control T_{60} impinger was sampled and plated in duplicate. Remaining volumes of PBDW/spore suspension were measured and recorded for each impinger.

Table 5 shows survival of BSN as CFU and as a per cent recovery as compared to the control impingers. At 20 mins, mean BSN spore survival was 26%, falling to 5% at 40 mins and 11% at 60 minutes. These data indicate that *B. subtilis* var. *niger* spores do not remain viable in AGI-30 impingers for extended sampling periods, and that as sampling time increases, so also does collection fluid loss.

Table 5. Survival of Uninjured Spores of *B. subtilis* var. *niger* in All Glass Impingers

Sample	Counts (CFU)	CFU/ml	Vol (ml)	Total CFU	% Recovery	
					trial[a]	mean[b]
C T_0	111/140	1255	20.0	25100		
C T_{60}	141/118	1295	20.0	25900		
A T_{20}	52/59/59	567	14.0	7940	31	
B T_{20}	34/47/35	387	13.9	5380	21	26
A T_{40}	9/7/6	73	9.5	690	3	
B T_{40}	21/20/20	203	9.8	1990	8	5
A T_{60}	93/104/85	940	5.1	4790	19	
B T_{60}	20/11/16	157	4.8	750	3	11

[a] = Total CFU of trial/mean of total CFU of C T_0 and C T_{60}
[b] = Mean of total CFU A and B/mean of total CFU of C T_0 and C T_{60}

B. stearothermophilus spore survival is given in Table 6. There is little loss of viability until after 40 mins of operation of the impingers. At 60 mins, 62% of the spores were still viable.

Table 6. Survival of Uninjured Spores of *B. stearothermophilus* in All-Glass Impingers

Sample	Counts (CFU)	CFU/ml	Vol (ml)	Total CFU	% Recovery	
					trial[a]	mean[b]
C T_0	93/118	910	20.0	21100		
C T_{60}	61/84/144	963	20.0	19300		
A T_{20}	115/114/145	1297	14.0	17500	87	
B T_{20}	125/155/122	1340	13.9	18600	92	89
A T_{40}	184/153/180	1723	9.5	16300	81	
B T_{40}	N.D./138/146	1420	9.8	13900	69	75
A T_{60}	214/230/251	2317	5.1	11800	58	
B T_{60}	279/N.D.	2790	4.8	13400	66	62

[a] = Total CFU of trial/mean of total CFU of C T_0 and C T_{60}
[b] = Mean of total CFU A and B/mean of total CFU of C T_0 and C T_{60}
N.D. = No data

3.6.2 Survival of Injured Spores

As it is assumed that microorganisms emitted from medical waste treatment processes are injured to some degree, the survival of injured spores in the impingers was investigated. This experiment was identical to the survival experiment with fresh, uninjured spores with a few exceptions. Both control impingers (C T_0 and C T_{60}) were sampled before and after the vacuum pumps were operated. The control samples were plated in triplicate, rather than in duplicate. Based on the results of BSN survival above, the sample periods were shortened to 5, 10, and 20 minutes. To simulate

spore injury by thermal treatment, the spore suspension (4.5 ml @1 x 10^5 CFU/ml) was heated to 100°C for 20 mins immediately prior to the experiment.

BSN spores did not survive the heat treatment. Two CFU were recovered from control impinger samples, and two CFU were recovered from the six trial impinger samples (data not shown). As expected, BST spores were very resistant to moist heat inactivation. Following heat treatment of the BST stock, 84% of the spores remained viable. Survival of heat-treated spores in operating impingers was also high. Table 7 shows that after 5 mins of impinger operation, no spore viability was lost. After 10 and 20 mins of operation, spore recovery was 86% and 82%, respectively.

Table 7. Survival of Heat Treated Spores of *B. stearothermophilus* in All-Glass Impingers

Sample	Counts (CFU)	CFU/ ml	Vol (ml)	Total CFU	% Recovery	
					trial[b]	mean[c]
CA T_0	92/N.D./115	1035	20.0	20700		
CB T_0	110/100/89	997	20.0	19900		
A T_5	104/89/151	1147	18.8	21600	107	
B T_5	142/96/105	1143	18.2	20800	103	105
A T_{10}	89/111/87	957	18.0	17200	85	
B T_{10}	105/98/85	960	18.3	17600	87	86
A T_{20}	117/103/105	1083	15.3	16600	82	
B T_{20}	100/89/134	1077	15.3	16500	82	82
CA T_{20}	85/101/112	993	20.0	19900		
CB T_{20}	100/99/105	1013	20.0	20300		

