10	CE-10

Day 6: Clean SS

Sampler3	Sampler1	Sampler2	Sampler5	Sampler4
SS-10	SS-100	SS-100	SS-10	SS-100
SS-10	SS-10	SS-10	SS-100	SS-10
SS-100	SS-10	SS-10	SS-100	SS-100
SS-100	SS-100	SS-10	SS-100	SS-100
SS-10	SS-10	SS-100	SS-10	SS-10
SS-100	SS-100	SS-100	SS-10	SS-100
SS-10	SS-10	SS-100	SS-100	SS-10
SS-100	SS-100	SS-10	SS-10	SS-10

2.2 Dirty Sample Variability Experimental Design

Table 2.4 shows the experimental plan for the sampling from dirty surfaces. The dirty surface experiment was performed exactly as the clean surface experiment. For each day only one material was sampled and this order was randomly determined. For each day, the order of the samplers was randomized. Each sampler sampled eight total coupons, four with 10 CFU/coupon and four with 100 CFU/coupon. These eight coupons were randomly ordered and the sampler was blind to the spore surface concentration. There were a total of six days of sampling, three days for each of the two materials.

Table 2.4. Experimental Plan for Sampling from Dirty Surfaces

Day 1: Dirty SS

Sampler 2	Sampler 3	Sampler 4	Sampler 5	Sampler 1
SS-100	SS-10	SS-10	SS-10	SS-100
SS-100	SS-100	SS-100	SS-10	SS-100
SS-10	SS-100	SS-100	SS-100	SS-10
SS-100	SS-10	SS-10	SS-100	SS-10
SS-10	SS-100	SS-100	SS-100	SS-10
SS-100	SS-100	SS-10	SS-10	SS-100
SS-10	SS-10	SS-100	SS-100	SS-100
SS-10	SS-10	SS-10	SS-10	SS-10

Day 2: Dirty CE

Sampler 1	Sampler 5	Sampler 2	Sampler 3	Sampler 4
CE-10	CE-100	CE-10	CE-100	CE-10
CE-100	CE-10	CE-10	CE-10	CE-100
CE-100	CE-10	CE-100	CE-100	CE-10
CE-100	CE-100	CE-10	CE-10	CE-100
CE-10	CE-100	CE-100	CE-100	CE-10
CE-10	CE-10	CE-10	CE-10	CE-100
CE-100	CE-100	CE-100	CE-100	CE-100
CE-10	CE-10	CE-100	CE-10	CE-10

Day 3: Dirty SS

Sampler 3	Sampler 4	Sampler 5	Sampler 2	Sampler 1
SS-100	SS-100	SS-100	SS-100	SS-100
SS-10	SS-10	SS-10	SS-10	SS-10
SS-10	SS-100	SS-10	SS-100	SS-10
SS-100	SS-10	SS-10	SS-100	SS-100
SS-100	SS-10	SS-100	SS-100	SS-10
SS-10	SS-100	SS-100	SS-10	SS-100
SS-10	SS-10	SS-100	SS-10	SS-10
SS-100	SS-100	SS-10	SS-10	SS-100

Table 2.4 continued. Experimental Plan for Sampling from Dirty Surfaces

Day 4: Dirty CE

Sampler 5	Sampler 3	Sampler 4	Sampler 2	Sampler 1
CE-10	CE-100	CE-10	CE-10	CE-100
CE-100	CE-10	CE-10	CE-10	CE-100
CE-100	CE-10	CE-10	CE-100	CE-10
CE-10	CE-10	CE-100	CE-10	CE-10
CE-10	CE-10	CE-10	CE-10	CE-10
CE-100	CE-100	CE-100	CE-100	CE-100
CE-100	CE-100	CE-100	CE-100	CE-100
CE-10	CE-100	CE-100	CE-100	CE-10

Day 5: Dirty CE

Sampler 1	Sampler 5	Sampler 4	Sampler 2	Sampler 3
CE-100	CE-100	CE-100	CE-10	CE-10
CE-10	CE-10	CE-10	CE-100	CE-100
CE-100	CE-10	CE-10	CE-10	CE-10
CE-100	CE-100	CE-100	CE-100	CE-100
CE-10	CE-10	CE-100	CE-10	CE-100
CE-10	CE-100	CE-10	CE-100	CE-10
CE-10	CE-10	CE-100	CE-10	CE-100
CE-100	CE-100	CE-10	CE-100	CE-10

