



Assessment of the Effects of Holding Time on Fecal Coliform and *Salmonella* Concentrations in Biosolids

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Disclaimer

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Executive Summary

This report presents the results of the U.S. Environmental Protection Agency's (EPA's) biosolids holding time study (the "Study") of most probable number (MPN) procedures for the analysis of fecal coliform (using EPA Methods 1680 and 1681) concentrations in Class A and Class B matrices and *Salmonella* (using EPA Method 1682) concentrations in Class A matrices.

The purpose of this study was to determine whether biosolid samples can be held for 24 hours without significant change in bacterial densities for these analytes.

Since historical data indicated that ambient concentrations of fecal coliforms and *Salmonella* were not present in most of the Class A matrices (alkaline-stabilized, heat dried, and thermophilically digested) evaluated, fecal coliform and *Salmonella* samples were spiked. However, it was not necessary to spike most of the compost matrices analyzed for fecal coliforms because there were a sufficient number of ambient fecal coliforms to assess the effect of holding time. Spiking was not necessary for Class B matrices (aerobically digested, anaerobically digested) evaluated during the Study. Samples were analyzed at 6 and 24 hours after sample collection or spiking.

Four hundred laboratories were contacted for potential study participation in an effort to obtain six or more useable, valid datasets for every matrix/method combination. A number of reasons, including, but not limited to, limited production of some matrices, laboratory proximity to biosolids facilities, scheduling conflicts etc., resulted in limited data for some matrix/method combinations.

Fecal Coliforms

Based on the results of this study, Class A composted, Class B aerobically digested, and Class B anaerobically digested matrices analyzed for fecal coliforms by Methods 1680 and 1681 may be analyzed 24 hours after sample collection and still generate data comparable to those generated at 6 hours after sample collection.

All other matrices analyzed for fecal coliforms using Methods 1680 and 1681 should not exceed a 6-hour maximum sample transport holding time, and must be processed within 2 hours of receipt at the laboratory. The data for these matrix/method combinations generally showed a tendency to decrease with extended sample holding times.

Salmonella

Based on the results of this study and the limited data generated, Class A matrices analyzed for *Salmonella* using Method 1682 may not be analyzed beyond 6 hours after sample collection and still generate data comparable to those generated at 6 hours after sample collection. In general, *Salmonella* concentrations had a tendency to decrease with extended sample holding times.

SECTION 1.0 INTRODUCTION

Land application of biosolids is a critical component of solid waste management. Under Subpart D of Title 40 Code of Federal Regulations (CFR) Part 503, sewage sludges (biosolids) are required to be processed prior to land application in order to minimize pathogen levels and the potential public health risks associated with contact or exposure. Subpart D further defines and classifies sewage sludge (biosolids) for land application purposes based on pathogen concentrations. EPA has validated Methods 1680 (Reference 8.1) and 1681 (Reference 8.2) for fecal coliforms in Class A and B biosolids and Method 1682 (Reference 8.3) for *Salmonella* in Class A biosolids to support monitoring requirements. The objective of this study was to determine whether biosolid samples being analyzed for fecal coliforms and *Salmonella* could be held for 24 hours prior to sample analysis without a significant change in bacterial concentrations.

Holding time. Bacterial analysis of non-potable waters (i.e., wastewater) for compliance monitoring requires that sample analysis begin within 6 hours of sample collection (40 CFR part 136.3, Table II). The present study addresses the effect of holding time on fecal coliform and *Salmonella* concentrations in biosolids. Samples were analyzed at 6 and 24 hours after sample collection or sample spiking.

1.1 Summary of EPA Method 1680 for Fecal Coliforms

Fecal coliforms were evaluated in Class A and Class B biosolids using Method 1680 (Reference 8.1). Biosolid samples are homogenized and inoculated into lauryl tryptose broth (LTB) a presumptive medium. Following incubation at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 and 48 ± 3 hours, growth from positive tubes is transferred to EC broth (confirmatory medium) and incubated at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours. All tubes exhibiting turbidity and gas production are considered positive for fecal coliforms.

1.2 Summary of EPA Method 1681 for Fecal Coliforms

Fecal coliforms were evaluated in Class A and Class B biosolids using Method 1681 (Reference 8.2). Biosolid samples are homogenized and inoculated into A-1 medium and incubated at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours. All tubes exhibiting turbidity and gas production are considered positive for fecal coliforms.

1.3 Summary of EPA Method 1682 for *Salmonella*

Salmonella were evaluated in Class A matrices using Method 1682 (Reference 8.3). The MPN method requires enrichment, followed by selection and biochemical/serological confirmation. Biosolid samples are homogenized and inoculated into tryptic soy broth (TSB) an enrichment medium. After incubation, TSB is spotted onto selective modified semisolid Rappaport-Vassiliadis (MSRV) medium. Presumptively identified colonies from MSRV are streaked onto xylose-lysine desoxycholate agar (XLD) for isolation. Biochemical confirmation includes lysine-iron agar (LIA), triple sugar iron agar (TSI), and urease test medium, followed by serological confirmation using polyvalent O antisera.

SECTION 2.0 STUDY OBJECTIVES

The following study objective was established for the holding time study:

- Evaluate the effect of holding time across multiple laboratories and biosolid matrices on fecal coliform concentrations using EPA Methods 1680 and 1681 and on *Salmonella* concentrations using EPA Method 1682.

To accomplish these objectives, qualified volunteer laboratories analyzed unspiked and/or spiked biosolid samples for fecal coliforms and *Salmonella* at 6 and 24 hours from either sample collection or sample spiking.

The following data quality objective was established for this study:

- Data produced under this study must be generated according to the analytical and QA/QC procedures as described in each of the analytical methods or approved changes to these procedures in order to ensure that data will be of known and reliable quality.

SECTION 3.0 TECHNICAL APPROACH

3.1 Identification of Laboratories

Participant laboratories were representative of the general user community, with experience analyzing biosolid samples for bacteria and had access to representative biosolid matrices. Because the study assessed holding times, laboratories were located within driving distance (2 hours) of the facility providing the biosolid sample.

To reduce cost, volunteer laboratories were recruited, from a pool of 400 laboratories contacted for potential participation in the study. To reduce the burden on the participant laboratories and encourage volunteer participants, EPA provided the media, reagents, and disposable supplies required for the study.

3.2 Sample Collection, Storage Conditions, and Holding Times

A single bulk sample of at least 1000 g was collected and transported to the laboratory on ice and maintained at <10°C and above freezing. At the laboratory, the bulk sample was split into replicates, and spiked (if necessary), and stored in the refrigerator at <10°C and above freezing. After 6 and 24 hours from sample collection or spiking, replicates were analyzed by the appropriate procedure (**Table 1**).

3.3 Methods

The holding time for fecal coliforms was evaluated in both Class A and Class B matrices using Methods 1680 and 1681 and Class A matrices for *Salmonella* using Method 1682.

3.4 Study Design and Analyses

During the holding time study, all three methods (1680, 1681, and 1682) were used to analyze unspiked and spiked samples at multiple laboratories. Table 1 summarizes the number and type of samples that were evaluated during the holding time study to meet the objectives listed in Section 2.0.

Table 1. Study Analysis Summary

Matrix	Analyte	Method	No. of Replicates per Holding Time ¹	
			6 hours	24 hours
Alkaline- stabilized	fecal coliforms	1680/1681	10	10
	<i>Salmonella</i>	1682		
Compost	fecal coliforms	1680/1681	10	10
	<i>Salmonella</i>	1682		
Aerobically digested	fecal coliforms	1680/1681	10	10
Anaerobically digested	fecal coliforms	1680/1681	10	10
Heat Dried	fecal coliforms	1680/1681	10	10
Thermophilically digested	fecal coliforms	1680/1681	10	10

¹Background levels of fecal coliform bacteria were analyzed during preliminary analyses to determine if spiking was necessary. All matrices analyzed for *Salmonella* were spiked.

3.4.1 Preliminary Analyses

Preliminary analyses were conducted on unspiked and spiked Class A and Class B biosolid matrices, as appropriate, prior to the start of the holding time study. The time between preliminary and holding time study analyses was used to resolve issues that were observed during preliminary analyses. In addition, preliminary analyses results were used to determine whether spiking was necessary. Holding time samples were spiked if the ambient fecal coliform concentrations were ≤ 300 MPN/ g dry weight.

3.4.2 Fecal Coliform Sample Holding Time Analyses

Aerobically digested, alkaline-stabilized, anaerobically digested, compost, heat-dried, and thermophilically digested matrices were analyzed according to Methods 1680 and 1681 and holding time study instructions (Appendices B and C), as appropriate.

Unspiked Holding Time Samples and QC Samples. A single bulk sample was collected and transported to the laboratory on ice. The bulk sample was split into 20, 30 g replicates at the laboratory. Sixteen replicates remained unspiked (8 per holding time) and four replicates were spiked (2 per holding time) with laboratory-prepared *Escherichia coli* (ATCC #25922) spiking suspension according to the fecal coliform spiking procedure (Appendix E). Samples were immediately refrigerated after spiking at $<10^{\circ}\text{C}$ and above freezing. After 6 and 24 hours from sample collection, 10 replicates were removed from the refrigerator and analyzed according to Method(s) 1680 and/or 1681.

