1.0 INTRODUCTION

Microorganisms present in medical waste treatment systems represent a broad spectrum of groups ranging from frank pathogens to innocuous types normally present in indoor and outdoor environments. Both types and concentrations of organisms will var.y with each load of waste. When sampling emissions from these systems, the level of detectable organisms may reflect the success of the treatment process, the hardiness of the microbe, the culturability of the microbe, or the quantity of a particular organism (especially pathogens) in the waste. Many microorganisms are ubiquitous in the indoor and outdoor environment, and therefore, common to the health care setting and its medical waste. If selected indictor organisms sampled during emissions monitoring are indigenous to the waste and the environment, the possibility of contamination of emissions samples by environmental organisms could render the interpretation of monitoring results difficult.

For these reasons, emissions monitoring should be performed using indicator organisms seeded into the waste to be processed. Seeding should include concentrations of organisms typically expected to be present or exceeding those numbers in regulated medical waste. Indicator organisms for these processes should be:

- non-pathogenic
- intrinsically resistant to heat and chemical inactivation
- easily culturable for efficient recovery
- easily differentiated from other microorganisms through selective growth requirements or other distinguishing characteristics (such as pigmentation)
- effectively recovered by existing sampling methods
- not normally present in medical waste

2.0 BACKGROUND

The recommended indicator organisms are the spores of *Bacillus subtilis* var. *niger* (*B. subtilis*) and *B. stearothermophilus*. *B. stearothermophilus* is routinely used as a indicator of heat sterilization for steam autoclaves, and has recently been used as an indicator of effectiveness of a mechanical/chemical medical waste treatment system (Cole et al, unpublished results). *B. subtilis* spores are used as indicators of dry heat sterilization and have had many roles in aerobiology. They are used for testing leakage of airborne microorganisms from biosafety cabinets (NSF, 1987), and have been used extensively during development of standard bioaerosol recovery devices such as the all glass impinger (Tyler and Shipes, 1959), and the Andersen cascade impactor (Andersen, 1958). They have also been used as surrogate viruses in transport and recovery studies (Spendlove and Fannin, 1982).

These spores have other desirable characteristics of effective indicators. *B. stearothermophilus* can be selectively cultivated at 55°C, well above the temperatures at which most organisms can grow. *B. subtilis* var. *niger* is easily distinguished from other organisms that grow at 37°C by its characteristic orange pigmentation.

3.0 APPROACH

The bioaerosol literature is replete with data on recovery of *B. subtilis* var. *niger*, but *B. stearothermophilus* spore behavior in aerosol samplers is less well known. This report describes laboratory experimentation to evaluate the behavior of both indicator spores when in an operating, primary, aerosol sampling device -- the AGI-30 impinger. Also, growth of the two organisms on different, solid media are compared in order to identify optimum conditions for recovery and quantitation.

Organisms escaping from medical waste treatment systems may have been exposed to incomplete or ineffective treatment processes. These organisms may be assumed to be injured by heat, chemicals, or other agents. Recovery media should be such that test organisms can effectively repair and grow. Candidate media are those formulated for recovery of injured or fastidious microorganisms, such as FDA Bacteriostasis Agar and Trypticase Soy Agar. Pigmented *B. subtilis* var. *niger* can be cultured on media that enhances pigment production as well as injury recovery. The use of multiple media may ensure maximum recovery and quantitation, and differentiate indicator microorganisms from transient contamination.

Some medical waste treatment processes require extended treatment times. Emissions sampling should span the treatment times, but impingers are generally used for periods of 15 mins or less. To determine the losses of viability due to extended residence times in the AGI-30's, injured and non-injured spores of both types were placed in impingers that were then operated for up to one hour. Losses due to passage or slippage of the spores through the impingers were also estimated by connecting impingers in sequence and determining numbers of spores transferred from the first to the second impinger in each series. In this way, comparative behavior of the two spores and maximum sampling times could be established.

4.0 TEST ORGANISMS

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

B. stearothermophilus (BST) is a thermophilic bacterium whose spores are used as an indicator for moist heat sterilization and disinfection processes. Typical cream-colored colonies can be grown selectively by incubation at 55°C, well above temperatures at which most other organisms can grow. BST spores were prepared by Difco Laboratories, Detroit, Michigan.