Day 6: Dirty SS

Sampler 3	Sampler 4	Sampler 5	Sampler 2	Sampler 1
SS-100	SS-10	SS-10	SS-100	SS-100
SS-10	SS-100	SS-10	SS-10	SS-10
SS-100	SS-10	SS-10	SS-10	SS-100
SS-100	SS-100	SS-100	SS-100	SS-10
SS-10	SS-10	SS-100	SS-10	SS-100
SS-100	SS-100	SS-10	SS-100	SS-100
SS-10	SS-100	SS-100	SS-100	SS-10
SS-10	SS-10	SS-100	SS-10	SS-10

3.0 Experimental Procedures

This section discusses the experimental procedures and statistical analyses that were used in this study. Section 3.1 discusses the surrogate and how it was produced. Section 3.2 discusses the surface materials and how they were prepared. Section 3.3 discusses how the grime was produced and sprayed onto the coupons for the dirty samples. Section 3.4 discusses the sample, process, and analysis protocols followed during the experiment. Section 3.5 discusses the statistical methods that were used in the analyses.

3.1 Bacterial Culture Conditions

B. atrophaeus Nakamura ATCC 9372 (BG) was purchased from American Type Culture Collection (Manassas, VA). A culture of BG was grown overnight in tryptic soy broth (TSB; #286220; BD, Franklin Lakes, NJ) at 30 °C prior to being diluted 1:100 in 1.6 % nutrient broth with CCY salts (Buhr, et al. 2008). The culture was sporulated with shaking at 200 rpm for 7 days at 30 °C and then harvested by centrifugation for 10 min at 10,000 × *g* at 4 °C. Pellets were resuspended in sterile water and stored at 4 °C for 7 days (to enhance vegetative cell lysis), then washed three times in ultra-pure (18 Ω) sterile water. Spores were passed through a 41-µm filter to remove remaining cellular debris. Final spore suspensions were >95% phase bright. To reduce preparation variability, three independent spore cultures were grown and pooled.

3.2 Surface Materials

Eighteen gauge stainless steel sheets (316L) were cut to the desired coupon size, 25.4 by 25.4 cm (645.16 cm^2). Glazed porcelain floor and wall tile (UPC 737104028264) was purchased from Home Depot and cut to size. Surface materials were washed in a 1% solution of Liqui-nox® (Alconox Inc., New York, NY), rinsed three times in deionized water, and then air dried. After washing, all coupons were sterilized by autoclaving on a dry cycle for 90 minutes in Chex-All® Sterilization Pouches (Propper Manufacturing Company Inc., Long Island City, NY).

3.3 Coupon Grime Coating

A grime mixture was applied to clean test coupons to mimic background dust. The mixture was prepared by combining 97 grams of Arizona Road Dust (ISO 12103-1, A1 Ultrafine Test Dust, Analysis 5430, Powder Technology Inc., www.powdertechnologyinc.com) and 3 grams of biological mixture. The biological mixture was made by combining: Lycopodium powder, (Catalog No. 215B, Duke Scientific, Palo Alto, CA), 50% w/w; and Ragweed pollen, (Catalog No 214B, Duke Scientific), 50%. The mixture was sterilized by autoclaving two times on a liquid 90 minute cycle. Grime (12.5 grams) was resuspended in 1 liter of 70% denatured reagent alcohol (Ricca Chemical 2546705) and mixed vigorously on a stir plate. To provide a uniform coating, stainless steel or ceramic coupons were pre-coated with a light spray of 70% alcohol. The grime/alcohol mixture was then applied by pipetting with a targeted grime mass of

250 mg/coupon. Coupons were air dried overnight then stored in a coupon holder as to not disrupt the grime. The total mass of grime deposited was 230 mg ± 50 mg (data not shown).

