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Evaluation of Options for Interpreting Environmental Microbiology Field Data Results having Low Spore Counts

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Disclaimer

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List of Acronyms and Abbreviations

95% UCL	95% upper confidence limit
BBT	Butterfield Buffer with Tween
BCA	bias-corrected accelerated
Bg	*Bacillus atrophaeus* subspecies *globigii*
CFU	colony forming units
CLT	central limit theorem
cm^2	square centimeter
EPA	U.S. Environmental Protection Agency
L	liter
min	minute
MLE	maximum likelihood estimation
MVUE	minimum variance unbiased estimate
n	number of samples
PBST	phosphate-buffered saline with Tween® 20
ROS	regression on order statistics
Sd	standard deviation
SKC	vendor of air sampling equipment (SKC Inc.)
sponge	cellulose sponge-stick
swab	macrofoam swab
TNTC	too numerous to count
TSA	tryptic soy agar
UCL	upper confidence limit
µm	micrometer
vacuum	vacuum sock
wipe	Versalon® wipe

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Executive Summary

Following a widespread environmental release of a biological agent, such as *Bacillus anthracis,* remediation of contaminated facilities or areas may be needed to eliminate or reduce the risk of exposure. Decision makers may look to microbial exposure assessment using field data collected during remediation efforts (site characterization and/or post-decontamination sampling) to better inform decisions regarding identifying exposures, reducing hazards, selecting decontamination strategies, and facility clearance (Parkin, 2007; Nichols et al., 2006). However, estimating the magnitude of potential exposure using microbial data collected from the field can be complicated by the lack of guidelines for interpreting such data, especially when sample results fall below the limits of detection or quantification of the analytical method used to analyze the samples.

The number of bacterial spores in an environmental sample is often estimated by culturing bacteria from the sample extract on an appropriate growth medium and observing the number of colonies (colony forming units [CFU]) that grow through spread plating and/or filter plating. Conventionally, only spread plates with colony counts in the range of 30-300 CFU are used (although some method ranges differ slightly) because high colony counts might prevent accurate counting, which can lead to under-representing the actual count, and high variability is expected with low colony counts (Breed and Dotterrer, 1916). The countable range for filter plating is often reported as 20 to 200 colonies (SMC, 2011) although some methods have established slightly different ranges. Both spread plate and filter plate analyses can detect 1 CFU. If replicate plates are used, the detection limit is 1 CFU divided by the number of replicate plates used.

In cases where a sample result is reported as “not detected”, “below the detection limit”, or “below the limit of quantitation”, there is little information on how that result should be interpreted. An analytical measurement that can be expressed only as less than the established quantification limit is classified as “censored” (more precisely, “left censored”) at that limit. A “not detected” result is considered to be less than the method detection limit, or the lowest value for which it is known with high confidence that the characteristic is present in the sample and is classified as “censored” at that limit. Similar to a result that is less than the quantification limit, a non-detected result does not necessarily imply that the actual sample value is zero (Gilbert, 1987). When encountering censored data within an exposure assessment, EPA (1992) noted that a variety of data interpretation options could be used. Some researchers have compared various options for treating censored data, including but are not limited to; substitution, imputation methods, maximum likelihood estimation, regression on order statistics, and Kaplan-Meier methods.

This report documents the evaluation of six options for representing culture-based/microbial count data when no colonies were observed and/or when colonies were observed but were below the limits of quantification of the filter plating or spread plating techniques (i.e., censored data). The six options included: use of the mean spread plate count, even if under the limit of quantitation; two options for substitution; and three options for left censoring data at the quantitation and/or the detection limit. Secondary data that were used for this evaluation were generated from a previous interagency decontamination study (EPA, 2013). These data included indoor air and surface samples that were collected post-decontamination (when low numbers of viable and culturable *Bacillus atrophaeus* subspecies *globigii* (*Bg*) spores were expected within the samples) and analyzed for *Bg* spores using both spread plating and filter plating techniques. Mean plate counts were adjusted by multiplying by the elution spore suspension (for filter and spread plating) and serial dilution (for spread plating) to estimate the number of CFU in the sample. The sample concentration was also determined for both air (CFU/m^3) and surface samples (CFU/m^2). The higher filter plate or spread plate result was used to represent the sample. Each of the six data interpretation options evaluated in this report were applied to the paired spread plate and filter plate *Bg* spore data to compare summary statistics and to evaluate which options might be more useful for interpreting data when low spore counts and left censoring are present.

Based on the criteria set out in this study, results of this evaluation suggest that when the reported (unadjusted) mean spread plate count is nonzero but <30 CFU, the actual CFU value should be used if possible, rather than substituting a different value (e.g., 0 or 15 CFU) based on quantification limits. That is, all nonzero results should be used instead of using substitution methods. The reason is that substituting 0 CFU when spores are present understates the results. Substituting 15 CFU can understate or overstate the results, depending on whether the actual CFU is greater or less than 15 CFU. In addition, based on the results from the data included in this evaluation, if high variability and uncertainty in low concentration estimates is considered acceptable, then the use of a censoring option in which all nonzero, unadjusted mean spread plate counts are used in addition to censoring spread plate and filter plate results that were reported as 0 CFU at the detection limit could be the best option for handling censored observations. This option maximally utilizes all available information to provide conservative estimates of concentrations and indicates uncertainty associated with non-detection. However, if high variability and uncertainty in low concentration estimates is considered unacceptable, then censoring both spread plates and filter plates with counts below the quantitation limit at the quantitation limit could be the most useful option for handling censored observations. This option would require appropriate justification for the quantification and detection limits that are used to represent censored outcomes.

1.0 Introduction

Following a widespread environmental release of a biological agent, such as *Bacillus anthracis,* remediation of contaminated facilities or areas may be needed to eliminate or reduce the risk of exposure. Remediation efforts might include both site characterization sampling, to determine the extent of contamination, and post-decontamination sampling to determine decontamination efficacy (EPA, 2012a). Decision makers may look to microbial exposure assessment using data collected from the field to better inform decisions regarding identifying exposures, reducing hazards, selecting decontamination strategies, and facility clearance (Parkin, 2007; Nichols et al., 2006). However, estimating the magnitude of potential exposure using microbial data collected from the field can be complicated by the lack of guidelines for interpreting such data, especially when sample results fall below the limits of detection or quantification of the analytical method used to analyze the samples.

The number of bacterial spores in an environmental sample is often estimated by culturing bacteria from the sample extract on an appropriate growth medium and observing the number of colonies (colony forming units [CFU]) that grow through spread plating and/or filter plating. Conventionally, only spread plates with colony counts in the range of 30-300 CFU are used, although some method ranges differ slightly, e.g., 25-250 CFU (Sutton, 2006). The practice of using a specified countable range for spread plates originates from the historic bacterial examination of milk, as high colony counts (e.g., >300 CFU) might prevent accurate counting, which can lead to under-representing the actual count, and low colony counts (e.g., <30 CFU) were associated with high variability (Breed and Dotterrer, 1916). Filter plating provides a direct bacterial count based on the development of colonies that grow on the surface of a membrane filter. A sample is filtered through the membrane, which retains the bacteria. The membrane is then placed on medium where colony forming units are counted. The countable range for filter plating is often reported as 20 to 200 colonies (SMC, 2011) although some methods have established slightly narrower ranges (20-80 CFU; ASTM, 2004) or slightly higher ranges (50 to 300 CFU/filter; Clark et al., 1951).

Both spread plate and filter plate analyses can detect 1 CFU. As the U.S. Environmental Protection Agency's (EPA) *Method Validation of U.S. Environmental Protection Agency Microbiological Methods of Analysis* (2009a) document noted, when no organisms are observed upon applying culture methods to a particular plate, the result is reported as <1 CFU rather than 0 CFU. If replicate plates are used, the detection limit is 1 CFU divided by the number of plates. For example, because spread plate analysis of a given sample involves use of three replicate plates, the detection limit for spread plating is 0.33 CFU (i.e.,

1 CFU detected on one of three replicate plates). For filter plate analysis, the detection limit is either 1 CFU or 0.5 CFU, depending on whether one or two replicate filter plates are used to analyze a sample.

In cases where a sample result is reported as "not detected", "below the detection limit", or "below the limit of quantitation", there is little information on how that result should be interpreted. An analytical measurement that can be expressed only as less than the established quantification limit is classified as "censored" (more precisely, "left censored") at that limit. Likewise, an analytical measurement that is classified as "not detected" is considered to be "censored" at the detection limit. A "not detected" result is considered to be less than the method detection limit, or the lowest value for which it is known with high confidence that the characteristic is present in the sample. Similar to a result that is less than the quantification limit, a non-detected result does not necessarily imply that the actual sample value is zero (Gilbert, 1987).

When encountering censored data within an exposure assessment, EPA (1992) noted that a variety of data interpretation options could be used, and "selecting the appropriate method requires consideration of the degree of censoring, the goals of the exposure assessment, and the accuracy required." Some researchers have compared various options for treating censored data, and these options have continued to evolve in recent years beyond traditional (and problematic) substitution approaches as the ability to handle more computer-intensive analytical techniques has increased. For example, Gleit (1985) evaluated multiple options for small (n = 5 to 15) normal environmental data sets and found that a "fill-in with expected values" approach worked better than a maximum likelihood estimation (MLE) or "fill-in with constants" approach. Smeets et al. (2007) used various approaches for dealing with non-detect *Cryptosporidium* oocyst concentrations in water (including non-detects being set to zero and non-detects being set to the detection limit); however, the best non-detect concentration estimate was identified from log-linear extrapolation. For non-detects of *Campylobacter* spp. in water, Signor et al. (2005) compared a "nonparametric modified log-probability regression model" presented by El-Shaarawi (1989) and the "fill-in with expected values" technique developed by Gleit (1985). Although both methods generated similar mean concentrations, Signor et al. (2005) reported that the method proposed by El-Shaarawi (1989) produced a more conservative probability distribution function in the upper tail then the method proposed by Gleit (1985) and was the more preferred method. However, given a small dataset, Signor et al. (2005) noted that the preferred method may result in excessive rather than conservative estimates, and that an educated judgement would have to be made to determine the appropriateness of the variability analysis results.

Wong et al. (2009) used regression on order statistics (ROS) for non-detects in developing predictive models of enteric virus contamination at recreational beaches. Wong et al. (2009) noted that imputation methods, "which fill in values for non-detects without assigning them all the same value," provide better descriptions of censored data than substitution approaches. Imputation methods include MLE, Kaplan-Meier, and ROS. Wong et al. (2009) noted that ROS imputation is known to work better than MLE for data sets with <50 detected values. As noted by Helsel (2005), the more accurate methods for computing statistics (modern MLE, ROS, and Kaplan-Meier methods) are now available in statistical software.

This report documents the evaluation of six options for interpreting culture-based/microbial count data sets that include left censored data, or measurements that are less than established quantification limits and/or detection limits. The six options attempt to use the filter plating result along with the spread plating result for a given sample in order to improve the precision of the final sample result, and to consider different approaches to handling censored outcomes. However, while the evaluation considered paired spread plate and filter plate results, these options are applicable even when only one set of results is available for a sample.

The secondary data that were provided to Battelle for analysis in this study were generated from a previous interagency decontamination study (EPA, 2013), in which indoor air and surface samples were collected and split samples were analyzed for *Bacillus atrophaeus* subspecies *globigii* (*Bg*) spores using both spread plating and filter plating techniques (Section 2.2). Each of the six data interpretation options evaluated in this report were applied to paired spread plate and filter plate *Bg* spore data associated with air and surface samples collected post-decontamination in this study to compare summary statistics and to evaluate which options are more appropriate for use in making conclusions from the data when low spore counts and left censoring are present. (These six options are introduced in Section 2.3)

1.1 Terminology Used in This Report

The terms "count" and "mean plate count" refer to the number of CFU observed on replicate plates under a given method, then averaged across the plates. These counts are considered "unadjusted" until multiplied by the volume of the elution suspension and the dilution factor, as applicable, resulting in "adjusted CFU." (Mean filter plate counts were multiplied by the volume of the elution suspension; mean spread plate counts were multiplied by the dilution factor and volume of the elution suspension.) The term "result" (or equivalently, "concentration") refers to the adjusted CFU per volume of air sampled or per area of the surface sampled. Thus, in this context, "result" is equivalent to a *Bg* spore concentration.

Among the summary statistics considered in this evaluation of data interpretation options in the presence of censored data is a 95% upper confidence limit (95% UCL) on the mean concentration. The 95% UCL on the mean is defined as the lowest value that is expected to equal or exceed the true (unknown) mean of the distribution 95% of the time, if the experiment was to be continuously repeated under the same conditions. The 95% UCL is a measure of uncertainty in the mean, rather than a measure of variability in the data, which makes it distinct from the 95^{th} percentile of the data distribution. As described in EPA's Risk Assessment Guidance for Superfund (EPA, 1989), the 95% UCL on the mean is traditionally used in human health risk assessments as a point estimate of reasonable maximum exposure, or equivalently, the exposure point concentration (i.e., the contaminant concentration at the point of contact by humans). Haas et al. (1993) have noted that microbial exposures could be conducted under the same framework used for chemical risk assessments. Although distributional data are generally preferred over point estimates in microbial assessments (EPA, 2012b), 95% UCL on the mean values have been used to describe environmental microbiology data. For example, Goodwin et al. (2012) used the 95% UCL on the mean to describe infrequently detected methicillin-resistant *Staphylococcus aureus* in seawater and beach sand samples. Hamilton et al. (2006) calculated the 95% UCL on the mean for the annual probability of enteric virus infection associated with the ingestion of uncooked vegetables grown using reclaimed water.

2.0 Methods

2.1 Source of Paired Spread Plate and Filter Plate Data

Paired spread plate and filter plate data from an EPA study (EPA, 2013) of the decontamination of *Bg* released inside of a facility were used for this investigation involving the six data interpretation options. In the EPA decontamination study, indoor air samples were collected with SKC BioSampler® (SKC, Eighty Four, PA) units (liquid impingers) with the sample contents deposited directly into phosphate-buffered saline with Tween® 20 (PBST). Air samples were collected from two rooms within the contaminated building at three distinct heights and at three locations per room. These air samples were collected over 15 minutes in a staggered fashion (0-15, 15-30, and 30-45 minutes) to represent the level of *Bg* air contamination following decontamination. Indoor surface samples were collected with cellulose sponge-sticks (sponge), macrofoam swabs (swab), vacuum socks (vacuum), and Versalon® (Pall Corporation, Port Washington, NY) wipes (wipe). The sampled surface area varied considerably among the methods: 0.00258 m^2 for the swab, 0.0645 m^2 for the sponge and wipe, and 0.3716 m^2 for the vacuum. *Bg* spores were extracted from surface samples in PBST, and the volume of the elution spore suspension was determined. For sponge, vacuum, and wipe samples, the elution suspension was centrifuged and concentrated to reduce the volume. For both air and surface samples, the EPA study cultured portions of the elution suspension via spread plating and filter plating to estimate the number of *Bg* spores as CFU (EPA, 2013).

