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United States
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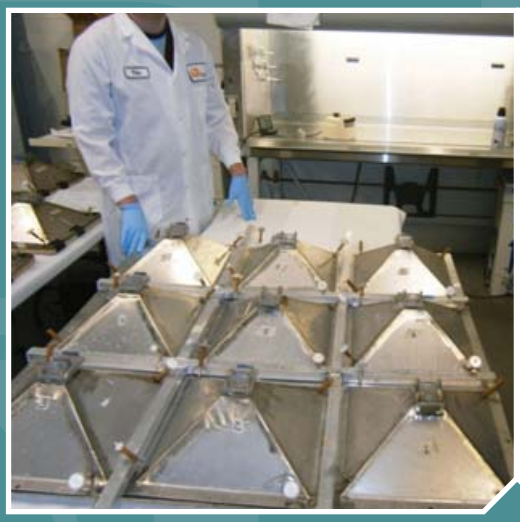
Systematic Evaluation of Aggressive Air Sampling for *Bacillus anthracis* Spores

Assessment and Evaluation Report



Office of Research and Development
National Homeland Security Research Center

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Systematic Evaluation of Aggressive Air Sampling for *Bacillus anthracis* Spores

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National Homeland Security Research Center
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

Disclaimer

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Questions concerning this document or its application should be addressed to:

Sang Don Lee, Ph.D.
Decontamination and Consequence Management Division
National Homeland Security Research Center
U.S. Environmental Protection Agency (MD-E343-06)
Office of Research and Development
109. T.W. Alexander Drive
Research Triangle Park, NC 27711
Phone: 919-541-4531
Fax: 919-541-0496
E-mail: lee.sangdon@epa.gov

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Project Team:

Sang Don Lee, Ph.D. (Principal Investigator)
National Homeland Security Research Center, Office of Research and Development,
US Environmental Protection Agency
Research Triangle Park, NC 27711

M. Worth Calfee, Ph.D.
National Homeland Security Research Center, Office of Research and Development,
US Environmental Protection Agency
Research Triangle Park, NC 27711

Shawn P. Ryan, Ph.D.
National Homeland Security Research Center, Office of Research and Development,
US Environmental Protection Agency
Research Triangle Park, NC 27711

Leroy Mickelsen
Office of Emergency Management, Office of Solid Waste and Emergency Response,
US Environmental Protection Agency
Research Triangle Park, North Carolina, United States

Stephen Wolfe
United States Environmental Protection Agency, Region 5, West Lake, Ohio, United States

Jayson Griffin
Office of Emergency Management, Office of Solid Waste and Emergency Response,
US Environmental Protection Agency
Research Triangle Park, North Carolina, United States

Rebecca Connell
Office of Emergency Management, Office of Solid Waste and Emergency Response,
US Environmental Protection Agency
Washington, DC, United States

Richard Rupert
Region 3

US Environmental Protection Agency
Philadelphia, PA, United States

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List of Acronyms and Abbreviations

AAS	Aggressive Air Sampling
ADA	Aerosol Deposition Apparatus
APPCD	Air Pollution Prevention and Control Division
ATCC	American Type Culture Collection
B.	*Bacillus*
BOTE	Bioresponse Operational Testing and Evaluation
CBRN	Chemical, Biological, Radiological, and Nuclear
CFU	Colony Forming Units
CM	critical measurement(s)
CMAT	Consequence Management Advisory Team
COC	chain of custody
COMMANDER	Consequence Management and Decontamination Evaluation Room
DCMD	Decontamination Consequence and Management Division
DHS	Department of Homeland Security
DPG	Dugway Proving Grounds
DQI	Data Quality Indicator
DQO	Data Quality Objective
ECBC	Edgewood Chemical Biological Center
EPA	U. S. Environmental Protection Agency
cm	centimeter
cm^2	square centimeter
FRM	Federal Reference Method
Hi-Vol	high volume
ISO	International Organization for Standardization
LPM	liters per minute
MDI	Metered Dose Inhaler
MOP	Miscellaneous Operating Procedure
m	meter
mph	miles per hour
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OEM	Office of Emergency Management
ORD	Office of Research and Development
PBST	Phosphate Buffered Saline Tween20
PM	Particulate matter
PPE	Personal Protective Equipment
ppm	part(s) per million
ppmv	part(s) per million by volume
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
RH	Relative Humidity
rpm	revolutions per minute
slpm	Standard liters per minute
SOP	Standard Operating Procedure
S&T	Science and Technology (Directorate)
VHP®	Vaporous Hydrogen Peroxide
WAM	Work Assignment Manager
WARRP	Wide Area Recovery and Resiliency Program

Executive Summary

The primary objectives of this project were to evaluate the Aggressive Air Sampling (AAS) method for use in sampling *Bacillus anthracis* spores. Unlike surface sampling, AAS gives airborne concentrations of respirable size particles that can be used to assess risk of inhalation exposure and as a result, risk of inhalation anthrax. Surface sampling does not differentiate particle size nor does it allow for determining the fraction of particles that could become re-aerosolized. The ability to ensure safe re-entry and reoccupation of spaces after a decontamination event relies on the ability to make accurate measurements of any residual contamination. The purpose of this evaluation was to identify the relative sampling efficacy of the AAS method for spore sampling as a function of surface type, spore surface concentration, and dissemination method. The AAS test results were compared to other currently-used surface sampling methods (i.e., vacuum socks or sponge wipes).

This study was designed and conducted to characterize the AAS technique. The first part of this characterization entailed use of AAS on surfaces with relatively high spore (*Bacillus atrophaeus)* surface loadings (i.e., ~1 x 10^4 colony forming units (CFUs) per cm^2), deposited via aerosol deposition apparatus (ADA) pyramids). Secondly, the efficacy of the AAS for clearance sampling (i.e., lower spore surface loadings) was determined using the initially loaded coupons that had been subjected to various targeted Vaporous Hydrogen Peroxide (VHP®) fumigation cycles (250 parts per million [ppm] hydrogen peroxide for 30, 60, or 90 minutes).

Three Federal Reference Method (FRM) PM-10 high volume samplers (Hi-Vols) were used to sample in sequence (three tandem 20-minute intervals) following aggressive aerosolization of material coupons that had been inoculated with spores. Use of the Hi-Vols in sequence allowed for a sampling duration of one hour at a total volumetric rate that would be desirable or required during an actual large-scale field event. Most, if not all, recovered spores were collected by the first Hi-Vol sampler (1 air exchange); analysis of samples collected by the second and third samplers showed near or less than background levels. The AAS procedure can be further improved by optimizing the air sampling flow rate, the number of air samplers, and the surface agitation method depending on the sampling site characteristics. Further, a breakdown of recovery from the impactor plate and the filter of the Hi-Vol sampler (D_{50} of 10 µm) showed that most of the recovery was typically from the filter fraction under the tested conditions. This means that 50% of 10 µm particles penetrate through the impactor and the other 50% are collected on the impaction plate.

The results of this study demonstrated that AAS may be a viable option to sample *B. anthracis* spores. The overall test results showed AAS results may vary depending on the surface spore characteristics (e.g., spore size distribution) and environmental conditions (e.g., relative humidity, fumigation, other activities that may impact the surface condition). When the AAS results were compared as a function of surface types, AAS recoveries were lowest from the carpet among three tested materials. The laminate surface showed the highest spore recoveries. The comparative recoveries ranged 0.37% to 5.84% for pre-fumigation and 0.004% to 1.032% for post-fumigation AAS.

1 Introduction

The U.S. Department of Homeland Security (DHS) is committed to using cutting-edge technologies and scientific talent in its quest to protect human health and the environment. The DHS Science and Technology Directorate (S&T) is tasked with researching and organizing the scientific, engineering, and technological resources of the United States and leveraging these existing resources into technological tools to help protect the homeland. Through this project, the U.S. Environmental Protection Agency's (EPA's) National Homeland Security Research Center (NHSRC) supports this effort through DHS's Wide Area Recovery and Resiliency Program (WARRP) S&T program.

Accurate measurements of residual contamination are needed to inform decisions on re-entry and reoccupation of spaces following site decontamination. For a wide area contamination incident, traditional surface sampling methods (i.e., wipe, swab and vacuum sock) used for clearance sampling may require an extensive number of samples, which may be time and labor intensive to achieve a reasonable confidence. Judgmental sampling is an alternate strategy which can reduce the total number of samples. However, statistical analyses of results from this sampling strategy are not applicable. Innovative techniques, such as Aggressive Air Sampling (AAS), may prove useful as an additional method to currently-used surface sampling methods and, with additional research, may be used as an alternative method in certain situations (i.e., detection of spore presence from unknown hot spots, wide spread contamination with concentration close to detection limit for surface sampling methods, etc.), and effectively shorten the timeline to recovery and/or reduce the sampling burden during a response. AAS could be used for numerous building interiors for rapid sampling with fewer required personnel, and in certain scenarios such as covert wide area contamination, could reduce the total number of samples per unit area compared to the current surface sampling methods. Given that surface sampling is one of the critical bottlenecks in the remediation process, AAS could ultimately result in a decrease in overall cleanup time. Unlike surface sampling, AAS indicates airborne concentrations of respirable size particles that can be used to assess risk of inhalation exposure and as a result, risk of inhalation anthrax. Surface sampling does not differentiate particle size nor does it allow for determining the fraction of particles that could become re-aerosolized.

This project evaluated the AAS method to determine whether this technique is appropriate for *Bacillus (B.) anthracis* spore sampling. The AAS method was used as a supplement to surface sampling following building decontamination in response to the 2001 intentional *B. anthracis* spore contamination incident in the U.S.[1-6] However, AAS was not used as a primary method for sampling due to a lack of a systematic and rigorous evaluation of this technique. The intent of this project is to empirically evaluate the AAS method for sampling *B. anthracis* surrogate spores.

Results from this project may be used by EPA's Office of Emergency Management (OEM) and other stakeholders to decide upon preferred characterization and clearance sampling methods to be employed during remediation efforts.

1.1 Process

The general process investigated in this project was AAS of selected surfaces contaminated with *B. atrophaeus* spores (i.e., surrogate for *B. anthracis* spores). The AAS method was evaluated for carpet, laminate, and painted drywall to determine whether this technique may be effective for *B. anthracis* spore

sampling from common surfaces. The AAS Standard Operating Procedure (SOP) used during the Bioresponse Operational Testing and Evaluation (BOTE, Phase I) [7, 8] project was used as the starting point for this study. Following deposition of the surrogate spores onto the target surface materials via aerosolization and settling, spore sampling efficacy of AAS was determined before and after fumigation with hydrogen peroxide. Similarly, the currently- recommended *B. anthracis* spore surface sampling methods (i.e., vacuum socks or sponge wipes) were used to quantify the spore abundance on additional replicates of the test surfaces. These recoveries were then compared to AAS recoveries. This evaluation identified the relative sampling efficacy of the AAS method for spore sampling as a function of surface type and spore surface loading.

1.2 Project Objectives

The primary objectives of this project were to evaluate the AAS method for sampling *B. anthracis* surrogate spores.

1.3 Experimental Approach

The experimental approaches used to meet the objectives of this project were:

- Use of controlled chambers, standardized sections of environmental surfaces, and precise spore inoculums;
- Contamination of materials via aerosol deposition of bacterial spores;
- Quantitative assessment of spore contamination by sampling representative sections of material before decontamination;
- Application of a prescribed AAS procedure to the test sections;
- Quantitative assessment of residual contamination by sampling test sections and procedural background level after AAS;
- Determination of AAS effectiveness (comparison of results from positive control samples and test sections); and
- Documentation of operational considerations (e.g., cross-contamination, procedural time, impacts on materials and personnel).