3.4 Sample Collection, Processing, and Analysis

Spore stock solutions were enumerated prior to testing by dilution series in phosphate buffered saline (#10010049; Invitrogen, Waltham, MA) containing 0.02% Tween-80 (#P4780; Sigma-Aldrich, St. Louis, MO), which is denoted PBS-T. To reduce experimental variability, a single person deposited spores in 20 droplets (0.05 ml each) onto the coupons as previously reported (Krauter et al. 2012). The inoculated materials were dried for approximately 2 hours.

The CDC protocol ("CDC - Surface Sampling Procedures for *Bacillus anthracis* Spores from Smooth, Non-Porous Surfaces - NIOSH Workplace Safety and Health Topic" 2015) for surface sampling *B. anthracis* spores from smooth, nonporous surfaces with a cellulose sponge was used in this study. Sampler training (~30 minutes long) included reading the CDC protocol and active training using test surfaces. Each test coupon was sampled by a single person with a 3M™ Sponge-Stick (3M, St. Paul, Minnesota; catalog number SSL10NB) using an overlapping 'S' pattern with horizontal strokes, the sponge was turned and the same area was wiped again using vertical 'S' strokes, the sponge was then turned on the edge to wipe using diagonal 'S' strokes, and finally the tip of the sponge was used to sample the material perimeter. The sponge was then handed to the sample processing person and transferred to a stomacher bag (Seward; catalog number BA6141). Ninety mL of PBS-T was added to the bag and the bag was processed in the stomacher for 1 minute at 260 rpm (Seward Stomacher 400 Circulator). Excess liquid was squeezed out of the wipe and the wipe was discarded. The final suspension was transferred to 50 mL conical tubes and then centrifuged for 15 minutes at 3,500 x *g*. The supernatant was removed carefully by pipetting and the remaining solution (~3 mL each tube) was vortexed for 30 seconds and sonicated for 30 seconds two times. The two aliquots were pooled and the final volume was recorded (Krauter et al. 2012). One mL of spore suspension was plated on TSA in triplicate; if there was no growth on the three plates a fourth sample was plated the following day. The same sample processing person was used for all experiments to reduce extraction and analysis variability. Additionally, a negative coupon, process control, and 10 inoculation controls were collected during each experimental run.

3.5 Statistical Methods

One focus of this study was to understand how recovery efficiency and the variability in the recovery efficiency measurements are influenced by other experimental factors. The factors that were investigated can be found in Table 2.2 and results of these investigations are found in Section 4.1.

In order to determine which factors are influencing recovery efficiency, analysis of variance (Peterson 1985) was used. In the event of possible unbalancing in the treatment sample sizes, Type III sums of squares were used in the analysis of variance (ANOVA) tables. The significance level (α) of 0.05 was used to determine statistical significance (i.e. p-values < 0.05 were considered significant). Two-way and three-way interactions were included in the original analyses; however, all two-way and three-way interactions that had p-values > 0.10 (deemed non-significant) were removed from the model and pooled

in with the error term. If a factor was determined to be statistically significant, it means that changes in the value (or level) of the factor has a significant effect on recovery efficiency. Interactions that were determined to be statistically significant indicate that as the values (or levels) of one variable change there is not a consistent change in recovery efficiency as the values (or levels) of another variable change. The bottom two plots in Figure 4.1 graphically show what constitutes an interaction.

It is important to note that because clean samples and dirty samples were not randomized (clean performed first, followed by dirty samples), a split plot error structure could be appropriate for the analyses. Split plot was not employed for this analysis because day to day variability was found to be non-significant in both clean and dirty sections of the experiment, indicating that the split plot nature of the error structure was likely not an issue. This decision allowed for the rolling up of more of the non-significant interactions, resulting in more statistical power to determine significant effects and interactions.

The other focus of this study was to better understand and estimate the variability in the sampling process. The factors in the sampling process that were identified and for which variability estimates made were: 1) random variability due to variability across human samplers; 2) random variability within each human sampler; 3) random variability due to differences between days; and 4) random variability in the triplicate measures taken within each sample. Results from these analyses are found in Section 4.2.

