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 (A)djusted (T)heoretical (C)hallenge (ATC) of test microorganisms or spores in a treatment test load and the number of (V)iable test microorganisms of spores recovered from that treatment test load (A)fter (T)reatment (VAT).

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

A - **Direct inoculation technique**
B - **Carrier system technique**

DIRECT INOCULATION TECHNIQUE

RECOVERY TEST RUN:

The purpose of a recovery test run is to determine the **percent of microorganisms or spores that can be recovered from an inoculated test load.** During the recovery test run, the factor that causes microbial destruction is omitted. A recovery test run shall be performed for the spore and each microorganism. In addition, the recovery test loads shall consist of the same waste types in the same combination as the treatment test loads that will be used in the efficacy test runs.

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

Theoretical challenge cfu/g (TC) is the known number of microorganisms or spores per gram of waste in the recovery test load. This number shall be determined by enumerating the stock solution of each microorganism or spore at the time each test load is inoculated. The enumeration shall be 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 shall be used to calculate the number of microorganisms or spores per gram of waste in the test load.

Recovered cfu/g (R) is the number of viable test microorganisms, on a per gram basis, 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 must be at least 1.0×10^6 for mycobacteria and at least 1.0×10^5 for spores. When calculating the amount of inoculum to use to seed a test load it is important to consider the different factors, such as inherent treatment unit dilution and potential adherence of the microorganism or spore to the items in the test load.

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

TREATMENT TEST RUNS:

An **adjusted theoretical challenge (ATC)** must be calculated for each treatment test load. Upon inoculation of a test load with the microbial or spore suspension, the stock suspension of microorganism or spore must be enumerated to determine the theoretical challenge, on a per gram basis, of the treatment test load. The adjusted theoretical challenge (ATC) for that treatment test load is then calculated using the theoretical challenge for the run and %R determined from the recovery test run.

Calculation: cfu/g TC X %R = cfu/g ATC

The samples of a treatment test load shall be obtained and processed per the requirements set forth in this rule to determine the (V)iable microorganisms or spores remaining in the test load (A)fter (T)reatment (VAT). Upon determination of the VAT for the treatment test load, the Log_{10} reduction in viable microorganisms or spores, for that specific treatment test load, is calculated as follows:

Calculation: Log_{10}(cfu/g ATC) - Log_{10} (cfu/g VAT) = Log_{10} Reduction

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

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Example Calculations of Infectious Waste Treatment Efficacy

This example is typical of treatment technologies that grind or shred infectious waste as a part of the treatment process. Please note that this example is not intended to employ all of the requirements found in Rule 3745-27-38 of the Ohio Administrative Code.

Test Organism – *Bacillus subtilis* spores in suspension

Weight of Test Load = 50.0 pounds, or 22,700 grams. The size of the test load is representative of the actual full load capacity of the treatment unit per the time it takes for the waste to be processed through the machine.

Amount and Concentration of Inoculum – A liquid spore suspension containing approximately 1.0×10^8 spores/ml was obtained. The minimum theoretical challenge (TC) for a 50 pound test load was calculated to be 2.27×10^9 spores (22,700 grams $\times$ 1.0×10^5 spores/gram). Therefore, 22.7 mls of inoculum would be needed to obtain the necessary theoretical challenge in a 50 pound test load. Since the percentage of recovery has not yet been calculated, the amount of inoculum was doubled to 45.4 mls (4.54×10^9 spores) to assure the attainment of the required adjusted theoretical challenge (ATC).

In order to increase the chance that the entire waste load would be equally inoculated, the 45.4 mls of stock spore suspension was added to 954.6 mls of an appropriate buffer solution. Subsequently, the one liter of spore suspension, containing a total of approximately 4.54×10^9 spores, was evenly divided into 20 screw cap plastic test tubes (50 mls each) and distributed throughout the recovery test load. To verify the number of spores present in the stock suspension, three samples of the stock suspension were serially diluted and the 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions were plated in triplicate.

Upon processing the recovery test run, nine (9) 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. Two hundred and seventy milliliters of appropriate neutralizing buffer were added to the composite sample. (NOTE: These steps were performed immediately upon retrieval of every third sample.) Using a waring blender, 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 following counts observed after incubation:

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Table 1: Enumeration of the stock 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^{-5}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-6}	135	129	130	132	134	135	131	132	131
10^{-7}	14	12	15	13	13	12	11	13	15
10^{-8}	1	0	1	1	2	1	0	1	2

By properly using the 10^{-6} dilution plates, which contain between 30 and 300 colony forming units, the stock spore suspension was enumerated:

$$\frac{(135+129+130)+(132+134+135)+(131+132+131)}{9} \times 10^{6} = 132111111 \text{ spores/ml}$$

Number of spores in = 1.32×10^{8} spores/ml stock suspension

Note: the spore stock suspension contained more than estimated amount of 1×10^{8} spores/ml.