Spiked Holding Time Samples and QC Samples. A single bulk sample was collected and transported to the laboratory on ice. The bulk sample was split into 20, 30 g replicates at the laboratory. Four replicates remained unspiked (2 per holding time) and sixteen replicates were spiked (8 per holding time) with laboratory-prepared *Escherichia coli* (ATCC #25922) spiking suspension according to the fecal coliform spiking procedure (Appendix E). Samples were immediately refrigerated after spiking at $<10^{\circ}\text{C}$ and above freezing. After 6 and 24 hours from sample collection, 10 replicates were removed from the refrigerator and analyzed according to Method(s) 1680 and/or 1681.

3.4.3 Salmonella Sample Holding Time Analyses

Compost and alkaline-stabilized matrices were analyzed according to Method 1682 and *Salmonella* holding time study instructions (Appendix D). For each matrix, a single bulk sample was collected and transported to the laboratory on ice. The bulk sample was split into 20, 30 g replicates at the laboratory. Four replicates remained unspiked (2 per holding time) and sixteen replicates (8 per holding time) were spiked with laboratory-prepared *Salmonella typhimurium* (ATCC #14028) spiking suspension according to the *Salmonella* spiking procedure (Appendix G). Samples were immediately refrigerated after spiking at $<10^{\circ}\text{C}$ and above freezing. After 6 and 24 hours from sample spiking, 10 replicates were removed from the refrigerator and analyzed according to Method 1682.

3.5 Quality Control (QC) Analyses

Participating laboratories completed the following QC requirements: media sterility checks, dilution water sterility checks, blender jar sterility check, method blanks, positive controls, and negative controls.

Table 2 summarizes the positive and negative control cultures used during the study.

Table 2. Positive and Negative Control Cultures

Method	Medium or Test	Positive Control	Negative Control
1680/1681	EC, A-1	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>
1680	LTB	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
1680/1681	mEndo	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>
1682	MSRV, XLD, TSI, LIA, Polyvalent O	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
1682	Urease	<i>Proteus vulgaris</i>	<i>Salmonella typhimurium</i>

SECTION 4.0 STUDY IMPLEMENTATION

4.1 Study Management

This study was designed under the direction of the Office of Science and Technology, Engineering and Analysis Division within the U.S. Environmental Protection Agency's (EPA's) Office of Water (OW). The EPA technical lead was Robin K. Oshiro. Coordination of activities for the Study was performed by the CSC Microbiology and Biochemistry Studies Group.

4.2 Study Schedule

Fecal coliform analyses were conducted between February 2006 and August 2006. *Salmonella* analyses were conducted between May 2006 and August 2006. All laboratories that analyzed biosolid matrices for *Salmonella* elected to complete fecal coliform analyses prior to moving on to the *Salmonella* analyses.

Prior to analyzing holding time study samples each laboratory was required to analyze four initial precision and recovery (IPRs) samples, one unspiked reference matrix sample, and one matrix spike (MS) sample to demonstrate method proficiency. Some laboratories repeated IPR analyses due to problems enumerating the spiking suspension using the spread plate technique. In addition, some of the laboratories had to repeat the holding time analyses due to fluctuating ambient fecal coliform concentrations to obtain uncensored data. Discussion of censored data is provided in Section 5.3.

4.3 Participant Laboratories

The 28 participating laboratories involved in the Study are shown in **Table 3**, below. No endorsement of these laboratories is implied, nor should any be inferred. Participant laboratories were randomly assigned numbers for purposes of presenting data in this report.

Table 3. Laboratories Participating in the Biosolids Holding Time Study

<p>A&L Great Lakes Laboratories Julie Speelman 3505 Conestoga Drive, Fort Wayne, IN 46808</p>	<p>Environmental Protection Agency, National Risk Management Research Laboratory Mark Meckes, Laura Boczek, Cliff Johnson 26 West Martin Luther King Drive, Cincinnati, OH 45268</p>
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SECTION 5.0 DATA REPORTING AND VALIDATION

5.1 Data Reporting

Laboratories submitted the following data to CSC for review and validation:

- Completed cover sheet with sample collection and QC information
- Completed sample-specific reporting forms
- Documentation of any additional information that would assist in evaluating the data

5.2 Data Validation

Data review checklists were used to ensure that each data package was complete and to ensure that each sample result met the study-specific and method-specific requirements. Items reviewed for each sample included the following:

- Confirmation that original forms were submitted
- Confirmation that all QC checks were performed and exhibited the appropriate response
- Confirmation that all method-specific incubation times and temperatures were met
- Confirmation that all media and reagents were used within expiration dates
- Confirmation that samples were spiked with appropriate dilution
- Confirmation that all calculations were correct

This process was performed independently by two data reviewers, each of whom entered the results into separate spreadsheets designed for data review and validation for this study. The results were compared to verify consistency and identify potential data entry errors.

The following issues were encountered during laboratory analyses:

Laboratory 12 (Method 1680, Class B, aerobically digested): Did not meet the QC acceptance criteria for OPR and MS samples during the holding time study, which may have been due to TSA enumeration difficulties, which likely resulted in underestimating the spike concentration. TSA counts were considerably lower compared to other laboratories. and Laboratory 12 results obtained during preliminary analyses. As a result, data was considered invalid and not included in subsequent data analyses.

Laboratory 16 (Method 1680, Class A, compost): Did not meet OPR and matrix spike (MS) criteria. As a result, data from Laboratory 16 was considered invalid and not included in subsequent data analyses.

Laboratory 17 (Method 1680, Class B, anaerobic): During the week of Class B sample analyses, the laboratory spiked OPR and MS samples according to the Class A spiking instructions, resulting in censored values for the OPRs (less-than values) and 0 percent recoveries for matrix spike samples. Since the laboratory failed to meet the Class B OPR and MS criteria as a result of spiking according to Class A spiking instructions, data was considered invalid and not included in subsequent data analyses.

Laboratory 23 (Method 1681, Class A, compost): Did not meet OPR and matrix spike (MS) criteria; recoveries for both OPR and MS samples were less than 1 percent. In addition, most of the analytical results observed during the holding time study were censored (less-than) values. As a result, data from Laboratory 23 was considered invalid and not included in subsequent data analyses.

Most laboratories were not familiar with the reference matrix (Milorganite®) which resulted in some laboratories not meeting IPR criteria for Methods 1680 and 1681 during preliminary analyses. These laboratories generally experienced difficulties homogenizing the Milorganite® samples. For samples being analyzed by Method 1681, excessive foaming during homogenization made it difficult to interpret A-1 results. As a result of the expedited study schedule, some laboratories moved forward with the holding time study, despite having failed to meet IPR criteria as specified by the method. However, unless specifically noted in this section all of the laboratories that failed the IPR criteria did meet the OPR criteria during the holding time study. While it would have been preferable for the laboratories to repeat the IPR analyses prior to moving forward with the holding time study, it was not possible due to scheduling constraints, and since the laboratories met OPR criteria, data from these laboratories was considered valid and included in subsequent data analyses.

Laboratory 6 (Methods 1680 and 1681, Class A alkaline-stabilized): As expected, fecal coliform results for all of the replicates analyzed at 6 and 24 hours were censored (less-than) values, because samples were not neutralized prior to spiking. Although results were censored, this data was included in subsequent data analyses.

Laboratory 1: A-1 (Method 1681) and MSR/V (Method 1682) media was expired (less-than 5 days beyond expiration date); all controls exhibited the appropriate response so data was included in subsequent data analyses.

Laboratory 2: EC tubes (Method 1680) were read 48 hours after incubation (instead of being read at 24 and 48 hours), results were considered valid and included in subsequent data analyses.

Laboratory 13 (Method 1681, compost): Did not analyze MS samples during the holding time study. However, the laboratory did analyze MS samples during the week of preliminary analyses, and because the laboratory analyzed less than 20 matrix samples during the study, the laboratory met the method-specific MS criteria. Although the laboratory did not meet the study-specific requirements for performance of MS samples during the week of the holding time study, data from this laboratory was considered valid and included in subsequent data analyses because method-specific QC criteria were met.

Laboratory 19 (Method 1681, Class B, anaerobically digested): Both OPR and MS samples resulted in censored (less-than) values during the holding time study. Evaluation of the TSA spike enumeration indicated that the *E. coli* spiking suspension was not viable. The laboratory did meet IPR and MS criteria during the preliminary analyses. Since the spiking suspension used during the holding time study was not viable and because the laboratory was clearly in-control and performing acceptably throughout the study, data was considered valid and included in subsequent data analyses.

Laboratories 1 and 5 (Method 1682, compost): Analyzed samples that were shipped from facilities not within driving distance during the holding time study. Because all matrices were spiked with *S. typhimurium*, results from these samples were included in subsequent data analyses.

5.3 Censored Data

During the evaluation of holding time study samples, results below the analytical range of the method (less-than results) were observed for all of the spiked alkaline-stabilized (Class A) replicates evaluated by Methods 1680 and 1681 at Laboratory 6 and unspiked compost replicates evaluated by Method 1680 at Laboratory 15. Censored (less-than) values were observed for all of the unspiked samples analyzed for *Salmonella*, regardless of matrix. In addition, all of the spiked alkaline-stabilized replicates analyzed for *Salmonella* resulted in censored (less-than) values. All censored values were set to one-half the detection limit (less-than value) prior to data analyses.