The estimated variance for the first three variability factors listed above are determined by using variance component analysis (Qu et al. 2013). A mixed model is created with recovery efficiency for each sample being the response variable and the factors investigated in the analysis of variance (Table 2.2) as fixed effects and day and sampler as random effects. There are various model fitting techniques available to use in variance component estimation. This paper uses profiled restricted maximum likelihood (PREML) to fit the model (Qu et al. 2013). Models were fit and variance estimates calculated for each combination of levels for each of the investigated factors, as well as for combining the levels of each factor. For example, variance component estimates are calculated when the concentration is 10 CFU/coupon, samples are clean and from ceramic coupons. They are also calculated when the concentration is 10 CFU/coupon, the samples are either clean or dirty, and the samples are taken from either ceramic or stainless steel coupons. The only factor that is not ever combined is concentration. Variance estimates were very different between 10 CFU/coupon and 100 CFU/coupon, so all results were reported separately for each.

The results from variance component analyses are different than just calculating variances from the means of the different levels of each random-effects factor. These variances include many of the variances due to other effects and therefore, do not isolate the variance due only to the factor. Variance component analysis calculates the variances properly by isolating the variance that is only due to the factor of interest. For example, the mean recovery efficiencies of five human samplers would be different, making one believe that there is a significant variance between samplers. However, there could be other factors in the experiment, like day of week or surface material, which could be responsible for the variance, resulting in an actual variance between the samplers that is close to 0.

The estimated variance for the last variability factor (triplicate measures) is calculated differently. A recovery efficiency value is calculated for each sample based upon the triplicate measures. With three values taken for each sample, it is possible to calculate the simple variance of those three measures for each sample. The overall mean of those variances is then used as the estimate of the variance in the triplicate measures.

4.0 Results

This section discusses the results of this experiment. Section 4.1 looks at the effects that the varied factors had on recovery efficiency. Section 4.2 discusses the estimated variance components for the variances of interest for recovery efficiency.

4.1 Experimental Factor Analysis Results

This experiment was designed to look at the effect of the varied factors on recovery efficiency. These varied factors were material (stainless steel and ceramic), spore surface concentration (10 and 100 CFU/coupon), and interfering material (clean and dirty surfaces). ANOVA was used to perform a statistical analysis of these factors and all two-way and three-way interactions. Only those interactions that were significant (p-value < 0.05) or close to significant (p-value < 0.10) were included in the analysis. Table 4.1 summarizes the ANOVA results.

A significance level of 0.05 (95% confidence) was used to determine statistical significance (i.e. p-value < 0.05 means a significant difference or interaction). There were no statistically significant differences between materials (p-value = 0.1504) or between conditions (p-value = 0.7562) (condition concerns interfering materials – clean versus dirty surfaces). There were also no significant differences between the human samplers (p-value = 0.6439). As expected, there was a significant difference in recovery efficiency for concentrations (p-value < 0.0001), where 100 CFU samples had a higher recovery efficiency than 10 CFU samples. This difference in recovery efficiency for each concentration is shown in the top plot of Figure 4.1. The dashed lines in this plot represent the standard deviation.

Interactions between factors indicate that changes in the levels of one factor does not produce consistent recovery efficiencies when changing levels of the other factor. The interaction between concentration and condition (dirty vs. clean) is statistically significant (p-value < 0.0001) and can best be understood by the middle plot in Figure 4.1. At 10 CFU the clean samples had slightly higher recovery efficiency than the dirty samples, while at 100 CFU, the dirty samples had much higher recovery efficiency. The interaction between material and condition (interfering material) was nearly significant (p-value = 0.0597) at the 95% confidence level. The bottom plot of Figure 4.1 shows that the ceramic dirty samples had a higher recovery efficiency, but when sampling stainless steel, there wasn't much difference between clean and dirty samples.

