APPENDIX

CALCULATING LOG REDUCTIONS FOR INFECTIOUS WASTE TREATMENT TECHNOLOGIES

Infectious Waste Treatment Efficacy is evaluated by determining a specific "Log_{10} Reduction." "Log_{10} Reduction" is defined as the difference between the logarithm of the adjusted theoretical challenge (ATC) of bacterial spores in a test waste load and the number of viable bacterial spores recovered from that test waste load after treatment (VAT), calculated as follows:

$$Log_{10} \text{ Reduction} = Log_{10} (\text{ATC cfu/g}) - Log_{10} (\text{VAT cfu/g})$$

An applicant for an alternative infectious waste treatment technology approval should select the appropriate example depending on the method the applicant chooses to inoculate the waste, either:

A - Direct inoculation technique, or
B - Carrier system technique

A. DIRECT INOCULATION TECHNIQUE

RECOVERY TEST RUN:

The purpose of a recovery test run is to determine the amount of bacterial spore loss as a result of the physical aspects of the treatment unit and the ability to retrieve the bacterial spores from the waste load. During the recovery test run, the factor that causes microbial destruction is omitted. A recovery test run is performed for the bacterial spores, and the recovery test waste loads consist of the same waste types in the same combination as the test waste loads that will be used in the efficacy test runs, calculated as follows:

$$\%R = \frac{\text{R cfu/g}}{\text{TC cfu/g}} \times 100$$

Recovered (R) cfu/g is the number of viable bacterial spores per gram of waste recovered from the processed solid portion of the recovery test run, or the liquid

portion if the technology is designed to treat only infectious liquids. Note that this number is at least 1.0×10^8 for bacterial spores. When calculating the amount of inoculum to use to seed a test waste load it is important to consider the different factors, such as inherent treatment unit dilution and potential adherence of the bacterial spores to the items in the test waste load.

Theoretical challenge (TC) cfu/g is the known number of bacterial spores per gram of waste in the recovery test waste load. This number is determined by enumerating the stock solution of bacterial spores at the time each test waste load is inoculated. The enumeration is performed by serial dilution and triplicate plating of the appropriate dilutions on culture medium. The average number of colony forming units per milliliter of suspension is used to calculate the number of bacterial spores per gram of waste in the test waste load.

Percent Recovery (%R) is calculated by dividing the number of bacterial spores recovered from the processed recovery test waste load by the theoretical bacterial spore challenge of the recovery test waste load and then multiplying the result by one hundred. This percentage is used to determine the adjusted theoretical challenge of bacterial spores in the subsequent test waste loads.

Note: “cfu/g” is an expression for colony forming units per gram of waste solids.

TREATMENT TEST RUNS:

An **Adjusted Theoretical Challenge (ATC) cfu/g** is calculated for each test waste load. Upon inoculation of a test waste load with the bacterial spore suspension, the stock suspension of bacterial spores is enumerated to determine the TC per gram of waste of the test waste load. The ATC for that test waste load is then calculated using the TC for the run and %R determined from the recovery test run, as follows:

$$\text{ATC cfu/g} = \text{TC cfu/g} \times \%R$$

The samples of a test waste load are obtained and processed per the requirements set forth in this rule to determine the VAT. Upon determination of the VAT for the test waste load, the Log_{10} reduction in bacterial spores for that specific test waste load is calculated as follows:

$$\text{Log}_{10}\text{Reduction} = \text{Log}_{10}(\text{ATC cfu/g}) - \text{Log}_{10}(\text{VAT cfu/g})$$

EXAMPLE CALCULATIONS OF INFECTIOUS WASTE TREATMENT EFFICACY

This example is typical for treatment technologies that grind or shred infectious waste as a part of the treatment process.

Test organism – *Bacillus subtilis* bacterial spores in suspension.

Weight of test waste load is 50.0 pounds or 22,700 (2.27×10^4) grams. The size of the test waste load is representative of the actual full load capacity of the treatment unit for a single treatment cycle.

Amount and Concentration of Inoculum – A liquid bacterial spore suspension containing approximately 1.0×10^8 bacterial spores/milliliter was obtained. In this example, the minimum TC for a 50-pound test waste load was calculated to be 2.27×10^9 cfu (2.27×10^4 g $\times$ 1.0×10^8 cfu/g). Therefore, 22.7 milliliters of inoculum would be needed to obtain the necessary TC in a 50-pound test waste load. Since the percentage of recovery has not yet been calculated, the amount of inoculum was doubled to 45.4 milliliters (4.54×10^9 bacterial spores) to assure the attainment of the specified ATC.