SECTION 6.0 RESULTS, DATA ANALYSIS, AND DISCUSSION

This section includes results of unspiked and spiked biosolid samples which were analyzed for fecal coliforms or *Salmonella*. Only valid results are included in this section; a detailed description of data invalidation information is included in Section 5.0.

6.1 Fecal Coliform (Methods 1680/1681) Holding Time Study Results for Class A and Class B Biosolid Samples

All fecal coliform results were natural log-transformed prior to performing any statistical analyses as a result of the skewed distribution of the results, as recommended in Statistical Methods for Environmental Pollution Monitoring (Reference 8.4). Results were stratified by EPA Methods (1680 and 1681), matrix, matrix class, and spike type (i.e., spiked or unspiked). For each stratification, a two-way Analysis of Variance (ANOVA) model was fit to assess the effect of holding time and laboratory on the log-transformed concentrations, and to assess whether there was a significant interaction between holding time and laboratory. For 14 of the 17 method/matrix/matrix class/spike types, there was no significant interaction between holding time and laboratory, and, as a result, a single comparison was performed in a single ANOVA model using data from all laboratories for these 14 method/matrix/matrix class/spike type combinations. Pairwise comparisons were not necessary for this analysis because samples were analyzed at only two holding times. For the other three method/matrix/matrix class/spike type combinations, there was a significant interaction between holding time and laboratory, and therefore a single two-sample t-test was performed for each laboratory separately.

Fecal coliform results are summarized in **Tables 4** through **9**. Significant decreases in bacterial concentrations at 24-hour compared to 6-hour results are indicated by a “D”.

Based on statistical analysis of the fecal coliform results, the following observations were made:

Alkaline-Stabilized Class A Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were significantly decreased by the 24-hour holding time compared to the 6-hour holding time at Laboratory 7. Only two laboratories analyzed alkaline-stabilized Class A samples during the holding time study resulting in two data sets for both methods.

Alkaline-Stabilized Class B Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were significantly decreased by the 24-hour holding time compared to the 6-hour holding time at Laboratory 22. A wide variation of fecal coliform concentrations was observed in alkaline-stabilized Class B matrices during the holding time study. This might be attributed to variability of pH across replicates, as a result of the treatment process. In general, replicates with a consistent pH of 12 would significantly reduce bacterial levels, while replicates with a lower pH or uneven distribution of pH could result in replicates with much higher bacterial levels or delayed die-off.

During preliminary analyses, fecal coliform concentrations were considerably lower than Class A regulatory limits (<1000 MPN/g dry weight), even though the matrix evaluated by Laboratory 22 generally falls into the Class B category. Alkaline-stabilized matrices were not neutralized prior to spiking to ensure that the matrices evaluated were representative of real-world alkaline-stabilized matrices. Spiked replicates analyzed at 6 hours resulted in fecal coliform concentrations that ranged from <65 MPN/g dry weight to 6.0×10^6 MPN/g dry weight, despite having been spiked at approximately 3.0×10^6 MPN/g dry weight. These censored values (<65 MPN/g dry weight) for samples spiked at such high-levels would only be expected if the pH of the replicate was very high

(e.g., pH 12). Conversely, the 6.0×10^6 MPN/g dry weight would only have been observed if there were high ambient concentrations; which would not be possible in samples with an extremely high pH. Additionally, laboratories reported observing chunks of lime in some (but not all) of the replicates. The preliminary results, the 6-hour results, the laboratory's observations, and the expectation that this was a Class B matrix supports the supposition that the treatment process resulted in a highly variable pH. The significant decrease in fecal coliform density at 24 hours may have simply been a result of pH variability in the replicates spiked and analyzed, with replicates with a wide range of pH being analyzed at the 6-hour holding time and replicates with high pH being analyzed at the 24-hour holding time.

Composted Class A Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were not significantly different at the 24-hour holding time compared to the 6-hour holding time at all five laboratories (3, 13, 15, 17, and 29) that analyzed a composted matrix during the holding time study.

Heat Dried Class A Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were significantly decreased by the 24-hour holding time compared to the 6-hour holding time at Laboratory 7. Only two laboratories analyzed heat dried samples during the holding time study resulting in two data sets for Method 1680 and one data set for 1681.

Thermophilically Digested Class A and Class B Matrices Analyzed Using Method 1680

- Only two thermophilically digested matrices were evaluated during the study, both by Method 1680. Fecal coliform concentrations were not significantly different at the 24-hour holding time compared to the 6-hour holding time regardless of matrix class.

Aerobically Digested Class B Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were not significantly different at the 24-hour holding time compared to the 6-hour holding time at all five laboratories (3, 8, 24, and 27).

Anaerobically Digested Class B Matrices Analyzed Using Methods 1680 and 1681

- Fecal coliform concentrations were not significantly different at the 24-hour holding time compared to the 6-hour holding time at all twelve laboratories (1, 5, 9, 11, 14, 18, 19, 20, 25, 26, and 28).

Table 4. Fecal Coliform Mean Results (MPN/g dry weight) for Alkaline-Stabilized Samples

Laboratory	Class	Method 1680 (LTB/EC)		Method 1681 (A-1)	
		6 hours	24 hours	6 hours	24 hours
6	A	24	24	24	24
7	A	24,342	3,875 D ^a	27,242	3,516 D ^a
2	B	128,508	19,205	22,320	1,954
22	B	1,028,871	5,925 D ^a		
Mean	A	12,183	1,949	13,633	1,770
RSD ^b	A	58	56	25	40
Mean	B	578,689	12,565	22,320	1,954
RSD ^b	B	192	230	155	114

Shading indicates laboratories did not conduct sample analyses with corresponding methods

^a Fecal coliform concentrations were significantly lower at 24-hours compared to 6-hour results as indicated by "D"

^b Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

Table 5. Fecal Coliform Mean Results (MPN/g dry weight) for Composted Samples

Laboratory	Method 1680 (LTB/EC)		Method 1681 (A-1)	
	6 hours	24 hours	6 hours	24 hours
3	1	3	0	1
13			1	1
15	0	0		
17 ^a	46,153	28,858		
29 ^a			744	1,168
Mean (unspiked)	0	2	1	1
RSD ^b (unspiked)	56	150	29	130
Mean (spiked)	46,153	28,858	744	1,168
RSD ^b (spiked)	76	59	43	44

Shading indicates laboratories did not conduct sample analyses with corresponding methods

^a Laboratory analyzed spiked replicates; preliminary analyses indicated spiking was necessary

^b Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

Table 6. Fecal Coliform Mean Results (MPN/g dry weight) for Heat Dried Samples

Laboratory	Method 1680 (LTB/EC)		Method 1681 (A-1)	
	6 hours	24 hours	6 hours	24 hours
7	8,128	5,664	7,228	1,463 D ^a
21	149	89		
Mean	4,139	2,876	7,228	1,463
RSD ^b	123	84	55	47

Shading indicates laboratories did not conduct sample analyses with corresponding methods

^a Fecal coliform concentrations were significantly lower at 24-hours compared to 6-hour results as indicated by “D”

^b Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

Table 7. Fecal Coliform Mean Results (MPN/g dry weight) for Thermophilically Digested Samples

Laboratory	Class	Method 1680 (LTB/EC)	
		6 hours	24 hours
10	A	2,228	9,305
22	B	226,951	111,353
Mean	A	2,228	9,305
RSD ^a	A	71	75
Mean	B	226,951	111,353
RSD ^a	B	93	78

None of the participant laboratories evaluated thermophilically digested samples using method 1681

^a Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

Table 8. Fecal Coliform Mean Results (MPN/g dry weight) for Aerobically Digested Samples

Laboratory	Method 1680 (LTB/EC)		Method 1681 (A-1)	
	6 hours	24 hours	6 hours	24 hours
3	128,138	105,693	122,108	81,852
8			5,368	6,051
24	4,242	4,079		
27	8,509,642	7,932,045		
Mean	2,880,674	2,680,606	63,738	43,951
RSD ^a	50	41	70	72

Shading indicates laboratories did not conduct sample analyses with corresponding methods

^a Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

Table 9. Fecal Coliform Mean Results (MPN/g dry weight) for Anaerobically Digested Samples

Laboratory	Class	Method 1680 (LTB/EC)		Method 1681 (A-1)	
		6 hours	24 hours	6 hours	24 hours
1	B	221,480	353,130	132,072	179,295
5	B			276,318	209,209
9	B	79,316	103,986		
11	B	141	125	69	211
14	B	3,936	3,685		
18	B	818,406	532,167		
19	B			23,446	11,532
20	B	59,211	31,417		
25 ^a	A			937	3,253
26	B			101,974	146,687
28	B	319,430	295,914		
Mean	A			937	3,253
RSD ^b	A			85	198
Mean	B	214,560	188,632	106,776	109,387
RSD ^b	B	81	115	109	143

Shading indicates laboratories did not conduct sample analyses with corresponding methods

^a Laboratory analyzed a spiked Class A anaerobically digested sample

^b Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

6.2 *Salmonella* (Method 1682) Holding Time Study Results for Biosolid Samples

All *Salmonella* results were natural log-transformed prior to performing any statistical analyses as a result of the skewed distribution of the results, as recommended in Statistical Methods for Environmental Pollution Monitoring (Reference 8.4). Results were stratified by matrix. For the alkaline-stabilized matrix, a single comparison was performed using a two-sample t-test based on the data from the one laboratory that performed analyses for the given matrix. For the composted matrix, a two-way Analysis of Variance (ANOVA) model was fit to assess the effect of holding time and laboratory on the log-transformed concentrations, and to assess whether there was a significant interaction between holding time and laboratory. There was no significant interaction between holding time and laboratory for compost samples, and, as a result, a single comparison was performed in a single ANOVA model using data from all laboratories for that matrix. Pairwise comparisons were not necessary for this analysis because samples were analyzed at only two holding times.