This data investigation used paired spread plate and filter plate results for 18 indoor air samples and 136 surface samples from the EPA study, for a total of 154 samples. These samples were collected post-decontamination and resulted in a high prevalence of low *Bg* counts (compared to pre-decontamination levels), including results falling below quantification or detection limits (i.e., left censored at these limits). Therefore, in order for a data investigation option to be considered among the better performers in this evaluation, it needed to be relevant and valid when samples contained low spore counts.

2.2 Overview of Spread Plating and Filter Plating

Spread Plating. For spread plating, 0.1 mL of the elution suspension (or subsequent 10-fold dilution) was applied to each of three replicate tryptic soy agar (TSA) plates. The sample was mechanically spread across the TSA plates using a cell spreader. Once the TSA plates were incubated, the CFU were counted on each plate. A series of 10-fold dilutions of the initial sample were also prepared and spread plated as

well. The use of dilutions in the spread plating approach allowed estimation of the number of *Bg* spores when densities were high in the undiluted sample. Spread plating was performed in triplicate (that is, three spread plates prepared for each dilution).

Equation 1 shows the formula for calculating the adjusted CFU from spread plating (i.e., the estimated number of *Bg* spores collected in the sample after adjustment per the volume of the elution suspension and the serial dilution). Equation 1 was used to estimate the number of *Bg* spores collected in the total elution suspension sample by accounting for the dilution factor and volume of the elution suspension. While the adjusted CFU is a count variable, the calculation does not necessarily result in an integer value, and in such situations, the result is rounded to the nearest integer.

Equation 1. Calculation of Adjusted CFU from Spread Plating

$$\textbf{Adjusted CFU} = \textbf{Mean Spread Plate Count}\ (\textbf{CFU/mL}) \times \textbf{Serial Dilution}\ (\textbf{unitless}) \times \textbf{Volume}\ (\textbf{mL})$$

where:

Adjusted CFU = estimated number of *Bg* spores (CFU) collected in the sample; rounded to the nearest integer.

Mean Spread Plate Count = (unadjusted) average CFU across three replicate spread plates, per volume of elution suspension plated.

Serial Dilution = dilution factor (unitless); a factor of 10 (from 10 to 10,000) that accounted for plated volume (0.1 mL) and any associated 10-fold dilutions used for spread plating. The serial dilution with an unadjusted mean spread plate count of 30 to 300 CFU was selected for calculation of the adjusted CFU. Note: when the mean spread plate count was identified as censored (i.e., below the quantification limit or detection limit), a dilution factor of 10 was applied, reflecting the most undiluted suspension plated.

Volume = volume of elution suspension (mL).

Historically for spread plating, the dilution that led to an unadjusted mean spread plate count between 30 and 300 CFU (with some guidance recommending slightly different ranges. If a dilution led to counts above this range, it was not used. This was because colony overlap or crowding may prevent accurate counting (Breed and Dotterrer, 1916). Alternatively, if a dilution led to an unadjusted mean spread plate count of less than 30 CFU, variability becomes high. Therefore, to help maximize accuracy and precision, the dilution with an unadjusted mean spread plate count of 30 to 300 CFU (quantifiable range) was selected for reporting the final spread plating result for a sample. In practice, however, it is possible to detect a single spore on one of the three spread plates, and thus, to report a nonzero CFU result for the sample.

Colony counts (CFU) provide an estimate of spore levels in a sample and could arise from one or several spores (Sutton, 2006). Colony plate counts can underestimate the true number of organisms in the sample if they are clumped together (Almeida et al., 2008). However, Baron et al. (2008) noted that when flow-enhanced spores were allowed to settle on agar plates and subsequently dispersed using a spreader and Butterfield Buffer with Tween (BBT), this provided a better estimate of spore numbers compared to un-manipulated agar plates and agar plates exposed to BBT solution after spore settling. Spreading likely separated spore clumps, thereby reducing the chance that multiple spores were contributing to a single CFU. Baron et al. (2008) hypothesized that spreading/BBT techniques would be similar to approaches where surface samples were collected and then cultured from liquid suspensions.

Filter Plating. For filter plating, 1 mL or more of undiluted sample (i.e., the elution suspension) was vacuum filtered through a 0.45 µm Microfunnel that prevented the passage of *Bg* spores. After filtration was completed, the filter was placed onto a TSA plate and incubated to support colony growth. The elution suspension was then applied to either one or two replicate TSA filter plates. As with spread plating, all CFU counts ≤300 were reported on a given plate. If the CFU count was >300 on a plate, the result would be reported as "too numerous to count" (TNTC), although this outcome did not occur among the sample results considered in this evaluation.

Equation 2 shows the calculation for the adjusted CFU from filter plating. Like spread plating, the adjusted CFU is a count variable and thus is rounded to the nearest integer if necessary. Clark et al. (1951) noted that filter plating is advantageous when determining the number of CFU in samples containing low densities of culturable bacteria (<30 CFU/mL). When spore numbers are high in the elution suspension, filter plating will result in too many colonies to achieve accurate counts (Clark et al., 1951).

Equation 2. Calculation of Adjusted CFU from Filter Plating

$$\textbf{Adjusted CFU} = \textbf{Mean Filter Plate Count}\ (\textbf{CFU/mL}) \times \textbf{Volume}\ (\textbf{mL})$$

where:

Adjusted CFU = estimated number of *Bg* spores (CFU) collected in the sample; rounded to the nearest integer.

Mean Filter Plate Count = the (unadjusted) CFU count if a single filter plate was used for the sample, or the average CFU count if two filter plates were used for the sample, relative to the volume of elution suspension plated.

Volume = volume of elution suspension (mL).

2.3 Options for Interpreting Censored Microbiological Data

For a given air or surface sample considered in this assessment, the reported (adjusted) CFU value was often too low to be quantifiable under a given analysis technique (filter plating or spread plating). This assessment considered six different options for handling these non-quantifiable outcomes when calculating summary statistics and the 95% UCL on the mean. Results of these calculations were compared across these six options, called "data interpretation options," and the outcome of this comparison provided knowledge to inform future data decisions. These options were applied to paired spread plate and filter plate data for a given air or surface sample, although having paired data is not required to implement these options. Table 1 defines these six options.

Table 1. Six Data Interpretation Options Evaluated for Censored Microbiological Data

Data Interpretation Option	Description
1. Substitute 0 - Treat as Detect	• 0 CFU was substituted for unadjusted mean spread plate counts <30 CFU. • Mean filter plate counts and mean spread plate counts of 0 CFU were treated as reported. • The filter plate result was used to represent the sample when greater than the spread plate result. • Results of 0 CFU/m^2 (or CFU/m^3) were possible, and all results were treated as detects for the calculation of summary statistics and 95% UCL on the mean.
2. All Spread - Treat as Detect	• No substitution was made for unadjusted mean spread plate counts, even if <30 CFU. • Mean filter plate counts and mean spread plate counts were treated as reported. • The filter plate result was used to represent the sample when greater than the spread plate result. • Results of 0 CFU/m^2 (or CFU/m^3) were possible, and all results were treated as detects for the calculation of summary statistics and 95% UCL on the mean.
3. Substitute 15 - Treat as Detect	• 15 CFU (i.e., half the quantification limit) was substituted for unadjusted mean spread plate counts <30 CFU. • Mean filter plate counts were treated as reported. • The filter plate result was used to represent the sample when greater than the spread plate result. • All results were nonzero and treated as detects for the calculation of summary statistics and 95% UCL on the mean.

Data Interpretation Option	Description
4. < Quantification - Identify if "Less-Than"	• Unadjusted mean spread plate counts less than the quantification limit were identified as being <30 CFU, and the associated results were calculated assuming a dilution factor of 10 and identified as being censored. • Unadjusted filter plate counts of 0 CFU were identified as either <1 CFU (if one filter plate was used) or <0.5 CFU (if two plates were analyzed), and the associated results were identified as being censored. • The sample result was represented by the analysis method (spread plate or filter plate) with the higher detected result. If both methods yielded results identified as censored, the lower result (i.e., filter plate) was used to represent the sample. • Summary statistics were based on the detected results only, while the 95% UCL on the mean was based on the detected and censored results.
5. < Detection - Identify if "Less-Than"	• When nonzero, unadjusted mean spread plate counts, even if <30 CFU, were used as reported and treated as detected. • Unadjusted mean spread plate counts of 0 CFU were identified as being <0.33 CFU and the associated results were calculated assuming a dilution factor of 10 and identified as censored. • Unadjusted filter plate counts of 0 CFU were identified as either <1 CFU (if one filter plate was used) or <0.5 CFU (if two plates were analyzed), and the associated results were identified as being censored. • The sample result was represented by the analysis method (spread plate or filter plate) with the higher detected result. If both methods yielded results identified as censored, the lower result was used to represent the sample. • Summary statistics were based on the detected results only, while the 95% UCL on the mean was based on the detected and censored results.
6. < Quantification - Both Methods -- Identify if "Less-Than"	• Unadjusted mean spread plate counts less than the quantification limit were identified as being <30 CFU, and the associated results were calculated assuming a dilution factor of 10 and identified as being censored. • Unadjusted mean filter plate counts less than a quantification limit of 20 CFU were identified as being <20 CFU, and the associated results were identified as being censored. • The sample result was represented by the analysis method (spread plate or filter plate) with the higher quantifiable result. If both methods yielded results identified as censored, the lower result (i.e., filter plate) was used to represent the sample. • Summary statistics were based on the quantifiable results only, while the 95% UCL on the mean was based on the quantifiable and censored results.

Each of these data interpretation options yielded a separate set of sample results (as described in Section 2.4). Summary statistics including the 95% UCL on the mean were generated for each set using EPA's ProUCL software (as described in Section 2.5). Each of the six data interpretation options is described in more detail below, as well as the historic basis for its consideration.

(1.) Substitute 0 – Treat as Detect. This option, also referred to as "Substitute 0", treated samples with unadjusted mean spread plate counts <30 CFU as having 0 CFU. However, in determining the sample result, the sample's filter plate result was used if it was detected and greater than the spread plate result. Because nonzero filter plate counts <30 CFU are considered "detects", this option generally defaulted to the filter plate result whenever the unadjusted mean spread plate count was <30 CFU.

Brown et al. (2007a, 2007b, and 2007c) reportedly only considered spread plates with CFU counts of 30-300 when measuring *Bg* surface contamination following aerosol deposition. Several researchers sampling for spores on surfaces used filter plate results alone or in combination with other plating techniques. Hodges et al. (2006) and Estill et al. (2009) evaluated various surface sampling methods for *Bacillus anthracis* spores based on counting CFU on filter plates. Krauter et al. (2012) studied the recovery of *Bg* spores with wipe samples and used filter plating if no growth occurred after standard serial dilution and plating. Calfee et al. (2012) studied the decontamination of *Bg* spores deposited on surfaces, and when <30 CFU were counted on a TSA plate, the remaining wipe sample extract was analyzed by filter plating.

(2.) All Spread – Treat as Detect. This option, also referred to as "All Spread", used the reported unadjusted mean spread plate count, even when the value was less than quantification limits (<30 CFU), and treated all samples as having detected results. For a given sample, this option assigned the final sample result as the larger of the filter plate or spread plate result.

ASTM (2004) methods for water state that all colonies on spread plates should be counted when microbial counts are low. Sutton (2006) also noted that plate counting guidance varies by organization, and some report colonies below the countable range, e.g., <30 CFU for spread plates as an estimated count.

Some research involving surface sampling of spores failed to document approaches with regard to a quantification limit or minimum acceptable spread plate count, simply indicating that CFU were counted (Sanderson et al., 2002; Frawley et al., 2008; Valentine et al., 2008). Sanderson et al. (2002) noted that when the CFU counts were >300, the results were reported as too numerous to count.

(3.) Substitute 15 – Treat as Detect. For this option (also referred to as "Substitute 15"), unadjusted mean spread plate counts less than the quantification limit (i.e., <30 CFU) were substituted with one-half

of the quantification limit (15 CFU), and the result was always treated as detected. For a given sample, this option assigned the final sample result as the larger of the filter plate result or spread plate result.

Rodda et al. (1993) evaluated the substitution of one-half the detection limit for a risk assessment of enteric viruses in water. The associated daily risk of enteric virus infection was considerably higher than the mean yearly risk recommended by EPA. Rodda et al. (1993) acknowledged that the identification of low risk levels in drinking water was inhibited by small sample volumes and high detection limits. Signor et al. (2005) used one-half of the detection limit for one result (one out of 16 daily samples) in a study to quantify the impact of rain events on *Cryptosporidium* concentrations in surface water. Substituting with one-half of the detection limit was a common practice for purposes of conducting a chemical risk assessment historically (for example, when analyzing measured concentrations of chemicals in the environment), as it simplified the data analysis, but this practice is diminishing as recent increases in standard computing power allow for more sophisticated data analysis techniques to be implemented.

(4.) < Quantification – Identify if "Less-Than". Unlike the previous three options that treated each sample result as detected when calculating summary statistics (including 95% UCL on the mean), this option (also referred to as "< Quantification") retained information on whether the sample result was less than the quantification limit of 30 CFU (for the spread plate) or less than the detection limit of either 1.0 or 0.5 CFU (for the filter plate). (Filter plates generally do not have an associated quantification limit, as all countable CFU on a filter plate are considered valid.) For the data summaries and analyses, such unadjusted mean plate counts were identified as censored at either <1 CFU for one filter plate, <0.5 CFU for two replicate filter plates, or <30 CFU for the spread plates. The censored counts were then adjusted to account for elution suspension volumes and dilution factor per Equations 1 and 2. If both the associated spread plate and filter plate results were censored, the lower result (i.e., the filter plate result) was used to represent the sample and was identified as censored. Otherwise, the higher detected result was used, and the sample was identified as a detect. ProUCL includes statistical methods such as Kaplan-Meier (Kaplan and Meier, 1958) for computing the 95% UCL on the mean for data sets with censored data.

(5.) < Detection – Identify if "Less-Than". This option, also referred to as "< Detection", utilized the same approach as the < Quantification option (4), except that the unadjusted mean spread plate count was censored only when less than the detection limit (i.e., <0.33 CFU). Unadjusted mean spread plate counts of at least 1 CFU but less than 30 CFU (i.e., less than the quantification limit) were not treated as censored, and therefore, the reported mean spread plate count was used. If both the associated spread

plate and filter plate results were identified as being censored, the lower result was used to represent the sample. Otherwise, the higher detected result was used.

(6.) < Quantification – Both Methods – Identify if "Less-Than". This option, also referred to as <Quantification – Both Methods, was essentially the < Quantification option (4), augmented by implementing a quantification limit for the filter plating method as well as the spread plating method. Here, a quantification limit of 20 CFU, which has sometimes been used for filter plating (ASTM, 2004), was used. Because this limit is less than the quantification limit of 30 CFU for spread plating, and because filter plating does not require multiplying the result by a dilution factor like spread plating, the result for filter plating was always less than that for spread plating for a given sample. Thus, when both results were censored, the filter plating result was adopted under this option (as it was the lower result).