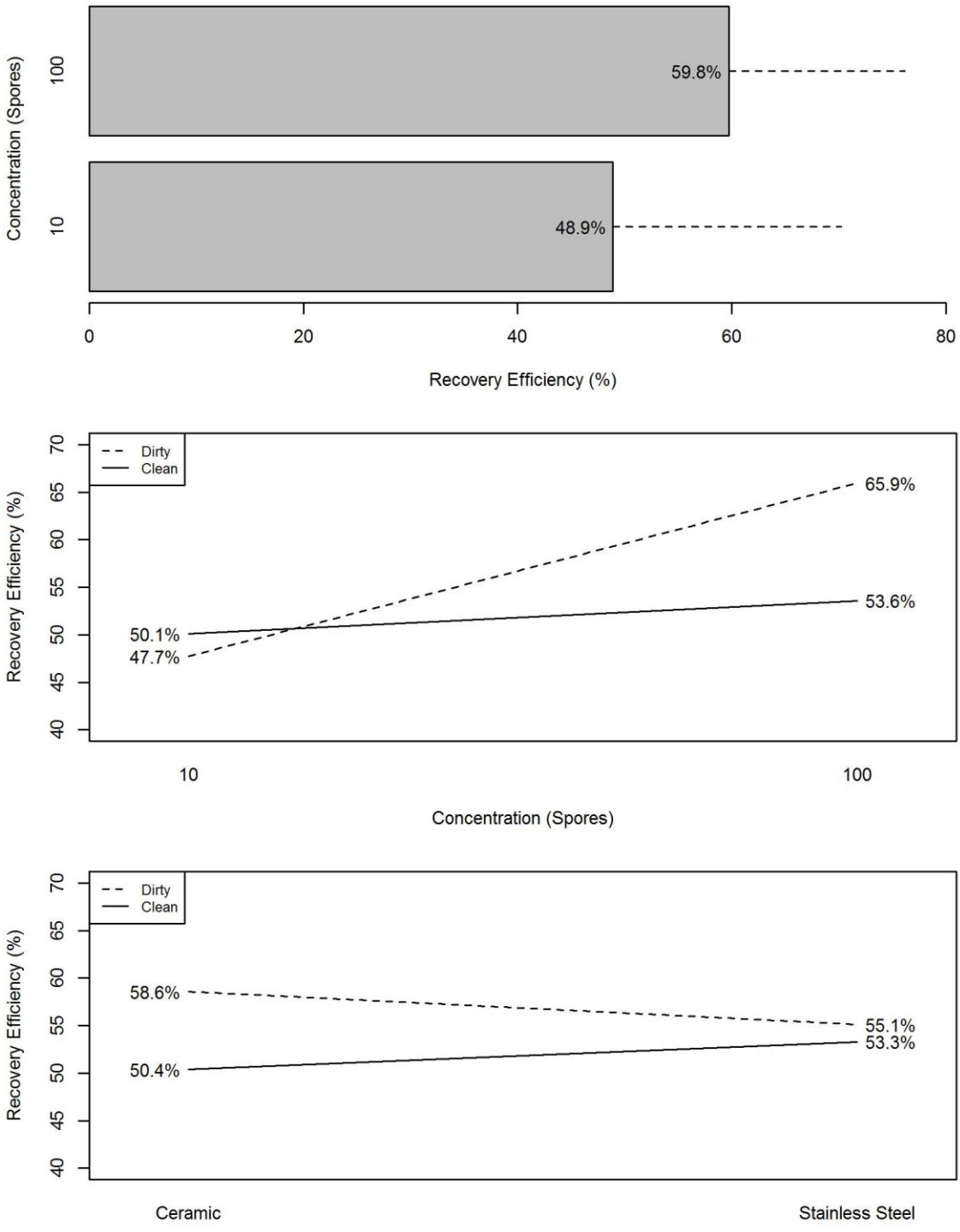

Figure 4.1. Bar Chart (bar is the mean and dashed line is the standard deviation) and Interaction Plots for Significant and Nearly Significant Factors and Interactions

Table 4.1. ANOVA Table Showing Statistical Significance for the Tested Factors and Significant Interactions

Source	DoF [a]	Mean Sq [b]	F-value [c]	P-value [d]
Material	1	715	2.07	0.1504
Concentration	1	19873	57.66	<0.0001
Condition[e]	1	33	0.10	0.7562
Material · Condition	1	1228	3.56	0.0597
Concentration · Condition	1	6459	18.74	<0.0001
Sampler	5	232	0.67	0.6439
Error	468	345		

[a] Degrees of freedom

[b] Mean squares (estimates of variance)

[c] F Test Statistic (from ANOVA)

[d] Probability when assuming the means are equal, that a random sample could result in means as far apart as observed in this experiment