In order to increase the chance that the entire waste load would be equally inoculated, the 45.4 milliliters of stock bacterial spore suspension was added to 954.6 milliliters of an appropriate buffer solution. Subsequently, the one liter of bacterial spore suspension, containing a total of approximately 4.54×10^9 bacterial spores, was evenly divided into 20 screw cap plastic test tubes (50 milliliters each) and distributed throughout the recovery test waste load. To verify the number of bacterial spores present in the stock suspension, three samples of the stock suspension were serially diluted and the 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} dilutions were plated in triplicate, with the counts observed after incubation recorded in Table 1.

Upon processing the recovery test run, nine separate 10.0-gram samples of processed solids were collected at equal time intervals as the waste exited the treatment unit. Upon collection of every third 10.0-gram sample, the three samples were combined to make a 30-gram composite sample. 270 milliliters of appropriate neutralizing buffer were added to the composite sample. (NOTE: These steps were performed immediately upon retrieval of every third sample.) The composite sample was blended to produce a homogenous 10^{-1} dilution of the composite sample. The remaining samples of processed waste were prepared in the same manner. Serial dilutions of the three composite samples were made and plated in triplicate, with the counts observed after incubation recorded in Table 2.

Table 1: Enumeration of the stock bacterial spore suspension:

	Sample #1			Sample #2			Sample #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-6}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-7}	135	129	130	132	134	135	131	132	131
10^{-8}	14	12	15	13	13	12	11	13	15
10^{-9}	1	0	1	1	2	1	0	1	2

By using the 10^{-7} dilution plates, which contain between 30 and 300 colony forming units, the stock bacterial spore suspension was enumerated as follows:

$$\frac{(135+129+130)+(132+134+135)+(131+132+131)}{9} \times 10^{7} = 1.32 \times 10^{9}$$

bacterial spores per milliliter

Number of bacterial spores in the stock suspension is 1.32×10^{9} bacterial spores per milliliter of stock suspension.

TC of the recovery test waste load was calculated as follows:

(1.32×10^{9} bacterial spores/milliliter)(45.4 milliliter suspension) = 5.99×10^{10} bacterial spores added to recovery test waste load.

$$\frac{5.99 \times 10^{10} \text{ bacterial spores}}{2.27 \times 10^{4} \text{ grams of test waste load waste}} = 2.64 \times 10^{6} \text{ bacterial spores/g}$$

TC = 2.64×10^{6} bacterial spores/g of waste recovery run

Table 2: Recovery Test Run Results

	Composite #1			Composite #2			Composite #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-3}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-4}	138	140	143	150	153	148	145	140	140
10^{-5}	12	15	13	17	17	16	15	13	12
10^{-6}	1	2	2	3	2	2	1	2	1

By using the 10^{-4} dilution plates, which contain between 30 and 300 colony forming units, the mean number of viable bacterial spores recovered (R) from the recovery test run was calculated as follows:

$$\frac{(138+140+143)+(150+153+148)+(145+140+140)}{9} \times 10^4 = 1.44 \times 10^6 \text{ cfu/g}$$

$$R = 1.44 \times 10^6 \text{ cfu/g}$$

%R of bacterial spores from the recovery test waste load was calculated as follows:

$$\frac{1.44 \text{ X } 10^6 \text{ cfu/g}}{2.64 \text{ X } 10^6 \text{ cfu/g}} \times 100$$

$$\%R = 54.5\%$$

TREATMENT RUN RESULTS:

Enumeration of the stock bacterial spore suspension used in this treatment run was performed and calculated as described above. The stock bacterial spore suspension contained 1.0×10^9 bacterial spores/milliliter.

The test waste load was inoculated with 45.4 milliliter of stock bacterial spore suspension. The TC per gram of waste in the test waste load was 2.18×10^6 bacterial spores. However, it was discovered in the recovery test run that only 54.5% of the number of bacterial spores processed through the unit can be recovered from the waste.

The ATC is calculated as follows:

$$ATC = TC\ cfu/g \times \%R = 2.64 \times 10^6\ cfu/g \times 54.5\% = 1.44 \times 10^6\ cfu/g$$

Note: The test waste load for the subsequent treatment test run was prepared and processed in the same manner as the recovery test waste load, except that the factor that causes microbial destruction was included.