2.4 Equations for Calculating Sample Concentrations

For each of the six data interpretation options described in Table 1 and Section 2.3, summary statistics and the 95% UCL on the mean were calculated from sample results expressed either as CFU/m^3 (for air samples) or CFU/m^2 (for surface samples). The sample concentration was calculated from the adjusted CFU using Equations 3 and 4 (below) for air and surface samples, respectively. While the sample-specific input parameters (e.g., plate counts, dilution factors, elution suspension volumes) are not provided in this report, the adjusted CFU counts for both the spread plate and filter plate (as calculated using Equations 1 and 2) are provided in Appendix A Tables A-1 and A-2, as are the individual sample concentrations associated with each data interpretation option (as calculated using Equations 3 and 4).

Equation 3. Calculation of Air Sample Concentration (CFU/m^3)

$$\text{Air Sample Concentration } (\text{CFU}/\text{m}^3) = \frac{\text{Adjusted CFU}}{[\text{Flow Rate of Air Sampler } (\text{L}/\text{min}) \times 15 \text{ min Sample Duration} \times \text{m}^3/1000\text{L}]}$$

where:

Adjusted CFU = estimated number of *Bg* spores (CFU) collected in the sample from Equation 1 for spread plating or Equation 2 for filter plating.

Flow Rate of Air Sampler (L/min) = flow rate of the SKC BioSampler® unit (sample-specific, but ranged from 11.65 to 13.39 L/min for the data used in this report).

15 min Sample Duration = all air samples were collected for a 15 minute duration.

m^3/1000L = conversion factor to convert L to m^3.

Equation 4. Calculation of Surface Sample Concentration (CFU/m^2)

$$\textbf{Surface Sample Concentration } \left(\textbf{CFU/m}^{\textbf{2}}\right) = \frac{\textbf{Adjusted CFU}}{\textbf{Sample Area } \left(\textbf{m}^{\textbf{2}}\right)}$$

where:

Adjusted CFU = estimated number of *Bg* spores (CFU) collected in the sample from Equation 1 for spread plating or Equation 2 for filter plating.

Sample Area (m^2) = surface area sampled: 0.00258 m^2 for the swab, 0.0645 m^2 for the sponge and wipe, and 0.3716 m^2 for the vacuum.

Note that the sample concentrations calculated from Equations 3 and 4 are continuous in nature over the set of non-negative real numbers and are not rounded to the nearest integer.

2.5 Overview of Statistical Approach to Calculating Summary Statistics and 95% UCLs on the Mean

Summary statistics of the sample concentration results were generated separately for air and surface samples and for each of the six data interpretation options using Version 4.1 of EPA's ProUCL software (EPA, 2010)[1]. In addition, ProUCL was used to calculate the 95% UCL on the mean. ProUCL provides several state-of-the-art parametric and nonparametric statistical methods for calculating the 95% UCL on the mean from data sets containing both uncensored and censored data. These methods, which are detailed in EPA (2007), include the following:

Parametric Methods (all results detected/quantifiable)

- Student's t-statistic – assumes normality or approximate normality
- Approximate gamma upper confidence limit (UCL) – assumes gamma distribution
- Adjusted gamma UCL – assumes gamma distribution
- Land's H-Statistic – assumes lognormality
- Chebyshev Theorem using the minimum variance unbiased estimate (MVUE) of the parameters of a lognormal distribution (denoted by Chebyshev (MVUE)) – assumes lognormality

Nonparametric Methods (all results detected/quantifiable)

- Modified t-statistic – modified for skewed distributions

[1] Downloaded from http://www.epa.gov/osp/hstl/tsc/software.htm.

- Central limit theorem (CLT) – to be used for large samples
- Adjusted central limit theorem (adjusted-CLT) – adjusted for skewed distributions and to be used for large samples
- Chebyshev Theorem using the sample arithmetic mean and standard deviation (Sd) (denoted by Chebyshev (Mean, Sd))
- Jackknife method – yields the same result as Student‘s t-statistic for the UCL of the population mean
- Standard bootstrap
- Percentile bootstrap
- Bias-corrected accelerated (BCA) bootstrap
- Bootstrap t
- Hall’s bootstrap

Nonparametric Methods (some non-quantifiable results)
Different techniques are available in ProUCL to estimate the 95% UCL under the Kaplan-Meier method, including the following:

- Using a percentile bootstrap method
- Using a BCA bootstrap method
- Using the Chebyshev inequality
- Using a Student-*t* cutoff value

Results of statistical tests for goodness-of-fit for the normal, lognormal, and gamma distributions are presented in Appendix B for each data interpretation option. As a result of these tests and upon viewing plots of the data, because the data were inherently count-based rather than continuous in nature, and because it was preferred to make a common distributional assumption for all analyses if possible, a nonparametric approach was taken to estimate the 95% UCL on the mean for each option and each sample type.

The Substitute 0, All Spread, and Substitute 15 options use substitution techniques and/or actual observed plate counts to represent the sample. When calculating summary statistics or the 95% UCL on the mean, these options treat all data as detected, including results of 0 CFU/m^3 or 0 CFU/m^2. Therefore, only those methods in ProUCL that are relevant to data sets containing 100% detected results are considered for these three options. Furthermore, because the first two of these three options can yield results of 0 CFU,

parametric techniques for calculating 95% UCL on the mean that require all data to be nonzero (e.g., lognormal, gamma) were not applicable.

For data interpretation options that identify results as censored limits (< Quantification and < Detection), ProUCL calculates summary statistics on detected results only, while the 95% UCL on the mean is calculated using statistical techniques that account for the presence of censored values. These methods include Kaplan-Meier and ROS, both of which allow for multiple detection/quantification limits among samples within the same data set.

Statistical Outliers. The statistical approaches used in this report to calculate a 95% UCL on the mean were applied both with and without identified statistical outliers included in the analysis. Outliers are those sample values which are identified as extreme in a particular direction relative to the distribution of the remaining sample values; they are labeled as statistical outliers based on the results of a statistical hypothesis test (Gilbert, 1987). For each data interpretation option having at least three detected values, an outlier test available within ProUCL was applied to data that were not labeled as censored limits. Dixon's extreme value test (Dixon, 1950) was applied to the indoor air data due to the small sample size (n=18), while Rosner's test (Rosner, 1983) was applied to the surface sample data (n=136). Note that both tests assume a normal distribution to the data, which does not generally hold for these data interpretation options. Therefore, the results of these tests were used only as a guide for identifying extreme data values. When outliers with large values are included in data analyses, they can contribute to inflated summary statistics and can unduly impact the results of goodness-of-fit tests and the decision on the approach used. However, they do not warrant automatic exclusion from analysis simply due to their magnitude – some verification that their values are invalid is typically needed. Thus, if there is insufficient evidence that outliers are invalid, analyses are typically performed, as was done here, with and without the outliers included, to assess the impact on the analysis outcomes (e.g., 95% UCL on the mean).

3.0 Results

For each of the six data interpretation options in Table 1, the results of paired spread plate and filter plate analyses were evaluated for each sample. In general, the higher detected result between the two analysis methods was adopted as the count (CFU) estimate for the sample, and sample concentration was calculated from the CFU value. Of the two, the higher result when detected was considered a more precise estimate of the actual count. However, if both spread plate and filter plate results were left censored (that is, both results were expressed as a "less-than" value), the lower value was selected and treated as censored. Here, the lower value was selected because that result provided more information on the actual sample count. For example, if two methods reported values of <30 and <60 CFU for a given sample, the value of <30 is considered more precise given neither method can quantify the result. Air sample results (*Bg* spores in the air) were presented in units of CFU/m^3 based on the air sampling flow rate and sample duration. Surface sample results (*Bg* spores on surfaces) were presented in units of CFU/m^2 based on the surface area sampled.

Appendix A contains tables of the results under each of the six data interpretation options for each individual sample. Table A-1 contains results for the 18 indoor air samples, while Table A-2 contains the 136 surface sample results. The following findings were noted before any substitutions or designations of censoring were made within a particular data interpretation option:

- Unadjusted mean spread plate counts were <30 CFU for all 18 indoor air samples and for all but one of the 136 surface samples.
    - Only one surface sample did not have a mean spread plate count of <30 CFU. Its unadjusted count equaled 30 CFU; after adjusting for dilution factor and sample volume and dividing by sample area, its result was 3,991 CFU/m^2.
- Seven of the 18 indoor air samples reported mean spread plate counts >0 CFU (before the application of any substitutions) – the remaining 11 samples reported a result of 0 CFU.
    - The largest mean spread plate count among these seven samples was 1.33 CFU (i.e., one count in each of two plates, and two counts in the third plate), prior to multiplying by the sample volume.
    - One of these seven samples had a single CFU observed in the second serial dilution. Although no colonies were observed at the first dilution, application of a dilution factor of 100 was required, leading to the largest result (for an option not associated with substitution) of 1,758 CFU/m^3). All others air samples were based on a dilution factor of 10, and the next largest result (for an unsubstituted option) was 714 CFU/m^3.

- 41 of the 136 surface samples (30%) reported mean spread plate counts >0 CFU (before the application of any substitutions) – the remaining samples reported a result of 0 CFU.
    - Excluding the one detected surface sample noted above (with an unadjusted mean spread plate count = 30 CFU), the largest unadjusted mean spread plate count was 10 CFU.
    - Two of these 41 samples required a dilution factor of 100, while all others used a dilution factor of 10. As described above for the air sample based on a dilution factor of 100, no colonies were observed at the first dilution. The adjusted CFU for both samples was 167 CFU, which was within the range observed for the other 39 samples (15 to 1,483 CFU). However, with the application of the small sampling area for a swab (0.00258 m^2), one sample based on a dilution factor of 100 had the largest unsubstituted result (which occurs by implementing the All Spread option) – 64,729 CFU/m^2. The next largest sample result was 38,760 CFU/m^2, also for a swab sample. The other sample based on a dilution factor of 100 was from a vacuum sample with a result of 449 CFU/m^2.
- Only two of the 18 indoor air samples (11%) had a filter result of 0 CFU. In contrast, 55% of the surface samples had a filter result of 0 CFU, or 75 of the 136 surface samples.
    - The filter results for these 77 samples (i.e., the two indoor air and 75 surface samples with filter result of 0 CFU) were identified as less-than values for the < Quantification options (4 and 6).
    - Of these 77 samples, the two indoor air sample filter results and 12 of the surface samples representing wipe samples were analyzed using a single filter plate and thus were based on a detection limit of 1 CFU.
    - All of the remaining surface samples (i.e., 63 vacuum, sponge, and swab samples) were analyzed using two plates and thus were based on a detection limit of 0.5 CFU.
- Only one of the 18 indoor air samples (6%) had a result of 0 CFU for both spread plate and filter plate, compared to 66 of the 136 surface samples (49%).

3.1 Summary Statistics and Histograms

Tables 2a and 2b present summary statistics, calculated by ProUCL, on the results for the indoor air samples and the surface samples, respectively. As noted in Table 1, under the < Quantification and < Detection options, the summary statistics in these tables were calculated only on detected results, while all sample results are used to calculate the summary statistics for the other three options. Several parameters were included in Tables 2a and 2b to help describe/differentiate the data sets being evaluated including: the number of samples, the number of samples represented by the spread plate and filter plate

Table 2a. Summary Statistics[a] for Air Sample Concentrations (CFU/m^3), by Data Interpretation Option

	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. < Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. < Quantification - Both Methods -- Identify if "Less-Than"
# Samples	18	18	18	18	18	18
# Filter / # Spread[b]	16 / 2	12 / 6	0 / 18	18 / 0	13 / 5	18 / 0
% Detected	100%	100%	100%	88.9%	94.4%	0.0%
# of 0 CFU/m^3 Results	2	1	0	NA	NA	NA
# of Censored Results	NA	NA	NA	2[c]	1[d]	18
Mean (CFU/m^3)	104	278	8,323	117	294	--
Standard Deviation	78	409	531	72	416	--
Coefficient of Variation	0.75	1.47	0.06	0.62	1.41	--
Skewness	0.87	3.12	1.77	1.07	3.08	--
Minimum	0	0	7,733	51	51	--
25th Percentile	52	56	7,956	55	57	--
50th Percentile	86	158	8,110	108	159	--
75th Percentile	147	271	8,623	158	288	--
90th Percentile	213	523	8,742	215	550	--
95th Percentile	229	871	8,963	236	923	--
Maximum (CFU/m^3)	288	1,758	9,957	288	1,758	--
Standard Deviation of Log-Transformed Results	NA	NA	0.061	0.595	1.015	--

CFU, colony forming units, NA = not applicable.

[a] Calculated only on detected results. For the Substitute 0, All Spread, and Substitute 15 options, all data were considered to be detected (including results of 0 CFU/m^3) and therefore all results were included in the calculation of the summary statistics. The less-than values associated with the < Quantification and < Detection options were not included in the summary statistics as these results were not specifically known.

[b] The number of filter plate results and spread plate results selected to represent the 18 air samples.

[c] The two less-than samples were <55 and <59 CFU/m^3; see Table A-1, which identifies detected and less-than results.

[d] The one less-than sample was <59 CFU/m^3; see Table A-1, which identifies detected and less-than results.

Table 2b. Summary Statistics[a] for Surface Sample Concentrations (CFU/m^2), by Data Interpretation Option

	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. < Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. < Quantification - Both Methods -- Identify if "Less-Than"
# Samples	136	136	136	136	136	136
# Filter / # Spread[b]	60 / 76	36 / 100	0 / 136	135 / 1	102 / 34	135 / 1
% Detected	100%	100%	100%	44.8%	51.5%	3.7%
# of 0 CFU/m^2 Results	75	66	0	NA	NA	NA
# of Censored Results	NA	NA	NA	75[c]	66[c]	131[d]
Mean (CFU/m^2)	465	1,181	36,785	1,037	2,295	2,159
Standard Deviation	2,810	6,506	76,912	4,143	8,957	1,202
Coefficient of Variation	6.04	5.51	2.09	3.99	3.90	55.67
Skewness	10.83	8.47	2.93	7.32	6.06	0.68
Minimum	0	0	2,018	8	8	681
25th Percentile	0	0	2,018	31	47	1,705
50th Percentile	0	8	11,395	170	233	2,093
75th Percentile	95	249	15,407	450	1,039	2,326
90th Percentile	582	1,372	46,512	2,093	2,481	3,991
95th Percentile	1,977	2,791	290,698	2,481	4,745	3,991
Maximum (CFU/m^2)	32,171	64,729	290,698	32,171	64,729	3,991
Standard Deviation of Log-Transformed Results	NA	NA	1.397	1.936	2.017	0.645

CFU, colony forming units; NA = not applicable.

[a] Calculated only on detected results. For the Substitute 0, All Spread, and Substitute 15 options, all data were considered to be detected (including results of 0 CFU/m^2) and therefore all results were included in the calculation of the summary statistics. The less-than values associated with the < Quantification and < Detection options were not included in the summary statistics as these results were not specifically known.

[b] The number of filter plate results and spread plate results selected to represent the 136 surface samples.

[c] The less-than samples ranged from <8 to <1,163 CFU/m^2; see Table A-2, which identifies detected and less-than results.

[d] The results for the five samples with quantifiable outcomes were 681, 1,705, 2,093, 2,326, and 3,991 CFU/m^2.

results, the percentage of results treated as detects, the number of zero results (i.e., 0 CFU/m^3 or CFU/m^2), and the number of censored results.