[e] Condition is presence of interfering material (dirty versus clean surface)

4.2 Variance Component Analysis Results

Before a variance component analysis was performed the variability measured within a sample was investigated. Each sample was analyzed in triplicate with the mean of those analytical measurements being the measurement recorded for the sample (the number of CFU recovered). Table 4.2 shows the within-sample variability that was calculated due to the triplicate measures. The within-sample variance was calculated for each condition of sample (clean and dirty), as well as each combination of material and concentration. The condition and material had a minimal effect on the variability, as shown in the ANOVA performed in the previous section. There was a significant difference in concentrations and this is evident in Table 4.2. The yellow cells show the overall average within sample variance for each concentration. This table shows that there is high variability across the triplicate measures for 10 CFU test (variance = 1238 when combining across condition and material), but this variability was greatly reduced for 100 CFU test (variance = 231 when combining across condition and material). These results were generally expected and one of the reasons that samples are analyzed in triplicate. The three analyses are averaged to get one measure that would be more accurate than if only one analysis was performed.

Table 4.2. Average Variance within a Sample (Recovery Efficiency with Triplicate Measures) for Each Condition/Concentration/Material

	10 CFU/coupon			100 CFU/coupon		
Condition	Ceramic	Stainless Steel	Both Materials	Ceramic	Stainless Steel	Both Materials
Clean	1099	1237	1169	230	240	235
Dirty	1314	1298	1306	241	214	227
Combined	1208	1267	1238	235	227	231

Variance component analysis is used to assess the amount of variability in a response variable that is associated with random-effects variables. The random-effects variables that were included in this study are: human sampler, day, and sample (replication). The sample effect measures the within sample variability; the day effect measures the between day variability; the sampler effect measures the between human sampler variability. The response variable in this study was recovery efficiency.

Table 4.3 summarizes the variance component estimates for each combination of material, condition (clean/dirty), and concentration. Because the differences between materials was minimal, there is a combined column in Table 4.3 which reports the mean of both materials. This was also done for condition (clean/dirty). There were significant differences in variance component estimates for the two concentrations, so there are no combined estimates. The yellow shaded cells show the variance estimates for the 10 spores and 100 spores concentrations when combining all other factors.

From Tables 4.3 and 4.4 it is apparent that the within human sampler variance is the largest and it is much larger for 10 CFU versus 100 CFU. This is consistent with other low contamination studies previously performed (Krauter et al. 2011). The between day variance estimates are much smaller with a minimal effect due to concentration level. The between day variance measured the day to day variability and is most likely a measure of how close the actual spore surface concentrations were to the targeted loadings.

The between human sampler variance estimates are very small with combined estimates of 0 for 10 CFU and 1.2 for 100 CFU. Variance component analyses are performed to separate the sources of variability. This means that although the samplers did not always obtain the same recovery efficiency when sampling coupons under similar conditions, the differences that occurred were generally due to other factors and not who was doing the sampling. For this study the five samplers were trained so that sampling techniques were similar. Proper training and following the same sample collection protocol is most likely the biggest factor in having minimal variability between samplers.

Table 4.3. Variance Component Estimates for Each Measure for Each Condition/Concentration/Material

Source of Variability	Condition	10 CFU/coupon			100 CFU/coupon		
		Ceramic	Stainless Steel	Both Materials	Ceramic	Stainless Steel	Both Materials
Within Sampler	Clean	480.9	306.6	390.7	163.7	149.4	156.6
	Dirty	408.8	498.9	455.7	113.1	230.2	178.8
	Combined	444.5	404.7	426.2	145.3	198.4	173.1
Between Sampler	Clean	0	4.5	5.1	0	0	0
	Dirty	0	0	0	16.2	31.1	15.2
	Combined	0	0	0	0	5.5	1.2
Between Day	Clean	0	0	0	134.4	58.9	96.6
	Dirty	0	207.0	100.7	47.0	83.9	65.1
	Combined	0	103.3	42.1	90.4	70.9	78.9

Table 4.4. Percent of Total Variance for Each Source Measured for Each Concentration Amount

Source of Variability	10 CFU/coupon	100 CFU/coupon
Within Sampler	91.0%	68.4%
Between Sampler	0%	0.5%
Between Day	9.0%	31.2%

5.0 Conclusions

There are many factors that contribute to the variability in surface sample recovery efficiency. The main focus of this study was to understand and estimate the amount of variability due to differences between individuals performing the sampling (samplers). In the process of doing this, other sources of variability were identified and measured. Three sources of variability were measured in this study: 1) within sampler variability – the variability in measurements between samples for each human sampler; 2) between sampler variability – the variability in measurements between the different human samplers; and 3) between day variability – the variability in measurements taken from different days.