Salmonella results are summarized in **Table 10**. Significantly different holding times are indicated by a “D,” indicating a significant decrease in concentration compared to 6-hour results.

Based on statistical analysis of the *Salmonella* results, the following observations were made:

Alkaline-Stabilized Class A Matrix

- *Salmonella* concentrations were not significantly different at the 24-hour holding time compared to the 6-hour holding time.

Composted Class A Matrices

- *Salmonella* concentrations were significantly decreased by the 24-hour holding time compared to the 6-hour holding time at Laboratories 1 and 5.

Table 10. *Salmonella* Mean Results (CFU/ 4g dry weight) from Biosolid Samples

Laboratory	Matrix	Method 1682	
		6 hours	24 hours
6	Alkaline-stabilized	0.29	0.29
1	Compost	9.95	5.34 D ^a
5	Compost	7.78	5.22 D ^a
Mean	Alkaline-stabilized	0.29	0.29
RSD ^b	Alkaline-stabilized	0	0
Mean	Compost	8.87	5.28
RSD ^b	Compost	48.50	73.80

^a *Salmonella* concentrations were significantly lower compared to “6” hour results

^b Pooled within-lab relative standard deviation was determined by calculating the square root of the weighted mean of the squared lab RSDs

SECTION 7.0 CONCLUSIONS

Land application of biosolids is a critical component of solid waste management. Under Subpart D of the 40 Code of Federal Regulations (CFR) Part 503, sewage sludges (biosolids) are required to be processed prior to land application in order to minimize pathogen levels and the potential public health risks associated with contact or exposure. Subpart D further defines and classifies biosolids for land application purposes based on pathogen concentrations. EPA has validated Methods 1680 and 1681 for fecal coliforms in Class A and B biosolids and Method 1682 to support the monitoring requirements for *Salmonella* in Class A biosolids. The main objective of this study was to determine whether biosolid samples being analyzed for fecal coliforms and *Salmonella* can be held for 24 hours prior to sample analysis without a significant change in bacterial concentrations.

Four hundred laboratories were contacted for potential study participation in an effort to obtain six or more useable, valid datasets for every matrix/method combination. A number of reasons, including, but not limited to, limited production of some matrices, laboratory proximity to biosolids facilities, scheduling conflicts etc., resulted in limited data for some matrix/method combinations.

Fecal Coliforms

Based on the results of this study, Class A composted, Class B aerobically digested, and Class B anaerobically digested matrices analyzed for fecal coliforms by Methods 1680 and 1681 may be analyzed 24 hours after sample collection and still generate data comparable to those generated at 6 hours after sample collection.

All other matrices analyzed for fecal coliforms using Methods 1680 and 1681 should not exceed a 6-hour maximum sample transport holding time, and must be processed within 2 hours of receipt at the laboratory. The data for these matrix/method combinations generally showed a tendency to decrease with extended sample holding times.

Salmonella

Based on the results of this study and the limited data generated, Class A matrices analyzed for *Salmonella* using Method 1682 may not be analyzed beyond 6 hours after sample collection and still generate data comparable to those generated at 6 hours after sample collection. In general, *Salmonella* concentrations had a tendency to decrease with extended sample holding times.

SECTION 8.0 REFERENCES

- 8.1** US EPA. 2005. EPA Method 1680: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using Lauryl Tryptose Broth (LTB) and EC Medium
- 8.2** US EPA. 2005. EPA Method 1681: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple Tube Fermentation using A-1 Medium
- 8.3** US EPA. 2005. EPA Method 1682: *Salmonella* in Sewage Sludge (Biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium
- 8.4** Gilbert, R.O. 1987. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand Reinhold, New York.

SECTION 9.0 ACRONYMS

LTB	Lauryl tryptose broth
LIA	Lysine iron agar
MPN	Most probable number
MSRV	Modified semisolid Rappaport-Vassiliadis medium
QC	Quality control
RSD	Relative standard deviation
SAS	Statistical analysis software
SD	Standard deviation
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSI	Triple sugar iron agar
XLD	Xylose-lysine desoxycholate agar

**Appendix A:
Laboratory Capabilities Checklist**

Laboratory Capabilities Checklist Biosolids Holding Time Study

(March 13, 2006)

EPA plans to invite utility and commercial laboratories to participate in a study to evaluate the effect of holding time on fecal coliforms in biosolids using EPA Methods 1680 and 1681 and *Salmonella* concentrations in biosolids using EPA Method 1682. Samples will be analyzed at 6 and 24 hours following collection and/or spiking. In addition, the study will include an assessment of false positive and false negative confirmation rates for EPA Methods 1680 and 1681. Since Method 1682 includes serological confirmation, assessment of false positive and false negative confirmation rates will not be done during this study. EPA will provide the media, reagents, and disposable supplies necessary for this study. Volunteer laboratories and participants will be acknowledged in the study reports.

If your laboratory is interested in participating in this study, please provide the requested information below and fax the signed, completed checklist to Darcy Gibbons at 703.461.8056. Darcy will confirm receipt of the checklist. If you have any questions pertaining to the information requested below or the holding time study, please do not hesitate to contact Yildiz Chambers at 703.461.2165 or [ychambers@csc.com](mailto:y Chambers@csc.com).

Section 1. Laboratory Capabilities and Experience

- A. Please complete the requested laboratory capabilities and experience information below, if this information has not been previously provided to CSC. The information requested in **Table 1** pertains to experience with a given method for biosolids analyzed.

Table 1. Analyst Experience

Analyst	Years of experience or estimated number of samples analyzed		
	1680 (LTB/EC)	1681 (A-1)	1682 (MSRV)

- B. Name of primary analyst participating in study: _____
- C. Primary analyst's years of experience performing biosolids analyses: _____
- D. What certifications does your laboratory have for microbial analyses?
- E. Additional comments:

Section 2. Background Information

- A. Does your laboratory have access to biosolid samples? Yes No
If yes, please indicate in Table 2, below.
- B. Is your laboratory located within two hours driving distance of the biosolid facility (sample collection site)? Yes No
- C. Has your laboratory ever participated in a biosolids study? Yes No
- D. If your laboratory has experience analyzing biosolid samples for fecal coliforms and/or *Salmonella*, please place a check “✓” next to the biosolid type which you have analyzed and indicate both: the method(s) used for analysis, and typical concentrations of each analyte (**Table 2, below**).

Table 2. Fecal coliforms and *Salmonella*

Access?	Biosolids Type	Class A or B	Monitoring Frequency	Fecal coliforms		<i>Salmonella</i>	
				Methods	Typical Range	Methods	Typical Range
<i>Example</i> ✓	<i>Composted</i>	<i>A</i>	<i>2 times per month</i>	<i>1680</i>	<i>30 x 10⁵</i>	<i>1682</i>	<i>12 x 10³</i>
	Heat-dried						
	Composted						
	Thermophilically digested						
	Alkaline-stabilized						
	Aerobically digested						
	Anaerobically digested						

- E. CSC recognizes that your laboratory may have access to multiple facilities with biosolid matrices. For each facility, please describe the treatment process (e.g., composted, anaerobically digested). Please be as descriptive as possible.

Facility 1:

Facility 2:

Facility 3:

- F. Will your laboratory be prepared to collect and analyze biosolid samples within the 6-hour holding time?
Yes No

I certify that the information provided above is accurate and complete:

Primary Analyst: _____

Phone Number: _____

Email Address: _____

Lab Manager: _____

Phone Number: _____

Email Address: _____

Laboratory Name: _____

Laboratory FedEx Shipping Address: _____

Signature (lab manager): _____

Date: _____

**Appendix B:
Instructions for the Biosolids Holding Time Study
for Methods 1680/1681 (Class A)**

Instructions for the Biosolids Holding Time Study Method 1680 and 1681: Class A Fecal Coliforms

Holding Time Study Schedule

The same analytical procedures described for preliminary analyses will be used during the Biosolids Holding Time Study. The following schedule may be used as a time-line for sample analyses. *Note:* During the holding time study, unspiked samples with growth will be submitted to verification as described in the verification instructions.