The following information can be gained from the summary statistics in Tables 2a and 2b:

- The Substitute 0 option was equivalent to using the filter plate result for all samples but the one surface sample where the unadjusted mean spread plate count equaled 30 CFU.
    - Because all unadjusted mean spread plate results were <30 CFU except for one surface sample, all nonzero filter plate results were used to determine the sample result except for the one surface sample.
    - Nonzero filter plate results represented 16 of 18 air samples and 60 of 136 surface samples.
    - More than half of the surface samples (75 of 136) were assigned a result of 0 CFU/m^2 under this option. Two of 18 air samples were assigned a result of 0 CFU/m^3.
    - While the largest observed surface result (swab) was 32,171 CFU/m^2 under this option, the next largest result (vacuum sock) was 3,991 CFU/m^2 (i.e., the sample using the spread plate result).
    - As a note, if filter plate data were not available and spread plate results <30 CFU were considered to have 0 CFU, then all air sample results would be 0 CFU/m^3, and only one surface sample result would be nonzero (i.e., 3,991 CFU/m^2). Without consideration of the filter plate data, the associated mean level of *Bg* spore contamination on the indoor surfaces would be 29 CFU/m^2 compared to 465 CFU/m^2 with the filter plate data.

- The All Spread option showed results of 0 CFU/m^3 or 0 CFU/m^2 only when both the spread plates and filter plates had unadjusted plate counts of 0 CFU. Compared to the Substitute 0 option, the sample result under the All Spread option was obtained more frequently from the spread plate results, and fewer results of zero were encountered.
    - Nonzero filter plate results were selected to represent 12 of 18 air samples and 36 of 136 surface samples.
    - Slightly under half of the surface samples (66 of 136) were assigned a result of 0 CFU/m^2 under this option. One of 18 air samples was assigned a result of 0 CFU/m^3.
    - For five of the seven indoor air samples having nonzero values for unadjusted mean spread plate count, and for 34 of the 41 such surface samples, the result was based on the spread plate outcome.

- The Substitute 15 option used substitution techniques in all cases where unadjusted mean spread plate counts were less than quantification limits, and thus, there were no outcomes equal to zero.
    - Spread plate results were selected to represent all air samples and all 136 surface samples.
    - These substitutions imply that samples having true 0 CFU counts will be represented by some positive count, leading to overestimates. As a result, the summary statistics were orders of magnitude higher than for the other options.
    - For the surface samples, the range of results did not overlap among samples with different sampling methods:
        - Swab samples: Each of the 11 swab samples had a result of 290,698 CFU/m^2 (the spore content of each sample was <30 CFU which was therefore substituted by 15 CFU, each had an elution suspension volume of 5 mL, and each had a sample area of 0.00258 m^2).
        - Wipe samples: The result for each of the 20 wipe samples was 46,512 CFU/m^2 (the spore content of each sample was <30 CFU thus substituting 15 CFU for the result, each had an elution suspension volume of 20 mL, and each had a sample area of 0.0645 m^2).
        - Sponge samples: Results for 69 samples ranged from 6,744 to 16,512 CFU/m^2 (the result for each of the 69 sponge samples was non-detected and thus was substituted with 15 CFU; each had a sample area of 0.0645 m^2, but elution suspension volumes ranged from 2.9 to 7.1 mL among these samples).
        - Vacuum samples: Except for one vacuum sample, each sample result equaled 2,018 CFU/m^2 (which resulted from a substitution of 15 CFU due to being a non-detect, each had an elution suspension volume of 5 mL, and each had a sample area of 0.3716 m^2) – the one vacuum sample with a detected outcome had a result of 3,991 CFU/m^2 (See table A-2).
    - For the air samples, the results ranged from 7,733 to 9,957 CFU/m^3; all 18 samples underwent substitution with 15 CFU and all sample durations were 15 minutes, but elution suspension volumes ranged from 10.2 to 11.6 mL and air sampling flow rates ranged from 11.65 to 13.39 L/min.

- Like the Substitute 0 option , the < Quantification (Spread only) option was equivalent to using the filter plate result for all samples but the one surface sample where the unadjusted mean spread plate count equaled 30 CFU. (This was because the other spread plate sample results were

identified as less than the quantification limit, and the detection limit for the filter plate was always less than the quantification limit for the spread plate.)

- For 2 of 18 air samples and 75 of 136 surface samples, the results were identified as being censored. Censored values were identified only with those samples that have non-detected outcomes for the filter analysis. The nonzero results of the Substitute 0 option matched the detected results used in the < Quantification option.
- For some samples, results were identified as censored at limits that fell above the outcomes of some detected samples, and the censored limits differed considerably among samples. For example, occurrences of censored results among surface samples (taken from filter analysis) were as follows:
  - Vacuum samples: <8 CFU/m^2 (0.3716 m^2 sample area, elution suspension volume of 5 mL, average of two plates – i.e., the detection limit was based on a potential CFU count of 0.5)
  - Sponge samples: <31, <47, or <62 CFU/m^2 (0.0645 m^2 sample area, elution suspension volumes ranged from 3.4 to 7.0 mL, average of two plates)
  - Wipe samples: <310 CFU/m^2 (0.0645 m^2 sample area, elution suspension volume of 20 mL, only one plate – i.e., the detection limit was based on a potential CFU count of 1)
  - Swab samples: <1,163 CFU/m^2 (0.00258 m^2 sample area, elution suspension volume of 5 mL, average of two plates)
- Less-than values based on the spread plates at the quantification limit, were at least two orders of magnitude greater than the associated less-than values based on the filter plate detection limits.

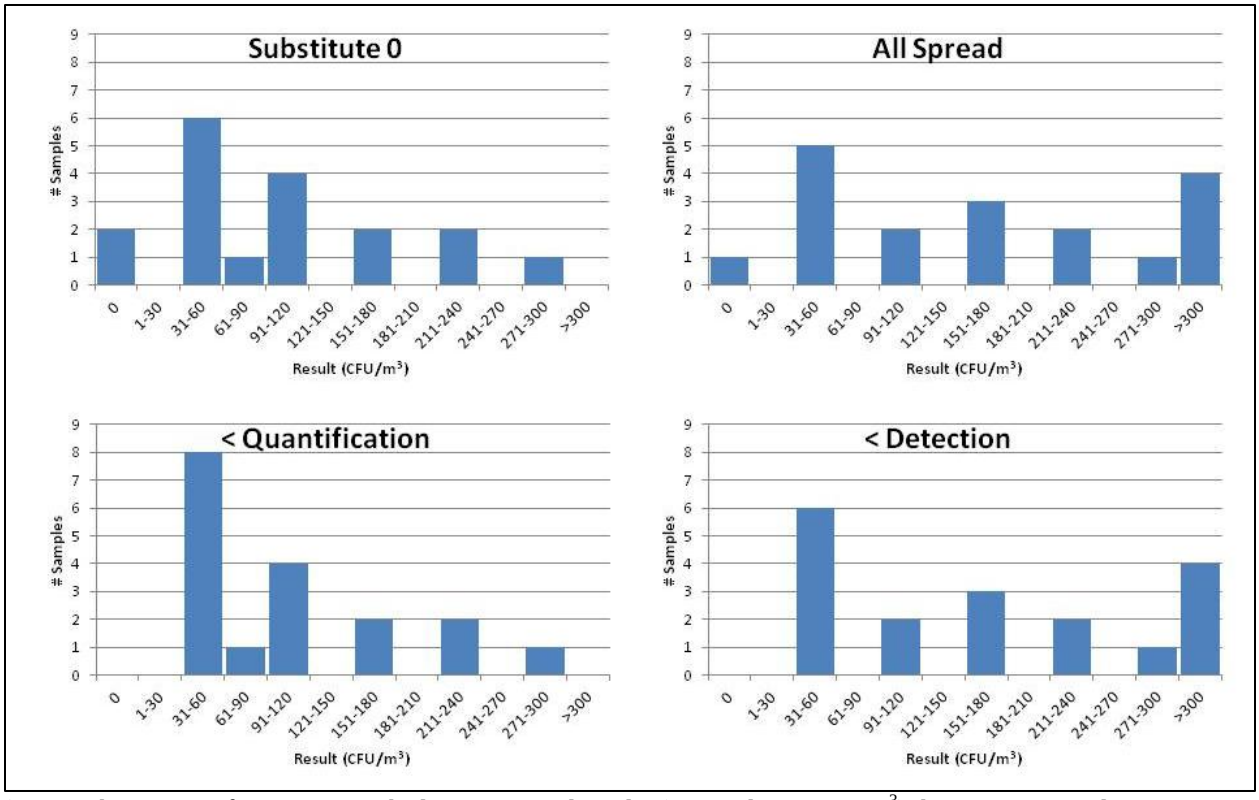
- Under the <u>< Detection</u> option , all results identified as detected matched those nonzero results used in the All Spread option for the given sample, while all results labeled as censored (i.e., not detected for both spread plate and filter plate analyses) were censored at the same limit as in the < Quantification option. This is due to the filter plate analysis always resulting in the lower censoring limit for samples under both options.
  - Despite the spread plate censored value declining from the < Quantification option to the < Detection option (as its interpretation shifts from a quantification limit to a detection limit), the censored value associated with the filter plate was still used in all instances when the result was non-detected under both analyses.

- As expected, the < Detection option resulted in fewer sample results identified as censored compared to the < Quantification option, due to the decline in the number of samples whose spread plate result is classified as censored.
- Generally, when the sample outcome changed from the < Quantification option (either from a censored result to a detected result, or among detected results), the outcome was higher for the < Detection option. As a result, the summary statistics were higher under the < Detection option than for the < Quantification option, especially in the upper tail of the distribution.

- The < Quantification – Both Methods option resulted in outcomes falling below quantifiable limits (i.e., left-censored) for all 18 indoor air samples, and for all but five of the 136 surface samples. Because no quantifiable outcomes occurred among the indoor air samples under this option, no summary statistics are presented for this option in Table 2a (and no 95% UCL calculations can be performed), and the summary statistics in Table 2b are based on only five sample measurements.
  - All vacuum samples below quantification limits are portrayed as <269 CFU/m^2. (Two vacuum samples had quantifiable results: 681 CFU/m^2 and 3,991 CFU/m^2.)
  - All sponge samples fall between 501 and 2,500 CFU/m^2. (Three sponge results were quantifiable: 1,705 CFU/m^2, 2,093 CFU/m^2, and 2,326 CFU/m^2.)
  - All wipe samples are portrayed as <6,202 CFU/m^2.
  - All swab samples are portrayed as <38,760 CFU/m^2.

Figures 1a through 1c (for air samples) and Figures 2a through 2c (for surface samples) present the results in histograms by data interpretation option. In these histograms, the vertical axis represents the number of samples having results within the range specified on the horizontal axis (beneath each bar). In these histograms, results falling below quantification or detection limits are portrayed by their respective limits.

To facilitate comparison of the data distribution and trends across the four data interpretation options in Figures 1a and 2a, the range of the vertical axis and the categories on the horizontal axis are consistent among the four histograms. However, for both air and surface samples, the histograms for the Substitute 15 option (Figures 1b and 2b) and the < Quantification – Both Methods option (Figures 1c and 2c) have different axis ranges from the other options as their data were considerably higher in magnitude and thus different from the others.



(Note: The < Quantification option had two censored results (<55 and <59 CFU/m^3) that were reported as 0 CFU/m^3 for the Substitute 0 option. The < Detection option had one less-than result (<59 CFU/m^3) that was reported as 0 CFU/m^3 for the All Spread option. These samples are represented by their censored results in the bottom two histograms.)

Figure 1a. Histograms of <u>Air Sample Concentrations</u> (CFU/m^3) for Four Data Interpretation Options (n=18)

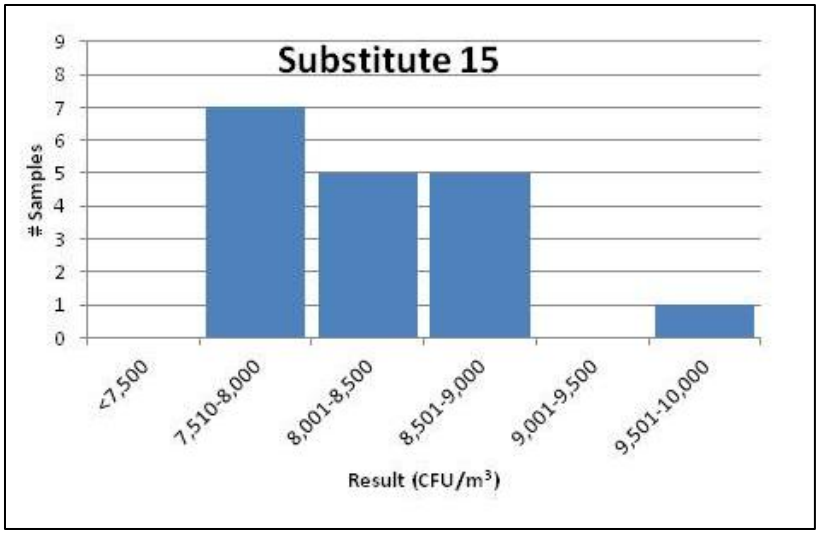


Figure 1b. Histogram of Air Sample Concentrations (CFU/m^3) for the Substitute 15 Data Interpretation Option (n=18)