These variability calculations were made under different testing situations, to help determine the effect other factors have on variability. This study was performed at two different concentration amounts (10 and 100 CFU/coupon), with two materials (ceramic tile and stainless steel), and with both clean samples and dirty samples (samples with interfering material). Table 4.3 list the variance estimates for each of these sources, with each combination of the other factors. The within human sampler variance is the largest and makes most of the total variability (Table 4.4). The between human-sampler variability is negligible in each case. There is a little variability between days, but it is a small percentage of the total.

There was low variability between the recoveries from ceramic tiles and stainless steel surface materials. The dirty samples often had slightly higher variance estimates than the clean samples. There was a strong significant difference in the amount of variability between the two spore surface concentrations. The 10 CFU runs had significantly higher variance. Some of this variability may be due to the fact that it is difficult to deposit exactly 10 spores (measured as CFU) onto a coupon. Specifically, being off just one or two CFU from the target of 10 CFU amounts to a difference of 10% to 20% from the expected number of 10 CFU on a coupon. This would cause the variability estimate to be higher. With 100 CFU tests, being just a few spores from the target would result in only a small percentage from the target; thus having less effect on the variability estimates.

Because this experiment was designed to study the sources of variability during different conditions, it was possible to look at how these different conditions affected recovery efficiency. Analysis of variance was used to look for factors and interactions that significantly influenced recovery efficiency during the experiment. The effect of spore surface concentration was significant with a recovery efficiency of nearly 60% with 100 CFU/coupon, and only about 49% with 10 CFU/coupon (see top plot on Figure 4.1). Again, the effect of attempting to get exactly 10 CFU on each coupon, may be partly responsible for this effect. Another factor that might be influencing this effect can be noted in the significant interaction between concentration amount and presence of interfering material (condition). At 10 CFU, there was little difference in recovery efficiency between the clean and dirty samples. However at 100 CFU, the dirty samples had a recovery efficiency rate at 65.9%, while the clean samples were only at 53.6% (see middle plot in Figure 4.1). This indicates the possibility that recovery efficiency is improved in the presence of interfering material, especially at higher spore surface concentrations (100 CFU instead of just 10 CFU).

One other interaction showed some possible effect on recovery efficiency. The interaction between material type and presence of interfering material was not statistically significant at the 95% confidence level, but was at the 90% confidence level (see bottom plot on Figure 4.1). With stainless steel coupons, the recovery efficiency was very close to the same for dirty and clean samples; however, on ceramic coupons, the recovery efficiency was higher for dirty samples (58.6%) than for the clean samples (50.4%).

6.0 References

Buhr T.L., D.C. McPherson, B.W. Gutting. 2008. Analysis of broth-cultured *Bacillus atrophaeus* and *Bacillus cereus* spores. J Appl Microbiol **105**:1604–1613.

Krauter, P.A., G.F. Piepel, R. Boucher, M. Tezak, B.G. Amidan, and W. Einfeld. 2012. False negative rate and other performance measures of a sponge-wipe surface sampling method for low contaminant concentrations. Appl. Environ. Microbiol. **78**:846-854.

Peterson, R.G. 1985. Design and Analysis of Experiments. Marcel Dekker, INC. New York.

Qu L, Guennel T, Marshall SL. 2013. Linear Score Tests for Variance Components in Linear Mixed Models and Applications to Genetic Association Studies. Biometrics. **69** (4): 883–892.

Rose L.J,, **L. Hodges, H. O'Connell, J. Noble-Wang.** 2011. National validation study of a cellulose sponge wipe-processing method for use after sampling *Bacillus anthracis* spores from surfaces. Appl Environ Microbiol **77**:8355–8359.

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