Theoretical Challenge (TC) of the recovery test load was calculated as follows:

$(1.32 \times 10^{8}$ spores/ml)(45.4 ml suspension) = 5.99×10^{9} spores added to recovery test load.

$$\frac{5.99 \times 10^{9} \text{ spores}}{22{,}700 \text{ grams of test load waste}} = 2.64 \times 10^{5} \text{ spores/g}$$

TC = 2.64×10^{5} 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^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-3}	138	140	143	150	153	148	145	140	140
10^{-4}	12	15	13	17	17	16	15	13	12
10^{-5}	1	2	2	3	2	2	1	2	1

By properly using the 10^{-3} dilution plates, which contain between 30 and 300 colony forming units, the mean number of viable spores recovered (R) from the recovery test run was calculated:

$\frac{(138+140+143)+(150+153+148)+(145+140+140)}{9} \times 10^3 = 144000$ cfu/gram

$R = 1.44 \times 10^5$ spores/gram

Percent recoverability (%R) of spores from the recovery test load was:

$\frac{1.44 \times 10^5 \text{ cfu/gram R}}{2.64 \times 10^5 \text{ cfu/gram TC}} \times 100 = 54.5\%$

%R = 54.5%

Treatment Run Results:

Enumeration of the stock spore suspension used in this treatment run was performed and calculated as described above. The stock spore suspension contained 1.09×10^8 spores/ml.

The treatment test load was inoculated with 45.4 ml of stock spore suspension. The TC per gram of waste in the test load was 2.18×10^5 spores. However, it was discovered in the recovery test run that only 54.5% of the number of spores processed through the unit can be recovered from the waste. Therefore, the ATC is 1.19×10^5 spores/gram of waste.

Note: The treatment test load for the subsequent treatment test run was prepared and processed in the same manner as the recovery test 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	91*
10^{-2}	11	14	15	4	6	6	9	9	8
10^{-3}	1	1	0	0	0	0	1	0	1

By properly selecting the dilution with plate counts between 30 and 300, the mean recovery of spores from the treatment test load was:

$$\frac{(84+80+81)+(68+66+65)+(72+75)}{8^*} \times 10^2 = 740 \text{ cfu/gram}$$

$R = 7.40 \times 10^2$ cfu/gram

*** Note that the replicate plate containing 91 colonies was not used in the calculations as dictated by Paragraph (E)(10) of this Rule.**

Log_{10} Reduction:

$Log_{10}(1.19 \times 10^5 \text{ cfu/g}) - Log_{10}(7.40 \times 10^2 \text{ cfu/g}) = Log_{10}$ Reduction

5.076 - 2.869 = 2.207

A Log_{10} Reduction = 2.207 is insufficient to meet the 4 log reduction requirement for spores. Therefore, the technology would have to be altered in order to meet the reduction standard.

CARRIER SYSTEM TECHNIQUE

RECOVERY TEST RUN:

The purpose of a recovery test run is to determine the **percent of microorganisms or spores that can be recovered from utilizing a carrier system.** During the recovery test run the factor that causes microbial destruction is omitted. A recovery test run shall be performed for the spore and each microorganism. In addition, the recovery test loads shall consist of the same waste types in the same combination as the treatment test loads that will be used in the efficacy test runs.

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

Theoretical challenge cfu/g (TC) is the known number of microorganisms or spores present on each carrier in the recovery test load. The number shall be determined by enumerating the carrier directly at the time each test load is inoculated. The enumeration of a representative sampling of carriers shall be performed by serial dilution and triplicate plating of the appropriate dilutions on culture medium. The lowest average number of

colony forming units shall be used to calculate the number of microorganisms or spores in the test load.

Recovered cfu/g (R) is the number of viable test microorganisms 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 must be at least 1.0×10^6 for mycobacteria and at least 1.0×10^5 for 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 and potential adherence of the microorganism or spore to the items in the test load.

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

TREATMENT TEST RUNS:

An **adjusted theoretical challenge (ATC)** must be calculated for each treatment test load. Upon inoculation of a test load with the microbial or spore carrier, a representative sampling of carriers must be enumerated to determine the theoretical challenge of the treatment test load. The number of microorganism or spores shall be determined by enumerating the carrier directly. This number shall be determined by enumerating a representative sampling of carriers to be used of each microorganism or spore at the time each test load is inoculated. The enumeration shall be performed by serial dilution and triplicate plating of the appropriate dilutions on culture medium. The lowest average number of colony forming units shall be used to calculate the adjusted theoretical challenge (ATC). The adjusted theoretical challenge (ATC) for that treatment test load is then calculated using the theoretical challenge for the run and %R determined from the recovery test run.

Calculation: cfu TC X %R = cfu ATC

The samples of a treatment test load shall be obtained and processed per the requirements set forth in this Rule to **determine the (V)iable microorganisms or spores remaining in the test load (A)fter (T)reatment (VAT).** Upon determination of the VAT for the treatment test load, the Log_{10} reduction in viable microorganisms or spores, for that specific treatment test load, is calculated as follows:

Calculation: Log_{10}(cfu ATC) - Log_{10}(cfu VAT) = Log_{10} Reduction

Note: “cfu” is an expression for colony forming units.

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Example Calculations of Infectious Waste Treatment Efficacy

This is a typical example of any treatment technology that would utilize a carrier system. Please note that this example is not intended to employ all of the requirements found in Rule 3745-27-38 of the Ohio Administrative Code.

Test Organism - *Bacillus subtilis* spores in suspension

Weight of Test Load = 90.0 pounds. The size of the test load is representative of the actual full load capacity of the treatment unit per the time it takes for the waste to be processed through the machine.

Amount and Concentration of Carriers - A liquid spore suspension containing approximately 1.0×10^8 spores/ml was obtained. The minimum carrier number is one carrier per ten pounds of test waste load. A 90 pound test load should contain a minimum of nine (9) carriers. Each carrier would need to contain 1.0×10^5. Since the percentage of recovery has not yet been calculated, the amount of carrier inoculum was doubled to 2×10^5 spores to assure the attainment of the required adjusted theoretical challenge (ATC).

To verify the number of spores present on each carrier, three carriers containing the initial stock suspension were serially diluted and the 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated in triplicate.

Upon processing the recovery test run, the nine (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 three (3) carrier composite sample. One hundred milliliters of an appropriate neutralizing buffer were added to the composite sample to wash the 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 following counts observed after incubation:

Table 1: Enumeration of the stock 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^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-3}	135	129	130	132	134	135	131	132	131
10^{-4}	14	12	15	13	13	12	11	13	15
10^{-5}	1	0	1	1	2	1	0	1	2

By properly using the 10^{-3} dilution plates, which contain between 30 and 300 colony forming units, the stock spore suspension was enumerated:

$\frac{(135+129+130)+(132+134+135)+(131+132+131)}{9} \times 10^3 = 132111111$ spores

Number of spores = 1.32×10^5 spores per carrier

Note: the individual spore carriers contained more than estimated amount of 1×10^5 spores/ml.

Theoretical Challenge (TC) of the recovery test load was calculated as follows:

TC = 1.32×10^5 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^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-3}	98	100	103	110	113	108	105	100	100
10^{-4}	12	15	13	17	17	16	15	13	12
10^{-5}	1	2	2	3	2	2	1	2	1

By properly using the 10^{-3} dilution plates, which contain between 30 and 300 colony forming units, the mean number of viable spores recovered (R) from the recovery test run was calculated:

$\frac{(98+100+103)+(110+113+108)+(105+100+100)}{9} \times 10^3 = 104111$ cfu

R = 1.04×10^5 spores

Percent recoverability (%R) of spores from the recovery test load was:

$\frac{1.04 \times 10^5 \text{ cfu/gram R}}{1.32 \times 10^5 \text{ cfu/gram TC}} \times 100 = 78.7\%$

%R = 78.7%

Treatment Run Results:

Enumeration of the stock spore suspension used in this treatment run was performed and calculated as described above. The stock spore suspension contained 1×10^8 spores/ml.

The treatment test load was inoculated with 9 carriers each with 1.32×10^5. However, it was discovered in the recovery test run that 78.7% of the number of spores processed through the unit can be recovered from the waste.

Calculation: TC(cfu) X %R = ATC(cfu)

1.32×10^5 cfu/gram TC X 78.7% = 1.03×10^5 ATC(cfu)

Therefore, the ATC is 1.03×10^5.

Note: The treatment test load for the subsequent treatment test run was prepared and processed in the same manner as the recovery test 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	91*
10^{-2}	11	14	15	4	6	6	9	9	8
10^{-3}	1	1	0	0	0	0	1	0	1

By properly selecting the dilution with plate counts between 30 and 300, the mean recovery of spores from the treatment test load was:

$$\frac{(84+80+81)+(68+66+65)+(72+75)}{8^*} \times 10^2 = 740 \text{ cfu/gram}$$

$R = 7.40 \times 10^2$ cfu/gram

*** Note that the replicate plate containing 91 colonies was not used in the calculation as dictated by Paragraph (E)(10) of this Rule.**

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Log_{10} Reduction:

$Log_{10}(1.03 \text{ X } 10^5 \text{ cfu/g}) - Log_{10}(7.40 \text{ X } 10^2 \text{ cfu/g}) = Log_{10}$ Reduction

5.012 - 2.869 = 2.143

A log_{10} Reduction = 2.143 is insufficient to meet the 4 log reduction requirement for spores. Therefore, the technology would have to be altered in order to meet the reduction standard.