B. subtilis var. *niger* (BSN) spores are resistant to both heat and chemical inactivation and produce distinctive, orange pigmented colonies when grown on appropriate media at 37°C. BSN spores were prepared by AMSCO, Erie, Pennsylvania.

5.0 MEDIA AND REAGENTS

Trypticase Soy Agar (TSA)	Beef Extract Glucose Agar (BEGA)
Nutrient Agar (NA)	Tyrosine Agar (TYR)
FDA Bacteriostasis Agar (FDA)	Phosphate Buffer Dilution Water (PBDW)

Trypticase Soy Agar, Nutrient Agar, and FDA Bacteriostasis Agar plates were prepared in the RTI Environmental Microbiology Laboratories using dehydrated media procured from Becton Dickinson Microbiology Systems, Cockeysville, Maryland. The BEGA and TYR media were prepared by supplementing the NA with 1.0% glucose and 0.5% L-Tyrosine, respectively. The PBDW was prepared according to the formulation of the Association of Official Analytical Chemists (AOAC).

6.0 EVALUATION OF RECOVERY MEDIA

Five candidate agar media were prepared to evaluate the germination and growth of BSN and BST spores. Candidate media were selected for their ability to produce distinctly pigmented colonies of BSN and to recover maximum numbers of viable organisms. Media formulations are listed in Table 1. The growth of BSN and BST was compared on Trypticase Soy Agar (TSA), FDA Bacteriostasis Agar (FDA), Nutrient Agar (NA), Beef Extract Glucose Agar (BEGA), and L-tyrosine agar (TYR). NA is a standard general agar used to recover and grow an extensive spectrum of bacteria and is the basis of BEGA and TYR. TSA and FDA are used to recover fastidious microorganisms from environmental samples. BEGA produces a more intensely pigmented orange BSN colony than other agars. TYR produces black colonies of BSN.

Table 1. Candidate Media for Spore Growth Tests

Trypticase Soy Agar (TSA)	0.5% Papaic Digest of Soybean Meal 0.5% NaCl 1.5% Pancreatic Digest of Casein 1.5% Agar
Nutrient Agar (NA)	0.3% Beef Extract 0.5% Peptic Digest of Gelatin 1.5% Agar
FDA Bacteriostasis Agar (FDA)	1.0% Peptic Digest of Animal Tissue 0.5% Beef Extract 0.5% NaCl
Beef Extract Glucose Agar (BEGA)	Nutrient Agar 1.0% Glucose
Tyrosine Agar (TYR)	Nutrient Agar 0.5% L-Tyrosine

6.1 Growth and Quantitation of Test Organisms

Growth of test organisms was evaluated by serially diluting stock suspensions in PBDW and plating replicate 0.1 ml inocula of a series of dilutions on test media. Initial tests were done with separate stocks of BST and BSN. To test for interference, the spore suspensions were mixed, diluted, and plated. Three replicates of each dilution and medium were incubated at each temperature (37° or 55°C).

BST spores grew well on all candidate media at 55°C with no striking pigmentation (Table 2). Colonies were all of a typical cream-color, but NA produced smaller colonies than other media, and growth on FDA was diffuse, probably due to excessive moisture on the agar surface. BST colonies on TYR were not countable because they were difficult to distinguish against the milky colored agar. The numbers of colony forming units, when countable, were essentially equivalent on the different media ($0.98\text{-}1.1 \times 10^8$ CFU/ml).

Table 2. Quantitation of *Bacillus stearothermophilus* Spore Suspension Using Four Candidate Recovery Media

Medium	Mean Spores/ml	Std. Dev.	Comment
Trypticase Soy Agar	1.1×10^8	2.3×10^7	cream colonies
Nutrient Agar	1.1×10^8	2.1×10^7	smallest colonies
Beef Extract Glucose Agar	9.8×10^7	5.9×10^6	cream colonies
Tyrosine Agar	N.D.		uncountable, cream colonies on milky, opaque agar
FDA Agar	N.D.		uncountable, cream growth

N.D. = No data

Quantitation and pigmentation of BSN spores on five candidate media are shown in Table 3 and Figure 1, respectively. Pigmented colonies of BSN were initially orange on all media. After 72 hr., the colonies on TYR turned black (Fig. 1). On NA, the colonies were very pale orange and some were grey. Again, FDA yielded diffuse colonial growth, perhaps due to excessive plate moisture. TSA gave the highest counts (2.5×10^7 CFU/ml), followed by TYR, NA, and BEGA (2.1×10^7, 1.9×10^7, and 1.6×10^7 CFU/ml, respectively).