1.3.1 Testing Approach

Tests consisted of three parts that were intended to be conducted in duplicate for a total of six tests. The test matrix, shown in Table 2.1, shows that each test included one loading level (i.e., inoculum concentration) for each surface type. The target spore surface loading was 1×10^4 CFU/cm^2. The three surface types selected for this study were carpet, laminate flooring, and painted dry wallboard. In addition to conducting AAS during each test, the laminate and painted dry wallboard coupons were sampled using the sponge wipe method (Section 2.5.1), and carpet was sampled using the vacuum sock method (Section 2.5.2). Culture-based methods were subsequently used to quantify the number of spores recovered by enumeration of CFUs on microbiological growth media after plating serially-diluted aliquots of sample extracts. Therefore, in this report “recovery” is defined by the number of CFUs observed following sample collection, extraction, and analysis (dilution-plating or filter-plating).

2 Materials and Methods

2.1 Testing Chamber

All testing was done in the Consequence Management and Decontamination Evaluation Room (COMMANDER). This is a specially constructed enclosed, single-access-point chamber (henceforth, chamber) within the current Homeland Security Enclosure located within High-Bay Room 130 (H130) at EPA's Research Triangle Park, NC campus. COMMANDER meets the following criteria:

- Supports repeated fabrication of an environment (e.g., furnished office room; outdoor setting) contained within the chamber;
- Allows for release of biological organisms or chemicals into the chamber;
- Allows for application of a decontamination technology (including fumigation with toxic, corrosive gases);
- Supports entry into the chamber during all of the above mentioned activities (in appropriate personal protective equipment [PPE]);
- Has external dimensions of 2.7 m x 3.7 m x 3 m high;
- Has nominal internal dimensions of 2.5 m x 3.4 m x 2.7 m; with about 41m^2 of floor and walls;
- Contains one air-tight entry/exit port with a window;
- Contains a 1.8 m x 1.8 m x 2.4 m high airlock with single entry/exit port with a window;
- Contains entry/exit ports in line with the enclosure double door to allow for large materials to be brought into or out of the chamber; and
- Complies with all relevant local and national codes.

The environmental conditions (relative humidity [RH] and temperature) both during the conditioning phases and during AAS and surface sampling were monitored by a Vaisala (Model 333, Vaisala, Helsinki, Finland) probe mounted on the side of the chamber. The COMMANDER was kept at a slightly negative pressure (~ 2" water) to prevent exfiltration of spores from COMMANDER into the general laboratory space.

2.2 AAS

During the AAS procedure, three consecutive aerosol samples (every 20 minutes) were collected using three Hi-Vol samplers (Thermo Fisher Scientific Inc., High Volume Air Sampler VFC-PM10.for FRM RFPS-1287-063, Pittsburgh, PA, (https://www.thermo.com/eThermo/CMA/PDFs/Various/File_52267.pdf accessed March, 2013)). Collection of the first 20-minute sample was initiated simultaneously with aggressive agitation of surfaces and forced resuspension of particles. Particle resuspension was maintained with turbulent air created by the operation of a stainless steel mixing fan (P/N 1729K11, McMaster-Carr, Atlanta) (shown in Figure 2-1). The resuspension process was performed using a leaf blower (Toro Power Sweep Electric Blower, Model # 51585) operated at the highest speed (approximately 260 km per hour (160 miles per hour)). The operating conditions of the leaf blower, described in Miscellaneous Operating Procedure (MOP) 3166 (Appendix A), were repeated for all the

tests and set at an angle of 45 degrees, a distance of less than 30 cm from the target coupon, and with a forced aerosolization time of 1 min per one 35.56 cm x 35.56 cm area (see Appendix A for copies of all associated MOPs). The leaf blower operator moved the tip of the blower back and forth across the coupon(s) at approximately 1 meter per second for the total operation time. The exhaust of the Hi-Vol samplers was recycled within COMMANDER.

For each test, three stainless steel (reference samples) and 15 material coupons (three sampled using currently-used methods and 12 sampled using AAS pre-fumigation). Following fumigation, the 12 material coupons were seperated into two groups, 9 for post-fumigation AAS, and 3 sampled with currently-used methods post-fumigation. Surface samples of coupon materials and stainless steel, used to characterize the inoculation, were collected on the same day of the AAS test. The coupons sampled with currently-used methods underwent exactly the same series of conditions, prior to the final sampling event, as the AAS test samples: inoculated, subjected to AAS, fumigated, and then sampled with the currently-used surface sampling methods rather than AAS.

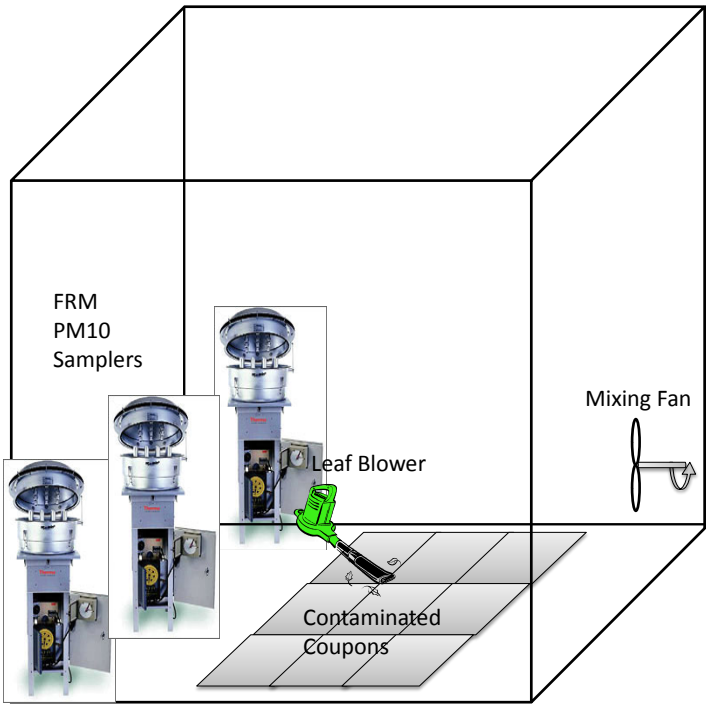


Figure 2-1. COMMANDER setup for AAS

2.3 Test Materials and Deposition

2.3.1 Test Coupons Preparation

Various surface material types (carpet, laminate flooring, and painted drywall) were represented by 35.56 cm x 35.56 cm and 107 cm x 107 cm coupons, while the reference stainless steel control coupons were 35.56 cm x 35.56 cm. Coupons were fabricated according to MOP 3150-All, and were sterilized by a 250 ppmv VHP® cycle for 4 hours inside COMMANDER. After sterilization, coupons were degassed for a

minimum of three days before testing. Coupons were inoculated in H-130 outside the COMMANDER facility. To prevent cross-contamination, the test coupons were transported and disassembled (uncovering the ADAs) in the sterilized COMMANDER airlock. Positive material and stainless steel control coupons also underwent the same transport to COMMANDER; however, they were disassembled and sampled in H-130, outside COMMANDER.

2.3.2 Bacillus Spore Preparation

The test organism for this work was a powdered spore preparation of *B. atrophaeus* (American Type Culture Collection, ATCC 9372) and silicon dioxide particles. This bacterial species was formerly known as *B. subtilis* var *niger* and subsequently *B. globigii*. The preparation was obtained from the U.S. Army Dugway Proving Grounds (DPG) Life Science Division. The preparation procedure is reported in Brown et al.[9] briefly, after 80 – 90 percent sporulation, the suspension was centrifuged to generate a preparation of about 20 percent solids. A preparation resulting in a powdered matrix containing approximately 1 x 10^{11} viable spores per gram was prepared by dry blending and jet milling the dried spores with fumed silica particles (Degussa, Frankfurt am Main, Germany). The powdered preparation was loaded into metered dose inhalers (MDIs) by the U.S. Army Edgewood Chemical Biological Center (ECBC) or by ARCADIS according to a proprietary protocol. Control checks for each MDI were included in the batches of coupons contaminated with a single MDI.

2.3.3 Coupon Inoculation

Coupons were inoculated with spores of *B. atrophaeus* from a MDI using the procedure detailed in MOP 3161-HD and in the article by Calfee *et al[10].* . Briefly, each 107 cm x 107 cm coupon was inoculated using an array of nine Aerosol Deposition Apparatus (ADA)s (see Figure 2-2), each designed to fit one 35.56 cm x 35.56 cm coupon or area of any thickness.

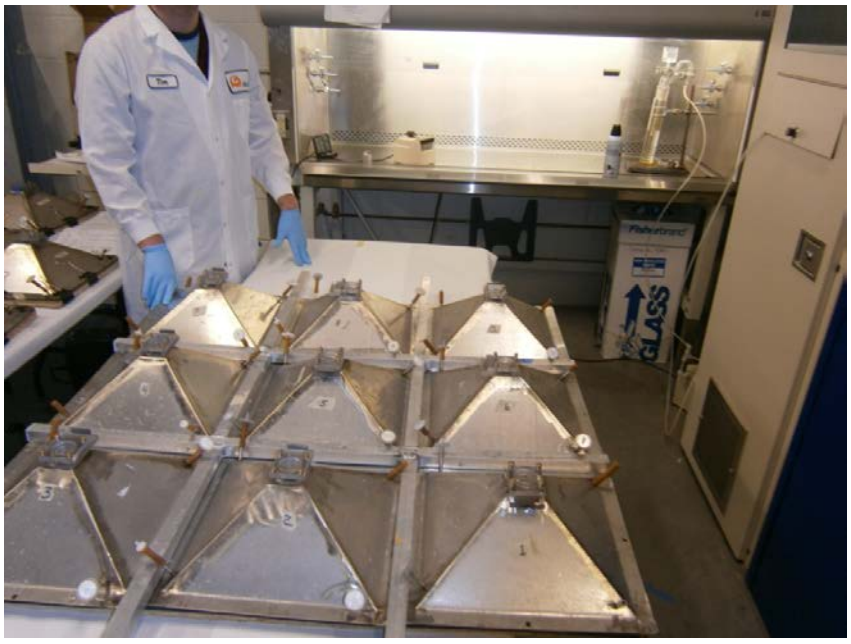


Figure 2-2. 3 x 3 grid of ADAs on a 107 cm x 107 cm coupon

Stainless steel and the other 35.56 cm x 35.56 cm material coupons were inoculated using an individual ADA. In accordance with MOP 3161-HD, the MDI was discharged a single time into each dosing chamber. The spores were allowed to settle onto the coupon surfaces for a minimum period of 18 hours. After the minimum 18-hour period, the large (107 cm x 107 cm) coupon was then moved to the COMMANDER airlock where the ADAs were removed. The uncovered coupons were then transferred to their positions inside COMMANDER. Care was taken not to touch the inoculated surface. Personnel changed garb (laboratory coat, P95 mask, hair net, boot covers) after handling the coupons to help prevent cross-contamination from the moving/handling operation. Three small (35.56 cm x 35.56 cm) coupons were also transported into COMMANDER, while an additional three remained in place until the following day, when surface samples were collected. These small coupons were then walked to the airlock in the same manner to simulate the transport undergone by test coupons. ADAs remained on the coupons until just before sampling.