Sample Analyses: LTB/EC and A-1

Option A: If ambient fecal coliform concentration is \leq 300 MPN / g dry weight

2 unspiked matrix samples per holding time
8 spiked matrix samples per holding time
1 unspiked OPR sample per holding time (Milorganite® [reference matrix])
2 spiked OPR samples per holding time (Milorganite® spiked with *E. coli* ATCC # 25922 spiking suspension)
Total = 13 samples per holding time

Option B: If ambient fecal coliform concentration is $>$ 300 MPN / g dry weight

8 unspiked matrix samples per holding time
2 spiked matrix samples per holding time
1 unspiked OPR sample per holding time (Milorganite® [reference matrix])
2 spiked OPR samples per holding time (Milorganite® spiked with *E. coli* ATCC # 25922 spiking suspension)
Total = 13 samples per holding time

Example Schedule

Monday (0800)

- Inoculate spiking suspension (see spiking protocol)

Tuesday (0800)

- Dilute spiking suspension and plate onto TSA (see spiking protocol)
- Collect a 1000 g bulk sample
- **Follow Option A instructions if ambient fecal coliform concentration is \leq 300 MPN / g dry weight**
- **Follow Option B instructions if ambient fecal coliform concentration is $>$ 300 MPN / g dry weight**
 - Weigh out 20, 30 g samples
 - Weigh out 6, 30 g samples of Milorganite® for OPR analysis (1 unspiked and 2 spiked per holding time)
- **Option A:** Spike 16 of the 20 samples
 - **Liquid samples:** 3 mL of spiking suspension “B” per 300 mL of sample
 - **Solid samples:** 3 mL of spiking suspension “B” per 30 g of sample
- **Option B:** Spike 4 of the 20 samples
 - **Liquid samples:** 3 mL of spiking suspension “B” per 300 mL of sample
 - **Solid samples:** 3 mL of spiking suspension “B” per 30 g of sample

- Refrigerate all samples at $< 10^{\circ}\text{C}$ and above freezing
 - **Option A:** Unspiked matrix: 2 (6 hour) and 2 (24 hour)
Spiked matrix: 8 (6 hour) and 8 (24 hour)
Unspiked OPR: 1 (6 hour) and 1 (24 hour)
Spiked OPR: 2 (6 hour) and 2 (24 hour)
 - **Option B:** Unspiked matrix: 8 (6 hour) and 8 (24 hour)
Spiked matrix: 2 (6 hour) and 2 (24 hour)
Unspiked OPR: 1 (6 hour) and 1 (24 hour)
Spiked OPR: 2 (6 hour) and 2 (24 hour)

Tuesday - 6 Hour Hold Time (1400)

- Spike 2, 30 g Milorganite® samples (OPRs)
- **Option A:** Remove 2 unspiked and 8 spiked samples from refrigerator
- **Option B:** Remove 8 unspiked and 2 spiked samples from refrigerator
- Homogenize by adding 30 g samples to 270 mL of sterile buffer water
- Inoculate LTB and/or A-1 tubes with the following undiluted and diluted homogenate
 - **Unspiked liquid:** 1.0 mL, 10^{-1} , 10^{-2} , 10^{-3}
 - **Unspiked solid:** 10 mL, 1.0 mL, 10^{-1} , 10^{-2}
 - **Spiked liquid:** 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
 - **Spiked solid:** 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}
- **Unspiked OPR:** Analyze 1 unspiked Milorganite® sample by inoculating 5 tubes of 2X media with 10 mL undiluted homogenate
- Incubate LTB tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours
- Incubate A-1 tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 3 hours ± 15 minutes
- Transfer A-1 tubes to a water bath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 21 ± 2 hours

Wednesday - 24 Hour Hold Time (0800)

- Spike 2, 30 g Milorganite® samples (OPRs)
- **Option A:** Remove 2 unspiked and 8 spiked samples from refrigerator
- **Option B:** Remove 8 unspiked and 2 spiked samples from refrigerator
- Homogenize by adding 30 g samples to 270 mL of sterile buffer water
- Inoculate LTB and/or A-1 tubes with the following undiluted and diluted homogenate
 - **Unspiked liquid:** 1.0 mL, 10^{-1} , 10^{-2} , 10^{-3}
 - **Unspiked solid:** 10 mL, 1.0 mL, 10^{-1} , 10^{-2}
 - **Spiked liquid:** 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
 - **Spiked solid:** 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}
- **Unspiked OPR:** Analyze 1 unspiked Milorganite® sample by inoculating 5 tubes of 2X media with 10 mL undiluted homogenate
- Incubate LTB tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours
- Incubate A-1 tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 3 hours ± 15 minutes
- Transfer A-1 tubes to a water bath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 21 ± 2 hours
- Read TSA plates and record results (see spiking protocol)

Wednesday Cont. (1200 - 1400)

- Read A-1 (6 hour) tubes at 24 ± 2 hours and record results; refrigerate A-1 tubes from unspiked samples (with growth) for verification
- Read LTB (6 hour) tubes at 24 ± 2 hours and record results
- Transfer growth from LTB tubes with growth and gas production to EC tubes
- Reincubate LTB for an additional 24 ± 2 hours at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
- Incubate EC broth in the waterbath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Thursday (0600 - 1000)

- Read A-1 (24 hour) tubes at 24 ± 2 hours and record results; refrigerate A-1 tubes from unspiked samples (with growth) for verification
- Read LTB (24 hour) tubes at 24 ± 2 hours and record results
- Transfer growth from LTB tubes with growth and gas production to EC tubes
- Reincubate LTB for an additional 24 ± 2 hours at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
- Incubate EC broth in the waterbath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Thursday (1200 - 1600)

- Read LTB (6 hour) tubes at 48 ± 3 hours and record results
- Transfer growth to EC tubes and incubate at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours
- Read EC (24 hour) tubes at 24 ± 2 hours and record results; refrigerate EC tubes from unspiked samples (with growth) for verification

Friday (0600 - 1000)

- Read LTB (24 hour) tubes at 48 ± 3 hours and record results
- Transfer growth from presumptive tubes to EC
- Incubate EC tubes at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Saturday (0600 - 1000)

- Read EC tubes at 24 ± 2 hours and record results; refrigerate EC tubes from unspiked samples (with growth) for verification

Monday

- Remove EC and A-1 tubes from refrigerator and begin verifications (see verification instructions)

Note: Please fax and send a hard copy of results and any accompanying documentation from holding time study to Darcy Gibbons at **703-461-8056**.

**Appendix C: Instructions for the Biosolids Holding Time Study
for Methods 1680/1681 (Class B)**

Instructions for the Biosolids Holding Time Study Method 1680 and 1681: Class B Fecal Coliforms

Holding Time Study Schedule

During the holding time study, follow the same analytical procedures as described above for the preliminary analyses. Once preliminary analyses are complete, results may be used to determine appropriate dilutions to be analyzed. The following schedule may be used as a time-line for sample analyses. *Note:* During the holding time study, samples with growth will be submitted to verification as described in the verification instructions.

Sample Analyses: LTB/EC and A-1

8 unspiked matrix samples per holding time

2 spiked matrix samples per holding time

1 unspiked OPR sample per holding time (Milorganite® [reference matrix])

2 spiked OPR samples per holding time (Milorganite® spiked with *E. coli* ATCC # 25922 spiking suspension)

Total = 13 samples per holding time

Example Schedule

Monday (0800)

- Inoculate spiking suspension (see spiking protocol)

Tuesday (0800)

- Dilute spiking suspension and plate onto TSA (see spiking protocol)
- Collect a 1000 g bulk sample
- Weigh out 20, 30 g samples (8 unspiked matrix and 2 spiked matrix per holding time)
- Weigh out 6, 30 g samples of Milorganite® for OPR analysis (1 unspiked and 2 spiked per holding time) as follows
 - Unspiked matrix: 8 (6 hour) and 8 (24 hour)
 - Spiked matrix: 2 (6 hour) and 2 (24 hour)
 - Unspiked OPR: 1 (6 hour) and 1 (24 hour)
 - Spiked OPR: 2 (6 hour) and 2 (24 hour)
- Refrigerate all samples at < 10°C and above freezing

Tuesday - 6 Hour Hold Time (1400)

- Spike 2, 30 g Milorganite® samples (OPRs)
- Remove the 8 unspiked and 2 spiked samples from refrigerator
- Homogenize by adding 30 g samples to 270 mL of sterile buffer water
- After homogenization for 1 minute, spike matrix and OPR samples with 3 ml of undiluted spiking suspension, then homogenize for 1 additional minute

- Inoculate LTB and/or A-1 tubes with the following serially diluted homogenate
 - **Unspiked liquid:** 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}
 - **Unspiked solid:** 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
 - **Spiked liquid:** 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}
 - **Spiked solid:** 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7}
- **Unspiked OPR:** Analyze 1 unspiked Milorganite® sample by inoculating 5 tubes of 2X media with 10 mL undiluted homogenate
- Incubate LTB tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours
- Incubate A-1 tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 3 hours ± 15 minutes
- Transfer A-1 tubes to a water bath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 21 ± 2 hours

Wednesday - 24 Hour Hold Time (0800)