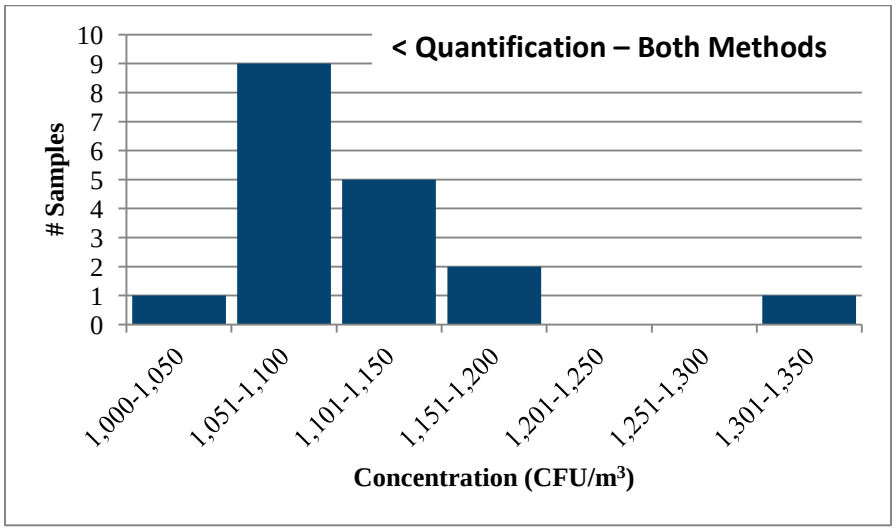
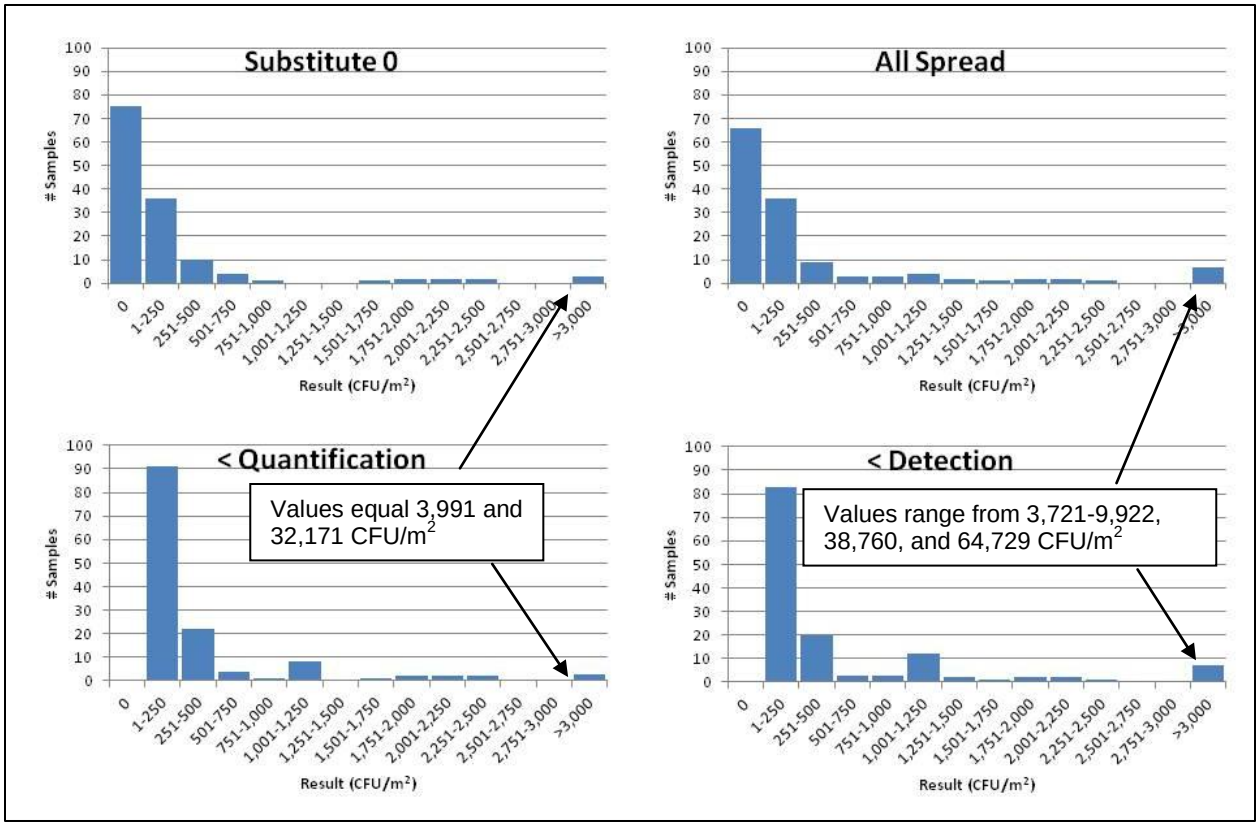


Figure 1c. Histogram of Air Sample Concentrations (CFU/m^3) for the < Quantification – Both Methods Option (n=18)



(Note: The < Quantification option had 75 censored results, all of which had results of 0 CFU/m^2 for the Substitute 0 option. The < Detection option had 66 censored results, all of which had results of 0 CFU/m^2 for the All Spread option. These samples are represented by their censored results [<8, <31, <47, <62, <310, and <1,163 CFU/m^2] in the bottom two histograms.)

Figure 2a. Histograms of <u>Surface Sample Concentrations</u> (CFU/m^2) for Four Data Interpretation Options (n=136)

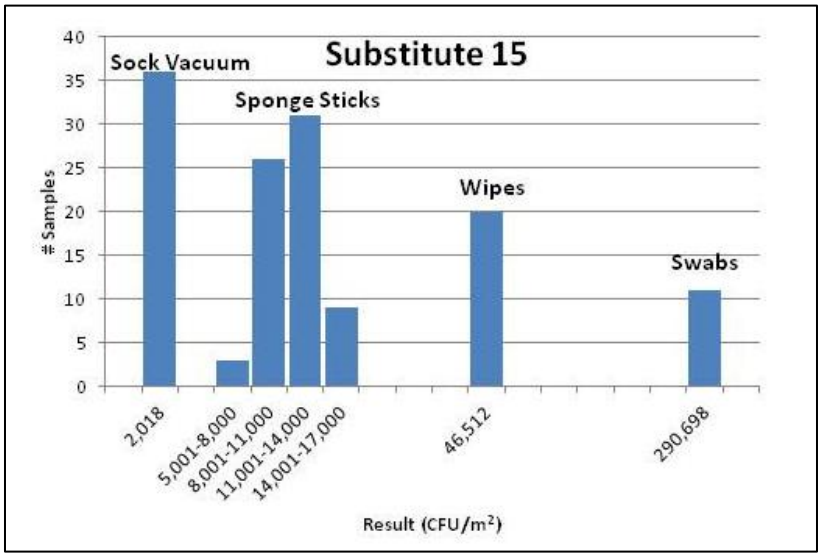


Figure 2b. Histogram of Surface Sample Concentrations (CFU/m^2) for the Substitute 15 Treatment Option (note: horizontal axis not to scale) (n=136)

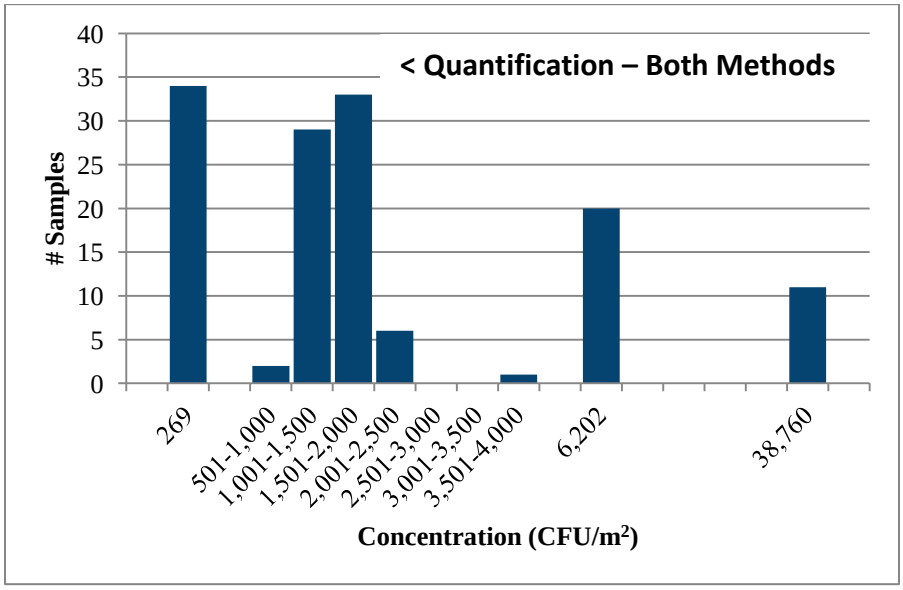


Figure 2c. Histogram of Surface Sample Concentrations (CFU/m^2) for the < Quantification – Both Methods Option (note: horizontal axis not to scale) (n=136)

The histograms for Substitute 15 option (Figures 1b and 2b) and the < Quantification – Both Methods option (Figures 1c and 2c) show that these options were prone to generating very high results for some samples. These very high results tended to be clustered, or separated from the distribution of results for the other samples, suggesting that all results do not adhere to a common normal, lognormal, or gamma distribution. For example, under the Substitute 15 option, the result for one air sample (9,957 CFU/m^3) was about 13% higher than the next highest air sample result. The separation was much more prominent for the surface sample results, where, as noted earlier, the swab results (290,698 CFU/m^2 for all 11 samples under the Substitute 15 option) were an order of magnitude higher than for all other sample types (due to very small swab surface area sampled), and the wipe results (46,512 CFU/m^2 for all 20 samples) were at least three times higher than sponge and vacuum samples. Thus, it is apparent from these histograms and data investigation that the method of substitution used in the Substitute 15 and < Quantification – Both Methods options not only yielded much higher results than the other options, but they were much more prone to yielding outliers that would have a large impact on the 95% UCL on the mean calculation. The data distribution depends heavily on the range of surface areas sampled under both options.

The All Spread option and the < Detection option were prone to generating more large outliers compared to the Substitute 0, < Quantification –Identify if Less then, and < Quantification-Both Methods options. Under the All Spread and < Detection options, the air sample with the highest result (1,758 CFU/m^3) exceeded the sample with the next highest result by more than double. The result of 1,758 CFU/m^3 was due in part to the need to take a 100-fold dilution when analyzing this sample, versus a 10-fold dilution that was taken for all other air samples. Two surface samples had results that were more than 4 times higher than the other samples; both samples had small surface areas, and one of the two samples had a 100-fold dilution. This shows that when any counts are noted among the replicate plates for a sample of a relatively small area, the result under either of these options will likely be very high when expressed as CFU per unit area.

3.2 95% UCLs on the Mean

ProUCL was used to generate estimates for the 95% UCL on the mean for each data interpretation option and each data set (indoor air and surface), using the data summarized in Section 3.1 (and presented in Appendix A). While ProUCL offers many varied techniques that require various types and degrees of distributional assumptions, all of which are detailed in EPA (2010), the final set of recommended 95% UCL estimates was obtained without assuming a specific data distribution for the results, as a single

distribution type cannot be discerned to hold across all options and for the post-decontamination samples being utilized in this assessment. Estimates of the 95% UCL of the mean under other statistical techniques (and assuming different distributional assumptions) are presented in Appendix C.

For indoor air samples, Table 3a presents estimates of the 95% UCL on the mean, while estimates for surface samples are presented in Table 3b. Within these tables, the Substitute 0 option and the <Quantification option are grouped together, as they are based on the same data, except the latter option identified results as being less than the associated quantification limit rather than substituting zeroes. The All Spread and < Detection options are similarly grouped. Note that the < Quantification – Both Methods option is excluded from Table 3a as all results were censored for the 18 indoor air samples under this option, and thus, no 95% UCLs could be calculated.

Table 3a. Recommended 95% Upper Confidence Limit (UCL) on the Mean, Using Air Sample Concentrations (CFU/m^3) for Each of the Six Data Interpretation Options

	Data Interpretation Options				
	1. Substitute 0 - Treat as Detect	**4. <Quantification - Identify if "Less-Than"**	**2. All Spread - Treat as Detect**	**5. < Detection - Identify if "Less-Than"**	**3. Substitute 15 - Treat as Detect**
Recommended UCL Calculation Method	Hall's Bootstrap UCL	Kaplan-Meier (BCA)	Chebyshev	Kaplan-Meier (Chebyshev)	Student's-t or Modified-t
	Outliers Included (n=18)				
95% UCL on the mean (CFU/m^3)	142	138	699	700	8,540 or 8,549
	Outliers Excluded[a] (n=17)				
95% UCL on the mean (CFU/m^3)	No outliers excluded		384	384	8,375

BCA, bias-corrected accelerated; CFU, colony forming units

[a] Outliers were 1,758 CFU/m^3 for All Spread and < Detection options, and 9,957 CFU/m^3 for Substitute 15 option. No outliers were excluded from the other options.

Table 3b. Recommended 95% Upper Confidence Limit (UCL) on the Mean, Using Surface Sample Concentrations (CFU/m^2) for Each of the Six Data Interpretation Options

	Data Interpretation Options					
	1. Substitute 0 - Treat as Detect	**4. <Quantification - Identify if "Less-Than"**	**2. All Spread - Treat as Detect**	**5. <Detection - Identify if "Less-Than"**	**3. Substitute 15 - Treat as Detect**	**6. <Quantification – Both Methods- Identify if "Less-Than"**
Recommended UCL Calculation Method	Chebyshev	Kaplan-Meier (Chebyshev)	Chebyshev	Kaplan-Meier (Chebyshev)	Chebyshev	Kaplan-Meier (Student-t or Percentile Bootstrap)
	Outliers Included (n=136)					
95% UCL on the mean (CFU/m^2)	1,516	1,533	3,613	3,638	65,533	826 or 2,326
	Outliers Excluded[a] (n=135)					
95% UCL on the mean (CFU/m^2)	467	481	2,026	2,617	No outliers excluded	No outliers excluded

CFU, colony forming units

[a] Outliers were 32,171 CFU/m^2 for Substitute 0 and < Quantification options, and 64,729 CFU/m^2 for All Spread and < Detection options. No outliers were excluded from the Substitute 15 option.

Note that in both tables, 95% UCL on the mean values were calculated both with and without outliers identified in the data sets. By excluding outliers, the value of the calculated 95% UCL on the mean can be reduced by over 50 percent. The identified outliers are specified in the footnotes to the tables.

Recall that the Substitute 0, All Spread, and Substitute 15 options (options 1 to 3) treat all results as detected, and that the first two of these three options permit results of zero. Thus, while ProUCL recommends a specific nonparametric approach for calculating the 95% UCL based on the size of the standard deviation of the log-transformed results, this is not possible with the Substitute 0 and All Spread options as results of zero are valid. For air samples (Table 3a), the Hall's Bootstrap was taken as the recommended approach for the 95% UCL calculation under the Substitute 0 option as its actual coverage probability tends to approach 95% and the method adjusts for bias and skewness (EPA, 2010). For the Substitute 15 option, the standard deviation of the log-transformed air sample results (Table 2a) falls below 0.5, and thus, ProUCL recommends either a Student-t or modified-t approach as the 95% UCL estimates under these approaches have good coverage probability even when no specific distributional form is assumed on the results. For the three options treating "less-than" values as censored, ProUCL indicates that those approaches which are recommended under a lognormal distribution are also applicable to data that are skewed but not necessarily lognormal (EPA, 2010). Thus, the recommended

95% UCL estimates for these three options are those that are relevant under a lognormal distribution assumption.

In general, for the samples included in this evaluation, using results identified as censored had a relatively small effect on the 95% UCL on the mean calculation, compared to how the spread plate results were used to represent the sample result (i.e., unadjusted mean spread plate counts less than the quantification limit (30 CFU) treated as zeroes, all counts <30 CFU treated as valid, or treated as 15 CFU). This was seen by noting that the 95% UCL on the mean calculations were similar between the two paired data interpretation options (i.e., the options within bolded lines within Tables 3a and 3b), but differed considerably between different pairs of options. The 95% UCL on the mean under the All Spread option was over twice the size of the UCL under the Substitute 0 option for surface samples (over four times the size when excluding the outlier), and over five times the size of the Substitute 0 option result for air samples.

The 95% UCLs on the mean results under Substitute 15 option were much higher (by an order of magnitude) than for the other options. For air samples, removal of the outlier had a minor effect on the calculation of the UCL. For surface samples, the 95% UCL estimates under < Quantification - Both Methods option are highly variable, as they are based on only five detected outcomes out of the 136 sample results. Thus, the two recommended estimates from ProUCL, both nonparametric-based, are an order of magnitude different (Table 3b). (To ensure appropriate coverage, the higher of the two recommended estimates should be selected as a conservative estimate.)