Table 3: Treatment Test Run Results:

	Composite #1			Composite #2			Composite #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-1}	84	80	81	68	66	65	72	75	74
10^{-2}	11	14	15	4	6	6	9	9	8
10^{-3}	1	1	0	0	0	0	1	0	1

By selecting the dilution with plate counts between 30 and 300, the mean recovery of bacterial spores from the test waste load was calculated as follows:

$$\frac{(84+80+81)+(68+66+65)+(72+75+74)}{9} \times 10^1 = 739\ cfu/g$$

$$VAT = 7.39 \times 10^2\ cfu/g$$

Log_{10} Reduction is calculated as follows:

$$Log_{10}(ATC\ cfu/g) - Log_{10}(VAT\ cfu/g)$$

$$Log_{10}(1.44 \times 10^6\ cfu/g) - Log_{10}(7.39 \times 10^2\ cfu/g) = 6.16 - 2.87 = 3.29$$

A Log_{10} Reduction equal to 3.29 is insufficient to meet the 6 log reduction requirement.

B. CARRIER SYSTEM TECHNIQUE

RECOVERY TEST RUN

The purpose of a recovery test run is to determine the amount of bacterial spore loss as a result of the physical aspects of the treatment unit and the ability to retrieve the bacterial spores when utilizing a carrier system. During the recovery test run, the factor that causes microbial destruction is omitted. A recovery test run is performed for the bacterial spores and the recovery test waste loads consist of the same waste types in the same combination as the test waste loads that will be used in the efficacy test runs, calculated as follows:

$$\%R = \frac{\text{R cfu/carrier}}{\text{TC cfu/carrier}} \times 100$$

Recovered (R) cfu/carrier is the number of bacterial spores recovered from the carriers. Note that this number is at least 1.0×10^7 for bacterial spores. When calculating the amount of inoculum to apply to a carrier system it is important to consider the different factors, such as inherent treatment unit dilution, potential introduction of artifact due to the thickness of the layer of adhered spores, and potential adherence of the bacterial spores to the items in the test waste load.

Theoretical challenge (TC) cfu/carrier is the known number of bacterial spores present on each carrier in the recovery test waste load. The number is determined by enumerating the carrier directly at the time each test waste load is inoculated. The enumeration of a representative sampling of carriers is performed by serial dilution and triplicate plating of the appropriate dilutions on culture medium. The lowest average number of colony forming units is used to calculate the number of bacterial spores in the test waste load.

Percent Recovery (%R) is calculated by dividing the number of bacterial spores recovered from the processed recovery test waste load by the theoretical bacterial spore challenge of the recovery test waste load and then multiplying the result by one hundred. This percentage is used to determine the adjusted theoretical challenge of bacterial spores in the subsequent test waste loads.

Note: “cfu/g” is an expression for colony forming units per gram of waste solids.

TREATMENT TEST RUNS:

An **Adjusted Theoretical Challenge (ATC)** cfu/carrier is calculated for each test waste load. Upon inoculation of a test waste load with the bacterial spore carrier, a representative sampling of carriers is enumerated to determine the TC of the test waste load. The number of bacterial spores is determined by enumerating the carrier directly. This number is determined by enumerating a representative sampling of carriers to be used for bacterial spores at the time each test waste load is inoculated. The enumeration is performed by serial dilution and triplicate plating of the appropriate dilutions on culture medium. The lowest average number of colony forming units is used to calculate the ATC. The ATC for that test waste load is then calculated using the TC for the run and %R determined from the recovery test run, as follows:

$$\text{ATC cfu/carrier} = \text{TC cfu/carrier} \times \%R$$

The samples of a test waste load are obtained and processed per the requirements set forth in this rule to determine the viable bacterial spores remaining in the test waste load after treatment (VAT). Upon determination of the VAT for the test waste load, the Log_{10} reduction in viable bacterial spores for that specific test waste load is calculated as follows:

$$Log_{10}\ \text{Reduction} = Log_{10}(\text{ATC cfu/carrier}) - Log_{10}(\text{VAT cfu/carrier})$$

EXAMPLE CALCULATIONS OF INFECTIOUS WASTE TREATMENT EFFICACY

This is a typical example of any treatment technology that would utilize a carrier system.

Test Organism – *Bacillus subtilis* bacterial spores in suspension.

Weight of test waste load is 90 pounds. The size of the test waste load is representative of the actual full load capacity of the treatment unit for a single treatment cycle.

Amount and Concentration of Carriers – A liquid bacterial spore suspension containing approximately 1.0×10^8 bacterial spores/milliliter was obtained. In this example, the minimum carrier number is one carrier per ten pounds of test waste load. A 90-pound test waste load should contain a minimum of 9 carriers. Each carrier would need to contain 1.0×10^7 bacterial spores. Since the percentage of recovery has not yet been calculated, the amount of carrier inoculum was doubled to 2×10^7 bacterial spores to assure the attainment of the specified ATC.

