Appendix F
Sample Preparation for Fecal Coliform Tests and *Salmonella* sp. Analysis

1. Sample Preparation for Fecal Coliform Tests

1.1 Class B Alternative 1

To demonstrate that a given domestic sludge sample meets Class B Pathogen requirements under alternative 1, the density of fecal coliform from at least seven samples of treated sewage sludge must be determined and the geometric mean of the fecal coliform density must not exceed 2 million Colony Forming Units (CFU) or Most Probable Number (MPN) per gram of total solids (dry weight basis). The solids content of treated domestic sludge can be highly variable. Therefore, an aliquot of each sample must be dried and the solids content determined in accordance with procedure 2540 G. of the 18th edition of Standard Methods for the Examination of Water and Wastewater (SM).

Sludge samples to be analyzed in accordance with SM 9221 E. Fecal Coliform MPN Procedure and 9222 D. Fecal Coliform Membrane Filter Procedure may require dilution prior to analysis. An ideal sample volume will yield results which accurately estimate the fecal coliform density of the sludge. Detection of fecal coliform in undiluted samples could easily exceed the detection limits of these procedures. Therefore, it is recommended that the following procedures be used (experienced analysts may substitute other dilution schemes as appropriate).

For Liquid Samples:

1. Use a sterile graduated cylinder to transfer 30.0 mL of well mixed sample to a sterile blender jar. Use 270 mL of sterile buffered dilution water (see Section 9050C) to rinse any remaining sample from the cylinder into the blender. Cover and blend for two minutes on high speed. 1.0 mL of this mixture is 0.1 mL of the original sample or $1.0X10^{-1}$.

2. Use a sterile pipette to transfer 11.0 mL of the blended sample mixture to 99 mL of sterile buffered dilution in a sterile screw cap bottle and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." 1.0 mL of this mixture is 0.010 mL of the original sample or $1.0X10^{-2}$.

3. Use a sterile pipette to transfer 1.0 mL of dilution "A" to a second screw cap bottle containing 99 mL of sterile buffered dilution water, and mix as before. This is dilution "B." 1.0 mL of this mixture is 0.00010 mL of the original sample or $1.0X10^{-4}$.

4. Use a sterile pipette to transfer 1.0 mL of dilution "B" to a sterile screw cap bottle containing 99 mL of sterile buffered dilution water, and mix as before. This is dilution "C." Go to step 5 for MPN analysis (preferred) or 7 for MF analysis.

5. For MPN analysis, follow procedure 9221 E. in SM. Four series of 5 tubes will be used for the analysis. Inoculate the first series of 5 tubes each with 10.0 mL of dilution "B." This is a 0.0010 mL of the original sample. The second series of tubes should be inoculated with 1.0 mL of dilution "B" (0.00010). The third series of tubes should receive 10.0 mL of "C" (0.000010). Inoculate a fourth series of 5 tubes each with 1.0 mL of dilution "C" (0.0000010). Continue the procedure as described in SM.

6. Refer to Table 9221.IV. in SM to estimate the MPN index/100 mL. Only three of the four series of five tubes will be used for estimating the MPN. Choose the highest dilution that gives positive results in all five tubes, and the next two higher dilutions for your estimate. Compute the MPN/g according to the following equation:

$$\text{MPN Fecal Coliform/g} = \frac{10 \times \text{MPN Index/100 mL}}{\text{largest volume} \times \% \text{ dry solids}}$$

Examples:

In the examples given below, the dilutions used to determine the MPN are underlined. The number in the numerator represents positive tubes; that in the denominator, the total number of tubes planted; the combination of positives simply represents the total number of positive tubes per dilution.

Example	0.0010 mL	0.00010 mL	0.000010 mL	0.0000010 mL	Combination of positives
a	5/5	5/5	3/5	0/5	5-3-0
b	5/5	3/5	1/5	0/5	5-3-1
c	0/5	1/5	0/5	0/5	0-1-0

For each example we will assume that the total solids content is 4.0%.

For example a:

The MPN index/100 mL from Table 9221.4 is 80. Therefore:

$$\text{MPN/g} = \frac{10 \times 80}{0.00010 \times 4.0} = 2.0 \times 10^6$$

For example b:

The MPN index/100 mL from Table 9221.4 is 110. Therefore:

$$\text{MPN/g} = \frac{10 \times 110}{0.0010 \times 4.0} = 2.8 \times 10^5$$

For example c:

The MPN index/100 mL from Table 9221.4 is 2. Therefore:

$$\text{MPN/g} = \frac{10 \times 2}{0.0010 \times 4.0} = 5.0 \times 10^3$$

5. Alternately the membrane filter procedure may be used to determine fecal coliform density. This method should only be used if comparability with the MPN procedure has been established for the specific sample medium. Three individual filtrations should be conducted in accordance with SM 9222 D. using 10.0 mL of dilution "C," and 1.0 mL and 10.0 mL of dilution "B." These represent 0.000010, 0.00010, and 0.0010 mL of the original sample. Incubate samples, and count colonies as directed. Experienced analysts are encouraged to modify this dilution scheme (e.g. half log dilutions) in order to obtain filters which yield between 20 and 60 CFU.

6. Compute the density of CFU from membrane filters which yield counts within the desired range of 20 to 60 fecal coliform colonies:

$$\text{coliform colonies/g} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample} \times \% \text{ dry solids}}$$

For Solid Samples:

1. In a sterile dish weigh out 30.0 grams of well mixed sample. Whenever possible, the sample tested should contain all materials which will be included in the sludge. For example, if wood chips are part of a sludge compost, some mixing or grinding means may be needed to achieve homogeneity before testing. One exception would be large pieces of wood which are not easily ground and may be discarded before blending. Transfer the sample to a sterile blender. Use 270 mL of sterile buffered dilution water to rinse any remaining sample into the blender. Cover and blend on high speed for two minutes. One milliliter of this sample contains 0.10 g of the original sample.

2. Use a sterile pipette to transfer 11.0 mL of the blender contents to a screw cap bottle containing 99 mL of sterile buffered dilution water and shake vigorously a minimum of 25 times. One milliliter of this sample contains 0.010 g of the original sample. This is dilution "A."

3. Follow the procedures for "Liquid Samples" starting at Step 3.

Examples:

Seven samples of a treated sludge were obtained prior to land spreading. The solids concentration of each sample was determined according to SM. These were found to be:

Sample No.	Solids Concentration (%)
1	3.8
2	4.3
3	4.0
4	4.2
5	4.1
6	3.7
7	3.9

The samples were liquid with some solids. Therefore the procedure for liquid sample preparation was used. Furthermore, the membrane filter technique was used to determine if the fecal coliform concentration of the sludge would meet the criteria for Class B alternative 1. Samples were prepared in accordance with the procedure outlined above. This yielded 21 individual membrane filters (MF) plus controls. The results from these tests are shown in Table 1

Table 1. Number of Fecal Coliform Colonies on MF Plates

Sample No.	0.000010 mL Filtration	0.00010 mL Filtration	0.0010 mL Filtration
1	0	1	23
2	2	18	TNTC
3	0	8	65
4	0	5	58
5	0	1	17
6	0	1	39
7	0	1	20

The coliform density is calculated using only those MF plates which have between 20 and 60 blue colonies whenever possible. However, there may be occasions when the total number of colonies on a plate will be above or below the ideal range. If the colonies are not discrete and appear to be growing together results should be reported as "too numerous to count" (TNTC). If no filter has a coliform count falling in the ideal range (20 - 60), total the coliform counts on all countable filters and report as coliform colonies/g. For sample number 2 the fecal coliform density is:

$$\text{coliform colonies/g} = \frac{(2+18)\times 100}{(0.000010 + 0.00010) \times 4.3} = 4.2 \times 10^6$$

Sample number 3 has two filters which have colony counts outside the ideal range also. In this case both countable plates should be used to calculate the coliform density/g. For sample number 3, the fecal coliform density is:

$$\text{coliform colonies/g} = \frac{(8 + 65) \times 100}{(0.00010 + 0.0010) \times 4.0} = 1.6 \times 10^6$$

Except for sample number 5, all of the remaining samples have at least one membrane filter within the ideal range. For these samples, use the number of colonies formed on that filter to calculate the coliform density. For sample number 1, the fecal coliform density is:

$$\text{coliform colonies/g} = \frac{23\times 100}{0.0010 \times 3.8} = 6.0 \times 10^5$$

Coliform densities of all the samples were calculated and converted to $\log_{10}$ values to compute a geometric mean. These calculated values are presented in Table 2.

Table 2. Coliform Density of Sludge Samples

Sample No.	Coliform Density	$\log_{10}$
1	6.0×10^5	5.78
2	4.2×10^6	6.63
3	1.6×10^6	6.22
4	1.4×10^5	6.14
5	4.0×10^5	5.60
6	1.0×10^6	6.02
7	5.1×10^5	5.71

The geometric mean for the seven samples is determined by averaging the $\log_{10}$ values of the coliform density and taking the antilog of that value.

$(5.78 + 6.63 + 6.22 + 6.14 + 5.60 + 6.02 + 5.71)/7 = 6.01$

The antilog of $6.01 = 1.03 \times 10^6$

Therefore, the geometric mean fecal coliform density is below 2 million and the sludge meets Class B Pathogen requirements under alternative 1.

1.2 Class A Alternative 1

Part 503 requires that, to qualify as a Class A sludge, treated sewage sludge must be monitored for fecal coliform (or *Salmonella* sp. and have a density of less than 1,000 MPN fecal coliform per gram of total solids (dry weight basis). The regulation does not specify total number of samples. However, it is suggested that a sampling event extend over two weeks and that at least seven samples be collected and analyzed. The membrane filter procedure may not be used for this determination. This is because the high concentration of solids in such sludges may plug the filter or, render the filter uncountable. The total solids content for each sample must be determined in accordance with procedure 2540 G. of SM.