Table 3. Growth of Spores of *Bacillus subtilis* var. *niger* on Five Candidate Media at 37°C

Medium	Titer	Std. Dev.	Comment
Trypticase Soy Agar	2.5×10^7	2.9×10^6	orange colonies
Nutrient Agar	1.9×10^7	3.4×10^6	pale orange to grey colonies
Beef Extract Glucose Agar	1.6×10^7	1.4×10^6	bright orange colonies
Tyrosine Agar	2.5×10^7	1.7×10^6	orange colonies turn black after 72 hr.
FDA Agar	N.D.	N.D.	uncountable, orange growth

N.D. = No data

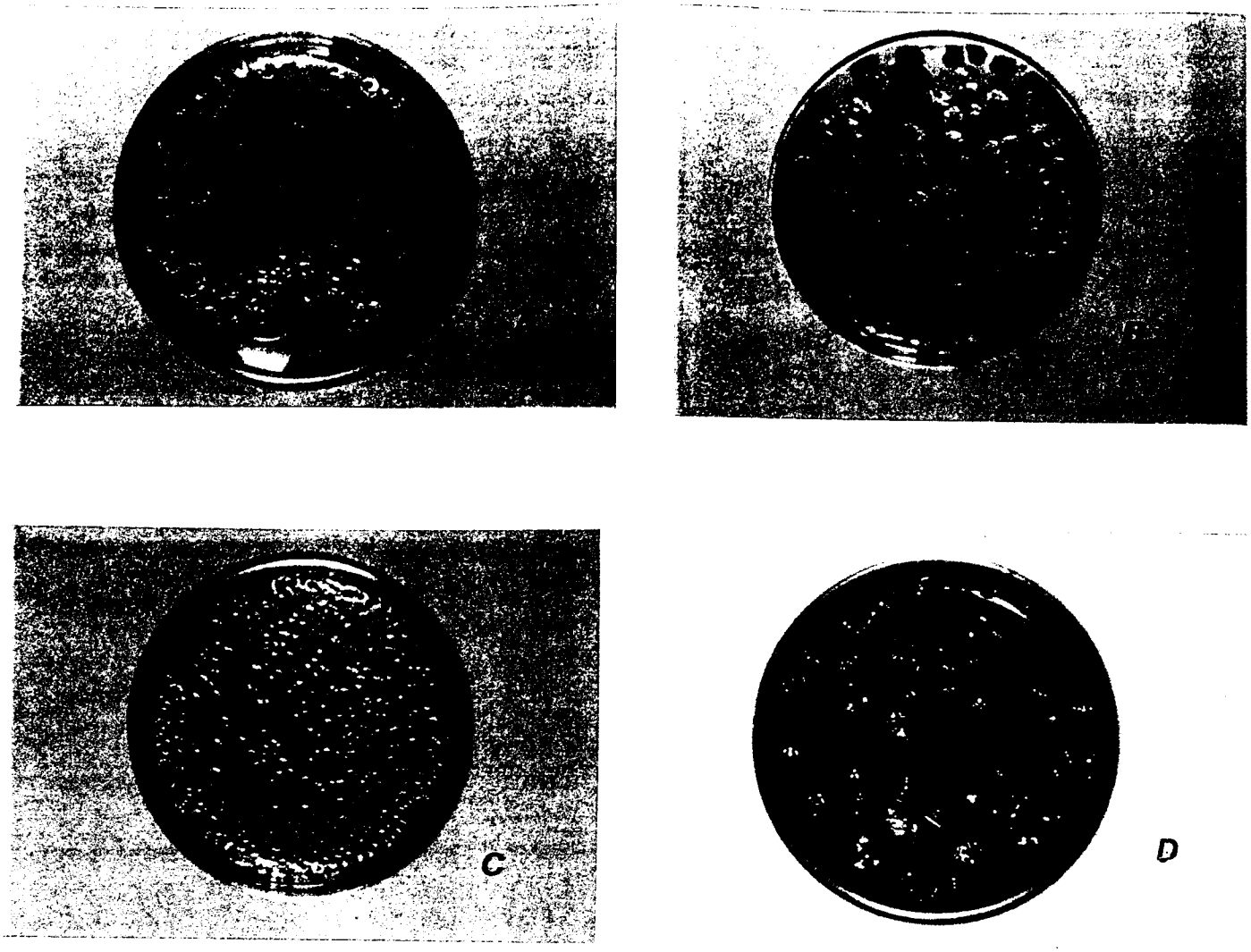

FIGURE 1. PIGMENTED GROWTH OF *B. SUBTILIS* VAR *NIGER* ON CANDIDATE MEDIA:

A. Trypticase Soy Agar

B. Nutrient Agar

C. Beef Extract/glucose Agar

D. Tyrosine Agar

Both organisms retained their characteristic colors and grew only at desired temperatures (cream @ 55°C for BST and orange @ 37°C for BSN) when plated in mixed suspensions as shown in Table 4. BST quantitation on FDA was 35% of that on TSA.

Table 4. Growth of Mixed Spore Suspension on Candidate Media

	B. stearothermophilus[a]		*B. subtilis* var. *niger*[b]		
Agar	**Mean spores (CFU/ml)**	**Std. Dev.**	**Mean spores (CFU/ml)**	**Std. Dev.**	**Comment**
TSA	9.3×10^6	1.3×10^6	1.1×10^7	4.0×10^5	no interference
BEGA	N.D.		1.4×10^6	6.8×10^5	no interference
TYR	N.D.		1.0×10^7	4.9×10^5	no interference
FDA	3.3×10^6	1.5×10^5	1.1×10^7	4.6×10^5	no interference

[a] = incubated 72 hr. @ 55°C
[b] = incubated 72 hr. @ 37°C
N.D. = No data

7.0 SPORE SURVIVAL DURING AEROSOL SAMPLING

The All-Glass impinger (AGI-30) is regarded as a standard bioaerosol sampler. It has been used to monitor medical waste bioemissions from steam autoclave (Barbeito and Brookey, 1976) and mechanical/chemical treatment (Denys, 1989), as well as medical waste compaction (Emery et al, 1992). The AGI-30 is required in the American Society for Testing Materials' (ASTM) *Standard Practice for Sampling Airborne Microorganisms at Municipal Solid-Waste Processing Facilities.* Additionally, the National Sanitation Foundation's Standard Number 49 (Class II Biohazard Cabinetry) requires the use of AGI-30s.

The AGI-30 is designed to draw air at 12.5 lpm through a curved inlet tube and then through a capillary stem jet, 30mm above the impinger base. When the pressure drop across the capillary attains a minimum of half an atmosphere, the flow through it becomes sonic and therefore rate limiting, and behaves as a critical orifice. The sampler is efficient for microbial particles in the respirable size range (0.8 - 15μm), impinging them into 20 ml of turbulent collection fluid. The turbulence provides for breakup of particle aggregates, important in the determination of total, discrete viable counts. As sampling time increases however, concern focuses on potential injury of collected organisms, as well as loss of aerosolized collection fluid and organisms. It was necessary to investigate the survivability and potential loss of collected indicator organisms over time to include those injured (treatment exposed) as well as uninjured (non-treatment exposed).

7.1 Survival of Uninjured Spores

Fresh stocks of both types of spores (BSN and BST) were diluted and mixed together to yield a suspension of approximately 10^5 CFU/ml of each. Eight sterile impingers were filled with 19.7 ml each of PBDW and labelled "Control T_0, Control T_{60}, A T_{20}, B T_{20}, A T_{40}, B T_{40}, A T_{60}, and B T_{60}", followed by addition of 0.3 ml of the spore mixture to each of the impingers. The impinger fluid was mixed and 1.0 ml was removed from Control T_0 for spore assays. One-tenth ml volumes of impinger fluid from Control T_0 were plated on TSA in duplicate for incubation at 55°C. The process was repeated on TSA for incubation at 37°C. After the control sample was taken, vacuum pumps connected to the impingers were operated at 41 mm Hg for 20, 40, and 60 mins (Fig. 2). At the end of each sample period, one pair of impingers was sampled as above and plated in triplicate. After the 60 min samples were plated, a control T_{60} impinger was sampled and plated in duplicate. Remaining volumes of PBDW/spore suspension were measured and recorded for each impinger.