[a] = (Total CFU of trial/mean of total CFU of all controls)x 100
[b] = (Mean of total CFU A and B/mean of all controls)x 100
N.D. = No data

3.6.3 Spore Slippage from AGI-30s

In addition to verifying spore survival during sampler operation, design of a monitoring approach requires a determination of degree of spore slippage through the impingers. Such slippage has been reported as 0.3 - 0.5% of BSN for the AGI-4 (Harstad and Filler, 1969; Tyler and Shipe, 1959), although no data have been found for BST or BSN slippage through AGI-30s. Because of the similar design, slippage was expected to be low for the AGI-30. To assess the rate of slippage, pairs of impingers were connected in series with the first impinger containing a spore suspension, and the second, or rear impingers, containing only PBDW. The impingers were operated for 20 mins followed by assay of the contents of the front and rear impingers.

Fresh stocks of BSN and BST spores were diluted and mixed to yield a suspension containing ~1 x 10^5 CFU/ml of each organism. Four impingers were labelled "front" and filled with 19.7 ml PBDW, and four were labelled "rear" and filled with 20 ml PBDW. Stock spore suspension was added to the front impingers (0.3 ml each) and sampled for enumeration as a control. Four pairs of front and rear impingers were connected in series with Tygon tubing and then connected to vacuum pumps, which were operated for 20 mins at 41 mm Hg. Front impingers were sampled and plated in triplicate on TSA for each organism as described above. The volume of impinger fluid remaining was measured and recorded. Impinger fluid from the rear impingers was divided into two portions, measured, and filtered through separate 0.2 µm membranes. The membranes were placed on TSA for incubation at 37°C and 55°C for growth of BSN and BST retained on the membranes.

Results showed very low slippage of spores from the front impingers for both organisms (Tables 8, 9). Slippage can be calculated based on the spores recovered from the front impinger (Surviving) or on the amount inoculated into the front impinger (Total). Using the more conservative calculation, slippage of surviving spores, BSN showed a mean slippage of 0.2% (Table 8), while mean BST slippage was 0.3% in four trials.

Table 8. Slippage of *B. subtilis* var. *niger* Through AGI-30 Impingers

Sample	Spore Counts (CFU)		Slippage (%)	
	Front	Rear	Surviving[a]	Total[b]
A	22000	4	0.02	0.01
B	7400	17	0.20	0.04
C	4700	<2	<0.04	<0.005
D	2700	16	0.60	0.04
Mean			.20	.02

[a] = (Rear CFU / Front recovered spores CFU) x 100
[b] = (Rear CFU / Applied Spores 3.8 x 10^4 CFU) x 100
< = At detection limit of assay, no colonies counted

Table 9. Slippage of *B. stearothermophilus* Through AGI-30 Impingers

Sample	Spore Counts (CFU)		Slippage (%)	
	Front	Rear	Surviving[a]	Total[b]
A	26900	73	0.3	0.2
B	23500	82	0.3	0.3
C	24800	38	0.2	0.1
D	20400	65	0.3	0.2
Mean			0.3	0.2

[a] = (Rear CFU / Front recovered spores CFU) x 100
[b] = (Rear CFU / Applied Spores 3.2 x 10^4 CFU) x 100

3.7 DISCUSSION

Methods for field evaluation of biological emissions from medical waste treatment devices can be selected based upon the above results. Bacterial endospores can be grown on Trypticase Soy Agar (TSA) and thermophilic *B. stearothermophilus* can be selectively grown at elevated temperature with no interference from other spore-forming bacteria. Similarly, a pigmented spore former, *B. subtilis* var. *niger*, can be grown and differentiated from endogenous organisms by its coloration on three different agars. BSN forms orange colonies on TSA, is more intensely pigmented on beef extract glucose agar, and becomes black on tyrosine agar. TSA provided the highest counts of the three agars.