4.0 Discussion

4.1 Statistical Approach

The generation of summary statistics on the *Bg* spore data set, including a 95% UCL on the mean, needed to account for two issues: (1) the presence of censored results, and (2) the count-based nature of the unadjusted data. Within this report, filter plate results were considered censored if no CFU were observed on the plates (i.e., non-detect), or in the case of one option, if unadjusted filter plate counts were <20 CFU (i.e., below a quantification limit). Spread plate results were considered censored if either no CFU were observed on the plates (i.e., non-detect), or if the unadjusted mean spread plate counts were <30 CFU (i.e., below the quantification limit).

If censored data are not handled appropriately (e.g., treated as detected, or use of various substitution approaches), this can result in descriptive statistics that do not adequately represent the true underlying data distribution (Helsel, 2005). For censored data sets, Helsel (2005) indicated that the following methods are among those preferred for generating statistics over substitution methods:

- MLE – Uses detected observations and censored limits to generate summary statistics that are expected to have produced both the detect and non-detect data.
- Imputation (e.g., ROS) – Non-detects are assigned values determined from the distribution of the detected data (e.g., probability plots of detects). Not all non-detects are assigned the same value.
- Kaplan-Meier – A nonparametric approach (i.e., does not assume a specific distributional model) that estimates the cumulative distribution function of data in the presence of multiple detection limits, then generates statistics that are based on this estimated distribution.

These and other methods have been incorporated into EPA's ProUCL software (Version 4.1), which was used in this evaluation. As described by EPA (2010), ProUCL provides several parametric and nonparametric methods that can be used with uncensored (100% detected) and censored (censored limits) observations at multiple quantification/detection limits, such as the Kaplan-Meier and ROS. ProUCL helps identify an appropriate statistical method by applying goodness-of-fit tests relative to a specified distributional model (e.g., normal, lognormal, gamma) and making recommendations on a method based on the outcome of these tests and other properties of the data (e.g., standard deviation, skewness, sample size, number of censored limits). Nonparametric methods are available and preferred if none of the distributional models are deemed adequate for the data being analyzed.

Methods for computing the 95% UCL on the mean using ProUCL are well established for chemical contamination in environmental samples. However, Brattin et al. (2012) remarked that some of the more common statistical procedures in ProUCL may not be applicable for calculating a 95% UCL on the mean for parameters measured using count-based analytical methods. In particular,

- Counts of zero prohibit ProUCL from evaluating gamma and lognormal data distributions or applying statistical procedures that assume these underlying distributions hold.
- ProUCL tests for goodness-of-fit to normal, lognormal, and gamma distributions which are continuous in nature, while count-based data are inherently discrete.
- ProUCL assumes only inter-sample variation, while count-based data may also consist of random Poisson counting variation.

The complicating nature of analyzing count-based data was also described by Petterson et al. (2001): "Statistical analysis of data from microbial count experiments has traditionally involved converting all counts to concentrations by dividing by sample volumes, followed by analysis assuming a continuous statistical distribution. At low concentrations, stochastic variability in sampling is not negligible and microbial counts from a single well-mixed suspension may not be assumed to be uniform. Analysis of the data using counting statistics (discrete rather than continuous distributions) allows for consideration of sampling variability, and differences in information content (low counts versus high counts) to be properly addressed."

If a Poisson distribution is assumed due to the counting nature of the unadjusted data, the mean is estimated by the arithmetic mean (as usual), but the standard deviation is estimated by the square root of the arithmetic mean, which tends to underestimate the actual standard deviation in these data when the mean is small. Furthermore, when the mean is large, the 95% UCL would be calculated in the same way as if a normal distribution was assumed, using the mean and standard deviation calculated under the Poisson distributional assumption.

The necessity of considering discrete microbial distributions versus continuous distributions may depend on the average level of contamination. For example, as just noted, a normal approximation to the Poisson (discrete) distribution can hold when the average contamination level is high. At very low average levels of contamination, heterogeneity becomes more important, as, for example, in exposure analyses.

4.2 All Spread Option

The All Spread option for interpreting the spore data selects the higher result from filter plating or spread plating to represent the sample, without qualifying the result as being below detection or quantification limits (i.e., all data are treated as detected). As such, this option is expected to yield more accurate results than the two substitution options (Substitute 0 or Substitute 15), although estimates of accuracy are made without explicitly known CFU levels. Substituting 0 CFU when spores are present understates the results. Substituting 15 CFU may understate or overstate the results, depending on whether the actual number of viable spores in samples with non-quantifiable spread-plate outcomes is greater than or less than 15 CFU. Because the data examined in this study were post-decontamination, the number of residual spores detected was low – substantially less than 15 CFU – and therefore substituting 15 CFU for non-quantifiable outcomes yielded results much higher than was observed as colonies on plates. Thus, the Substitute 15 option is most likely not appropriate for settings in which very low spore counts are typically present.

Filter plates are suitable for detection and quantification of spores especially at low environmental concentrations. For example, filter plates were used to analyze surface samples collected after a low (3 to 200 CFU/100 cm^2) loading with *Bacillus anthracis* spores (Estill et al., 2009). Krauter et al. (2012) used filter plating to maximize the detection of low numbers of *Bg* spores from surface samples; if no spores were observed on spread plates, a portion of the elution suspension was then filter plated.

ASTM (2004) methods for water state that all colonies on spread plates should be counted when microbial counts are low. Sutton (2006) also noted that plate counting guidance varies by organization, and some report colonies below the countable range for spread plates (e.g., <30 CFU) as an estimated count.

Helsel (2005) and other researchers have cautioned against using analytical data below the reporting limit as uncensored data, or using any type of substitution approach to analyze non-detects or non-quantifiable outcomes. From an analytical chemistry perspective, Helsel argued that readings below the reporting limit cannot be identified as being different from zero or from each other. It is uncertain if this concern is valid for microbiological spread plate and filter plate data as well, but for similar reasons, some researchers have avoided using spread plate results below the quantification limit, using filter plate results instead. A validation study (Rose et al. 2011) for the recovery of *Bacillus anthracis* spores from surfaces used filter plate results if the spread plate results fell below the quantification limit (<25 CFU). Rose et al. (2011)

filtered two 1-ml aliquots of spore elution suspensions to detect low numbers of spores. If the spread plate counts were <25 CFU, then the filter plate CFU counts were used for quantification. The authors considered this analysis of cellulose sponge wipe processing to be a validated method with oversight being provided by the Centers for Disease Control and Prevention Laboratory Response Network review committee. Calfee et al. (2012) used filter plates to detect *Bg* spores collected from surface samples if fewer than 30 CFU were detected on spread plates.

Given the bias noted for substitution approaches, it appears appropriate to use the reported unadjusted mean spread plate counts <30 CFU in determining the sample CFU results, without substituting alternate values for these outcomes.

4.3 Substitution Options

The options of substituting mean spread plate counts <30 CFU (i.e., below the quantification limit) with either 0 CFU or 15 CFU appeared to underestimate and overestimate results, respectively. For example, when using the Substitute 0 option, the mean sample results for air and surface samples were approximately half the results associated with the All Spread option. When using the Substitute 15 option, the mean sample results were 30 times the results associated with the All Spread option. When substituting results for all samples below the quantification limit with 15 CFU and treating them as detected outcomes, the results became considerably inflated, as CFU counts per spread plate and filter plate analyses were actually often below the substituted value. In this study, substituting 15 CFU for samples with <30 CFU on spread plates biased the results high. Depending on the actual number of CFU counts in the sample, substituting 15 CFU may result in a high or low bias.

EPA (2010) acknowledged that substituting one-half of the detection limit for non-detected outcomes can introduce bias, even with few (5-10%) non-detects. Substituting zero for <30 CFU on spread plates biases results in underestimates for the affected samples and thus does not reflect a conservative approach for post-decontamination sampling. Helsel (2005) also noted that substitution approaches can hide real trends while potentially introducing false trends in the data, and therefore, does not recommend substituting values for non-detects or non-quantified values and treating the outcome as detected. Helsel (2010) referred to substitution as a flawed method (except possibly when estimating a mean for a data set with only one less-than threshold) and clarified that substitution does not equate to imputation.

4.4 "Less-Than" Options

The three < Quantification and < Detection options qualified sample results as censored (less-than) if below the associated quantification limits or detection limit. Incorporating this qualification into the data analysis required alternative statistical approaches to calculate 95% UCL on the mean (e.g., Kaplan-Meier methods within ProUCL) compared to when all sample results were treated as detected. However, with the data set evaluated, 95% UCL on the mean values were similar to one of the other options that did not account for censoring. For example, the surface sample 95% UCL on the mean for the Substitute 0 option was 1,516 CFU/m^2 while the < Quantification option was 1,533 CFU/m^2; the 95% UCL on the mean values were 3,613 CFU/m^2 for the All Spread option and 3,638 CFU/m^2 for the < Detection option.

If quantification limits are associated with both filter and spread plate analyses (as with the <Quantification – Both Methods option) and these limits are high in value, then the majority, if not all, samples could have a final result that is censored at one or the other of these limits. As a result, this outcome would provide very little information to the calculation of a 95% UCL (or the calculation may not be possible), leading to large and unstable UCL estimates. This finding occurred with the <Quantification – Both Methods option in this analysis.

Although 95% UCL on the mean results were not very different from when censoring is accounted for, the incorporation of terminology such as "non-detects" or "censored limits" could help avoid implying more certainty in the data than if sample results were reported as a single number (e.g., 0 CFU/m^2). Even if the culturing of environmental samples yields 0 CFU, it is not necessarily true that the sampled medium was free of spores (Edmonds, 2009). Only a portion of the sample was likely cultured, and recovery and extraction efficiencies were not 100%. However, censoring may not necessarily be appropriate for count data. For example, Petterson et al. (2006) indicated that results of zero should be included when modeling a discrete distribution. Brattin et al. (2012) also noted that for count-based data, results with a count of zero are real observations that must be evaluated as such. However, counts of zero are only appropriate for the portion of the sample represented by the analyzed aliquot; it does not necessarily imply that no spores are present through the entire sample.

4.5 Data Validation

Given the potential importance of identifying data as non-detect and using data below the quantification limit, environmental microbiological data may benefit from undergoing a data validation process. Data

validation is traditionally an analyte- and sample-specific evaluation of data quality often conducted by a party independent of data generation and data use (EPA, 2002 and 2009b). Data validation, as noted here, primarily refers to the assigning of qualifiers (or flags) to the data in order to identify potential deficiencies about data quality. For example, data qualifiers typically associated with chemical data include:

- U – The analyte was not detected above the quantification limit
- J – The analyte is determined to be present in the sample, but its concentration is uncertain
- R – Sample results rejected

Data qualifiers for chemical data may also be associated with method blanks, surrogate recoveries, holding times, matrix spikes and matrix spike duplicates, and field duplicates (EPA, 2002 and 2009b). Unique data qualifiers may be needed for microbiological data, although procedures for validating microbiological data were not identified in the literature. A data validation step where microbiological analytical results are qualified and flagged may aid in the interpretation of environmental microbiology data. For example, a validation process could distinguish microbiological results below quantification limits. Appropriate quality control procedures, such as use of split samples or duplicate samples, could also contribute to information on the quality of microbiological results.

Validation could also draw attention to somewhat anomalous results that might influence the data set. As described in Section 3.0, there were three samples that each had a single CFU observed in the second serial dilution during spread plating. Although no colonies were observed at the first dilution, application of a dilution factor of 100 was required, (leading to the some of the largest results observed for the unsubstituted options). Two of the associated results were also identified as potential outliers (1,758 CFU/m^3 and 64,729 CFU/m^2) in Tables 3a and 3b. Data validation flags associated with these results could have alerted the user to the uniqueness of these data. Processes for handling such unusual plate counting situations (such as two dilutions with countable colonies) can vary and whichever method is desired should be documented and justified within a standard operating procedure (Sutton, 2006). The approach in the current evaluation used the dilution with the largest mean spread plate count. Other than the three samples described in this paragraph that were based on a dilution factor of 100, all spread plate results were based on a dilution factor of 10.

4.6 Data Groupings

For this assessment, all surface data were combined across sampling methods (swab, wipes, etc.) when generating summary statistics, including the 95% UCL on the mean. However, large differences were observed in the data values among different types of surface samples, primarily due to the differences in sampled areas that are linked to the sampling method. Thus, in order to ensure the calculated 95% UCL is not dominated by differences among sampling methods, it may be necessary to select a single sampling method that most appropriately yields samples and results that will address the sampling objectives. For example, when CFU are present in swab and wipe samples, the very small area associated with these samples will lead to very high results when expressed per square meter, compared to vacuum and sponge samples that are collected over a wider area, and subsequently, the CFU counts on a per area basis are lower. Recovery efficiency (likely influenced by sampling method and material sampled) may also need to be accounted for when analyzing the data.

5.0 Summary

This study analyzed data from air and surface samples for which CFU of *Bg* spores were determined both by spread plate and filter plate methods. Having paired results for each sample representing the two methods provided an opportunity to assess the impact of alternative methods of addressing low or no CFU counts on filter plates and spread plates used to analyze environmental microbiological samples. Paired spread plate and filter plate results were available for 154 samples (18 indoor air samples collected with SKC BioSampler® units and 136 surface samples collected with sponges, swabs, vacuums, or wipes). These samples were collected following decontamination and resulted in relatively low level *Bg* detections with many results below detection or quantification limits. The data associated with these samples were used to evaluate each of six data interpretation options for analyzing censored microbiological data. The data interpretation options are summarized in Table 1 (Section 2.3).

Comments on the various data interpretation options are provided below and are based on comparison of the means and 95% UCL on the mean calculated using each option. The findings of this assessment are subject to the type of data that were considered – for example, the unadjusted mean spread plate count for all but one sample in the data set was reported to be less than the quantification limit (i.e., <30 CFU). Therefore, results and conclusions may be different if spread plate results (based on counts above the quantification limit) were more prevalent in the data. In addition, although conclusions related to estimation bias (i.e., underestimation, overestimation) were made for these data interpretation options, a more complete assessment of bias needs to consider data with a higher prevalence of samples with known CFU counts. Selection of a recommended option will depend on the type of analysis being conducted (e.g., a screening level assessment versus a more detailed analysis), whether the data are being treated as discrete or continuous, and whether results less than the quantification limit are deemed suitable for use.

Results for the Substitute 0 option indicated results were biased low as this option had the lowest means and 95% UCL on the mean results among all options evaluated. Here, all nonzero sample results (except one surface sample) were based on the filter plate results.

The All Spread option reflected the level of *Bg* spores collected more accurately than the Substitute 0 or Substitute 15 option. The All Spread mean and 95% UCL on the mean results were more than double that of the Substitute 0 option. Higher results imply greater accuracy because, in theory, colonies would only be present if culturable bacteria were actually present. Relative to the Substitute 0 option, fewer sample results were based on filter plate results and the number of zero results was reduced.

The Substitute 15 option biased the results rather high. The Substitute 15 option mean and 95% UCL on the mean results were an order of magnitude higher than the results of all other data interpretation options. All Substitute 15 results were based on spread plating, and other than the one surface sample with a mean spread plate count of 30 CFU, the sample results were based on a substituted mean spread plate count of 15 CFU. Although not used for this option, the reported unadjusted mean spread plate counts were all ≤10 CFU and most (146 of 154 samples) were ≤2 CFU, which is considerably lower than the substituted value of 15 CFU. If the true mean of the samples had been higher than 15 CFU, the Substitute 15 option would bias the results low.

The < Quantification option generated values for the 95% UCL on the mean that were similar to the Substitute 0 approach (likely biased low). Mean results were higher, especially for the surface samples, for the < Quantification option than the Substitute 0 option as mean results for the < Quantification option were only based on detected values. This option might be beneficial for generating 95% UCL on the mean for data sets where results below the quantification limit are deemed unusable.

The < Detection option generated values for the 95% UCL on the mean that were similar to the All Spread option (expected to most accurately reflect *Bg* spore contamination). The calculated means were higher (especially for the surface samples) for the < Detection option than the All Spread option as the mean results for the < Detection option were only based on detected values. This option may be beneficial to avoid the presentation of zero results, which might imply more certainty in the data than what actually may exist.

The < Quantification – Both Methods option yields unstable estimates for the 95% UCL, as this option leads to a large proportion of samples with non-quantifiable results. All air sample results were non-quantifiable in this analysis, and all but five surface sample results were non-quantifiable. Thus, 95% UCL estimates could be made only for surface samples. However, this option would be preferred if, in fact, any sample outcome should be deemed non-quantifiable when either spread plate or filter plate analysis are below their respective quantification limits.

Overall, in estimating the 95% UCL on the mean, ProUCL can handle spore concentration data at multiple censoring limits and offers nonparametric approaches (Kaplan-Meier-based) when distributional assumptions are not achieved.

Based on the results from the data included in this evaluation:

- If high variability and uncertainty in low concentration estimates is considered acceptable, then the < Detection option could be the best option for handling censored observations. The < Detection option maximally utilizes all available information to provide conservative estimates of concentrations and indicates uncertainty associated with non-detection.
- If high variability and uncertainty in low concentration estimates is considered unacceptable, then the < Quantification - Both Methods option could be the most useful option for handling censored observations This option would require appropriate justification for the quantification and detection limits that are used to represent censored outcomes.

Note that it was desired to identify an option that yielded the best estimate for the 95% UCL in the presence of censored outcomes while taking advantage of all available information on spread plate and filter plate results for each sample. Thus, this investigation favored one option over another based on its ability to generate an upper confidence limit on the mean whose expected coverage percentage was closest to 95%. It did not consider, for example, which option led to the most conservative estimate that it likely to have a higher coverage percentage than 95%.

Future work to further evaluate the accuracy and precision of these or other options for data interpretation is warranted because of the potential importance of low-level human exposures to biological agents. Accurately knowing the concentration of biological agents on surfaces or in air is necessary for performing meaningful exposure assessment. The design of the study could include larger numbers of samples containing accurately known concentrations of *Bg* spores that could be evaluated using alternative data interpretation options.

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Appendix A

Listings of Individual Sample Concentrations under the Six Data Interpretation Options

Table A-1. Listing of Individual Air Sample Concentrations under the Six Data Interpretation Options

Barcode	Volume (mL)	Flow Rate (L/min)	Dilution Factor	Un-adjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Sub-stitute 15 - Treat as Detect	4. <Quanti-fication - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quanti-fication – Both Methods -- Identify if "Less-Than"	4. <Quanti-fication - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quanti-fication – Both Methods -- Identify if "Less-Than"
4375	10.40	12.35	10	Yes	0	21	113	113	8421	113	113	1123	1	1	0
5028	10.20	12.89	100	Yes	340	20	103	1758	7913	103	1758	1055	1	1	0
5030	10.20	12.94	10	Yes	0	41	211	211	7883	211	211	1051	1	1	0
5033	11.60	11.65	10	Yes	77	12	69	441	9957	69	441	1328	1	1	0
5104	11.60	13.20	10	Yes	0	23	116	116	8788	116	116	1172	1	1	0
5110	10.20	13.19	10	Yes	0	31	157	157	7733	157	157	1031	1	1	0
5117	10.80	12.52	10	Yes	0	0	0	0	8626	59	59	1150	0	0	0
5133	10.20	12.80	10	Yes	0	10	52	52	7969	52	52	1063	1	1	0
5214	10.60	13.39	10	Yes	35	0	0	174	7916	55	174	1056	0	1	0
5258	10.40	13.08	10	Yes	0	10	51	51	7951	51	51	1060	1	1	0
5267	10.20	12.75	10	Yes	0	10	52	52	8000	52	52	1067	1	1	0
5300	11.20	12.98	10	Yes	37	56	288	288	8629	288	288	1150	1	1	0
5364	11.20	13.00	10	Yes	0	11	56	56	8615	56	56	1149	1	1	0
5382	11.40	13.07	10	Yes	76	23	117	388	8722	117	388	1163	1	1	0
5384	10.40	12.98	10	Yes	139	10	51	714	8012	51	714	1068	1	1	0
5409	10.40	12.96	10	Yes	0	31	159	159	8025	159	159	1070	1	1	0
5432	10.80	13.18	10	Yes	36	43	218	218	8194	218	218	1093	1	1	0
5959	10.80	12.78	10	Yes	0	11	57	57	8451	57	57	1127	1	1	0

CFU, colony forming units

Table A-2. Listing of Individual Surface Sample Concentrations under the Six Data Interpretation Options

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
1481	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1482	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1520	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1597	5	0.00258	100	Yes	167	8	3101	64729	290698	3101	64729	38760	1	1	0
1605	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1634	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1637	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1647	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1653	5	0.00258	10	Yes	0	0	0	0	290698	1163	1163	38760	0	0	0
1676	5	0.00258	10	Yes	100	83	32171	38760	290698	32171	38760	38760	1	1	0
1682	5	0.00258	10	Yes	0	5	1938	1938	290698	1938	1938	38760	1	1	0
1807	20	0.0645	10	Yes	67	20	310	1039	46512	310	1039	6202	1	1	0
1812	20	0.0645	10	Yes	267	140	2171	4140	46512	2171	4140	6202	1	1	0
1815	20	0.0645	10	Yes	0	60	930	930	46512	930	930	6202	1	1	0
1819	20	0.0645	10	Yes	67	20	310	1039	46512	310	1039	6202	1	1	0
1820	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1825	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1829	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1837	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1843	20	0.0645	10	Yes	67	120	1860	1860	46512	1860	1860	6202	1	1	0
1846	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1848	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1851	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1877	20	0.0645	10	Yes	0	20	310	310	46512	310	310	6202	1	1	0
1910	20	0.0645	10	Yes	67	20	310	1039	46512	310	1039	6202	1	1	0
1913	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0

Table A-2. (cont.)

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
1930	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1937	20	0.0645	10	Yes	133	160	2481	2481	46512	2481	2481	6202	1	1	0
1942	20	0.0645	10	Yes	133	0	0	2062	46512	310	2062	6202	0	1	0
1950	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
1955	20	0.0645	10	Yes	0	0	0	0	46512	310	310	6202	0	0	0
2070	5.5	0.0645	10	Yes	0	0	0	0	12791	47	47	1705	0	0	0
2072	6.1	0.0645	10	Yes	20	40	620	620	14186	620	620	1891	1	1	0
2231	4.5	0.0645	100	Yes	345	0	0	5349	10465	31	5349	1395	0	1	0
2233	6.4	0.0645	100	Yes	640	150	2326	9922	14884	2326	9922	2326	1	1	1
2234	6.4	0.0645	10	Yes	0	0	0	0	14884	47	47	1984	0	0	0
2235	5.9	0.0645	10	Yes	0	0	0	0	13721	47	47	1829	0	0	0
2236	7.1	0.0645	10	Yes	0	14	217	217	16512	217	217	2202	1	1	0
2240	6.4	0.0645	10	Yes	0	0	0	0	14884	47	47	1984	0	0	0
2241	5.3	0.0645	10	Yes	0	0	0	0	12326	47	47	1643	0	0	0
2266	5.2	0.0645	10	Yes	0	0	0	0	12093	47	47	1612	0	0	0
2436	4.8	0.0645	10	Yes	0	0	0	0	11163	31	31	1488	0	0	0
2566	4.5	0.0645	10	Yes	15	0	0	233	10465	31	233	1395	0	1	0
2577	7	0.0645	10	Yes	0	0	0	0	16279	62	62	2171	0	0	0
2582	5.2	0.0645	10	Yes	0	0	0	0	12093	47	47	1612	0	0	0
2599	4.9	0.0645	10	Yes	0	0	0	0	11395	31	31	1519	0	0	0
2601	4.4	0.0645	10	Yes	0	0	0	0	10233	31	31	1364	0	0	0
2606	5.1	0.0645	10	Yes	0	0	0	0	11860	47	47	1581	0	0	0
2652	6	0.0645	10	Yes	0	0	0	0	13953	47	47	1860	0	0	0
2653	5.7	0.0645	10	Yes	57	29	450	884	13256	450	884	1767	1	1	0
2654	5.1	0.0645	10	Yes	0	0	0	0	11860	47	47	1581	0	0	0
2679	4.8	0.0645	100	Yes	240	0	0	3721	11163	31	3721	1488	0	1	0
2721	6.1	0.0645	10	Yes	0	0	0	0	14186	47	47	1891	0	0	0

Table A-2. (cont.)

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
2722	5.9	0.0645	10	Yes	39	24	372	605	13721	372	605	1829	1	1	0
2723	3.4	0.0645	10	Yes	0	0	0	0	7907	31	31	1054	0	0	0
2724	6.5	0.0645	10	Yes	0	0	0	0	15116	47	47	2016	0	0	0
2725	5.5	0.0645	10	Yes	0	0	0	0	12791	47	47	1705	0	0	0
2741	4.7	0.0645	10	Yes	0	0	0	0	10930	31	31	1457	0	0	0
2742	5.8	0.0645	10	Yes	0	0	0	0	13488	47	47	1798	0	0	0
2743	6	0.0645	10	Yes	0	0	0	0	13953	47	47	1860	0	0	0
2749	5	0.0645	10	Yes	0	0	0	0	11628	47	47	1550	0	0	0
2794	5.2	0.0645	10	Yes	0	0	0	0	12093	47	47	1612	0	0	0
2825	5.2	0.0645	10	Yes	0	0	0	0	12093	47	47	1612	0	0	0
2827	4.5	0.0645	10	Yes	0	0	0	0	10465	31	31	1395	0	0	0
2839	3.8	0.0645	10	Yes	63	40	620	977	8837	620	977	1178	1	1	0
2840	4.9	0.0645	10	Yes	16	20	310	310	11395	310	310	1519	1	1	0
2844	4.5	0.0645	10	Yes	0	0	0	0	10465	31	31	1395	0	0	0
2900	4.4	0.0645	10	Yes	0	2	31	31	10233	31	31	1364	1	1	0
2914	5	0.0645	10	Yes	0	0	0	0	11628	47	47	1550	0	0	0
2916	6	0.0645	10	Yes	0	3	47	47	13953	47	47	1860	1	1	0
2957	3.7	0.0645	10	Yes	0	7	109	109	8605	109	109	1147	1	1	0
3013	3.5	0.0645	10	Yes	0	28	434	434	8140	434	434	1085	1	1	0
3063	3.9	0.0645	10	Yes	0	0	0	0	9070	31	31	1209	0	0	0
3065	3.8	0.0645	10	Yes	0	2	31	31	8837	31	31	1178	1	1	0
3066	4.3	0.0645	10	Yes	100	110	1705	1705	10000	1705	1705	1705	1	1	1
3076	6	0.0645	10	Yes	0	6	93	93	13953	93	93	1860	1	1	0
3077	5.5	0.0645	10	Yes	0	11	171	171	12791	171	171	1705	1	1	0
3218	4.4	0.0645	10	Yes	0	0	0	0	10233	31	31	1364	0	0	0
3238	2.9	0.0645	10	Yes	19	3	47	295	6744	47	295	899	1	1	0
3239	5.4	0.0645	10	Yes	18	24	372	372	12558	372	372	1674	1	1	0

Table A-2. (cont.)

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
3242	3.3	0.0645	10	Yes	0	2	31	31	7674	31	31	1023	1	1	0
3243	4.7	0.0645	10	Yes	94	35	543	1457	10930	543	1457	1457	1	1	0
3244	4	0.0645	10	Yes	0	0	0	0	9302	31	31	1240	0	0	0
3246	3.9	0.0645	10	Yes	0	14	217	217	9070	217	217	1209	1	1	0
3247	4.7	0.0645	10	Yes	0	0	0	0	10930	31	31	1457	0	0	0
3252	5.1	0.0645	10	Yes	0	0	0	0	11860	47	47	1581	0	0	0
3254	4.6	0.0645	10	Yes	0	0	0	0	10698	31	31	1426	0	0	0
3273	5.5	0.0645	10	Yes	73	135	2093	2093	12791	2093	2093	2093	1	1	1
3277	4.5	0.0645	10	Yes	0	0	0	0	10465	31	31	1395	0	0	0
3282	3.6	0.0645	10	Yes	0	0	0	0	8372	31	31	1116	0	0	0
3283	4	0.0645	10	Yes	0	2	31	31	9302	31	31	1240	1	1	0
3287	5	0.0645	10	Yes	0	5	78	78	11628	78	78	1550	1	1	0
3495	4	0.0645	10	Yes	0	4	62	62	9302	62	62	1240	1	1	0
3505	3.5	0.0645	10	Yes	0	0	0	0	8140	31	31	1085	0	0	0
3506	4.1	0.0645	10	Yes	0	4	62	62	9535	62	62	1271	1	1	0
3509	4.5	0.0645	10	Yes	45	0	0	698	10465	31	698	1395	0	1	0
3513	5	0.0645	10	Yes	83	10	155	1287	11628	155	1287	1550	1	1	0
3528	6	0.0645	10	Yes	0	15	233	233	13953	233	233	1860	1	1	0
3529	7	0.0645	10	Yes	0	28	434	434	16279	434	434	2171	1	1	0
3530	5	0.0645	10	Yes	0	0	0	0	11628	47	47	1550	0	0	0
3549	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3568	5	0.3716	100	Yes	417	253	681	1122	2018	681	1122	681	1	1	1
3569	5	0.3716	10	Yes	17	8	22	46	2018	22	46	269	1	1	0
3585	5	0.3716	10	Yes	17	3	8	46	2018	8	46	269	1	1	0
3592	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3593	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3594	5	0.3716	10	Yes	33	0	0	89	2018	8	89	269	0	1	0

Table A-2. (cont.)

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
3595	5	0.3716	10	Yes	0	5	13	13	2018	13	13	269	1	1	0
3598	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3599	5	0.3716	10	Yes	167	88	237	449	2018	237	449	269	1	1	0
3612	5	0.3716	10	Yes	0	18	48	48	2018	48	48	269	1	1	0
3616	5	0.3716	10	Yes	50	13	35	135	2018	35	135	269	1	1	0
3621	5	0.3716	10000	No	1483	465	3991	3991	3991	3991	3991	3991	1	1	1
3629	5	0.3716	10	Yes	67	0	0	180	2018	8	180	269	0	1	0
3631	5	0.3716	10	Yes	33	3	8	89	2018	8	89	269	1	1	0
3633	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3644	5	0.3716	100	Yes	167	0	0	449	2018	8	449	269	0	1	0
3648	5	0.3716	10	Yes	17	5	13	46	2018	13	46	269	1	1	0
3655	5	0.3716	10	Yes	0	3	8	8	2018	8	8	269	1	1	0
3658	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3660	5	0.3716	10	Yes	117	63	170	315	2018	170	315	269	1	1	0
3663	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3668	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3674	5	0.3716	10	Yes	0	5	13	13	2018	13	13	269	1	1	0
3676	5	0.3716	10	Yes	17	0	0	46	2018	8	46	269	0	1	0
3686	5	0.3716	10	Yes	0	8	22	22	2018	22	22	269	1	1	0
3689	5	0.3716	10	Yes	0	3	8	8	2018	8	8	269	1	1	0
3714	5	0.3716	10	Yes	0	10	27	27	2018	27	27	269	1	1	0
3718	5	0.3716	10	Yes	17	10	27	46	2018	27	46	269	1	1	0
3719	5	0.3716	10	Yes	50	8	22	135	2018	22	135	269	1	1	0
3720	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3722	5	0.3716	10	Yes	0	0	0	0	2018	8	8	269	0	0	0
3725	5	0.3716	10	Yes	67	38	102	180	2018	102	180	269	1	1	0
3868	5	0.3716	10	Yes	0	3	8	8	2018	8	8	269	1	1	0

Table A-2. (cont.)

Barcode	Volume (mL)	Sampled Area (m^2)	Dilution Factor	Unadjusted Mean Spread Plate Count <30 CFU?	Adjusted CFU		Concentration (CFU/m^3)						Indicator of Non-Censoring (i.e., Quantifiable and/or Detected) (1=Non-Censored, 0=Censored)		
					Spread Plate	Filter Plate	1. Substitute 0 - Treat as Detect	2. All Spread - Treat as Detect	3. Substitute 15 - Treat as Detect	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"	4. <Quantification - Identify if "Less-Than"	5. < Detection - Identify if "Less-Than"	6. <Quantification – Both Methods -- Identify if "Less-Than"
3978	5	0.3716	10	Yes	0	5	13	13	2018	13	13	269	1	1	0
3981	5	0.3716	10	Yes	0	5	13	13	2018	13	13	269	1	1	0

CFU, colony forming units

Appendix B

Distributional Goodness-of-Fit Tests Applied to Each Data Interpretation Option

Goodness-of-fit Testing. In order to ensure that the calculated UCL actually exceeds the unknown mean with 95% likelihood, the statistical method used to calculate this value must be appropriate for the given set of data. More specifically, if a statistical method requires the data to originate from a given statistical distribution such as a normal distribution, then that method is not appropriate for the given data if it can be demonstrated that the distribution of the data varies greatly from that assumed distribution. This is typically demonstrated through applying a goodness-of-fit statistical test and assessing graphs of the data. Therefore, ProUCL (Version 4.1 used for this evaluation) helps identify an appropriate statistical method by applying goodness-of-fit tests for normal, lognormal, and gamma distributions and making recommendations on a method based on the outcome of these tests and other properties of the data such as standard deviation, skewness, sample size, and number of censored values. These goodness-of-fit tests were performed on data for each of the six data interpretation options, as each option is based on the same amount of data (that is, no one option yields a larger dataset compared to the others). Samples with values falling below quantification or detection limits were represented by extrapolated values from a ROS method. Within ProUCL, the specific test applied for a given distributional model depended on whether or not the sample size exceeded 50; air samples had n=18, while surface samples had n=136:

- Test for Normality:
    - Shapiro-Wilk test for air samples (Shapiro and Wilk, 1965); Lilliefors test for surface samples (Lilliefors, 1967)
    - Normal ROS estimates were used to represent non-quantifiable sample results in these tests
- Test for Lognormality:
    - Shapiro-Wilk test on log-data for air samples; Lilliefors test on log-data for surface samples
    - Lognormal ROS estimates were used to represent non-quantifiable sample results in these tests
- Test for Gamma Distribution:
    - Kolmogorow-Smirnov test for both air and surface samples (D'Agostino and Stephens, 1986).
    - Gamma ROS estimates were used to represent non-quantifiable sample results in these tests

These tests assume the (null) hypothesis that the given distributional model holds and requires sufficient evidence from the data to reject this hypothesis. All goodness-of-fit tests were performed at the 0.05 significance level.

Table B-1. Results of Distributional Goodness-of-Fit Tests Applied to the Air Sample Data (n=18 samples) for Each Data Interpretation Option

Data Interpretation Option	% Detected or Quantifiable	Outcome of Distributional Tests		
		Test for Normality	Test for Lognormality	Test for Gamma Distribution
Substitute 0 – treat as detect	100%	Normality is Not Rejected	--	--
All Spread – treat as detect	100%	Data Not Normal	--	--
Substitute 15 – treat as detect	100%	Data Not Normal	Data Not Lognormal	Data Not Gamma Distributed
< Quantification (Spread only) – Identify if "Less Than"	89%	Data Not Normal	Data Not Lognormal	Data Not Gamma Distributed
< Detection – Identify if "Less Than"	94%	Data Not Normal	Lognormality is Not Rejected	Data Not Gamma Distributed
< Quantification – Both Methods – Identify if "Less Than"	0%	--	--	--

Test for Normality: Shapiro-Wilk test; Normal ROS estimates for non-quantifiable outcomes
Test for Lognormality: Shapiro-Wilk test on log-data; Lognormal regression on order statistics (ROS) estimates for non-quantifiable outcomes
Test for Gamma: Kolmogorow-Smirnov test; Gamma ROS estimates for non-quantifiable outcomes
All tests were performed at the 0.05 significance level.

Table B-2. Results of Distributional Goodness-of-Fit Tests Applied to the Surface Sample Data (n=136 samples) for Each Data Interpretation Option

Data Interpretation Option	% Detected or Quantifiable	Outcome of Distributional Tests		
		Test for Normality	Test for Lognormality	Test for Gamma Distribution
Substitute 0 – treat as detect	100%	Data Not Normal	--	--
All Spread – treat as detect	100%	Data Not Normal	--	--
Substitute 15 – treat as detect	100%	Data Not Normal	Data Not Lognormal	Data Not Gamma Distributed
< Quantification (Spread only) – Identify if "Less Than"	45%	Data Not Normal	Lognormality is Not Rejected	Data Not Gamma Distributed
< Detection – Identify if "Less Than"	51%	Data Not Normal	Lognormality is Not Rejected	Data Not Gamma Distributed
< Quantification – Both Methods– Identify if "Less Than"	4%	Data Not Normal	Data Not Lognormal	Data Not Gamma Distributed

Test for Normality: Lilliefors test; Normal regression on order statistics (ROS) estimates for non-quantifiable outcomes
Test for Lognormality: Lilliefors test; Lognormal ROS estimates for non-quantifiable outcomes
Gamma: Kolmogorow-Smirnov test; Gamma ROS estimates for non-quantifiable outcomes
All tests were performed at the 0.05 significance level.

References for Appendix B

D'Agostino, R.B. and M.A. Stephens, 1986. *Goodness-of-Fit Techniques*. Marcel Dekker, Inc.

Lilliefors, H., 1967. On the Kolmogorov–Smirnov test for normality with mean and variance unknown. Journal of the American Statistical Association. 62(318):399–402.

Shapiro, S.S. and M.B. Wilk, 1965. An analysis of variance test for normality (complete samples). Biometrika 52(3–4):591–611.

Appendix C

Estimates for 95% Upper Confidence Limit (UCL) on the Mean, Applying Various Statistical Methods for Each Data Interpretation Option

Table C-1a. Estimates for 95% Upper Confidence Limit (UCL) on the Mean, Applying Various Statistical Methods That Rely on a Specific Distributional Form, for Air Sample Data (CFU/m^3)

95% UCL Calculation Method	Data Interpretation Options				
	Substitute 0 – treat as detect	All Spread – treat as detect	Substitute 15 – treat as detect	< Quantification (Spread only) – Identify if "Less Than"	< Detection – Identify if "Less Than"
Methods Assuming a Normal Distribution					
Student-t[a]	136	446	8,540	137	447
Methods Assuming a Positively Skewed Distribution with 100% Quantifiable Outcomes					
Adj. Central Limit Theorem	138	513	8,584		
Modified-t	136	458	8,549		
Methods Assuming a Lognormal Distribution					
Chebyshev (MVUE)			8,844		
H-Statistic[a]				158	549
Methods Using Lognormal ROS Extrapolation for Non-Quantifiable Results					
Student-t				139	448
Percentile Bootstrap				138	452
BCA Bootstrap				141	564
H-UCL				149	515
Methods Assuming a Gamma Distribution					
Adjusted Gamma.			8,561	147	641

BCA, bias-corrected accelerated; CFU, colony forming units

[a] The Student-t (under the Normal Distribution assumption) and H-Statistic approaches assume that left-censored (non-quantifiable) observations are substituted by one-half of the detection or quantification limit.

Table C-1b. Estimates for 95% Upper Confidence Limit (UCL) on the Mean, Applying Various Nonparametric Statistical Methods, for Air Sample Data (CFU/m^3)

95% UCL Calculation Method	Data Interpretation Options[a]				
	Substitute 0 – treat as detect	All Spread – treat as detect	Substitute 15 – treat as detect	< Quantification (Spread only) – Identify if "Less Than"	< Detection – Identify if "Less Than"
Central Limit Theorem	134	437	8,528		
Student-t				139	448
Normal-Z				137	439
Jackknife	136	446	8,540	139	448
Standard Bootstrap	133	432	8,526		
Hall's Bootstrap	142	1,086	8,752		
Bootstrap t	141	713	8,626	146	704
BCA	138	525	8,574	136	469
Percentile bootstrap	133	446	8,536	138	450
Chebyshev	184	699	8,868	183	700

BCA, bias-corrected accelerated; CFU, colony forming units

[a] The options represented by the last two columns of the table, which lead to left-censored data, utilize a Kaplan-Meier approach to estimating the underlying distribution of the data.

Table C-2a. Estimates for 95% UCL on the Mean, Applying Various Statistical Methods That Rely on a Specific Distributional Form, for Surface Sample Data (CFU/m^2)

95% UCL Calculation Method	Data Interpretation Options					
	Substitute 0 – treat as detect	All Spread – treat as detect	Substitute 15 – treat as detect	< Quantification (Spread only) – Identify if "Less Than"	< Detection – Identify if "Less Than"	< Quantification – Both Methods – Identify if "Less Than"
Methods Assuming a Normal Distribution						
Student-t[a]	864	2,105	47,708	918	2,157	3,237
Methods Assuming a Positively Skewed Distribution with 100% Quantifiable Outcomes						
Adj. Central Limit Theorem	1,101	2,532	49,405			
Modified-t	902	2,173	47,984			
Methods Assuming a Lognormal Distribution						
Chebyshev (MVUE)			51,807			
H-Statistic[a]			41,905	584	1,381	2,954
Methods Using Lognormal ROS Extrapolation for Non-Quantifiable Results						
Student-t				873	2,116	238
Percentile Bootstrap				947	2,210	240
BCA Bootstrap				1,262	2,646	262
H-UCL				1,380	4,083	192
Methods Assuming a Gamma Distribution						
Adjusted Gamma.			45,117	877	2,152	179

BCA, bias-corrected accelerated; CFU, colony forming units

[a] The Student-t (under the Normal Distribution assumption) and H-Statistic approaches assume that left-censored (non-quantifiable) observations are substituted by one-half of the detection or quantification limit.

Table C-2b. Estimates for 95% Upper Confidence Limit (UCL) on the Mean, Applying Various Nonparametric Statistical Methods, for Surface Sample Data (CFU/m^2)

95% UCL Calculation Method	Data Interpretation Options[a]					
	Substitute 0 – treat as detect	All Spread – treat as detect	Substitute 15 – treat as detect	< Quantification (Spread only) – Identify if "Less Than"	< Detection – Identify if "Less Than"	< Quantification – Both Methods – Identify if "Less Than"
Central Limit Theorem	862	2,099	47,633			
Student-t				879	2,125	826
Normal-Z				876	2,119	825
Jackknife	864	2,105	47,708	877	2,122	1,396
Standard Bootstrap	867	2,082	47,296			
Hall's Bootstrap	2,209	6,041	49,029			
Bootstrap t	2,170	6,078	49,556	2,143	6,067	818
BCA	1,381	2,674	50,003	975	2,282	2,344
Percentile bootstrap	932	2,218	48,292	941	2,244	2,326
Chebyshev	1,516	3,613	65,533	1,533	3,638	944

BCA, bias-corrected accelerated; CFU, colony forming units

[a] The options represented by the last three columns of the table, which lead to left-censored data, utilize a Kaplan-Meier approach to estimating the underlying distribution of the data.

Table C-3 is taken from Table 2-5 of EPA (2010). It indicates those approaches for estimating the 95% UCL that ProUCL recommends when all results are considered positive and detected, and no specific distributional form is assumed for these results other than the presence of skewness. The recommendations are based on the value of the standard deviation of the log-transformed results and the sample size (n).

Table C-3. Summary of ProUCL Recommended Approaches for Calculating the 95% Upper Confidence Limit (UCL) on an Unknown Mean When All Results are Positive and Detected and Taken from a Skewed Dataset Without a Discernable Distribution

Standard Deviation of Log-Transformed Results	Sample Size (n)	ProUCL Recommended Approach
Less than or equal to 0.5	All sample sizes	Student-t, modified-t, or H-UCL
Between 0.5 and 1.5	All sample sizes	95% Chebyshev (mean, SD) UCL
Between 1.5 and 2.0	Less than 20	99% Chebyshev (mean, SD) UCL
	Equal or greater than 20	95% Chebyshev (mean, SD) UCL
Between 2.0 and 2.5	Less than 10	Hall's Bootstrap UCL
	Between 10 and 20	99% Chebyshev (mean, SD) UCL
	Between 20 and 50	97.5% Chebyshev (mean, SD) UCL
	Equal or greater than 50	95% Chebyshev (mean, SD) UCL
Between 2.5 and 3.0	Less than 10	Hall's Bootstrap UCL
	Between 10 and 30	99% Chebyshev (mean, SD) UCL
	Between 30 and 70	97.5% Chebyshev (mean, SD) UCL
	Equal or greater than 70	95% Chebyshev (mean, SD) UCL
Between 3.0 and 3.5	Less than 15	Hall's Bootstrap UCL
	Between 15 and 50	99% Chebyshev (mean, SD) UCL
	Between 50 and 100	97.5% Chebyshev (mean, SD) UCL
	Equal or greater than 100	95% Chebyshev (mean, SD) UCL
Greater than 3.5	All sample sizes	99% Chebyshev (mean, SD) UCL

Source: Table 2-5 of EPA (2010).

When censored data are present and no discernable distribution is assumed for the results, ProUCL indicates that the UCL computational method recommended for a normal distribution could be used if the distribution of observed results resembles a symmetric distribution, and for a lognormal or gamma distribution if the distribution of observed results is skewed. Sections 4.10.3 and 4.10.4 of EPA (2010) provide the ProUCL-recommended approaches for the gamma and lognormal distribution, respectively.

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