- Spike 2, 30 g Milorganite® samples (OPRs)
- Remove the 8 unspiked and 2 spiked samples from refrigerator
- Homogenize by adding 30 g samples to 270 mL of sterile buffer water
- After homogenization for 1 minute, spike matrix and OPR samples with 3 ml of undiluted spiking suspension, then homogenize for 1 additional minute
- Inoculate LTB and/or A-1 tubes with the following serially diluted homogenate
 - **Unspiked liquid:** 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}
 - **Unspiked solid:** 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}
 - **Spiked liquid:** 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}
 - **Spiked solid:** 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7}
- **Unspiked OPR:** Analyze 1 unspiked Milorganite® sample by inoculating 5 tubes of 2X media with 10 mL undiluted homogenate
- Incubate LTB tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours
- Incubate A-1 tubes at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 3 hours ± 15 minutes
- Transfer A-1 tubes to a water bath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 21 ± 2 hours
- Read TSA plates and record results (see spiking protocol)

Wednesday (1200 - 1400)

- Read A-1 (6 hour) tubes at 24 ± 2 hours and record results; refrigerate A-1 tubes from unspiked samples (with growth) for verification
- Read LTB (6 hour) tubes at 24 ± 2 hours and record results
- Transfer growth from LTB tubes with growth and gas production to EC tubes
- Reincubate LTB for an additional 24 ± 2 hours at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
- Incubate EC broth in the waterbath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Thursday (0600 - 1000)

- Read A-1 (24 hour) tubes at 24 ± 2 hours and record results; refrigerate A-1 tubes from unspiked samples (with growth) for verification
- Read LTB (24 hour) tubes at 24 ± 2 hours and record results
- Transfer growth from LTB tubes with growth and gas production to EC tubes
- Reincubate LTB for an additional 24 ± 2 hours at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
- Incubate EC broth in the waterbath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Thursday (1200 - 1600)

- Read LTB (6 hour) tubes at 48 ± 3 hours and record results
- Transfer growth to EC tubes and incubate at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours
- Read EC (24 hour) tubes at 24 ± 2 hours and record results; refrigerate EC tubes from unspiked samples (with growth) for verification

Friday (0600 - 1000)

- Read LTB (24 hour) tubes at 48 ± 3 hours and record results
- Transfer growth from presumptive tubes to EC
- Incubate EC tubes at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 hours

Saturday (0600 - 1000)

- Read EC tubes at 24 ± 2 hours and record results; refrigerate EC tubes (with growth) for verification

Monday

- Remove EC and A-1 tubes from refrigerator and begin verifications (see verification instructions)

Note: Please fax and send a hard copy of results and any accompanying documentation from holding time study to Darcy Gibbons at **703-461-8056**.

**Appendix D: Instructions for the Biosolids Holding Time Study
for Method 1682**

Instructions for the Biosolids Holding Time Study

Method 1682: *Salmonella*

Holding Time Study Schedule

The same analytical procedures described for preliminary analyses will be used during the Biosolids Holding Time Study. The following schedule may be used as a time-line for sample analyses. *Note:* One positive and negative control tube/plate should be inoculated with the appropriate positive/negative control organism for each medium used during sample analysis.

Sample Analyses

2 unspiked matrix samples per holding time

8 spiked matrix samples per holding time

1 unspiked reference matrix (Milorganite®) sample per holding time

2 spiked reference matrix (Milorganite® spiked with *Salmonella typhimurium* ATCC # 14028 spiking suspension) samples per holding time (OPR)

Total = 13 samples per holding time

Example Schedule

Sunday (1200)

- Inoculate spiking suspension (see spiking protocol)

Monday (1200)

- Dilute spiking suspension and plate onto TSA (see spiking protocol)
- Collect a single bulk sample of approximately 1000 g and transport to the laboratory on ice; maintain sample at < 10°C and above freezing
- Weigh out 20, 30 g samples (2 unspiked and 8 spiked per holding time)
- Weigh out 6, 30 g samples of Milorganite® for OPR analysis (1 unspiked and 2 spiked per holding time)
- Spike 16 of the 20 matrix samples with 0.5 mL of spiking suspension “D” (10^{-6} of the original spiking suspension); see spiking protocol
- Refrigerate all samples at < 10°C and above freezing
 - Unspiked matrix: 2 (6 hour) and 2 (24 hour)
 - Spiked matrix: 8 (6 hour) and 8 (24 hour)
 - Unspiked OPR: 1 (6 hour) and 1 (24 hour)
 - Spiked OPR: 2 (6 hour) and 2 (24 hour)

Monday - 6 Hour Hold Time (1800)

- Spike 2, 30 g Milorganite® samples (OPRs)
- Remove 2 unspiked and 8 spiked matrix samples from refrigerator
- Homogenize by adding each 30 g sample to 270 mL sterile buffer
- Inoculate TSB tubes with the following volumes of homogenized unspiked or spiked samples
 - Add 20 mL homogenized sample to 10 mL 3X TSB
 - Add 10 mL homogenized sample to 5 mL 3X TSB
 - Add 1 mL homogenized sample to 10 mL 1X TSB
- Unspiked OPR: Inoculate 5 tubes of 3X media with 20 mL homogenized sample
- Incubate inoculated TSB tubes at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 24 ± 2 hours

Tuesday - 24 Hour Hold Time (1200)

- Spike 2, 30 g Milorganite® samples (OPRs)
- Remove 2 unspiked and 8 spiked matrix samples from refrigerator
- Homogenize by adding each 30 g sample to 270 mL sterile buffer
- Inoculate TSB tubes with the following volumes of homogenized unspiked or spiked samples
 - Add 20 mL homogenized sample to 10 mL 3X TSB
 - Add 10 mL homogenized sample to 5 mL 3X TSB
 - Add 1 mL homogenized sample to 10 mL 1X TSB
- Unspiked OPR: Inoculate 5 tubes of 3X media with 20 mL homogenized sample
- Incubate inoculated TSB tubes at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 24 ± 2 hours
- Examine TSA plates (spiking suspension enumeration) and record results (see spiking protocol)

Tuesday (1600 - 2000)

- Read TSB (6 hour) tubes at 24 ± 2 hour incubation and record results
- Transfer 6, 30 μl drops from each TSB tube (6 hour) to corresponding MSR/V plates; allow drops to absorb for approximately 1 hour at room temperature
Do not invert MSR/V plates
- Incubate MSR/V plates (6 hour) at $42^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 16 - 18 hours in a humidity-controlled incubator

Wednesday (0800 - 1200)

- Read TSB (24 hour) tubes at 24 ± 2 hour incubation and record results
- Transfer 6, 30 μl drops from each TSB tube (24 hour) to corresponding MSR/V plates; allow drops to absorb for approximately 1 hour at room temperature
Do not invert MSR/V plates
- Incubate MSR/V plates (24 hour) at $42^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 16 - 18 hours in a humidity-controlled incubator

Wednesday (1200-1400)

- Remove MSR/V plates (6 hour) from incubator and examine plates for the appearance of a “whitish halo” of growth; record results
- Choose 2 target colonies from MSR/V plates (6 hour) and streak growth onto 2 separate XLD plates
- Incubate XLD plates (6 hour) at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 18 to 24 hours

Thursday (0600)

- Remove MSR/V plates (24 hour) from incubator and examine for the appearance of a “whitish halo” of growth; record results
- Choose 2 target colonies from MSR/V plates (24 hour) and streak growth onto two separate XLD plates
- Incubate XLD plates (24 hour) at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 18 to 24 hours

Thursday (1200)

- Remove XLD plates (6 hour) from incubator; refrigerate one of the XLD plates at $< 10^{\circ}\text{C}$ and above freezing and submit the other plate to biochemical confirmation. *Note:* Both XLD plates may be refrigerated at $< 10^{\circ}\text{C}$ and above freezing over the weekend prior to submitting to biochemical confirmation.

Friday (0600)

- Remove XLD plates (24 hour) from incubator; refrigerate one of the XLD plates at $< 10^{\circ}\text{C}$ and above freezing and submit the other plate to biochemical confirmation. *Note:* XLD plates may be refrigerated at $< 10^{\circ}\text{C}$ and above freezing over the weekend prior to submitting to biochemical confirmation.

Monday (0800)

Biochemical Confirmation

- Remove one XLD plate from refrigerator (6 hour and 24 hour) and examine for growth
- Pick a well isolated colony from each XLD plate (6 hour and 24 hour) exhibiting *Salmonella* morphology and inoculate TSI, LIA, and Urease broth by stabbing the butt and streaking the slant; use the same XLD colony to inoculate all three media
- TSI/LIA/Urease broth: Incubate (6 hour and 24 hour) slants and broth at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 18 to 24 hours

Tuesday (0800)

- Remove TSI/LIA/Urease slants (6 hour and 24 hour) from the incubator and examine for positive/negative reactions; record results

Serology

- Emulsify growth on the slant portion of TSI; place two discrete drops of emulsified growth onto a slide
- To the first drop of emulsified growth, add one drop of polyvalent O antiserum
- To the second drop of emulsified growth, add one drop of sterile saline (as a visual comparison)
- Observe slide under magnification and record results

Note: In order for the original TSB tube to be considered positive for *Salmonella*, the associated inoculations must be MSR/V positive, XLD positive, either TSI or LIA positive, urease negative, and polyvalent-O positive. Correlate all positive plates and tubes to original TSB tube and record results.

Note: Please fax and send a hard copy of results and any accompanying documentation from holding time study to Darcy Gibbons at **703-461-8056**.