Because the primary spore sampling method is impingement, spore survival and slippage were also determined. Slippage was minimal (≤0.6%), and consistent with published results for the AGI-4. BSN spores proved more labile than BST spores, with loss of viability approaching 90% after one hour of cycling in the impingers compared to a 38% loss for BST. At shorter cycling times, losses of both spores were less. After 20 mins, 89% of BST spores remained viable, as did 26% of BSN spores. Heat treatment inactivated nearly all BSN spores, but did not affect BST. At least 80% of heat treated BST spores were recovered after twenty mins of cycling in impingers. Although both organisms survived simulated sampling in the impingers, BST was clearly more stable than BSN, particularly after heat treatment. For field monitoring of emissions, *B. stearothermophilus* is the better choice for thermal treatment devices.

3.8 INDICATOR MICROORGANISM RECOVERY RECOMMENDATIONS

Based upon the data generated from the studies described, it is recommended that Trypticase Soy Agar be used as the primary medium for recovery of indicator spores from air and fluid monitoring of potential medical waste treatment bioemissions. Confirmation of *B. subtilis* var. *niger* recovery and growth can be made by subculture to Tyrosine Agar and/or Beef Extract/Glucose Agar.

In order to maximize airborne spore recovery, it is recommended that air monitoring with AGI-30s not exceed 20 minutes. Large volume air samples should be collected with replicate samples.

4.0 SAMPLING APPROACH

The primary objective of this study was to assess the bioemission potential of selected alternative medical waste treatment technologies, using both spiked and non-spiked regulated medical waste. Spiked waste was seeded with large numbers of resistant bacterial endospores. The monitoring of each treatment system focused on demonstrating the presence or absence of the indicator spores from previously identified exhaust air and fluid emission points during waste treatment. Bioaerosol monitoring was conducted using samplers required or recommended in published standards, primarily the American Society for Testing Materials (ASTM) *Standard Practice for Sampling Airborne Microorganisms at Municipal Solid-Waste Processing Facilities* (E884-82).

4.1 INDICATOR MICROORGANISMS

Bacillus stearothermophilus spores ATCC 12980
Bacillus subtilis var. *niger* ATCC 9372

Commercially prepared spore suspensions were purchased and used as indicator organisms for all medical waste emission testing. *Bacillus stearothermophilus* (BST) spore suspensions were purchased from Difco Laboratories, Detroit, MI, Lot# 23731. The spore suspensions were in 1 ml vials and reportedly contained 1.0×10^8 spores per ml. Spore concentrations were verified by laboratory enumerations. Ten x 1 ml BST vials were combined and on two occasions serial dilutions of three aliquots were plated in triplicate. The suspension was also used to inoculate 9 x 0.2 µm polycarbonate filters with 1 ml of suspension on each. Dried BST spores were recovered and serial dilutions were plated in triplicate on three of the filters at 0, 24, and 48 hrs. This provided an initial laboratory quantitation and verification of survival of spores that were dried onto filters and used as the test challenge in steam autoclaves. Another BST vial enumeration was performed by combining two vials and plating serial dilutions of duplicate aliquots in triplicate. All of the vial enumeration results prior to field testing were averaged and used to calculate all field test spore challenges. Field test spore challenges were calculated by averaging initial spore vial or filter laboratory enumerations with spore vials or filters which were taken to the test site, returned with the test samples, and enumerated in the laboratory. All reported spore test challenges represent a minimum number of spores used in the testing.

Bacillus subtilis var. *niger* (BSN) spore suspensions were purchased from AMSCO, Erie, PA, Lot# LG013C. The spore suspensions were supplied in 10 ml vials and reportedly contained 2-4 x 10^8 spores per ml. The 10 ml vials were aliquoted into vials with 1 ml of suspension for field tests. Spore concentrations were verified by laboratory enumerations. For two of the 10 ml vials, three aliquots were serially diluted and plated in triplicate. For a third vial, two aliquots were serially diluted and plated in triplicate. All of the vial enumeration results prior to field testing were averaged and used to calculate all field test spore challenges. One of the vials was used to inoculate 9 x 0.2 μm polycarbonate filters with 1 ml each. Dried BSN spores were recovered and serial dilutions were plated in triplicate on three of the filters at 0, 24, and 48 hrs. This provided an initial laboratory quantitation and verification of survival of spores that were dried onto filters and used as the test challenge in steam autoclaves. Field test BSN spore challenges were calculated as were those of BST. All reported spore test challenges represent a minimum number of spores used in the testing.

4.2 MEDIA AND REAGENTS

Trypticase Soy Agar (TSA)
Tyrosine Agar (TYR)
Phosphate Buffer Dilution Water (PBDW)
API 50-CHB
API-20E

Trypticase Soy Agar was used as the primary medium for the recovery (isolation and growth) of collected BST and BSN indicator spores from air or fluid samples. Colonies of *Bacillus* growth presumptively identified as *B. subtilis* var. *niger*, were confirmed as BSN by isolation on L-tyrosine agar. On TYR, colonies of BSN will turn black after 72 hours. BST spore colonies were confirmed by their characteristic morphology and pigmentation, temperature growth requirements, and API 50-CHB and API-20E biochemical assays (Biomeriveux Vitek, Hazelwood, MO). Sterile PBDW (pH 7.2) served as the impinger collection fluid.

4.3 BIOAEROSOL SAMPLERS

All-Glass Impinger (AGI-30)
Mattson/Garvin Slit-To-Agar Sampler (M/G)
Andersen 2-Stage Cascade Sieve Impactor (AND -2)
Andersen N6 1-Stage Cascade Sieve Impactor (AND -1)

The AGI-30 operates at a flow rate of 12.5 liters/minute. The M/G, AND-2, and AND-1 impactors operate at a flow rate of 28.3 liters/minute.

4.4 GENERAL SAMPLING PLAN

For each of the treatment technologies evaluated, replicate samples of air from identified potential emission points were collected: 1) during ambient conditions without waste processing; 2) during waste processing of non-spiked medical waste; and, 3) during waste processing of medical waste spiked with indicator organisms. Length of sampling times and numbers of sample replicates were dependent to a large extent on the waste processing time for each technology. Fluid effluent samples were collected as generated from appropriate technologies, during the processing of both spiked and non-spiked wastes. (Specific sampling plans for each sampling site and technology are provided in sections 6.0 - 9.0), and were based on direct observations from prior, individual site visits.

The focus of all the emissions sampling was to attempt to recover any spores of *B. stearothermophilus* and/or *B. subtilis* var. *niger* that were seeded into medical waste being processed by each of the treatment technologies, as indicators of the release of potential pathogens to the surrounding environment. Air sampling during ambient conditions when no medical waste was treated provided for background levels of the indicator *Bacillus* spores in the natural environment. Likewise, sampling for the recovery of *Bacillus* spores during the treatment of non-spiked medical waste provided comparative background levels for assessment of the results of attempted recovery of indicator *Bacillus* spores from emissions during the treatment of spiked medical waste. Neither sampling under ambient nor non-spiked medical waste treatment conditions was done to characterize the natural airborne microbial flora in the medical waste treatment environment. Sampling was done solely to assess the potential for each technology to emit bioaerosols during the treatment process.

Ambient air sampling was conducted using Mattson/Garvin slit-to-agar samplers, in conjunction with Andersen two-stage cascade sieve impactors. Air emissions sampling during waste processing was conducted using AGI-30s, and Mattson/ Garvin and Andersen 2-stage samplers in exactly the same manner for both spiked and non-spiked wastes.

4.5 INDICATOR ORGANISM RECOVERY

Following daily monitoring, all samples were shipped in insulated containers to the RTI Environmental Microbiology Department laboratories and processed within 24 hours. Impinger and liquid effluent fluids were kept chilled during transport.

Impactor samples were collected for each incubation temperature (55°C and 37°C for *B. stearothermophilus* and *B. subtilis* var. *niger* respectively). All plates were incubated for at least six days.

All impinger fluids were processed for indicator organism recovery at 37°C and 55°C. Each fluid was thoroughly mixed, and 0.1 ml plated in duplicate on TSA for each incubation temperature. The remaining fluid was split in half, with each half filtered through a 0.2 μm membrane filter and placed on the surface of a TSA plate. For mechanical/chemical treatment, filters were rinsed with 0.1% sodium thiosulfate neutralizer. One membrane was incubated at 37°C and the other at 55°C. All plates were incubated for at least six days.

Waste treatment process fluids collected in duplicate at treatment facilities were vortex-mixed, and 0.1 ml was plated in duplicate. The remainder of each sample was filtered on a 0.2 μm cellulose nitrate membrane filter and placed on the surface of a TSA plate. For each set of duplicate samples, one was incubated at 37°C and the other at 55°C.

Colonies from the TSA at 37°C, that were presumptively identified as *B. subtilis* var. *niger*, were subcultured to Tyrosine agar for confirmation. Plates were incubated for at least 72 hours and resultant growth examined for the development of black pigmentation characteristic of *B. subtilis* var. *niger*.