The MDIs provide 200 discharges (50 µL each) per MDI. The number of discharges per MDI was tracked so that use did not exceed this value. Additionally, in accordance with MOP 3161-HD, the weight of each MDI was determined after completion of the contamination of each coupon. If an MDI weighed less than 10.5 g at the start of the contamination procedure described in MOP 3161-HD, this MDI was retired and a new MDI was used. For quality control of the MDIs, stainless steel contamination control coupons were inoculated as the first, middle, and last coupons within a single group of coupons inoculated by any one MDI within a single test. If the results from the contamination controls were outside the acceptance criteria, the results were immediately reviewed to determine the corrective action.

A log was maintained for each set of coupons that was dosed via the method of MOP 3161-HD. Each record included the unique coupon identifier, the MDI unique identifier, the date, the operator, the weight of the MDI before dissemination into the coupon dosing device, the weight of the MDI after dissemination, and the difference between these two weights. The coupon codes were pre-printed on the log sheet prior to the start of coupon inoculation (dosing). The ADA affixed to the coupon was labeled and remained on the coupon until immediately before sampling.

2.4 Test Matrix

The test matrix for Phase 1 testing is shown in Table 2-1. Each test includes a pre- and a post-fumigation sampling sequence as shown in Figure 2-5.

Table 2-1. Test Matrix

Test #	Test Material	Spore loading (CFU / cm^2)	Dissemination Method	Replicate Tests
4	Carpet	$10^3 \sim 10^4$	ADA	2
5	Laminate	$10^3 \sim 10^4$	ADA	2
6	Drywall	$10^3 \sim 10^4$	ADA	2

2.5 Sampling and Analytical Procedures

Within a single test, AAS of the material sections was completed for background coupons first before sampling of any test material sections was performed. Surface sampling was done either by sponge wipe sampling or vacuum sock sampling according to the protocols documented below. The sampled area by surface sampling methods was 35.56 cm x 35.56 cm (14" x 14") for each coupon.

Prior to the sampling event, all materials needed for sampling were prepared using aseptic techniques. The materials specific to each protocol are included in the relevant sections below. In addition, general sampling supplies were also needed. A sampling material bin was stocked for each sampling event. The bin contained enough sponge wipe sampling and vacuum sock sampling kits (prepared according to MOP 3141 A) to accommodate all required samples for the specific test. Additional kits of each type were included for back up. Sufficient prepared packages of gloves and bleach wipes were included in the bin. A sample collection bin was used to transport samples back to Biocontaminant Laboratory at EPA's facility in Research Triangle Park, NC. The exterior of the transport container was disinfected by wiping all surfaces with a bleach wipe or towelette moistened with a 0.5 to 0.8% hypochlorite (HOCl) solution (pH-adjusted bleach) prior to transport from the sampling location to the Biocontaminant Laboratory.

2.5.1 Sponge Wipe Surface Sampling and Extraction

Sponge wipe sampling is the currently-used method for sampling on nonporous-smooth surfaces such as ceramics, vinyl, metals, painted surfaces, and plastics. Sponge wipe (3M Sponge-Sticks™ (P/N SSL10NB), made of cellulose) sampling was used as the comparative method for smooth, nonporous surfaces, and was conducted according to MOP 3165 (for surface samples) or MOP 3169 (for impactor samples). The general approach is that a moistened sponge is used to wipe a specified area to recover surface-associated bacteria, viruses, and biological toxins. The impactor samples (see Figure 2-3) were representative of particulate matter (PM) greater in size than 10 µm. Due to the size and material characteristics (hard surface with holes) of the impactor, a separate sampling technique was required.

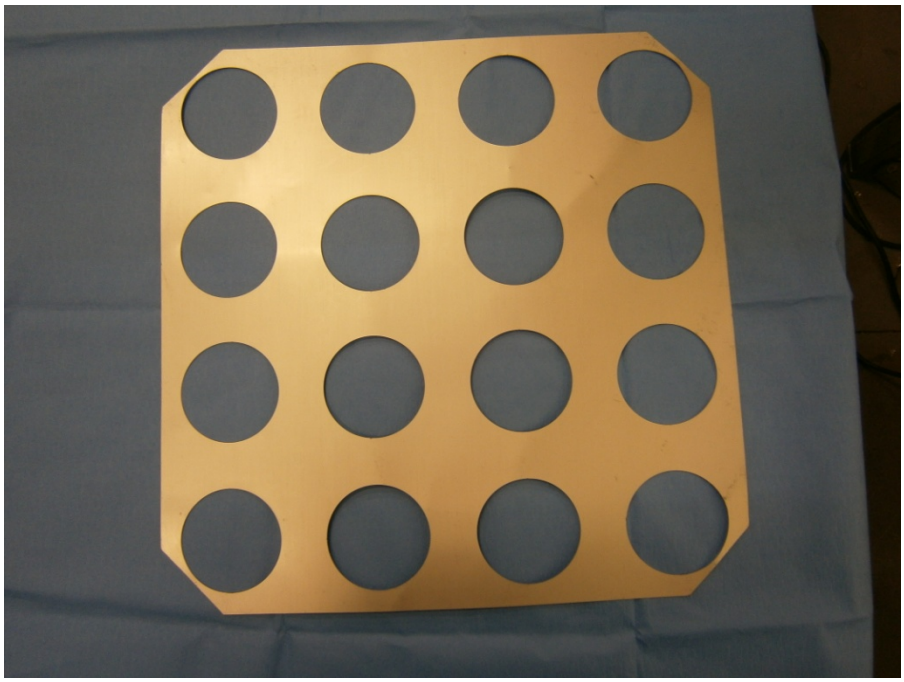


Figure 2-3. Hi-Vol Impactor Plate

Sponge wipe samples were extracted by stomaching (1 minute, 260 revolutions per minute [rpm]) in 90 mL of Phosphate Buffered Saline with Tween® 20 (PBST) using a Seward® Model 400 circulator (Seward® Laboratory Systems, Inc., Port Saint Lucie, FL), then concentrated with a centrifuge. Sponge wipe extraction is described in detail in MOP 6580. This MOP was developed based on the Laboratory Response Network (LRN) protocol.

2.5.2 Vacuum Sock Surface Sampling and Extraction

Vacuum sock sampling, the currently recommended sampling method for rough and/or porous surfaces, was used as the comparative method for carpet coupons. Vacuum sock sampling was conducted according to MOP 3145.

Vacuum sock samples were extracted by first wetting the collection (white) portion of the filter by dipping in PBST, then cutting the filter with sterile scissors (vertically, then horizontally) into small pieces (approximately 1 cm x 4 cm). As the filter was fractioned, the resulting pieces were allowed to fall into a 120 mL sterile specimen cup (Starplex Scientific LeakBuster Specimen Containers - Fisher Scientific #14-375-459, Pittsburgh, PA) containing 20 mL sterile PBST. The cups were then agitated (30 minutes, 300 rpm, ambient temperature) using an orbital platform shaker incubator (Lab-Line, Model 3625). Vacuum sock extraction is described in MOP 6572.

2.5.3 Hi-Vol Aerosol Sampling

Three Hi-Vol air samplers were used to collect aerosols from a large volume of air during each AAS sampling event. During each sampling campaign, each sampler was equipped with a new sterile 20.32 cm x 25.40 cm borosilicate (QM-A, Whatman) glass fiber filter. These Hi-Vol samplers use a volumetric

flow-controlled system for sampling of air (1020 to 1240 slpm) for the collection of particulate matter with diameter less than 10 µm (D50) onto the filter, and greater than 10 µm onto the impactor plate (shim). The three samplers were operated for 20 minutes each, in tandem. For instance, the samplers were not run concurrently, but sequentially, creating a total sampling duration of 1 hour.

Figure 2-4 shows the Hi-Vol sampler. On the top is an inlet designed to collect particulate matter in wind speeds of less than 30 miles per hour (mph). The 10 µm impactor plates (or shims) are placed in the opening seen in Figure 2-4. Below the inlet and impactor plates lies the borosilicate borosilicate glass fiber filter.

During sample recovery, the filters were folded using aseptic technique inside COMMANDER such that the side exposed to the spores was inside a double fold. The filter was then transferred to a sterile stomacher bag for extraction. The impactor plates, meant to capture all particles larger than 10 µm, were also sampled (using the sponge wipe method in accordance with MOP 3169). .

The filter samples were extracted by stomaching (2 minutes, 230 rpm) in 100 ml of PBST using a Seward® Model 400 circulator (Seward® Laboratory Systems, Inc, Port Saint Lucie, FL). Extraction of borosilicate filters is described in MOP 6586. To improve the detection limit, the pulp was removed using a sieve and the resultant liquid filtered according to MOP 6565.

Figure 2-4. Hi-Vol Air Sampler

2.5.4 Swab Surface Sampling

MOP 3135 was used for collecting swab samples. The general approach was to use a moistened swab to wipe a specified area to recover bacterial spores. Swab samples were collected from coupons before inoculation for quality assurance of the sterilization method. Typically, one coupon of each material type per sterilization batch was sampled.

2.6 Sampling Strategy

The AAS procedure consisted of the sequential events listed in Figure 2-5.

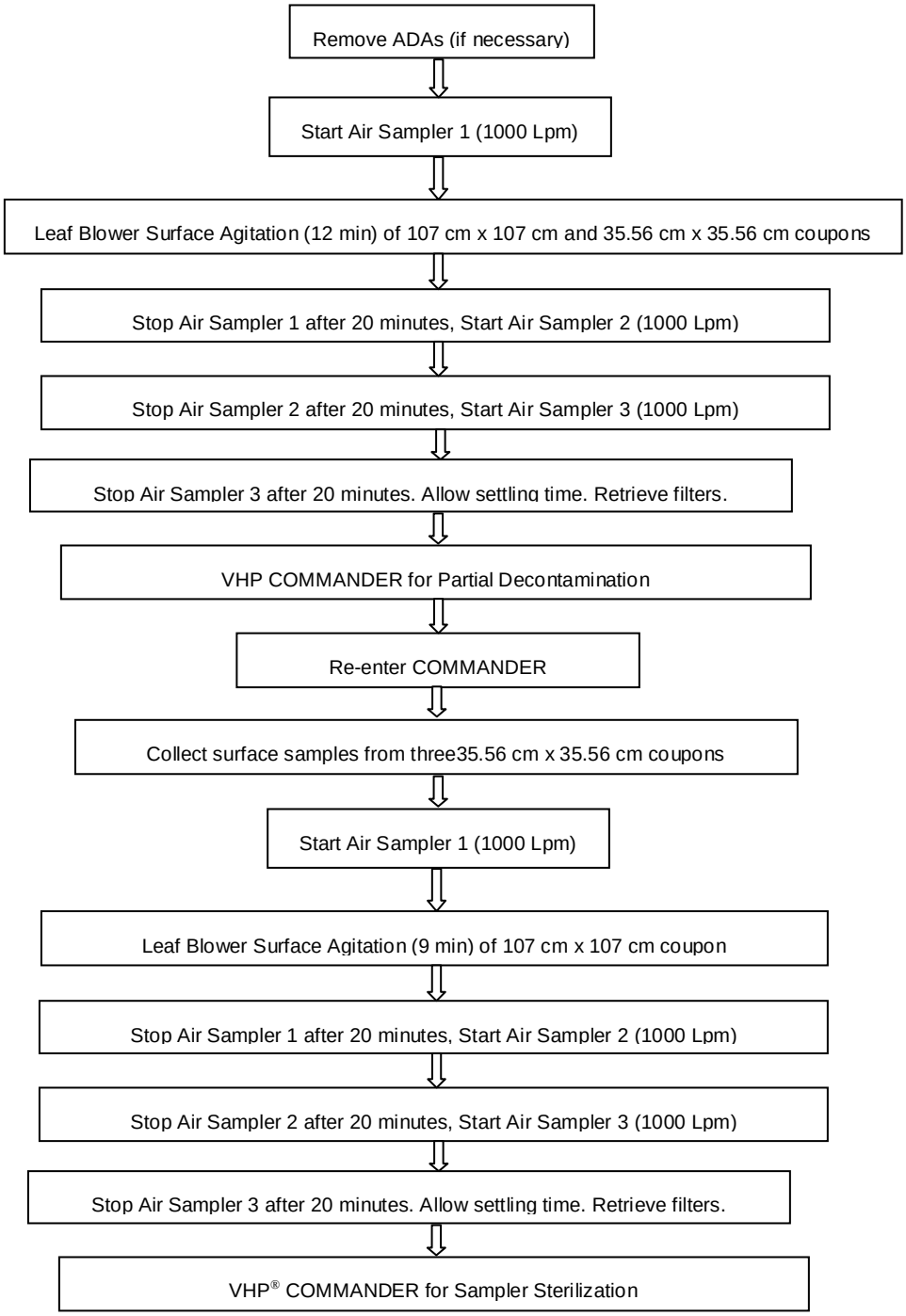


Figure 2-5. AAS pre- and post-fumigation procedure (The background AAS was conducted with three air samplers operating concurrently.)

Figure 2-6 shows the sampling sequence. The sampling sequence and samples collected are detailed below:

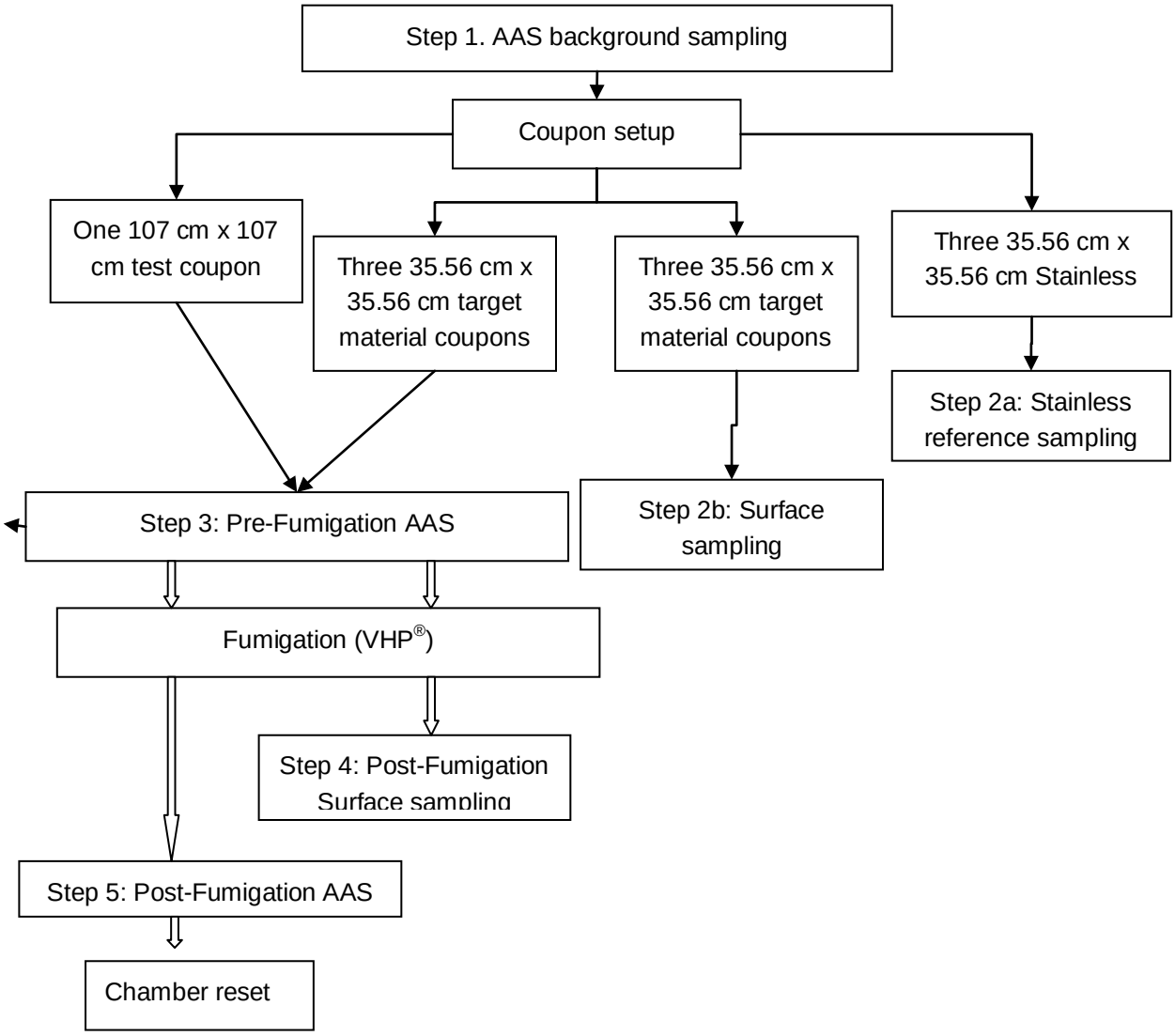


Figure 2-6. Phase 1 sampling sequence

1. Background Sampling. This sampling sequence occurred initially and between each of the tests following VHP® sterilization (250 ppmv H_2O_2 for four hours). One 107 cm x 107 cm sterilized material coupon, consisting of nine ADAs assembled side-by-side (3 x 3), were placed inside an empty, decontaminated COMMANDER during AAS. ADAs were removed and one background (negative) AAS sample was collected using all three Hi-Vol samplers concurrently. A leaf blower was used according to MOP 3165 to aerosolize spore contaminants, if any, from the sterilized coupon while the procedural aerosol background samples were collected. All MOPs associated with this testing can be found in Appendix A. This test sequence helped quantify any cross-contamination that may occur from test personnel.

2. Pre-Fumigation Surface Sampling. The pre-fumigation surface sampling involved the collection of samples from three replicate positive coupons (Figure 2-7 B), using the currently-used surface sampling methods. These sampling events occurred in H130, outside of COMMANDER.

3. MDI control surface reference samples. These control reference samples consisted of three 35.56 cm x 35.56 cm stainless steel coupons (Figure 2-7 A) that were inoculated at the same time and with the same spore loading as the test coupons, using the ADA deposition method. These samples were then subjected to surface reference sampling, performed using the sponge wipe method on the same day as the AAS test samples, but prior to the AAS phase. These samples were used to measure the stabilty of the MDI over the duration of the inoculation event.

4. Pre-Fumigation AAS. Three consecutive Hi-Vol samples (three tandem 20-minute intervals) were collected using three Hi-Vol samplers during and following aggressive resuspension of 12 (one 107 cm x 107 cm material coupon and three 35.56 cm x 35.56 cm coupons) test coupons (Figure 2-7 C) that were inoculated with spores. The coupon inoculation was performed using nine ADAs assembled side-by-side (3 x 3) outside of COMMANDER for the 107 cm x 107 cm coupons, and 3 individual ADAs for the 35.56 cm x 35.56 cm coupons, which were placed inside COMMANDER on the day of testing. Although the inoculation was performed horizontally for all coupons, the carpet and laminate flooring coupons were placed horizontally and the drywall coupons were placed vertically along the COMMANDER walls during AAS to mimic their real-world orientations. The Hi-Vol samplers were each operated for 20 minutes at 1000 Lpm (approximate 1 air exchange rate). The three consecutive 20-minute samples captured a 1-hour period of time, including the 12-minute agitation and 50-minute settling time.

5. Post- AAS / Post- VHP® Fumigation Surface Sampling. Three post-fumigation surface samples were collected from the three 35.56 cm x 35.56 cm target material coupons (Figure 2-7 E) before post-fumigation AAS on the 107 cm x 107 cm material coupons (Figure 2-7 D). These coupons were inoculated in the same manner as the 107 cm x 107 cm material coupon and underwent the same pre-fumigation AAS as the test coupon in Step 3.

6. Post-Fumigation AAS. Three consecutive Hi-Vol samples (every 20 minutes) were collected using three Hi-Vol samplers following an aggressive aerosolization of the 107 cm x 107 cm material coupon that was sampled during the pre-fumigation AAS (Step 3) and exposed to the VHP® fumigation process. The post-fumigation AAS was performed to determine the effectiveness of the technique at lower spore concentration and spores subjected to fumigation.

7. All materials were subjected to a VHP® cycle (250 ppmv for four hours) to reset for the next test.

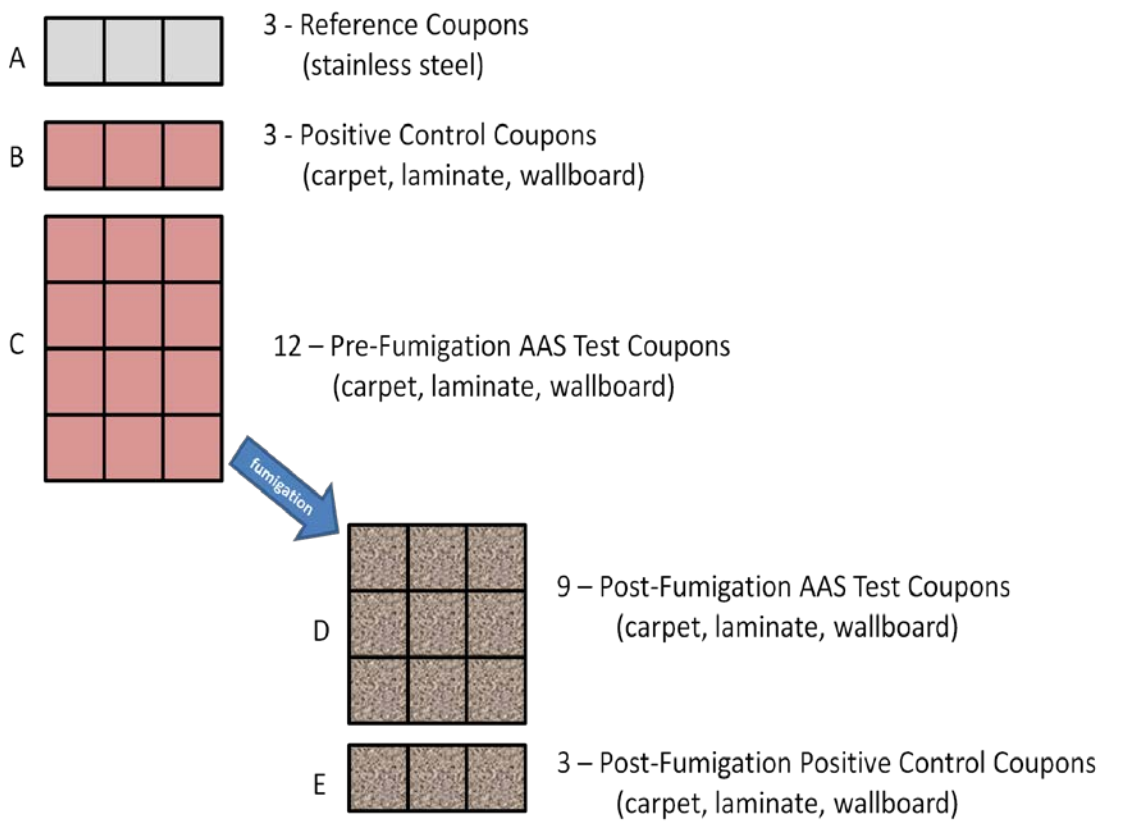


Figure 2-7. Schematic of control and test coupons utilized in each test

2.6.1 Sampling/Monitoring Points

All AAS operations were conducted in the COMMANDER. The COMMANDER environmental conditions were monitored by a Vaisala probe mounted onto the side of the chamber. The COMMANDER flow measurement was taken at the air outlet. Because COMMANDER is kept at negative pressure (~2" water) to prevent out-leakage, this sampling point included any in-leakage air. There were three sampling points inside COMMANDER during air agitation. These sampling points were in a well-mixed area due to the presence of a fan, and were considered co-located. Surface sampling took place both outside and inside COMMANDER. Surface samples of stainless steel used to characterize the inoculation, and the material positive controls were collected outside of COMMANDER on the day of AAS. Surface samples of the material coupons post VHP® fumigation were sampled inside COMMANDER. Air sampling during AAS was performed using three Hi–Vol Air Samplers.

Critical measurements during the sampling phase included duration of agitation with the leaf blower and duration of Hi-Vol operation. Pressure differential and flow rate data from the Hi-Vol samplers were collected to verify the correct operation of the blowers.

Environmental measurements included temperature (ranged 19 to 35 ^{o}C) and RH (ranged 48 to 66 %) of COMMANDER during all phases of tesing. The COMMANDER air exchange rate was constantly monitored.

Because current surface sampling techniques are intrusive, they also removed viable spores from the surface of the section. For example, the sampling method itself changed the spore concentration on the surface. Therefore each coupon could be sampled only once. Reference stainless steel control coupons were inoculated concurrently with test coupons and were conditioned at 40% RH along with test coupons, but did not undergo AAS. Surface sampling was also performed on coupons following AAS.

2.7 Sample Handling and Custody

2.7.1 Cross-contamination Prevention

Sampling presents a significant opportunity for cross-contamination of samples. In an effort to minimize the potential for cross-contamination, several management control practices were employed

- In accordance with aseptic technique, a sampling team was utilized, and consisted of a "sampler," a "support person," and a "sample handler."
- The sampler handled only the sampling media and the support person handled all other supplies. The sample handler removed ADAs before sampling and moved coupons after sampling. The sampler sampled the surface according to the appropriate procedure described in Section 2.5.
- The collection medium (e.g., sponge wipe or vacuum sock) was then placed into a sample container that was opened, held and closed by the support person.
- The sealed sample was handled only by the support person.
- All of the following actions were performed only by the support person, using aseptic technique:
    - The sealed bag with the sample was placed into another sterile plastic bag that was then sealed; that bag was then decontaminated using a bleach wipe.
    - The exterior of the transport container was decontaminated by wiping all surfaces with a bleach wipe or towelettes moistened with a solution of 5000 ppm hypochlorite prior to transport from the sampling location to the DCMD Biocontaminant Laboratory.

The sampling crew then changed their gloves in preparation for working with the next sample.

All sampling inside COMMANDER was done wearing clean garb (laboratory coat, P95 mask, hair net and boot covers) over the HazMat suits worn during leaf blower operation. Clean garb was changed between each activity (approximately every 10 minutes).

2.7.2 Sample Quantities

Table 2-2 lists the sample quantities for each test. Each aerosol sample consisted of two portions: the impactor and the borosilicate glass fiber filter.

Table 2-2. Sample Quantities per Test

Sample Type	Sample Quantity
Background samples	
Background – Negative aerosol samples	1
Background – Negative surface sample (test area)	3
Test Samples	
MDI control (stainless steel reference control) samples	4
Material positive control (Pre-AAS) samples	3
Pre-fumigation AAS aerosol samples	3
Post-fumigation surface samples	3
Post-fumigation AAS aerosol sample	3

2.7.3 Sample Containers

For each sponge wipe sample, the primary containment was a Seward Stomacher® bags (P/N BA6041/CLR, West Sussex, England). Secondary containment was sterile sampling bags. The primary, secondary, and tertiary containment of each vacuum sock sample consisted of separate-sterile sampling bags. Swabs were placed in the sterile swab containers (part of the swab sampling kit (BactiSwab® (Lenexa, Kansas)) as obtained from the supplier) and then bagged in two individual sterile sampling bags as secondary and tertiary containment. Hi-Vol filters were placed in a stomacher bag to be used for extraction, and then bagged in two individual sterile sampling bags as secondary and tertiary containment. All biological samples from a single test were then placed in a sterilized container. After samples were placed in the container for storage and transport to the Biocontaminant Laboratory, the container was wiped with a towelette saturated with a hypochlorite solution. A single container was used for storage in the decontamination laboratory during sampling and for transport to the Biocontaminant Laboratory.

2.7.4 Sample Identification

Each material section was identified by a description of the material and a unique sample number. The sampling team maintained an explicit laboratory log which included records of each unique sample number and its associated test number, contamination application, any preconditioning and treatment specifics, and the date treated. The sample codes eased written identification. Once the samples were transferred to the Biocontaminant Laboratory for extraction and analysis, each sample was additionally identified by a replicate plate identifier and dilution factor. Table 2-3 specifies the sample identification. The Biocontaminant Laboratory also included on each plate the date it was placed in the incubator.

Table 2-3. Sample Coding

Sample Identification: 28-TN-(X)M(AA)-C-S#		
Category	**Example Code**	
TN	1A	Test Number , followed by an A or B for duplicate tests
(X) M (Material)	X	Procedural Background sample
	C	Carpet
	L	Laminate Flooring
	W	Painted Drywall
	S	Stainless Steel (for QC purposes)
AA (aerosol samples)	AF	AAS aerosol sample (quartz filter)
	AS	AAS aerosol sample (impactor)
C (fumigation state)	1	Pre-fumigation
	2	Post-fumigation
S# (Sample Type)	F#	Field Blank
	L#	Laboratory blank
	R#	Replicate
DCMD Biocontaminant Laboratory Plate Identification: 28-TN-(X)M(AA)-C-S#-R-d		
28-TN-(X)M(AA)-C-S#	As above	
R (Replicate)	R	A – C
d(Dilution factor)	1	0 to 4, for 10E0 to 10E4

2.7.5 Sample Preservation

Following transfer to the Biocontaminant Laboratory, all samples were stored at 4 ± 2 °C until analyzed. All samples were allowed to equilibrate at room temperature for one hour prior to analysis.

2.7.6 Sample Holding Times

After sample collection for a single test was complete, all biological samples were transported to the Biocontaminant Laboratory immediately, with appropriate chain of custody (COC) form(s). Liquid samples were stored no longer than 24 hours prior to analysis. Samples of other matrices were stored no longer than five days before the primary analysis. Typical hold times, prior to analyses, for most biological samples was ≤ one day for liquid samples and ≤ three days for all others.

2.7.7 Sample Custody

Careful coordination with the Biocontaminant Laboratory was required to achieve successful transfer of uncompromised samples in a timely manner for analysis. Test schedules were confirmed with the Biocontaminant Laboratory prior to the start of each test. To ensure the integrity of samples and to maintain a timely and traceable transfer of samples, an established and proven chain of custody or possession is mandatory. Accurate records were maintained whenever samples were created, transferred, stored, analyzed, or destroyed. The primary objective of these procedures was to create an

accurate written record that could be used to trace the possession of the sample from the moment of its creation through the reporting of the results. A sample was in custody in any one of the following states:

- In actual physical possession
- In view, after being in physical possession
- In physical possession and locked up so that no one could tamper with it
- In a secured area, restricted except to authorized personnel; or
- In transit (by authorized personnel)

Laboratory test team members received copies of the test plans prior to each test. Pre-study briefings were held to apprise all participants of the objectives, test protocols, and COC procedures to be followed. These protocols were required to be consistent with any protocols established by EPA.

In the transfer of custody, each custodian signed, recorded, and dated the transfer on the COC. Sample transfer could be on a sample-by-sample basis or on a bulk basis. The following protocol was followed for all samples as they were collected and prepared for distribution:

- A COC record accompanied the samples. When turning over possession of samples, the transferor and recipient signed, dated, and noted the time on the record sheet. This record sheet allowed transfer of custody of a group of samples from High Bay Room H130-A to the Biocontaminant Laboratory.

- Samples were carefully packed and hand-carried between on-site laboratories. The COC record showing the identity of the contents accompanied all packages.

2.7.8 Sample Archiving

All samples and diluted samples were archived for a minimum of two weeks following completion of analysis. This time allowed for review of the data to be performed to determine if any re-plating of selected samples was required. Samples were archived by maintaining the primary extract at 4 ± 2 °C in a sealed extraction vessel.

3 Results and Discussion

3.1 Definition of Sampling Efficiency

Percent recovery (Equation 1) was calculated based on the recovery (CFU) from all inoculated coupons subjected to AAS, and the recovery (CFU) per coupon achieved by surface sampling methods. An equivalent of twelve coupons were present during pre-fumigation AAS (three individual coupons and one larger coupon containing a 3x3 grid), and an equivalent of nine coupons were present for the post-fumigation AAS (one coupon containing a 3 x 3 grid).

$$\%\, relative\ recovery\ = \frac{Total\ CFU\ recovered\ from\ FRM\ sample}{Average\frac{CFU}{coupon}\ from\ control\ coupons \times\ total\ number\ of\ coupons} * 100\% \qquad (1)$$

3.2 Inoculation and Spore Recovery

To demonstrate recovery from borosilicate filters, filters were spiked with 50 or 500 CFU, extracted according to MOP 6586, and sieved to remove the filter fibers suspensions generated by the extraction process. Recovery, based on the inoculum, ranged from 41 to 131%. This range of error is typical of microbiological recovery methods.

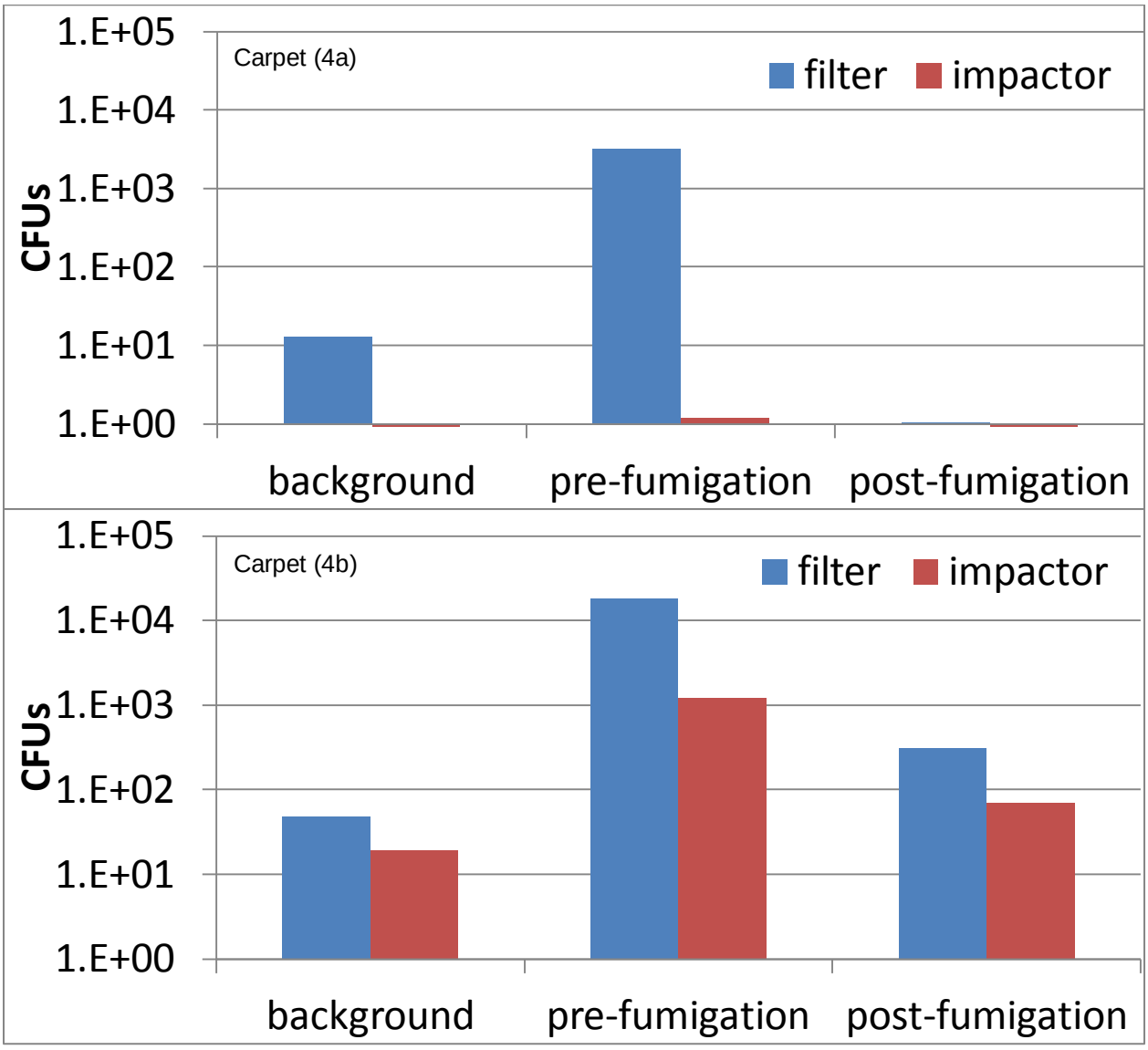
Stainless steel coupons were used to verify the magnitude and repeatability of spore loadings for every inoculation event, and to compare the recoveries from material coupons. Table 3-1 shows the average recovery (CFU) and the Relative Standard Deviation (RSD) for all six tests. Several different MDIs were used for inoculation of different tests, which explains some of the variability between tests. While all inoculation was utilized with *B. atrophaeus* spores prepared by Dugway Proving Ground, two different labs (ECBC and EPA) prepared MDIs with spores. MDIs (EPA1 and EPA2) prepared by EPA were loaded with spores of two different concentrations. Same inoculation source and method were used for both stainless steel and material coupons within each test. The results in Table 3-1 show that spore recoveries on laminate and drywall coupons were similar to those from the stainless steel coupons. However, recoveries from carpet coupons were lower than those on stainless steel.

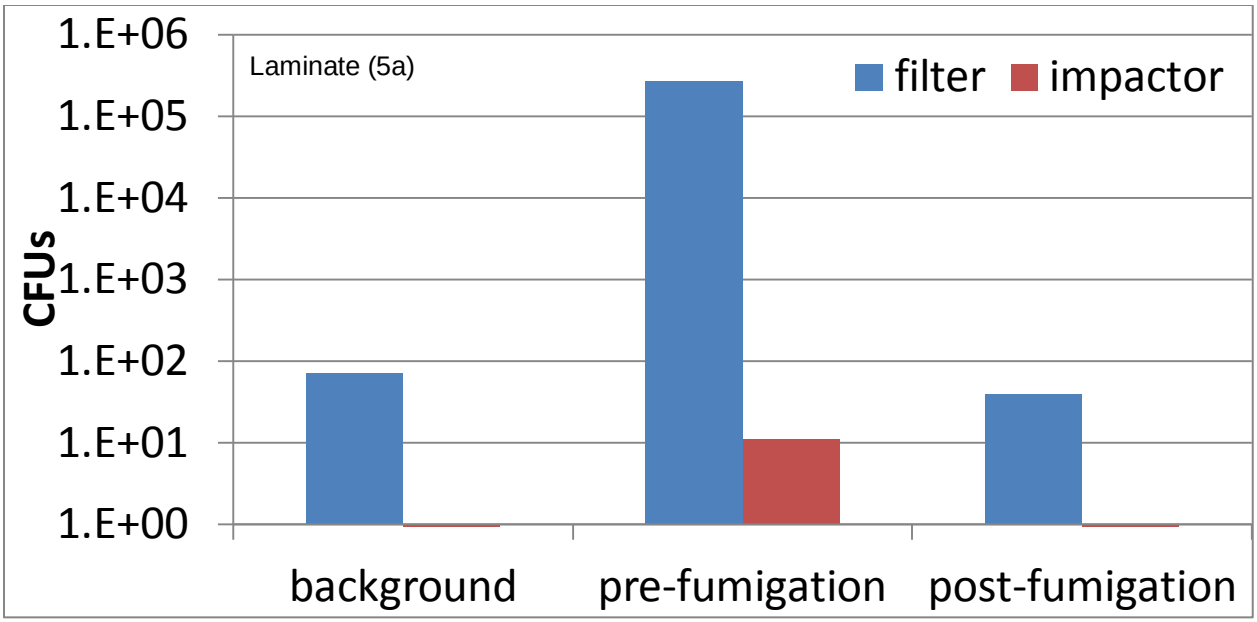
Table 3-1. Inoculation of Coupons

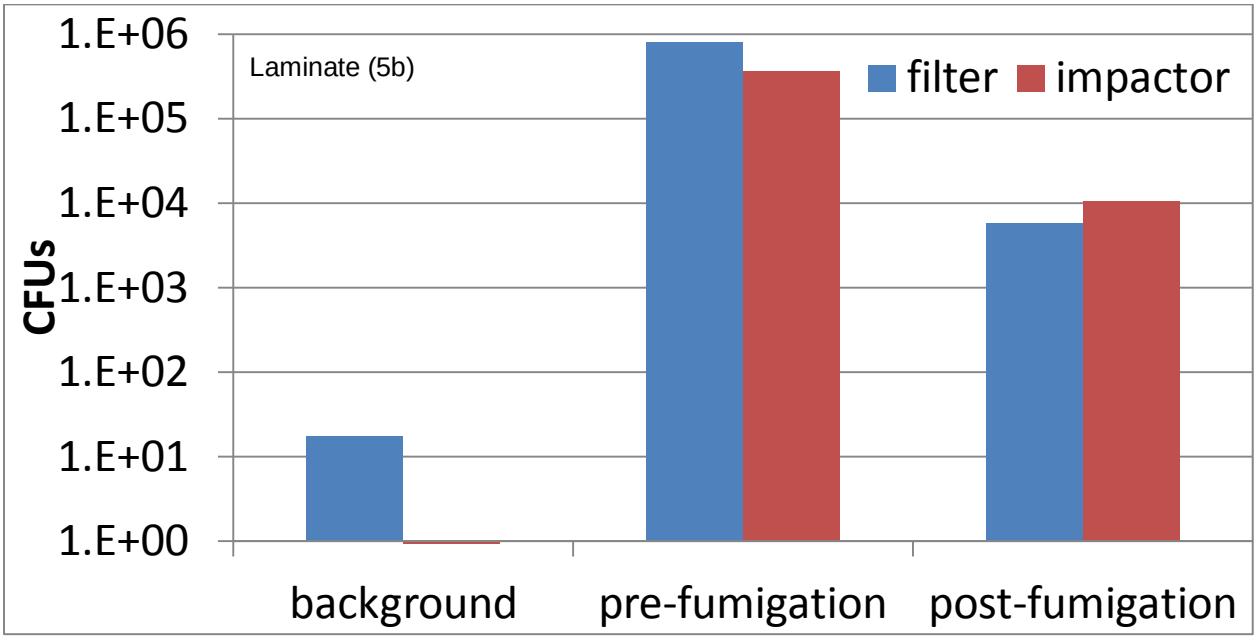
Test ID	Material	Spore preparation	Sample Method	Recovery (CFU) from Stainless Steel coupons (929 cm^2)		Recovery (CFU) from Material coupons (929 cm^2)	
				Avg.	RSD	Avg.	RSD
4a	Carpet	ECBC	Vacuum sock	8.28 X 10^6	17%	2.97 X 10^5	89%
4b	Carpet	EPA1	Vacuum sock	1.62 X 10^7	25%	1.77 X 10^6	69%
5a	Laminate	ECBC	Sponge wipe	8.80 X 10^6	25%	4.61 X 10^6	59%
5b	Laminate	EPA2	Sponge wipe	1.96 X 10^8	22%	3.01 X 10^8	47%
6a	Drywall	EPA1	Sponge wipe	1.23 X 10^7	32%	1.23 X 10^7	7%
6b	Drywall	EPA1	Sponge wipe	1.84 X 10^7	24%	1.24 X 10^7	33%

3.3 Aggressive Air Sampling Results

Three consecutive Hi-Vol samples were collected using three Hi-Vol samplers in sequence (20 minute intervals) for pre- and post-fumigation AAS. The recoveries from the second and third Hi-Vol samplers were less than 1% of the recoveries from the first Hi-Vol sampler for all tests. This implies that one air exchange rate (1000 lpm for 20 minutes) was sufficient to collect 99% of the total spores collected using AAS under the tested conditions. Background AAS was conducted with three Hi-Vol samplers running concurrently. The test results are shown in Figure 3-1.




Laminate (5a)
filter
impactor
CFUs
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
background
pre-fumigation
post-fumigation


Laminate (5b)
filter
impactor
CFUs
1.E+06
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1.E+04
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1.E+02
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1.E+00
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pre-fumigation
post-fumigation

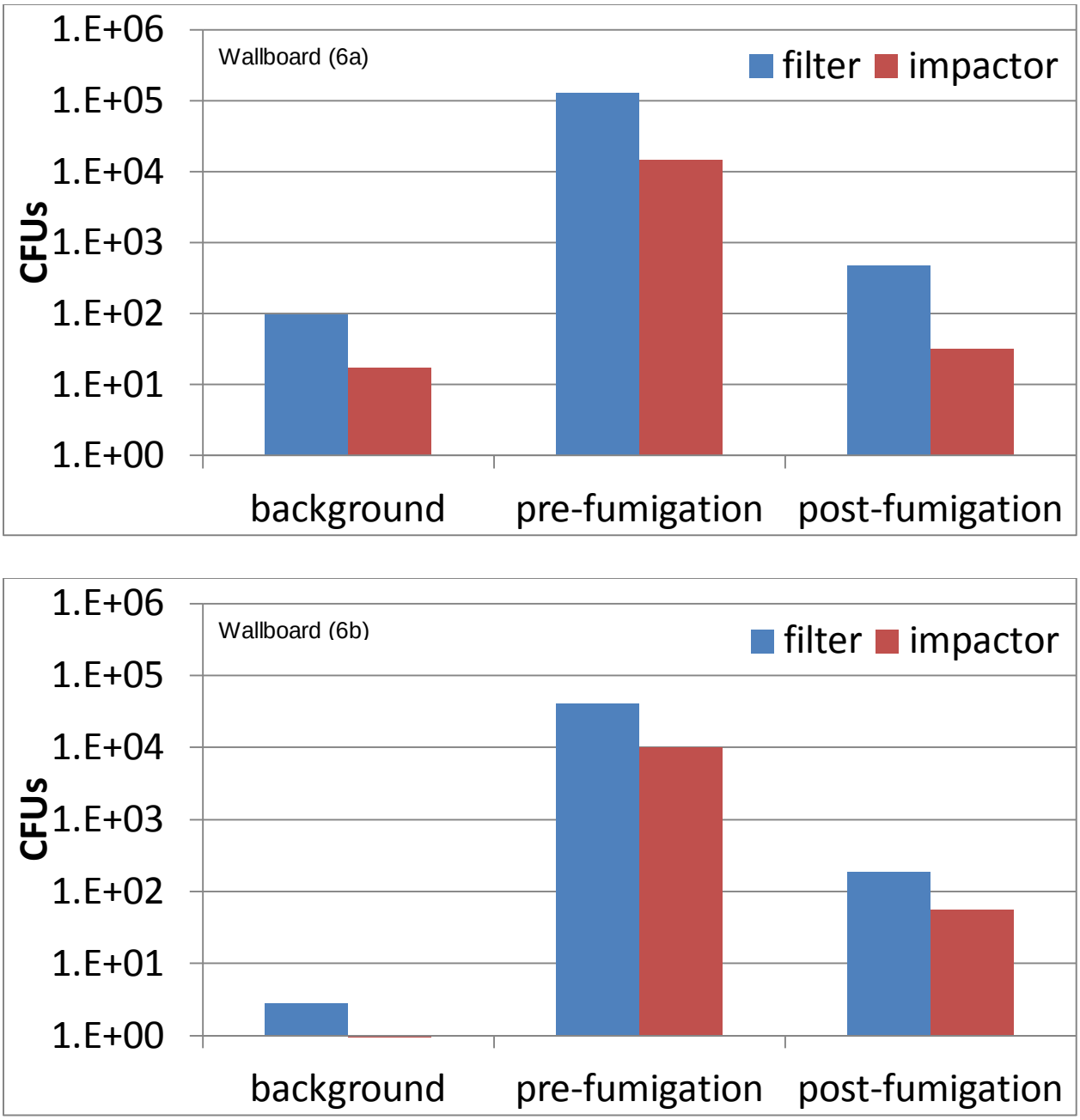

Figure 3-1. Recovery results from AAS tests

Figure 3-1 shows spore recoveries from filter (blue bars) and impactor (red bars) of background, pre- and post-fumigation AAS tests. The data shown in Figure 3-1 are the summation of all three samplers for background, pre- and post-fumigation AAS. Spores were detected from background AAS in all six tests. The total number of spores from background AAS tests was less than 100 CFUs (except 6a, approximately 110 CFUs) and this level is less than 0.1% of the pre-fumigation AAS recoveries. Therefore, the impact by the background spore levels was minimal to pre-fumigation AAS results. For

post-fumigation AAS, spore recoveries were adjusted by subtracting blank AAS spore recoveries for filter and impactor plate.

As a hypothesis, spore contamination in background AAS may be caused by the aerosol deposition using ADAs in an area adjacent to the test chamber. Test coupons were inoculated on a day before each AAS test and uncontrolled spores during deposition were released to the laboratory (close to COMMANDER) air. Released spores might be (re-)suspended into the air via activities in the laboratory. The spore-contaminated air might have been introduced to COMMANDER when the COMMANDER door was open to start the background AAS. Regardless, this detected background did not interfere with the interpretation of the results of this study.

Test results from carpet are shown in 4a and 4b of Figure 3-1. Test 4a was conducted with the MDI prepared by ECBC and EPA1 for Test 4b. The pre-fumigation spore recoveries from Test 4a were mostly from the filter sample (less than 1% from the impactor sample). However, the Test 4b results show that the spore recoveries were mostly (~95% of total recoveries) from the filter, but a more significant amount (~5% of total recoveries) of spores were recovered from the impactor plate. This difference may be due to the use of different MDIs for the two tests. To confirm this, the spore size distributions from two different MDIs were measured using a particle size instrument (Aerodynamic Particle Sizer, APS 3321, TSI Inc, Shorevies, MN). The mass mean diameter and standard deviation were 2.63 µm and 2.05 for the ECBC MDI and 4.91 µm and 6.22 for the EPA1 MDI. Since single *B. atrophaeus* spore size is approximately 0.8 µm, a larger number of agglomerated spores were inoculated to the coupons in Test 4b compared to Test 4a. The large cross sectional area of the agglomerated spores make for easier resuspension compared to the single spores. This is observed from the Test 4a and 4b results. The total number of spore recoveries from Test 4b was approximately 10 times higher than in Test 4a, despite the overall initial spore loadings being similar (Table 3-1).

Tests 5a and 5b in Figure 3-1 show the results of AAS on laminate surfaces. ECBC MDI was used for Test 5a and EPA2 for Test 5b. The AAS pre-fumigation results of Test 5a are similar to those of Test 4a. Most of spores were recovered from the filter sample, not from the impactor (less than 1% of total spore recovery). Test 5b pre-fumigation AAS results show that the amount of spores from the impactor is ~30% of the total spore recovery. As explained from the carpet test results, this impactor recovery difference between Test 5a and 5b is due to different MDI usage for inoculation (i.e., different particle sizes coming from the different MDIs). Tests 6a and 6b in Figure 3-1 show the results of AAS on painted wallboard surfaces. Both tests were conducted with EPA1 MDI. The results from both tests show significant spore recoveries from the impactor as seen in Tests 4b and 5b.

Table 3-2 shows the breakdown results of recovery from the impactor and the filter. The spore recoveries from the first Hi-Vol sampler were more than 95% of the total spore recoveries (sum of three Hi-Vol spore recoveries) for all six tests. The AAS test results were further compared to those of the currently-used sampling methods. The summary is shown in Table 3-2. The comparative recoveries ranged 0.37 to 5.84 %.

Table 3-2. Pre-Fumigation AAS Recovery

Test ID	Material	Blank AAS Recoveries[1]	Average Recoveries (Stainless Steel)	Average Recoveries (Material Coupons)	Total Filter Recovery	Total Impactor Recovery	Relative Recovery
		CFU/cm^2					%
4a	Carpet	0.05	8.90×10^3	3.20×10^2	3.38	0.001	1.06
4b	Carpet	0.09	1.74×10^4	1.91×10^3	19.62	1.30	1.10
5a	Laminate	0.05	9.47×10^3	4.96×10^3	290.01	0.01	5.84
5b	Laminate	0.02	2.11×10^5	3.30×10^5	841.50	386.13	0.37
6a	Drywall	0.26	1.32×10^4	1.32×10^4	134.80	15.46	1.13
6b	Drywall	0.06	1.98×10^4	1.33×10^4	43.64	10.40	0.40

[1] Total background spores detected during blank AAS prior to pre-fumigation AAS, value is the sum of three Hi-Vol samplers operated simultaneously (filter and impactor plate recoveries summed)

To determine the application of AAS at lower concentrations and under varied environmental conditions, the coupons were sampled by AAS before and after VHP® fumigation. Three control coupons were selected from the pre-fumigation AAS samples, which were fumigated at the same time as post-fumigation AAS test samples. These coupons were sampled using currently-used surface sampling methods consistent with each material type and then removed immediately prior to the initiation of the AAS operation on the test coupon. Varying cycles of VHP® (ppm-hours) fumigation were used to obtain differing amounts of post-fumigation surface spore concentrations (as a study parameter). Table 3-3 summarizes the results. Spore recoveries from two tests (4a and 5a) were below background spore recoveries. Collection signatures (percentage of spores collected on the impactor plate and total recoveries) were similar for pre- and post-fumigation AAS samples. No spores were recovered in Test 4a and Test 5a. These tests were initially conducted using high VHP® fumigation conditions (ppm-hours). Later tests (test 4b, 5b, 6a, and 6b) intentionally utilized less effective fumigation conditions in order to be able to detect the quantifiable amount of spores from coupon surfaces. The % relative recoveries ranged between 0.004 to 1.032 %. This % relative recovery values were lower than those from the pre-fumigation AAS tests. This low % relative recovery for post-fumigation AAS might be related to the impact from the application of AAS during the pre-fumigation tests and/or due to the VHP fumigation process. The initial AAS application might have removed a significant amount of loosely attached spores. This phenomenon would likely have a greater impact on AAS than the currently-used surface sampling methods. In addition, the surface coupons were exposed to hydrogen peroxide and higher relative humidity than the pre-fumigation AAS. These factors may increase the spore binding characteristics on surfaces. Therefore, the combination of these factors may have impacted the post-fumigation AAS.

Table 3-3. Post-Fumigation AAS Recovery

Test ID	Material	Fumigation (ppm·hours)	Average Recoveries (Material Coupons)	Total Filter Recovery[a]	Total Impactor Plate Recovery	Relative Recovery
			CFU/cm^2			%
4a	Carpet	495	0.01	B.B[b]	B.B	N.A.[c]
4b	Carpet	358	356.30	0.28	0.06	0.0960
5a	Laminate	501	0.00	B.B	B.B	N.A.
5b	Laminate	444	1679.22	6.10	11.23	1.032
6a	Drywall	188	3789.02	0.40	0.02	0.011
6b	Drywall	211	6986.01	0.20	0.06	0.004

[a] Filter and Impactor plate spore recoveries were adjusted by subtracting background AAS spore recoveries.
[b] Below background
[c] Not Applicable

4 Quality Assurance

This project was performed under an approved Category III Quality Assurance Project Plan titled *Systematic Evaluation of Aggressive Air Sampling for Bacillus anthracis Spores (January 2012)*.

4.1 Sampling, Monitoring, and Analysis Equipment Calibration

There were SOPs for the maintenance and calibration of all laboratory and DCMD Biocontaminant Laboratory equipment. All equipment was verified as being certified calibrated or having the calibration validated by the on-site (RTP, NC) Metrology Laboratory at the time of use. Standard laboratory equipment such as balances, pH meters, biological safety cabinets and incubators were routinely monitored for proper performance. Calibration of instruments was done at the frequency shown in Table 4-1. Any deficiencies were noted. The instrument was adjusted to meet calibration tolerances and recalibrated within 24 hours. If tolerances were not met after recalibration, additional corrective action was taken, possibly including recalibration or/and replacement of the equipment.

The Hi-Vol samplers use the pressure drop over an orifice to measure the volumetric flow rate. These were calibrated prior to testing by the APPCD Metrology Laboratory using a National Institute of Standards and Technology (NIST)-traceable ROOTS® meter.

Table 4-1. Sampling and Monitoring Equipment Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
Pipette	Gravimetric calibration twice per year	±0.1% weight
Thermometer	Compare to independent NIST thermometer (this is a thermometer that is recertified annually by either NIST or an International Organization for Standardization (ISO)-17025 facility) value once per quarter.	±1 °C
RH sensor	Compare to calibration salts once a week.	± 5%
Stopwatch	Compare against NIST Official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java once every 30 days.	±1 min/30 days
Clock	Compare to office U.S. Time @ time.gov every 30 days.	±1 min/30 days
Scale	Sartorius Bl 310 scale: Check calibration with Class 2 weights prior to each use.	±0.01 g

4.2 Data Quality

The primary objective of this project was to evaluate the AAS method to determine whether this technique is appropriate for *B. anthracis* spore sampling. This evaluation was to identify the relative sampling efficacy of the AAS method for spore sampling as a function of surface type, and spore surface concentration. The AAS test results were to then be compared to other currently-used surface sampling methods (i.e., vacuum socks or sponge wipes). This section discusses the Quality Assurance/Quality Control (QA/QC) checks (Section 4.3) and Acceptance Criteria for Critical Measurements (Section 4.4) considered critical to accomplishing the project objectives.

The Quality Assurance Project Plan (QAPP) in place for this testing was followed with several deviations, many of which were documented in the relevant sections of this report. Deviations included:

- Use of sieved filter liquid. When written, the QAPP documented the methods used for filtering a sample with low CFU counts from dilution plating. During testing it was determined that a maximum volume of 3 mL could be filter plated from quartz filter sample due to the presence of debris from the quartz. These samples were thus sieved before filtering to improve the limit of detection.

- Loss of data. Digitally acquired flow rate data were lost from a few tests, due to computer communication problems. However, flow rates at the beginning and end of each test were verified to confirm that samplers operated as expected during the sampling duration. The activation switch for each sampler was also verified to be in the “on” position following sampling initiation. For these reasons, the lost flow rate data were considered ancillary and only necessary to help define the operational parameters of Hi-Vol samplers.

- The MDIs in hand when the QAPP was written, manufactured by ECBC, failed to operate reliably. EPA, therefore, manufactured some replacements. Spores from different MDIs may have different characteristics; however for this study one MDI type (ECBC or EPA prepared) was used for all samples within a single test. Results obtained during each test were comparative (AAS compared to currently used sampling method); therefore results presented herein are not affected by MDI type.

4.3 QA/QC Checks

Uniformity of the test materials was a critical attribute to assuring reliable test results. Uniformity was maintained by obtaining a large enough quantity of material that multiple material sections and coupons could be constructed with presumably uniform characteristics. Samples and test chemicals were maintained to ensure their integrity. Samples were stored away from standards or other samples which could cross-contaminate them.

Supplies and consumables were acquired from reputable sources and were NIST-traceable when required. Supplies and consumables were examined for evidence of tampering or damage upon receipt and prior to use, as appropriate. Supplies and consumables showing evidence of tampering or damage were not used. All examinations were documented and supplies were appropriately labeled. Project

personnel checked supplies and consumables prior to use to verify that they met specified task quality objectives and did not exceed expiration dates.

Quantitative standards do not exist for biological agents. Quantitative determinations of organisms in this investigation did not involve the use of analytical measurement devices. Rather, the CFU were enumerated manually and recorded. Critical QC checks are shown in Table 4-2. The acceptance criteria were set at the most stringent level that could be routinely achieved and are consistent with the data quality objectives described in Section 4.4. Positive controls and procedural blanks were included, along with the test samples in the experiments so that well-controlled quantitative values were obtained. Background checks were also included as part of the standard protocol. Replicate coupons or tests were included for each set of test conditions. Qualified, trained and experienced personnel using SOPs/MOPs ensure data collection consistency. When necessary, training sessions were conducted by knowledgeable parties, and in-house practice runs were used to gain expertise and proficiency prior to initiating the research.

Sterility swabs from Test 6a and 6b coupons indicated that the sterilization procedures were insufficient to kill all the spores present, however contamination was minimal compared to the amount dosed onto test coupons and was consistent across all coupon types (test and control).

Table 4-2. QA/QC Sample Acceptance Criteria

Sample Type	Purpose	Acceptance Criteria	Corrective Actions	Frequency
Negative Aerosol Background Samples	Determine extent of cross-contamination in COMMANDER and from each sampling technique	No detectable spores	If CFU detected, discuss potential impact on results with EPA WAM. Repeat test if necessary after identifying and removing source of contamination	1 per sample per sampling technique per test
Negative coupon control sample	Determine extent of cross-contamination in COMMANDER	No detectable spores	Values on test coupons of the same order of magnitude will be considered to have resulted from cross-contamination. Discuss the potential impact on results with EPA WAM. Repeat test if necessary after identifying and removing source of contamination	3 per test
Field Blank	Verify the process of moving coupons does not introduce contamination	No detectable spores	Determine source of contamination and remove	1 per sampling type

Sample Type	Purpose	Acceptance Criteria	Corrective Actions	Frequency
Laboratory Materials	Verify the sterility of materials used to analyze viable spore count	No detectable spores	Determine source of contamination and remove	1-3 per material per test
Blank Tryptic Soy Agar Sterility Control (plate incubated, but not inoculated)	Controls for sterility of plates	No observed growth following incubation	All plates are incubated prior to use, any contaminated plates were discarded	All plates
Control Coupons (stainless steel and material)	Used to determine the extent of inoculation on the coupon.	1E6 CFU, ±0.5 log	Outside target range: discuss potential impact with EPA WAM; correct loading procedure for next test and repeat depending on decided impact	8 per test
Inoculation Reference Coupons (stainless steel)	Used to determine drift in the MDI	The CFU recovered from the first set of positive controls must be within 0.5 log of the second set of positive controls	Reject results and repeat test	3 per test
Biological Samples	Controls for outliers in colony growth	CFU counts between 30-300 Each CFU count must be within 100% of the other two replicates	Replate or filter plate if CFU outside criteria	Each sample

4.4 Acceptance Criteria for Critical Measurements

The Data Quality Objectives (DQOs) are used to identify the critical measurements (CM) needed to address the stated objectives and specify tolerable levels of potential errors associated with simulating the prescribed fumigation environments. The following measurements were deemed to be critical to accomplish part or all of the project objectives:

- enumeration of spores recovered from the surface of the coupons and aerosol filters

The Data Quality Indicators (DQIs) listed in Table 4-3 are specific criteria used to quantify how well the collected data met the DQOs. Failure to provide a measurement method or device that meets these goals results in the rejection of results derived from the CM. For instance, if the plated volume of a sample is not known (i.e., is not 100% complete), then that sample is invalid. When originally written, the QAPP specified the flow rate of the Hi-Vol as critical. However, results in this report are not based on volumetric terms, but in terms of a 20 minute sample. Therefore, failure to collect flow rate data in real time did not

invalidate the results as long as the duration of the sample could be determined. All Hi-Vol samplers passed pre- and post-test calibrations.

Table 4-3. Critical Measurement Acceptance Criteria

Critical Measurement	Measurement device	Accuracy	Precision	Detection Limit	Completeness
Plated Volume	Pipette	±2%	±1%	NA	100%
CFU/Plate	Manual counting	±10% (between 2 counters)	±5	1 CFU	100%
Sample time	Clock	±1 minute per hour	NA	NA	100%

Plated volume critical measurement goals were met. All pipettes are calibrated yearly by an outside contractor (Calibrate, Inc.) and verified to be within 2% tolerance by gravimetric analysis

Plates were quantitatively analyzed (CFU/plate) using a manual counting method. For each set of results (per test), a second count was performed (by a second technician) on 25 percent of the plates with significant data (data found to be between 30-300 CFU). All second counts were found to be within 10 percent of the original count.

There are many QA/QC checks used to validate microbiological measurements. These checks include samples which demonstrate the ability of the Biocontaminant Laboratory to culture the test organism, as well as to demonstrate that materials used in this effort do not themselves contain spores. The checks include:

- Negative control coupons: sterile coupons sampled just as inoculated ones were.
- Field blank samples: sample collection kits taken to the field and transferred as samples were.
- Laboratory material samples: includes all materials, individually, used by the DCMD Biocontaminant Laboratory in sample analysis
- Positive control coupons: coupons inoculated but not subjected to AAS
- Inoculation control coupons: stainless steel coupons inoculated at beginning, middle, and end of each inoculation campaign, not subjected to AAS, to assess the stability of the MDI during the inoculation operation.

The Vaisala RH meters were calibrated weekly and were within the factory specifications during each AAS operation.

4.5 Data Quality Audits

This project was assigned QA Category III and did not require technical systems or performance evaluation audits.

4.6 QA/QC Reporting

QA/QC procedures were performed in accordance with the QAPP for this investigation.

5 Summary and Recommendations

The results of this study demonstrated that AAS may be a viable option to sample *B. anthracis* spores. The overall test results showed AAS results may vary depending on the contamination characteristics (e.g., spore size distribution) and environmental conditions (e.g., RH, fumigation, other activities that may impact the surface condition). When the AAS recovery results were compared as a function of surface types, AAS recoveries were lowest from the carpet among three tested materials. The laminate surface showed the highest spore recoveries. The % relative recoveries ranged 0.37% to 5.84% for pre-fumigation and 0.004% to 1.032% for post-fumigation AAS.

AAS is composed of three major components: surface agitation for particle resuspension, resuspended particle mixing, and air sampling using filtration. The current test used a commercially available leaf lower (160 mph) to agitate the test surface. Agitation duration, pattern, and applied pressure should be improved to increase spore resuspension potential by investigating the surface agitation tool and method. To fully mix the air within the test chamber, one industrial size fan was used. However, excessive mixing can result in particle loss to walls while insufficient mixing can increase losses due to gravitational settling. The number of mixing fans, location, and fan speed should be optimized to reduce the resuspended particle loss. For air sampling, three Hi-Vol samplers were used in this study to sample in sequence during pre- and post-fumigation AAS. The first Hi-Vol sampler collected more than 95% of spores. The second and third samplers showed recoveries from inoculated coupons near or less than that of background levels. Further, a breakdown of recovery from the impactor and the filter of the high volume sampler showed that most of the recovery is typically from the filter; however, the recovery of spores on the impactors suggests some spore clumping or spores traveling on other particles of more than 10 µm in size (see Table 3-2). Background-adjusted recoveries from the first Hi-Vol sampler (first 20 minutes, including the occurrence of the leaf blower agitation) were higher than the two subsequent Hi-Vol samplers (second and third 20 minutes). The recoveries from the subsequent samplers were below background levels. Post-fumigation comparative recoveries were lower than pre-fumigation comparative recoveries for three tests (4b, 6a, 6b) by a factor of 10 to 1000. These lower recoveries are the results of the combination of all treatments on the test samples (e.g., AAS and fumigation). Hi-Vol samplers in the current study used ~1000 lpm for 20 min. This sampled volume represented 1 air exchange of COMMANDER volume. High sampling flow rate (less sampling duration) is recommended to minimize the loss of spores due to wall impaction and/or gravitational settling. Further the number of air samplers and location should be optimized by considering the surface and volume of sampling site.

Systematic investigations are needed to assess AAS for targeted sampling approaches. Future studies should seek to determine the application of AAS to varied dissemination scenarios (e.g., hot-spot, low levels over wide-area, unconfined spaces, etc.), environmental conditions, and application (e.g., characterization or clearance sampling).

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Appendix A: Miscellaneous Operating Procedures

MOP 3135	Procedure for Sample Collection using BactiSwab™ Collection and Transport Systems
MOP 3141A	Procedure for Assembling Irradiated Vacuum Sock Sampling Kits
MOP 3145	Procedure for HEPA Vacuum Sampling of Large and Small Coupons
MOP 3150-All	Procedure for Fabrication of35.56 cm x 35.56 cm, 71 cm x 71 cm, and 107 cm x 107 cm Material Coupons
MOP 3161-HD	Aerosol Deposition of Spores onto Material Coupon Surfaces using the Aerosol Deposition Apparatus (ADA) – High Dosing
MOP 3165	Sponge Sample Collection Protocol
MOP 3166	Aerosolization of Contaminated Coupons Using Toro Power Sweep Electric Blower for Aggressive Air Sampling (AAS)
MOP 3168	Aggressive Air Sampling (AAS) for WA 3-28: Phase I Sampling Approach
MOP 3169	Sponge Sample Collection Protocol for AAS Impactors
MOP 6535a	Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spore
MOP 6555:	Petri Dish Media Inoculation Using Beads
MOP 6562	Preparing Pre-Measured Tubes with Aliquots Amounts of Phosphate Buffered Saline with Tween 20 (PBST)
MOP 6563	Swab Streak Sampling and Analysis
MOP 6565	Filtration and Plating of Bacteria from Liquid Extracts
MOP 6572	Recovery of Spores from Vacuum Sock Samples
MOP 6580	Recovery of *Bacillus* Spores from 3M Sponge-Stick™ Samples
MOP 6586	Recovery of *Bacillus* Spores from Quartz Filters

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