Appendix E: Fecal Coliform Spiking Protocol (Class A)

Fecal Coliform Spiking Procedure

Class A Biosolids: Methods 1680 and 1681

The purpose of this protocol is to provide laboratories with a fecal coliform spiking procedure for the Method 1680 and 1681 Holding Time Study.

1.0 Preparation of *E. coli* Spiking Suspension

- 1.1 **Stock Culture.** Prepare a stock culture by inoculating a tryptic soy agar (TSA) slant (or other non-selective media) with *Escherichia coli* ATCC # 25922 and incubating at $35^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.
- 1.2 **1% Lauryl Tryptose Broth (LTB).** Prepare a 1% solution of LTB by combining 99 mL of sterile phosphate buffered dilution water and 1 mL of sterile single strength LTB in a sterile screw cap bottle or re-sealable dilution water container (Hardy Diagnostics D699 or equivalent). Shake to mix.
- 1.3 **Spiking Suspension (Undiluted).** From the stock culture of *E. coli* ATCC # 25922 in Section 1.1, transfer a small loopful of growth to the 1% LTB solution and vigorously shake a minimum of 25 times. Incubate at $35^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 20 ± 4 hours. The resulting spiking suspension contains approximately $1.0 \times 10^7 - 1.0 \times 10^8$ *E. coli* colony forming units (CFU) per mL. This is referred to as the “undiluted spiking suspension.” *Note:* Spiking suspension should be inoculated the day prior to preliminary and holding time study analyses.

2.0 Class A Biosolid Sample Spiking and Enumeration of Spiking Suspension

Homogenize the unspiked Class A biosolid sample according to study instructions. For preliminary analyses, four, 30 g Milorganite® samples (IPR) per method and one, 30 g matrix sample per method will be spiked prior to analyses. During the holding time study, four, 30 g Milorganite® samples (OPR) per method and either sixteen, 30 g matrix samples per method (Option A), or four, 30 g matrix samples per method (Option B) will be spiked.

2.1 Sample spiking: Class A

2.1.1 Dilute spiking suspension

- 2.1.1.1 Mix the spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension (from Section 1.3) to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “A.” A 1.0-mL volume of dilution “A” is 10^{-2} mL of the original undiluted spiking suspension.
- 2.1.1.2 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “A” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “B.” A 1.0-mL volume of dilution “B” is 10^{-3} mL of the original undiluted spiking suspension.
- 2.1.1.3 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “B” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “C.” A 1.0-mL volume of dilution “C” is 10^{-4} mL of the original spiking suspension.
- 2.1.1.4 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “C” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “D.” A 1.0-mL volume of dilution “D” is 10^{-5} mL of the original spiking suspension.

2.1.1.5 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “D” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “E.” A 1.0-mL volume of dilution “E” is 10^{-6} mL of the original spiking suspension.

2.1.2 Spike sample(s)

2.1.2.1 Liquid Samples

For every 300 mL of liquid sample, add 3.0 mL of spiking suspension “B”. *Note:* Laboratories spiking liquid samples will receive additional verbal instructions from CSC. Following spiking, inoculate appropriate medium according to the study instructions.

2.1.2.2 Solid Samples

For each 30-g sample, add 3.0 mL of spiking suspension “B”. Following spiking, inoculate appropriate medium according to the study instructions.

2.2 **Enumeration of undiluted spiking suspension (prepared in Section 1.3)**

2.2.1 Prepare TSA by adding 10 - 15 mL of TSA per 100 x 15 mm petri dish, and allow to solidify. For larger plates, adjust volume appropriately. Ensure that agar surface is dry. *Note:* To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

2.2.2 Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution “C” onto surface of pre-dried TSA plate [10^{-5} mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “D” onto surface of pre-dried TSA plate [10^{-6} mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “E” onto surface of pre-dried TSA plate [10^{-7} mL of the original spiking suspension].

2.2.3 Immediately after inoculating each spread plate, using a sterile bent glass rod or spreader, distribute inoculum over surface of medium by rotating the dish by hand or on a turntable. Shake spiking suspension 25 times before inoculating each plate.

2.2.4 After spreading, allow inoculum to absorb into the medium completely for approximately 1 - 3 minutes.

2.2.5 Invert plates and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 4 hours.

2.2.6 Count and record number of colonies per plate.

3.0 Calculate Concentration of *E. coli* (CFU / mL) in Undiluted Spiking Suspension

- 3.1 The number of *E. coli* CFU / mL in the spiking suspension will be calculated using all plates yielding counts within the ideal range of 30 - 300 CFU per plate.
- 3.2 If the number of colonies exceeds the upper range (i.e. >300) or if the colonies are not discrete, results should be recorded as “too numerous to count” (TNTC).
- 3.3 Calculate the concentration of *E. coli* (CFU / mL) in the undiluted spiking suspension according to the following equation. (Example calculations are provided in **Table 1.**)

$$EC_{\text{undiluted spike}} = \frac{CFU_1 + CFU_2 + \dots + CFU_n}{V_1 + V_2 + \dots + V_n}$$

Where

- EC_{undiluted spike} = *E. coli* CFU / mL in undiluted spiking suspension
- CFU = number of colony forming units from plates yielding counts within the ideal range of 30 - 300 CFU per plate
- V = volume of undiluted sample in each TSA plate yielding counts within the ideal range of 30 - 300 CFU per plate
- n = number of plates with counts within the ideal range

TABLE 1. EXAMPLE CALCULATIONS OF *E. COLI* SPIKING SUSPENSION CONCENTRATION

Examples	CFU / plate (triplicate analyses) from TSA plates			<i>E. coli</i> CFU / mL in undiluted spiking suspension (EC _{undiluted spike})*
	10 ⁻⁵ mL plates	10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	(275+250+30) / (10 ⁻⁵ +10 ⁻⁵ +10 ⁻⁶) = 555 / (2.1 x 10 ⁻⁵) = 26,428,571 = 2.6 x 10⁷ CFU / mL
Example 2	TNTC, TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	(299+109+32) / (10 ⁻⁶ +10 ⁻⁷ +10 ⁻⁷) = 440 / (1.2 x 10 ⁻⁶) =366,666,667 = 3.7 x 10⁸ CFU / mL

*EC_{undiluted spike} is calculated using all plates yielding counts within the ideal range of 30 - 300 CFU per plate

Appendix F: Fecal Coliform Spiking Protocol (Class B)

Fecal Coliform Spiking Procedure

Class B Biosolids: Methods 1680 and 1681

The purpose of this protocol is to provide laboratories with a fecal coliform spiking procedure for the Method 1680 and 1681 Holding Time Study.

1.0 Preparation of *E. coli* Spiking Suspension

- 1.1 **Stock Culture.** Prepare a stock culture by inoculating a tryptic soy agar (TSA) slant (or other non-selective media) with *Escherichia coli* ATCC # 25922 and incubating at $35^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.
- 1.2 **1% Lauryl Tryptose Broth (LTB).** Prepare a 1% solution of LTB by combining 99 mL of sterile phosphate buffered dilution water and 1 mL of sterile single strength LTB in a sterile screw cap bottle or re-sealable dilution water container (Hardy Diagnostics D699 or equivalent). Shake to mix.
- 1.3 **Spiking Suspension (Undiluted).** From the stock culture of *E. coli* ATCC # 25922 in Section 1.1, transfer a small loopful of growth to the 1% LTB solution and vigorously shake a minimum of 25 times. Incubate at $35^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 20 ± 4 hours. The resulting spiking suspension contains approximately $1.0 \times 10^7 - 1.0 \times 10^8$ *E. coli* colony forming units (CFU) per mL. This is referred to as the “undiluted spiking suspension.” *Note:* Spiking suspension should be inoculated the day prior to preliminary and holding time study analyses.

2.0 Class B Biosolid Sample Spiking and Enumeration of Spiking Suspension

Homogenize the unspiked Class B biosolid sample according to study instructions. For preliminary analyses, four, 30 g Milorganite® samples (IPR) per method and one, 30 g matrix sample per method will be spiked prior to analyses. During the holding time study, four, 30 g Milorganite® samples (OPR) per method and four, 30 g matrix samples per method will be spiked.

2.1 Sample spiking: Class B

2.1.1 Dilute spiking suspension

- 2.1.1.1 Mix the spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension (from Section 1.3) to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “A.” A 1.0-mL volume of dilution “A” is 10^{-2} mL of the original undiluted spiking suspension.
- 2.1.1.2 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “A” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “B.” A 1.0-mL volume of dilution “B” is 10^{-3} mL of the original undiluted spiking suspension.
- 2.1.1.3 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “B” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “C.” A 1.0-mL volume of dilution “C” is 10^{-4} mL of the original spiking suspension.
- 2.1.1.4 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “C” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “D.” A 1.0-mL volume of dilution “D” is 10^{-5} mL of the original spiking suspension.

2.1.1.5 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “D” to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “E.” A 1.0-mL volume of dilution “E” is 10^{-6} mL of the original spiking suspension.

2.1.2 Spike sample(s)

2.1.2.1 Liquid Samples

For every 300 mL of liquid sample, add 3.0 mL of the undiluted spiking suspension. *Note:* Laboratories spiking liquid samples will receive additional verbal instructions from CSC. Following spiking, inoculate appropriate medium according to the study instructions.