To verify the number of bacterial spores present on each carrier, three carriers containing the initial stock suspension were serially diluted and the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were plated in triplicate.

Upon processing the recovery test run, the 9 carriers were collected as the waste exited the treatment unit. Upon collection of every third carrier, the three carriers were combined to make a 3-carrier composite sample. One hundred milliliters of an appropriate neutralizing buffer were added to the composite sample to wash the bacterial spores from the carrier. (NOTE: These steps were performed immediately upon retrieval of every third carrier.) The composite sample was washed to produce a homogenous 10^{-1} dilution of the composite sample. The remaining carrier samples were prepared in the same manner. Serial dilutions of the three composite samples were made and plated in triplicate with the counts observed after incubation recorded in Table 1.

Table 1: Enumeration of the stock bacterial spore suspension:

	Sample #1			Sample #2			Sample #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-3}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-4}	135	129	130	132	134	135	131	132	131
10^{-5}	14	12	15	13	13	12	11	13	15
10^{-6}	1	0	1	1	2	1	0	1	2

By using the 10-4 dilution plates, which contain between 30 and 300 colony forming units, the stock bacterial spore suspension was enumerated as follows:

$$\frac{(135+129+130)+(132+134+135)+(131+132+131)}{9} \times 10^4 = 1.32 \times 10^6 \text{ bacterial spores}$$

Number of bacterial spores is 1.32×10^6 bacterial spores per carrier.

TC of the recovery test waste load was calculated as follows:

TC = 1.32×10^6 bacterial spores per carrier used in the recovery run

Table 2: Recovery Test Run Results

	Composite #1			Composite #2			Composite #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-3}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-4}	98	100	103	110	113	108	105	100	100
10^{-5}	12	15	13	17	17	16	15	13	12
10^{-6}	1	2	2	3	2	2	1	2	1

By using the 10^{-4} dilution plates, which contain between 30 and 300 colony forming units, the mean number of viable bacterial spores recovered (R) from the recovery test run was calculated as follows:

$$\frac{(98+100+103)+(110+113+108)+(105+100+100)}{9} \times 10^4 = 1.04 \times 10^6 \text{ cfu/carrier}$$

$$R = 1.04 \times 10^6 \text{ cfu/carrier}$$

%R of bacterial spores from the recovery test waste load was calculated as follows:

$$\frac{1.04 \times 10^6 \text{ cfu/carrier}}{1.32 \times 10^6 \text{ cfu/carrier}} \times 100 = 78.79\%$$

$$\%R = 78.79\%$$

TREATMENT TEST RUN RESULTS:

Enumeration of the stock bacterial spore suspension used in this treatment run was performed and calculated as described above. The stock bacterial spore suspension contained 1×10^9 bacterial spores/milliliter.

The test waste load was inoculated with 9 carriers each with 1.32×10^6 bacterial spores. However, it was discovered in the recovery test run that 78.79% of the number of bacterial spores processed through the unit can be recovered from the carriers.

The ATC is calculated as follows:

$$ATC = TC\ cfu/carrier \times \%R = 1.32 \times 10^6\ cfu/carrier \times 78.79\% = 1.04 \times 10^6\ cfu/carrier$$

Note: The test waste load for the subsequent treatment test run was prepared and processed in the same manner as the recovery test waste load, except that the factor that causes microbial destruction was included.

Table 3: Treatment Test Run Results

	Composite #1			Composite #2			Composite #3		
Dilution	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
10^{-1}	84	80	81	68	66	65	72	75	74
10^{-2}	11	14	15	4	6	6	9	9	8
10^{-3}	1	1	0	0	0	0	1	0	1

By selecting the dilution with plate counts between 30 and 300, the mean recovery of bacterial spores from the test waste load was calculated as follows:

$$\frac{(84+80+81)+(68+66+65)+(72+75+74)}{9} \times 10^1 = 739\ cfu/carrier$$

$$VAT = 7.39 \times 10^2\ cfu/carrier$$

Log_{10} Reduction is calculated as follows:

$$Log_{10}\ reduction = Log_{10}\ (ATC\ cfu/carrier) - Log_{10}\ (VAT\ cfu/carrier)$$

$$Log_{10}(1.04 \times 10^6\ cfu/carrier) - Log_{10}(7.39 \times 10^2\ cfu/carrier) = 6.017 - 2.869 = 3.148$$

A Log_{10} Reduction equal to 3.148 is insufficient to meet the 6 log reduction requirement.