For Liquid Samples:

1. Follow procedure 9221 E. in SM. Inoculate at least four series of five tubes using ten fold serial dilutions. Prepare the sample as described for "Class B Alternative 1, Liquid Samples," except inoculate each of the first series of tubes with 10.0 mL of the blender contents (the concentration of the enrichment broth must be adjusted to compensate for the volume of added sample). This is equivalent to adding 1.0 mL of sludge to the first series of tubes. Inoculate the remaining tubes and complete the analysis in accordance with SM.

2. Calculate the MPN as directed in Step 4 above.

For Solid Samples:

1. Follow procedure 9221 E. in SM. Inoculate at least four series of five tubes using ten fold serial dilutions. Prepare the sample as described for "Class B Alternative 1, Solid Samples," except inoculate each of the first series of tubes with 10.0 mL of the blender contents (the concentration of the enrichment broth must be adjusted to compensate for the volume of added sample). This is equivalent to adding 1.0 g of sludge (wet wieght) to the first series of tubes. Inoculate the remaining tubes and complete the analysis in accordance with SM.

2. Calculate the MPN as directed in step 4 above.

2. Sample Preparation for *Salmonella* sp. Analysis

Salmonella sp. quantification may be used to demonstrate that a sludge meets Class A criteria, instead of analyzing for fecal coliforms. Sludges with *Salmonella* sp. densities below 3 MPN/4 g total solids (dry weight basis) meet Class A criteria. The analytical method described in Appendix F of this document describes the procedure used to identify *Salmonella* sp. in a water sample. Similarly, the procedures for analysis of *Salmonella* sp. in SM (Section 9260 D) do not address procedures for sludges, the sample preparation step described here should be used, and the total solids content of each sample must be determined according to method 2540 G in SM.

For Liquid Samples:

1. Follow the same procedure used for liquid sample preparation for fecal coliform analysis described under "Class A Alternative 1." However, the enrichment medium used for this analysis should be dulcitol selenite broth (DSE) as described in Appendix G of this document or dulcitol selenite or tetrathionate broth as described in SM. Only three series of five tubes should be used for this MPN procedure. Use a sterile open tip pipette to transfer 10.0 mL of well mixed sample to each tube in the first series. These tubes should contain 10.0 mL of double strength enrichment broth. Each tube in the second series should contain 10.0 mL of double strength enrichment broth. These tubes should each receive 10.0

mL of the blended mixture. The final series of tubes should contain 10.0 mL of single strength enrichment broth. These tubes should each receive 1.0 mL of the blended mixture. Complete the MPN procedure as described in Appendix G or SM as appropriate.

2. Refer to Table 9221.IV. in SM to estimate the MPN index/100 mL. Calculate the MPN/4 g according to the following equation:

$$\textit{Salmonella}\text{ sp. MPN/4 g} = \frac{\text{MPN Index/100 mL} \times 4}{\%\text{ dry solids}}$$

For example:

If one tube in the first series was identified as being positive for *Salmonella* sp. and no other tubes were found to be positive, from Table 9221.IV one finds that a 1-0-0 combination of positives has an MPN index/100 mL of 2. If the percent of dry solids for the sample was 4.0, then:

$$\textit{Salmonella}\text{ sp. MPN/4g} \qquad \frac{2 \times 4}{4.0 = 2} = 2$$

For Solid Samples:

1. Follow the procedure for solid sample preparation for fecal coliform analysis described under Class A Alternative 1 above. However, the enrichment medium used for this analysis should be dulcitol selenite broth (DSE) as described in Appendix G or dulcitol selenite or tetrathionate broth as described in SM, and only three series of five tubes should be used for this MPN procedure. Use aseptic technique to weigh out and transfer 10.0 g of well mixed sample to each screw cap tube in the first series, shake vigorously to mix. These tubes should contain 10.0 mL of double strength enrichment broth. Likewise, each tube in the second series should contain 10.0 mL of double strength enrichment broth. These tubes should receive 10.0 mL of the blended mixture. The final series of tubes should contain 10.0 mL of single strength enrichment broth. These tubes should receive 1.0 mL of the blended mixture. Alternately, because the calculated detection limit is dependent upon the total solids content of the sample, samples with total solids contents >28% can be blended as described above and the blender contents can be used for inoculating the initial series of tubes. When this option is chosen, the final series of tubes will contain 0.1 mL of the blender contents. Complete the MPN procedure as described in Appendix G or SM as appropriate.

2. Refer to Table 9221.IV. in SM to estimate the MPN index/100 mL. Calculate the MPN/4 g according to the following equation:

$$\textit{Salmonella}\text{ sp. MPN/4g} = \frac{\text{MPN Index/1 00mL} \times 4}{\%\text{ dry solids}}$$