2.1.2.2 Solid Samples

For each 30-g sample, add 3.0 mL of the undiluted spiking suspension. Following spiking, inoculate appropriate medium according to the study instructions.

2.2 **Enumeration of undiluted spiking suspension (prepared in Section 1.3)**

2.2.1 Prepare TSA by adding 10 - 15 mL of TSA per 100 x 15 mm petri dish, and allow to solidify. For larger plates, adjust volume appropriately. Ensure that agar surface is dry. *Note:* To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

2.2.2 Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution “C” onto surface of pre-dried TSA plate [10^{-5} mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “D” onto surface of pre-dried TSA plate [10^{-6} mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “E” onto surface of pre-dried TSA plate [10^{-7} mL of the original spiking suspension].

2.2.3 Immediately after inoculating each spread plate, using a sterile bent glass rod or spreader, distribute inoculum over surface of medium by rotating the dish by hand or on a turntable. Shake spiking suspension 25 times before inoculating each plate.

2.2.4 After spreading, allow inoculum to absorb into the medium completely for approximately 1 - 3 minutes.

2.2.5 Invert plates and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 4 hours.

2.2.6 Count and record number of colonies per plate.

3.0 Calculate Concentration of *E. coli* (CFU / mL) in Undiluted Spiking Suspension

- 3.1** The number of *E. coli* CFU / mL in the spiking suspension will be calculated using all plates yielding counts within the ideal range of 30 - 300 CFU per plate.
- 3.2** If the number of colonies exceeds the upper range (i.e. >300) or if the colonies are not discrete, results should be recorded as “too numerous to count” (TNTC).
- 3.3** Calculate the concentration of *E. coli* (CFU / mL) in the undiluted spiking suspension according to the following equation. (Example calculations are provided in **Table 1.**)

$$EC_{\text{undiluted spike}} = \frac{CFU_1 + CFU_2 + \dots + CFU_n}{V_1 + V_2 + \dots + V_n}$$

Where

- EC_{undiluted spike} = *E. coli* CFU / mL in undiluted spiking suspension
- CFU = number of colony forming units from plates yielding counts within the ideal range of 30 - 300 CFU per plate
- V = volume of undiluted sample in each TSA plate yielding counts within the ideal range of 30 - 300 CFU per plate
- n = number of plates with counts within the ideal range

TABLE 1. EXAMPLE CALCULATIONS OF *E. COLI* SPIKING SUSPENSION CONCENTRATION

Examples	CFU / plate (triplicate analyses) from TSA plates			<i>E. coli</i> CFU / mL in undiluted spiking suspension (EC _{undiluted spike})*
	10 ⁻⁵ mL plates	10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	(275+250+30) / (10 ⁻⁵ +10 ⁻⁵ +10 ⁻⁶) = 555 / (2.1 x 10 ⁻⁵) = 26,428,571 = 2.6 x 10⁷ CFU / mL
Example 2	TNTC, TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	(299+109+32) / (10 ⁻⁶ +10 ⁻⁷ +10 ⁻⁷) = 440 / (1.2 x 10 ⁻⁶) = 366,666,667 = 3.7 x 10⁸ CFU / mL

*EC_{undiluted spike} is calculated using all plates yielding counts within the ideal range of 30 - 300 CFU per plate

Appendix G: *Salmonella* Spiking Protocol

Salmonella Spiking Procedure

Method 1682

The purpose of this protocol is to provide laboratories with a *Salmonella typhimurium* spiking procedure for the Method 1682 Holding Time Study.

1.0 Preparation of Laboratory-Prepared Spiking Suspensions

- 1.1 Stock Culture.** Prepare a stock culture by inoculating a tryptic soy agar (TSA) slant (or other non-selective media) with *Salmonella typhimurium* ATCC # 14028 and incubating at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 20 ± 4 hours. After incubation, the stock culture may be stored in the dark at room temperature for up to 30 days.
- 1.2 1% Tryptic Soy Broth (TSB).** Prepare a 1% solution of TSB by combining 99 mL of sterile phosphate buffered dilution water and 1 mL of sterile single-strength tryptic soy broth in a sterile screw cap bottle or re-sealable dilution water container. Shake to mix.
- 1.3 Spiking Suspension (Undiluted).** From the stock culture of *S. typhimurium* ATCC # 14028, in section 1.1, aseptically transfer a small loopful of growth to the 1% TSB solution and vigorously shake a minimum of 25 times. Incubate at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ for 20 ± 4 hours. The resulting spiking suspension contains approximately 1.0×10^7 to 1.0×10^8 *S. typhimurium* colony forming units (CFU) per mL. This is referred to as the “undiluted spiking suspension.”

2.0 Sample Spiking and Enumeration of Spiking Suspension

Homogenize the unspiked Class A biosolid sample according to study instructions. For preliminary analyses, four, 30 g Milorganite® samples (IPR) and one, 30 g matrix sample will be spiked prior to analyses. During the holding time study, four, 30 g Milorganite® samples (OPR) and sixteen, 30 g matrix samples will be spiked.

2.1 Sample spiking

2.1.1 Dilute spiking suspension

- 2.1.1.1** Mix the undiluted spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile phosphate buffered dilution water (Section 1.3), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “A”. A 1.0-mL volume of dilution “A” is 10^{-2} mL of the original undiluted spiking suspension.
- 2.1.1.2** Use a sterile pipette to transfer 1.0 mL of spiking suspension dilution “A” to 99 mL of sterile phosphate buffered dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “B”. A 1.0-mL volume of dilution “B” is 10^{-4} mL of the original undiluted spiking suspension.
- 2.1.1.3** Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “B” to 99 mL of sterile phosphate buffered dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “C”. A 1.0-mL volume of dilution “C” is 10^{-5} mL of the original undiluted spiking suspension.
- 2.1.1.4** Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution “C” to 99 mL of sterile phosphate buffered dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “D”. A 1.0-mL volume of dilution “D” is 10^{-6} mL of the original undiluted spiking suspension.

2.1.2 Spike sample(s)

2.1.2.2 For each 30 g sample, add 0.5 mL of spiking suspension “D”. Following spiking, inoculate appropriate medium according to the study instructions.

2.2 Enumeration of undiluted spiking suspension (prepared in Section 1.3)

2.2.1 Prepare TSA by adding 10 - 15 mL of TSA per 100 x 15 mm petri dish, and allow to solidify. For larger plates, adjust volume appropriately. Ensure that agar surface is dry. *Note:* To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

2.2.2 Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution “B” onto surface of pre-dried TSA plate [10⁻⁵ mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “C” onto surface of pre-dried TSA plate [10⁻⁶ mL of the original spiking suspension].
- Pipet 0.1 mL of dilution “D” onto surface of pre-dried TSA plate [10⁻⁷ mL of the original spiking suspension].

2.2.3 For each spread plate, using a sterile bent glass rod or spreader, distribute inoculum over surface of medium by rotating the dish by hand or on a turntable.

2.2.4 Allow inoculum to absorb into the medium completely for approximately 1 - 3 minutes.

2.2.5 Invert plates and incubate at 36°C ± 1.5°C for 24 ± 4 hours.

2.2.6 Count and record number of colonies per plate.

3.0 Calculate Concentration of *S. typhimurium* (CFU / mL) in Undiluted Spiking Suspension

3.1 The number of *S. typhimurium* CFU / mL in the undiluted spiking suspension will be calculated using all TSA plates yielding counts within the ideal range of 30 to 300 CFU per plate.

3.2 If the number of colonies exceeds the upper range (i.e., >300) or if the colonies are not discrete, results should be recorded as “too numerous to count” (TNTC).

3.3 Calculate the concentration of *S. typhimurium* (CFU / mL) in the undiluted spiking suspension according to the following equation. (Example calculations are provided in **Table 1**, below.)

$$\text{Salmonella}_{\text{undiluted spike}} = (\text{CFU}_1 + \text{CFU}_2 + \dots + \text{CFU}_n) / (V_1 + V_2 + \dots + V_n)$$

Where,

<i>Salmonella</i> _{undiluted spike}	=	<i>S. typhimurium</i> CFU / mL in undiluted spiking suspension
CFU	=	Number of colony forming units from TSA plates yielding counts within the ideal range of 30 to 300 CFU per plate
V	=	Volume of undiluted sample on each TSA plate yielding counts within the ideal range of 30 to 300 CFU per plate
n	=	Number of plates with counts within the ideal range

Table 1. Example Calculations of *S. typhimurium* Spiking Suspension Concentration

Examples	CFU / plate (triplicate analyses) from TSA plates			<i>Salmonella</i> CFU / mL in undiluted spiking suspension (<i>Salmonella</i> _{undiluted spike}) ^a
	10 ⁻⁵ mL plates	10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	$(275+250+30) / (10^{-5} + 10^{-5} + 10^{-6}) =$ $555 / (2.1 \times 10^{-5}) = 26,428,571 =$ 2.6 x 10⁷ CFU / mL
Example 2	TNTC, TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	$(299+109+32) / (10^{-6} + 10^{-7} + 10^{-7}) =$ $440 / (1.2 \times 10^{-6}) = 366,666,667 =$ 3.7 x 10⁸ CFU / mL

^a*Salmonella*_{undiluted spike} is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate