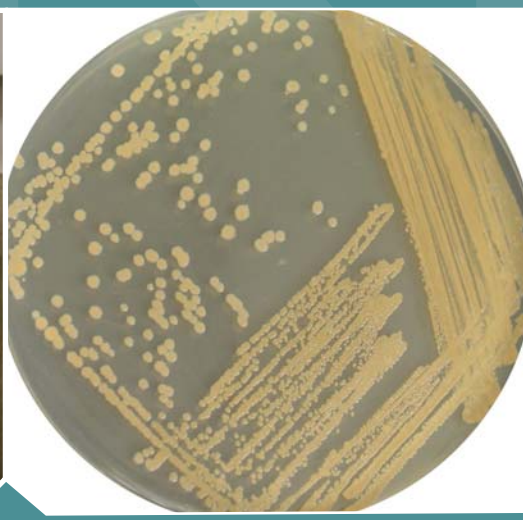
EPA/600/R-12/591 | August 2012 | www.epa.gov/ord

United States
Environmental Protection
Agency

Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Evaluation of Spray Method Parameters and Impact of Surface Grime



Office of Research and Development
National Homeland Security Research Center

EPA/600/R/12/591
August 2012

Disclaimer

The United States Environmental Protection Agency, through its Office of Research and Development's National Homeland Security Research Center, funded and managed this investigation through Contract #EP-C-09-027 WA 2-25 with ARCADIS, U.S. This report has been peer and administratively reviewed and has been approved for publication as an Environmental Protection Agency document. It does not necessarily reflect the views of the Environmental Protection Agency. No official endorsement should be inferred. The Environmental Protection Agency does not endorse the purchase or sale of any commercial products or services. This report includes photographs of commercially available products. The photographs are included for purposes of illustration only and are not intended to imply that Environmental Protection Agency approves or endorses the product or its manufacturer.

Questions concerning this document or its application should be addressed to the principal investigator on this effort.

M. Worth Calfee, 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: 919-541-7600
Fax: 919-541-0496
E-mail: Calfee.Worth@epamail.epa.gov

Foreword

Following the events of September 11, 2001, addressing the critical needs related to homeland security became a clear requirement with respect to EPA's mission to protect human health and the environment. Presidential Directives further emphasized EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological and/or radiological (CBR) attack. To support EPA's mission to assist in and lead response and recovery activities associated with CBR incidents of national significance, the National Homeland Security Research Center (NHSRC) was established to conduct research and deliver products that improve the capability of the Agency and other federal, state and local agencies to carry out their homeland security responsibilities.

One goal of NHSRC's research is to provide information on decontamination methods and technologies that can be used in the response and recovery efforts resulting from a CBR release over a wide area. The complexity and heterogeneity of the wide-area decontamination challenge necessitates the understanding of the effectiveness of a range of decontamination options. In addition to effective fumigation approaches, rapidly deployable or readily-available surface decontamination approaches have also been recognized as a tool to enhance the capabilities to respond to and recover from such an intentional CBR release.

Through working with Office of Research and Development's (ORD's) program office partners (EPA's Office of Emergency Management and Office of Chemical Safety and Pollution Prevention) and Regional on-scene coordinators, NHSRC is attempting to understand and develop useful surface decontamination procedures for wide-area remediation. This report documents the results of a laboratory study designed to better understand the effectiveness of surface cleaning and decontamination methods in an attempt to develop a readily-deployable treatment procedure for surfaces contaminated with, for example, *Bacillus anthracis* spores.

These results, coupled with additional information in separate NHSRC publications (available at www.epa.gov/nhsrc) can be used to determine whether a particular decontamination technology can be effective in a given scenario. NHSRC has made this publication available to the response community to prepare for and recover from disasters involving biological contamination. This research is intended to move EPA one step closer to achieving its homeland security goals and its overall mission of protecting human health and the environment while providing sustainable solutions to our environmental problems.

Jonathan Herrmann, Director
National Homeland Security Research Center

Acknowledgments

This effort was initiated following discussions with the U.S. EPA's Office of Solid Waste and Emergency Response's Office of Emergency Management (OEM) on high-priority research needs to support response and recovery following incidents involving chemical, biological, or radiological (CBR) agents or materials. Due to their regulatory responsibilities under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the U.S. EPA's Office of Chemical Substances and Pollution Prevention (OCSPP) was also interested in this effort. The management support from both program offices for the U.S. EPA's Office of Research and Development (ORD) regarding the contribution that research and development makes towards the U.S. EPA's preparedness in the homeland security area is greatly appreciated

This effort was managed by the principal investigator from ORD's National Homeland Research Center (NHSRC), and EPA's Office of Emergency Management, Chemical, Biological, Radiological and Nuclear (CBRN) Consequence Management Advisory Team.

Project Team:

M. Worth Calfee, Ph.D. (Principal Investigator)
Office of Research and Development, U.S. EPA
Research Triangle Park, NC 27711

R. Leroy Mickelsen, M.S., P.E.
Office of Solid Waste and Emergency Response, U.S. EPA
Research Triangle Park, NC 27711

Shawn P. Ryan, Ph.D.
Office of Research and Development, U.S. EPA
Research Triangle Park, NC 27711

This effort was completed under U.S. EPA contract #EP-C-09-027 with ARCADIS-US, Inc. The support provided by Tanya Medley (U.S. EPA/ORD/NHSRC) in acquiring the vast quantities of supplies required for the completion of this project is also acknowledged.

Additionally, the authors would like to thank the peer reviewers for their significant contributions. Specifically, the efforts of Joe Wood, Marissa Mullins, and Rebecca Connell are recognized.

Table of Contents

Appendices

List of Tables

List of Figures

List of Abbreviations & Acronyms

ADA	Aerosol Deposition Apparatus
ANOVA	Analysis of Variance
APPCD	Air Pollution Prevention and Control Division
ATCC	American Type Culture Collection
BSC	Biological Safety Cabinet
CBR	Chemical, Biological, and Radiological
CBRN	Chemical, Biological, Radiological, and Nuclear
CFU	Colony Forming Unit(s)
Cl_2	Chlorine
DCMD	Decontamination Consequence and Management Division
DHS	Department of Homeland Security
DI	Deionized
DPG	Dugway Proving Ground
DQI	Data Quality Indicator
DQO	Data Quality Objective
ECBC	Edgewood Chemical Biological Center
EPA	U. S. Environmental Protection Agency
FAC	Free Available Chlorine
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
g	gram
ft	Foot (Feet)
ft^2	Square Feet/Foot
HEPA	High Efficiency Particulate Air
H_2O_2	Hydrogen Peroxide
HSRP	Homeland Security Research Program
HVLP	High Volume Low Pressure
in	Inch(es)
in^2	square inch
INL	Idaho National Laboratory
ISO	International Organization for Standardization
kGy	kiloGray

kPa	kiloPascal
Lpm	Liter(s) per Minute
LR	Log Reduction
m^2	square meter
MDI	Metered Dose Inhaler
min	minute
mL	milliliter
MOP	Miscellaneous Operating Procedure
NFSA	National Fire Sprinkler Association
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
NRMRL	National Risk Management Research Laboratory
OCSPP	Office of Chemical Safety and Pollution Prevention
OEM	Office of Emergency Management
OSHA	Occupational Safety and Health Administration
ORD	Office of Research and Development
pAB	pH-Adjusted Bleach
PBS	Phosphate Buffered Saline (solution)
PBST	Phosphate Buffered Saline 0.05% TWEEN®-20
PI	Principal Investigator
PPE	Personal Protective Equipment
ppm	parts per million
ppmv	parts per million by volume
psi	pounds per square inch
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
RH	Relative Humidity
RSD	Relative Standard Deviation
sec	second
STS	Sodium Thiosulfate
TSA	Trypticase Soy Agar
TSP	Trisodium Phosphate
UV	Ultraviolet (light)

VHP®	Vaporous Hydrogen Peroxide

Executive Summary

This project supports the U.S. Environmental Protection Agency (EPA), through its Homeland Security Research Program (HSRP), to provide relevant information pertinent to the decontamination of contaminated facilities resulting from a bioterrorism-related incident. The primary focus of this project is to evaluate and improve the effectiveness and practical application of *in situ* expedient decontamination methods to remediate and restore areas contaminated by biological threat agents. These decontamination techniques would rely on equipment (e.g., garden hoses, portable chemical sprayers, power washers) and application of liquid decontaminant solutions that can be cost-effective and readily available on site.

The aim of this research was to optimize low-tech decontamination approaches using equipment and chemicals that should be readily available at local hardware stores, namely, the use of portable battery-powered chemical sprayers to dispense solutions of pH-adjusted bleach (pAB) onto contaminated surfaces. First, the temporal impact or the age of the pAB solution was investigated by evaluating the sporicidal efficacy on a small-scale. Nonvirulent *Bacillus* spores were used as surrogates for biological agents because they are easily used in laboratory tests without the risk of laboratory worker infection, and represent a conservative estimate of decontamination effectiveness. Rough-cut barnwood and primed and painted wallboard paper were chosen as test materials as they represent commonly occurring, yet challenging to decontaminate, surfaces likely encountered during an urban (indoor or outdoor) remediation. The decontamination solutions were pAB prepared fresh (used within 15 minutes after preparation) and pAB also used at 2, 4, 8, 24, and 32 hours after preparation. Potential negative bias of residual bleach was also evaluated for these two test materials at this inoculation level (7 ± 0.5 log CFU) and at lower inoculation levels (2-3 log ± 0.5 log CFU).

Second, the performance of pAB spray-based decontamination procedures was evaluated parametrically with respect to the physical removal, inactivation, and overall fate of spores on “medium-sized” (35.6 cm x 35.6 cm or 14 in by 14 in) drywall, pressure-treated wood, and concrete pieces (coupons). These materials were utilized neat (no added grime) and chosen because of their common occurrence in indoor and outdoor facilities. The decontamination procedures involved pAB sprayed onto the surface at two different flow rates and various reapplication rates. A wetted wipe decontamination approach was also tested on the medium drywall coupons.

Third, the impact of soiled surfaces (grime) on the sporicidal effectiveness of the low-tech decontamination procedure was assessed for the same “medium sized” coupons. The “medium-sized” coupons were evaluated in a custom-built test chamber.

Operational parameters such as processing time and physical impact on materials or decontamination crew were also determined. Further, to assess the potential for viable spores to be washed off the surfaces, all liquids used in the decontamination process were collected and analyzed quantitatively.

The results from this study indicate that aging of the pAB decontamination solution decreases its sporicidal effectiveness on surfaces that are typically difficult to decontaminate, such as those like wood with a higher organic content. In contrast, aging seems to have little or no effect (within the first four hours after

preparation) on the ability of pAB to decontaminate the nonporous drywall coupons. Solutions of pAB older than 4 hours show a marked decline in efficacy for either type of material tested.

The results from the decontamination procedure parametric tests indicated that greater than 6 log reduction in spores recovered from surfaces – a benchmark for determining efficacy of a decontamination procedure – can be obtained on grime-free drywall and concrete coupons with a single application of pAB (5 seconds (sec)/0.09 square meters (m^2) or 5 sec/square foot (ft^2)), when using the highest flow rate (1344 milliliters (mL)/minute (min)) tested. Further testing focused on developing decontamination procedures for the most challenging material, wood, where 6 log reductions could not be obtained with a single pAB spray application. For wood, increasing the pAB spray duration from 5 sec/ft^2 to 10 sec/ft^2 or increasing the frequency of spraying (two applications rather than a single application) did not provide any statistically significant improved efficacy in surface decontamination. Longer spray duration and/or additional applications were outside the scope of this study, in part because longer decontamination procedures prohibitively increase the cost and complexity of a full-scale response in a real-world scenario. However, based on these results, significant improvement in decontamination efficacy is not anticipated with longer spray duration and/or additional applications.

Tests of the decontamination procedures conducted at a medium level contamination challenge (1×10^4 to 1×10^5 CFU) on drywall and concrete coupon materials confirmed that a full decontamination (meaning no viable spores detected following decontamination) can be obtained with a single application of pAB (5 sec/0.09 m^2 or 5 sec/ft^2). However, no single decontamination procedure investigated within the scope of this study was found to be effective in inactivating/removing spores from low level (1×10^2 CFU) inoculated wood coupons.

The impact of grime (1 gram (g) per coupon) on decontamination efficacy was minimal for rough-cut barn wood and concrete coupons inoculated with 1×10^5 to 1×10^6 spores, when using either a pAB solution or a pAB/trisodium phosphate (TSP) solution as the decontaminant. The results with this grime loading suggest no particular advantage to the use of pAB with surfactant (TSP) versus pAB. Further studies should investigate the efficacy of pAB and pAB with surfactant when used to decontaminate heavily grimed surfaces (i.e., >1 g/0.09 m^2). Additional cleaning activities such as scrubbing or vacuuming did not increase decontamination efficacy, but may increase the chances that spores will be re-aerosolized. The wetted wipe decontamination procedures tested provided minimal to moderate decontamination efficacy (0.6 to 3.3 log reduction).

1. Introduction

The National Homeland Security Research Center (NHSRC) conducted this study in support of the EPA's Office of Research and Development's Homeland Security Research Program. The NHSRC aims to provide expertise and guidance on the selection and implementation of effective and efficient decontamination methods following biological contamination incidents. The Department of Homeland Security (DHS) is tasked to coordinate with other appropriate Federal departments and agencies to develop comprehensive plans which provide for seamless, coordinated Federal, state, local, and international responses to a biological attack. As part of these plans, EPA, in a coordinated effort with DHS, is responsible for developing strategies, guidelines, and plans for decontamination of persons, equipment, and facilities to mitigate the risks of contamination following a biological weapons attack. NHSRC provides expertise and products that can be widely used to prevent, prepare for, and recover from public health and environmental emergencies arising from terrorist threats and incidents.

In 2001, the introduction of a few letters containing anthrax spores into the U.S. Postal Service system resulted in the contamination of numerous government and private facilities. Although most of the facilities in which these letters were processed or received in 2001 were heavily contaminated, they were successfully remediated with approaches such as fumigation with chlorine dioxide or vaporous hydrogen peroxide (VHP®).[1] In addition to fumigation used primarily in heavily contaminated facilities, other cleaning methods were used in secondarily contaminated (e.g., cross-contaminated letters potentially in contact with the *Bacillus anthracis* spore-containing letters or tracked from primarily contaminated sites) areas or primarily contaminated facilities showing a minimal presence of spores.1 During the remediation in 2001, these methods included combinations of removal and disposal of contaminated items, vacuuming, and the use of liquid sporicides such as pH-adjusted bleach (pAB) solution to treat surfaces. Additionally, a set of combined mechanical and chemical procedures (vacuum, scrub/wash and bleach) was used successfully in the decontamination of a small wooden shed contaminated with *Bacillus anthracis* spores originating from animal hides during a drum-making process.[2] When effective, such a "lower-tech" approach involving washing and cleaning with readily available equipment and reagents would significantly increase EPA's readiness to respond to a wide area release. Accordingly, additional quick, effective, and economical decontamination methods having the capability to be employed over wide areas (outdoor and indoor) are needed to increase preparedness for a biological release on a larger spatial scale.

During the decontamination activities following the 2001 anthrax incidents, a combination of removal and *in situ* decontamination techniques was used. The balance between the two approaches was facility-specific and dependent upon many issues (e.g., physical state of the facility). One factor was that such remediation was unprecedented for the United States Government and no technologies had been proven for such use at the time (e.g., registered under the Federal Insecticide, Fungicide, Rodenticide Act [FIFRA]). The cost of disposal proved to be very significant and complicated by the nature of the waste (e.g., finding an ultimate disposal site). Since 2001, a primary focus for facility remediation has been improving the effectiveness and practical application of *in situ* decontamination methods and evaluating waste treatment options to optimize the decontamination/ disposal paradigm. This optimization, recognized as being site-specific, has a significant impact on reducing the cost and time for the remediation effort.

In previous studies conducted by NHSRC, data were generated to understand the effectiveness of different decontamination steps for surfaces inoculated with *Bacillus* spores via aerosol deposition[3]. This understanding was used to develop a recommended method for decontamination of a facility in Durham, NH, contaminated with natural *Bacillus anthracis* spores. These studies generated data to develop and test surface decontamination protocols based upon understanding the effectiveness of individual process steps. The current study was conducted to build upon the previously developed methods in order to (i) improve the ease of application (e.g., decrease reapplication time), (ii) improve overall effectiveness under application conditions of easier use, and (iii) to develop methods effective on grimed surfaces

1.1 Objectives

The primary objective of this study was to improve a series of low-tech decontamination remediation procedures applied to various surfaces contaminated with viable bacterial spores. The remediation approach used for this study focused on equipment (i.e., backpack sprayers, etc.) and chemicals (i.e., bleach, acetic acid (vinegar), etc.) that should be readily available at local hardware stores. Remediation activity parameters were chosen with the aim of improving the decontamination parameters by speeding up slow steps without reducing efficacy and by eliminating ineffective or counterproductive steps. The efficacy was evaluated by recovery of spores from the surfaces of materials while also considering the potential for cross-contamination. Operational parameters such as processing time, physical impact on materials or decontamination crew, and fate of the viable spores (e.g., re-aerosolization, contamination of equipment, wash water, filters) were also determined. This study was comprised of the following three tasks:

- **Task 1** - the impact on efficacy of the degradation of the pAB solution over time (up to 32 hours) was determined.
- **Task 2** - variations on the spray parameters of the decontaminant solution application procedure were investigated.
- **Task 3** - the impact of soiled surfaces (grime) on efficacy was determined.

1.2 General Approach

The general process investigated in this project was decontamination of surfaces contaminated with *Bacillus* spores (i.e., surrogates of *Bacillus anthracis*). Decontamination can be defined as the process of inactivating or reducing the amount of contamination in or on humans, animals, plants, food, water, soil, air, areas, or items through physical, chemical, or other methods to meet a cleanup goal. In terms of the surface of a material, decontamination can be accomplished by physical removal of the contamination or via inactivation of the contaminant with antimicrobial chemicals, heat, ultraviolet (UV) light, etc. Physical removal could be accomplished via *in situ* removal of the contamination from the material or physical removal of the material itself (i.e., disposal). Similarly, inactivation of the contaminant can be conducted *in situ* or after removal of the material for ultimate disposal.

In this research, the basis for the specific decontamination procedure was a process similar to that used by EPA in Region 1 to decontaminate a wooden shed in Danbury, CT. The process employed for the shed is documented in the “After Action Report – Danbury Anthrax Incident (U.S. EPA Region 1, September 19,

2008)"2 and discussed by Snook et al.[4] The resulting process that was the baseline for this project can be summarized in the following sequential procedural steps:

1. Vacuum surfaces with a wet/dry vacuum containing a High Efficiency Particulate Air (HEPA) filter.
2. Mist the surface with the pAB solution until it remains wetted; reapply as necessary to keep the surface wetted for a contact time of 10 minutes.
3. Scrub the surface using a brush with soap and water.
4. Rinse the surface with water.
5. Vacuum standing water from horizontal surfaces with the wet/dry vacuum.
6. Completely cover the surface with the pAB solution for the desired contact time (i.e., 30-60 min).
7. Rinse the entire surface with water.
8. Vacuum standing water from horizontal surfaces with the wet/dry vacuum.

Results from the previous study[3] , were used to develop refined decontamination procedures. Refinements were limited to eliminating steps that have no or negative efficacy and choosing the most effective duration for steps of variable times. Step 5 of the process, for instance, did not contribute to the efficacy of the procedure and could be a source for unintended re-aerosolization. Also, the 30 min of pAB exposure was sufficient to achieve a 6 log reduction of spores, so 30 min was the maximum exposure, instead of the 60 min exposure listed above. The current study attempted to improve the decontaminant application procedure further by investigating effects of varying pAB spray rates (1000 mL/min and 1300 mL/min) and re-applications (none, re-application after 5 minutes, and re-application after 15 minutes). The efficacy of these refined procedures, both with respect to the physical removal and the inactivation of spores, are evaluated on an operational-scale, with relevant test materials.

The general approach used to meet the objectives of this project was:

- use of controlled chambers, standardized coupons and spore inocula;
- inoculation of small or medium-sized uniform pieces of materials (coupons) with bacterial spores via aerosol deposition;
- quantitative assessment of initial viable spore surface concentration by sampling positive control coupons (coupons inoculated with the bacterial spores in the same manner as test coupons but not subjected to the decontamination treatment being tested);
- application of a prescribed decontamination procedure to the test coupons and procedural blanks;
- quantitative assessment of residual viable spore loading on each material type after application of combinations of decontamination procedures by sampling test coupons and procedural blanks;
- quantitative and qualitative analysis of viable spores that survive the various decontamination procedures through transfer to air (via aerosolization) and runoff or rinsate,

- qualitative analysis of viable spores that survive the various decontamination procedures through transfer to wet/dry vacuum filters and vacuum exhaust samples;
- determination of surface decontamination efficacy (comparison of concentrations of viable spores from positive controls and test coupons);
- determination of overall decontamination efficacy (accounting for viable spores transferred to other media [e.g., rinsate or air] during the decontamination process); and
- documentation of operational considerations (e.g., cross-contamination, procedural time, impacts on materials and personnel).

Small (18 mm [0.71 in.] diameter, area of 2.6 cm^2 [0.4 square inches (in^2)]) and medium-sized coupons (35.6 cm x 35.6 cm [14 in by 14 in]; area of 1265 cm^2 [196 in^2]) of relevant building materials were fabricated (see Section 2) and sterilized (see Appendix A) in groups identified by sterilization batch number. The small coupon size was chosen to maximize replicates and minimize sampling effects during the execution of the **Task 1** test matrix (see Section 3.1). The medium coupon size was chosen for application of the physical decontamination methods (i.e., vacuuming, spraying, and brushing) and field sampling methods (i.e., wipe and vacuum sock) used in this current study (**Tasks 2 and 3**). The materials used in this study included wallboard (primed and painted; vertical orientation to represent a wall), rough-cut barn wood (vertical orientation with wood slats oriented vertically), and concrete (vertical orientation). All materials used are considered porous with the exception of the painted wallboard (a sealed surface).

1.2.1 **Task 1**

In **Task 1**, two materials (rough-cut pine wood; primed and painted wallboard paper) were used to test the effectiveness of a pAB solution over time. The materials were inoculated with spores of *B. atrophaeus* (formerly, *B. globigii*) at 7 log CFU (± 0.5 log CFU). The decontamination solutions were freshly prepared pAB (used within 15 minutes after preparation), then used at 2, 4, 8, 24, and 32 hours after preparation. The coupons were sprayed at 0 and 15 minutes with pAB from a backpack sprayer at a flow rate of 1000 mL/min for a rate consistent with 5 sec/ft^2, as determined effective from previous studies[3,24]. The samples collected and analyzed for viable bacterial spores during this task included whole-coupon extractions from five (5) replicate test coupons of each material type and from two sets of positive controls (one per day of testing) and aliquots of rinsates. Samples were collected directly from the pAB solution for determination of pH and free available chlorine (FAC). In addition, aliquots of pAB were collected during spray application to determine post-spray FAC.

1.2.2 **Tasks 2 and 3**

In **Task 2**, the efficacy of spray decontamination approaches with various pAB application flow rates and reapplication scenarios were determined on three materials (rough-cut pine wood; primed and painted drywall, and concrete). These materials were utilized neat (no added grime) and chosen because of their common occurrence in indoor and outdoor facilities. Additionally, they represent a broad range of expected challenge for decontamination. The test materials were inoculated with spores of *B. atrophaeus* at

7 log CFU (± 0.5 log CFU). Recoveries from decontaminated coupons and positive control coupons were determined by wipe sampling, and used to evaluate decontamination efficacy. The main variables examined were the duration and frequency of application of decontamination solutions, which were either pAB or pAB with surfactant (TSP). Scrubbing and vacuuming steps were also investigated for their contribution to reduction of surface contamination.

1.2.3 **Task 3**

In **Task 3**, the impact of surface grime on decontamination efficacy was determined for rough-cut pine wood and concrete coupons. These materials were chosen due to their commonality and they represent materials that have been shown to present a challenge for decontamination. Neat (no grime) and grimed coupons were subjected to spray-based decontamination procedures that determined the additive effects of physical cleaning procedures (scrubbing and vacuuming) on surface decontamination efficacy. Similar to **Task 2**, coupons were inoculated with 7 log CFU (± 0.5 log CFU) spores of *B. atrophaeus.* Grime was applied to coupons at 1g per ft^2. Decontamination efficacy was determined by comparing recoveries from positive controls to that of coupons subjected to the treatment.

1.2.4 **Tasks 2 and 3** Procedure Overview

Although a preliminary test matrix was designed for **Tasks 2 and 3**, these matrices evolved greatly during this project as testing progressed and results were obtained (i.e., an adaptive management approach). Because of this evolution, the details of the testing conducted for these tasks are discussed in Section 3 immediately prior to reporting of the results. In this way, the reasoning behind the test progression will be presented to provide the logic used during the parametric tests.

The projected timeline and flow diagram for the testing approach for **Tasks 2 and 3** is shown in Figure 1-1. Details of the types and numbers of materials tested, as well as the procedures used for inoculation, decontamination, sampling, and testing are described in Section 2 and in the attached appendices.

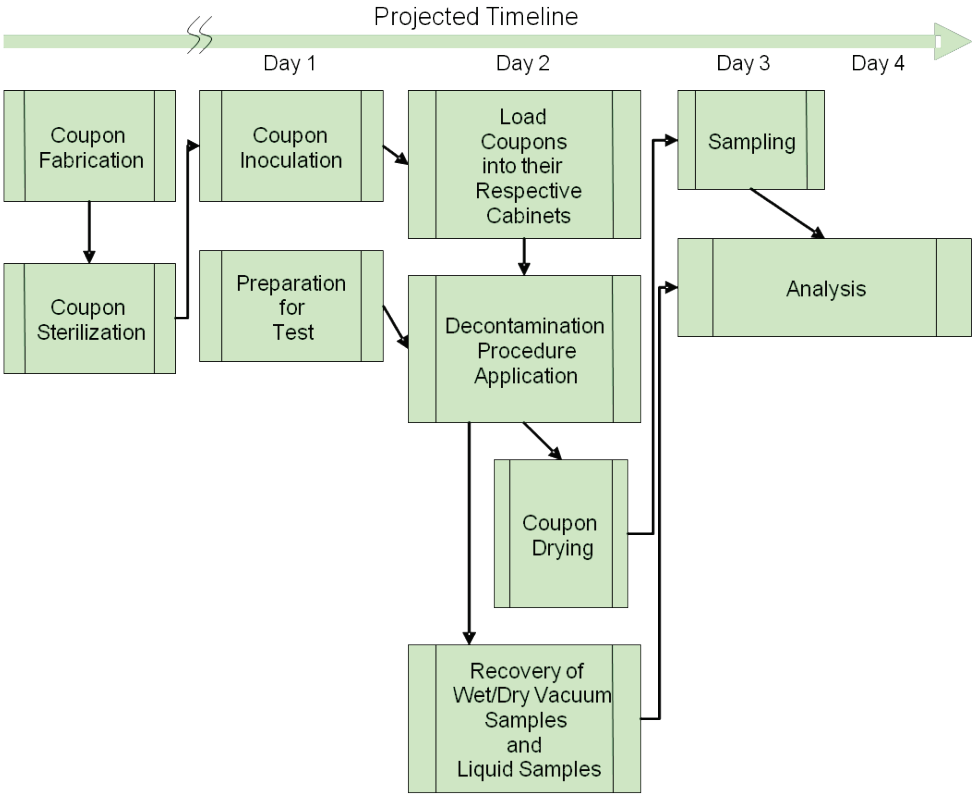


Figure 1-1. Typical Timeline and Flow Diagram for Each Test

Day 1 of testing involved coupon inoculation and preparation for testing on Day 2. The required number of test and positive control coupons were loaded with the target spores at least one day, but no more than four days, prior to the decontamination procedure. The coupons remained isolated in independent deposition devices throughout this time.

On Day 2, inoculated coupons were removed from the deposition devices and loaded into their respective cabinets (positive controls and test coupons into the Test Coupon Cabinets and the procedural blanks into the Procedural Blank Cabinet). As indicated above, positive control and test coupons were both inoculated with the target number of viable spores; the test coupons were subjected to the decontamination procedure being tested while the positive control coupons were not (and were maintained under ambient laboratory conditions). The purpose of the positive controls is to determine the starting viable spore load on each coupon type for comparison to the viable spore load on the test coupons after decontamination. Since the sampling process removes spores from the material surfaces, the procedure of using positive controls and test coupons to determine effectiveness is common.[11,13,14] Procedural blank coupons (negative controls) were the same materials as the test and positive controls. However, the procedural blanks were intentionally not inoculated with spores. The blanks were put through the same decontamination procedure as the test coupons for the purpose of elucidating any potential cross-contamination introduced during the testing procedure.

After the coupons were appropriately stored, sets of three coupons were then positioned in the decontamination chamber and the prescribed decontamination procedure was conducted. Coupons were placed in a vertical orientation. Procedural blank coupons were subjected to the decontamination procedure first. The blank coupons were then transferred to a dedicated storage/drying cabinet. Test coupons were then placed in the chamber for application of the decontamination procedure. The decontamination procedure was completed on all test coupons of one material type before moving on to the next material. After the decontamination procedure was applied to a coupon, the coupon was moved to the appropriate cabinet for drying (test coupons to the Decontaminated Coupon Cabinet and procedural blanks to the Procedural Blank Cabinet). To be consistent with field procedures, the decontaminated coupons were completely dry (allowed to dry for at least 24 hours) prior to sampling. After the completion of each set of coupons, the test chamber was cleaned in accordance with the procedure described in Appendix A. A coupon set included all blank coupons or all replicates of one material type.

The temperature and pH of the pAB solution and deionized (DI) water were measured at the initiation of a test and prior to the start of each test set (i.e., material type). The FAC of the pAB solution was also measured (see Section 2 for method). The flow rate from the backpack sprayer (SRS-540 Propack, SHURflo, Cypress, CA) was measured at the start and end of testing of each set of three coupons on which the sprayer was being used. The spray pattern for the backpack sprayer was confirmed (and adjusted as needed) prior to the start of a test. These measurements were made to ensure that such parameters were in accordance with the data quality objectives (DQOs) defined for the project (see Section 4) and did not confound test results and conclusions. Adjustments were made as necessary to achieve the desired set points within the acceptable tolerances.

Although surface sampling of the coupons did not occur until Day 3, several other samples were collected to obtain additional information on the fate of the spores. To assess the potential for viable spores to be washed off the surfaces, all liquid runoff (rinsate) generated in the decontamination process was collected and analyzed quantitatively for viable spores. Rinsate samples were a composite of the rinsate from all replicate coupons of a particular material type per test. Residual decontaminant within rinsates was neutralized *in situ* with sodium thiosulfate (STS) to prevent continued action of the pAB to reduce spore recovery (Section 2.6.7). Quantitative analysis was conducted on rinsate samples so that the magnitude of spore relocation could be determined. The volume of runoff liquid collected for each coupon set was measured after collection.

On Day 3, after at least 24 hours of drying, surface sampling of the coupons was performed. A sampled area of 0.12 m^2 (1.27 ft^2) per coupon for this study was created by sampling the interior section of each coupon; a template was used to cover the exterior 0.6 cm (0.25 in) of each coupon leaving a square 34.3 cm by 34.3 cm (13.5 in by 13.5 in) exposed for sampling. Surface sampling of each test coupon was conducted only once.

The analysis of the samples collected (coupon, filter, rinsate, and exhaust) occurred over a three-day period. In general, the NHSRC Biocontaminant Laboratory extracted the samples on the day of receipt, plated on the following day, and then counted colonies on the third day. However, instances occurred when it was possible to apply the decontamination procedure to only half of the coupons on the first day, with the remaining half decontaminated on the following day. For these tests, the later samples were analyzed over

a two-day period. Sample extraction and plating would occur on the day of receipt, or a day later, with colony counting the following day.

Appendix A describes the procedure for coupon, test chamber, and equipment cleaning and sterilization. Appendix B contains MOPs, including the aerosol deposition of spores. Appendices C, D, E and F contain additional details of the inoculation, decontamination, sampling and analysis procedures, respectively.

1.3 Definitions of Effectiveness

The "overall effectiveness (efficacy)" of a decontamination technique is a measure of the ability of the method to inactivate and/or remove the spores from contaminated building material surfaces (i.e., represented by coupons in this study) while taking into account viable spores that may be relocated to rinsate and/or aerosol fractions. Such fugitive biological emissions could result in secondary contamination that would necessitate additional remediation strategies.

1.3.1 Surface Decontamination Efficacy

The surface decontamination efficacy for each decontamination technique and surface material combination was evaluated by measuring the difference in the logarithm of the measured CFU before decontamination (determined from sampling the positive control coupons) and after decontamination (determined from sampling the test coupons) for that material. This value is reported as a log reduction on the specific material surface as defined in Equation 1-1.

$$\eta_i = \frac{\sum_{k=1}^{N_C} \log_{10}(CFU_{C,k})}{N_C} - \frac{\sum_{k=1}^{N_t} \log_{10}(CFU_{S,k})}{N_s} \qquad (1\text{-}1)$$

where:

η_i = Surface decontamination effectiveness: the average log reduction of spores on a specific material surface (surface material designated by *i*)

$\frac{\sum_{k=1}^{N_C} \log_{10}(CFU_{C,k})}{N_C}$ = The base 10 logarithm of the geometric mean, or the average of the base 10 logarithm of the number of viable spores (determined by CFU) recovered on the control coupons (C indicates control and N_C is the number of control coupons)

$\frac{\sum_{k=1}^{N_t} \log_{10}(CFU_{S,k})}{N_s}$ = The base 10 logarithm of the geometric mean, or the average of the base 10 logarithm of the number of viable spores (determined by CFU) remaining on the surface of a decontaminated coupon (S indicates a decontaminated coupon and N_s is the number of coupons tested).

When no viable spores were detected, a value of 0.5 CFU was assigned for $CFU_{S,k}$ (see Section 1.3.1.1) and the efficacy was reported as greater than or equal to the value calculated by Equation 1-1.

The standard deviation of the average log reduction of spores on a specific material (η_i) is calculated by Equation 1-2:

$$SD_{\eta_i} = \sqrt{\frac{\sum_{k=1}^{N_s}(x_k - \eta_i)^2}{N_S - 1}} \quad (1\text{-}2)$$

where:

SD_{η_i} = Standard deviation of η_i, the average log reduction of spores on a specific material surface

η_i = The average log reduction of spores on a specific material surface (surface material designated by *i*)

x_k = The average of the log reduction from the surface of a decontaminated coupon (Equation 1-3)

N_S = Number of test coupons of a material surface type.

$$x_k = \frac{\sum_{k=1}^{N_s}((\overline{\log_{10}(CFU_c)} - \log_{10}(CFU_{s,k}))}{N_s} \quad (1\text{-}3)$$

where:

$\overline{\log_{10}(CFU_C)} = \frac{\sum_{k=1}^{N_C}\log_{10}(CFU_{C,k})}{N_C}$ = Represents the "mean of the logs" (geometric mean), the average of the logarithm-transformed number of viable spores (determined by CFU) recovered on the control coupons (C = control coupons, N_c = number of control coupons, k = test coupon number and N_s is the number of test coupons)

$CFU_{s,k}$ = Number of CFU on the surface of the k^{th}

decontaminated coupon

N_s = Total number (1,k) of decontaminated coupons of a material type.

The average surface decontamination effectiveness of the decontamination technique for spores recovered on the surface of building materials, independent of the type of material, was evaluated by comparing the difference in the logarithm of the CFU before decontamination (from sampling of the positive control coupons) and after decontamination (from sampling of the test coupons) for all the tested materials. These data are calculated by determining the arithmetic mean of η for all material types according to Equation 1-4 and reported as log reductions of spores for each decontamination technique.

$$\eta_T = \frac{\sum_i \eta_i}{N_i} \quad (1\text{-}4)$$

where η_T is the overall surface log reduction efficacy for the technique, and N_i is the total number of coupon material types tested with that technique (*i* indicates coupon material type).

The standard deviation of η_T is calculated by Equation 1-5:

$$SD_{\eta_T} = \sqrt{\frac{\sum_i (\eta_i - \eta_T)^2}{N_i - 1}} \quad (1\text{-}5)$$

where:

SD_{η_T} = Standard deviation of η_T, the overall surface log reduction efficacy for the technique

η_T = Overall surface log reduction efficacy for the technique

η_i = The average log reduction of spores on a specific material surface (surface material designated by *i*)

N_i = Number of coupon material types.

While this method of calculating surface decontamination efficacy is useful for comparing decontamination methods, the indoor clearance criterion for a facility following actual bioterrorism events has generally been

no growth of the biocontaminant via culture for all environmental samples.[5] Thus, clearance sampling after use of a particular decontamination method in which CFU of the biocontaminant were detected would indicate that the decontamination was not adequately effective.

1.3.1.1 Detection Limits

Quantification of viable spores collected by surface sampling techniques was determined according to **MOP 6535a** (Appendix B). Quantification was accomplished by physical extraction of spores from sampling media, followed by plating 0.1 mL of serial dilutions of the extraction fluid in triplicate onto trypticase soy agar (TSA) (Difco,Franklin Lakes, NJ). Following plate incubation, CFU (30 to 300 colonies) were enumerated from the appropriate plate dilutions. The CFU per sample were calculated according to Equation 1-6. When fewer than 30 CFU (the quantitation limit) were present on the primary (no dilution) plates, the extracts were filter-plated as described in Section 2.7.12. When no detectable spores were found from the filter-plating, a value of 0.5 CFU was assigned as the detection limit for efficacy determinations (calculation of log reduction). The use of this detection limit value for samples with less than 30 CFU on the primary plates is consistent with other published methods.[6,7,8,9] For the current effort, this detection limit was considered for the plating and, hence, the multiplier of 200 (20 mL extraction fluid for wipe samples divided by 0.1 mL) was applied for all non-filter sample results. This procedure yielded an overall detection limit of 100 CFU/sample. The addition of the filter-plating method lowered the overall detection limit to the stated 0.5 CFU/sample due to analysis of the entire sample extract.

$$\frac{CFU}{sample} = \frac{average\ CFU\ from\ replicate\ dilution\ plates\ or\ on\ filter}{volume\ plated\ or\ filtered,mL} x \frac{1}{(tube\ dilution\ factor)} x(extract\ volume, mL) \quad (1\text{-}6)$$

The number of viable spores in the rinsate was calculated in a similar fashion when all of the rinsate was filtered. For aliquot sampling, a portion (typically 1, 10, and 89 mL) of the rinsate was filtered and the filter was directly plated. The enumerated CFU from these plates were then multiplied by the inverse fraction of the rinsate that was filtered. For example, if 100 mL of the 20,000 mL rinsate (the total volume collected after one coupon set of a test) were plated, then the CFU counts on the filter would be multiplied by 20000/100 (or 200) to represent the total number of spores in the rinsate. If no CFU were present on any filter plate, then a value of 0.5 CFU was assigned as the detection limit ($CFU_{S,k}$ in Equation 1.1). This detection limit was still subject to the multiplier, resulting in a detection limit of 100 CFU for the above example.

1.3.2 Overall Decontamination Effectiveness (Ultimate Fate of Spores)

The surface decontamination efficacy, as calculated in accordance with Equation 1-4, is a measure of the effectiveness of the procedure to mitigate the contamination on the surface of the materials. The measure of effectiveness is an aggregate value due to inactivation of the spores on the materials (i.e., due to the application of a sporicide) and/or physical removal of the spores from the material (e.g., washed/rinsed off or

removed by the vacuum process). When the spores are physically removed from the surface, viable spores may remain in the rinsate, become aerosolized by the physical contact of the decontamination procedure, or be collected on or in any equipment used in the decontamination procedure. Understanding the ultimate fate of the spores (or overall decontamination effectiveness) is critical to recognizing the utility or appropriate implementation of the specific decontamination process.

2. Materials and Methods

Coupon materials were chosen to represent common indoor and outdoor surfaces. Equipment and chemicals utilized for the decontamination methods were selected based upon their high availability at local hardware stores. Prior to use, all test equipment intended to come in contact with coupons or samples was sterilized via autoclave sterilization at 121 °C and 103 kiloPascals (kPa) (15 pounds per square inch (psi)), or by using a STERIS VHP® 1000ED (STERIS Corporation, Mentor, OH) hydrogen peroxide (H_2O_2) generator cycle at 250 parts per million by volume (ppmv) H_2O_2 for four hours. All laboratory work surfaces were covered with bench liner (Fisherbrand, P/N 14-127-47, Waltham, MA).

2.1 Coupon Preparation

For **Task 1**, test materials were 18 mm (0.71 in) diameter coupons prepared from primed and painted wallboard paper and rough-cut barn wood (Figure 2-1). For the wallboard coupons, the interior kraft facing was removed from ½-in thick gypsum wallboard (GOLD BOND, Home Depot SKU# 258-350). The paper was primed with Kilz2 Latex primer and painted with Behr Interior Enamel paint. Circles of 18 mm (0.71 in) diameter were punched from this primed and painted wallboard paper. Only the exterior surface of the wallboard was used because the presence of gypsum interferes with spore recovery during extraction. For the wood coupons, a hole saw (18 mm/0.71 in ID) was used to remove 0.3 cm (1/8 in)-thick circles from the surface of exterior rough-cut barn wood (2.5 cm x 15.2 cm [1 in x 6 in] pressure-treated Brazilian Pine Dog Ear Picket Fence lumber [Product Number 884-831, Home Depot, Durham, NC]). These circles (wood and wallboard paper) were then fastened to 18 mm (0.71 in) aluminum stubs (P/N 16119, Ted Pella, Inc., Redding, CA) using double sided tape (P/N 16073-2, Ted Pella, Inc., Redding, CA), resulting in a coupon. These coupons were sterilized prior to use by steam autoclave utilizing a gravity cycle program consistent with a NHSRC Biocontaminant Laboratory **MOP 6570**. Appendix B lists all associated MOPs for this project, which can be found in the project QAPP or associated Amendments (listed in Section 4.5). All 18 mm (0.71 in) diameter (2.6 cm^2 or 0.4 in^2) coupons were utilized in the vertical orientation during testing.

Figure 2-1. 18 mm Rough-Cut Barn Wood Coupon (Task 1)

For **Task 2**, test materials were 35.6 cm x 35.6 cm (14 in by 14 in) coupons of rough-cut barn wood, painted wallboard, and unsealed concrete. For **Task 3**, test materials were 35.6 cm x 35.6 cm (14 in by 14 in) coupons of rough-cut barn wood and unsealed concrete. Coupons were sterilized according to procedures found in Appendix A. All coupons for **Tasks 2 and 3** were tested in the vertical orientation. Coupon fabrication for both tasks is described below and outlined in **MOP 3150**:

- **Painted Wallboard** (Figure 2-2): A 35.6 cm x 35.6 cm (14 in by 14 in, 1267 cm^2 or 196 in^2) piece of 1.3 cm (0.5 in)-thick wallboard was cut from a 1.2 m by 2.4 m (4 feet (ft) by 8 ft) sheet (Product Number 258-350, Home Depot, Durham, NC). The cut edges were sealed by applying a skim coat of joint compound (Product Number 258-725, Home Depot, Durham, NC) to about 3.8 cm (1.5 in) of the backside edge of the coupon. Using joint tape (5.1 cm or 2 in) (Product Number 430-684, Home Depot, Durham, NC), one half of the tape (utilizing the factory fold) was applied to the back of the coupon. A second skim coat of joint compound was applied over the first coat. After the joint compound was dry, the coupon was turned over and a skim coat of joint compound was applied to the cut edge and about 2.5 cm (1 in) of the front edge. The tape was folded over the edge extending 1.3 cm (0.5 in) over the front side of the coupon. A second skim coat was applied and allowed to dry. Using a sanding block (Product Number 733-336, Home Depot, Durham, NC), any rough spots of the joint compound were removed. One coat of KILZ Latex Primer (Product Number 317-390, Home Depot, Durham, NC) was applied to the front side of the coupon and allowed to dry. This coat of primer was covered with one coat of Behr Premium Plus interior flat white latex paint (Product Number 135-992, Home Depot, Durham, NC). The back side of the coupon received one coat of Behr interior enamel paint (no primer was used on the back) (Product Number 374-776, Home Depot, Durham, NC).

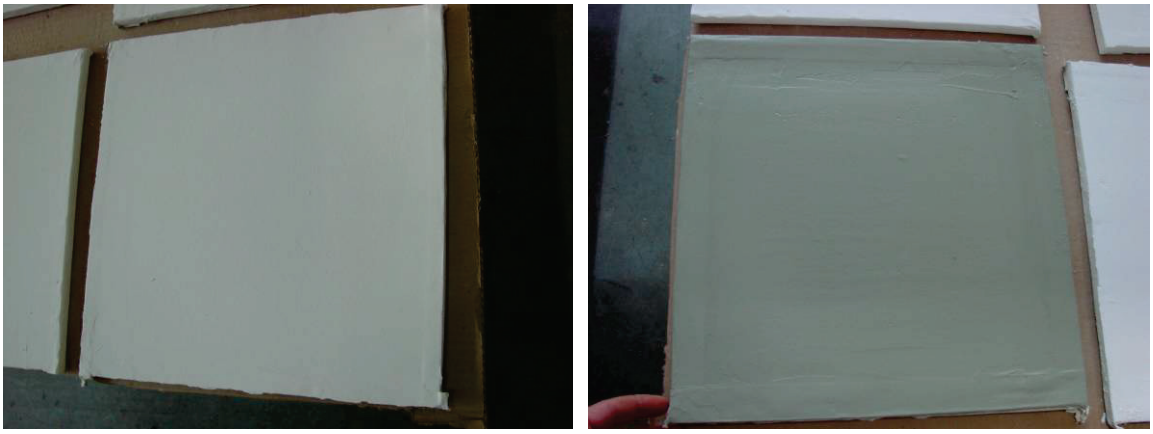


Figure 2-2. Wallboard Coupon Front (Left) and Back (Right)

- **Rough-Cut Barn Wood** (Figure 2-3): The material used to fabricate the 35.6 cm x 35.6 cm (14 in by 14 in) (1267 cm^2 or 196 in^2) coupons of rough-cut barn wood was 2.5 cm by 15.2 cm (1 in by 6 in)(nominal lumber size, 1.3 cm by 13.7 cm [0.5 in by 5.375 in], actual) pressure-treated Brazilian Pine Dog Ear Picket Fence lumber (Product Number 884-831, Home Depot, Durham, NC). The coupons were assembled using two 35.6 cm by 13.7 cm (14 in by 5.375 in) pieces of lumber, plus one 35.6 cm (14 in) long board ripped to 8.5 cm (3.35 in) wide, resulting in a 1267 cm^2 (196 in^2) coupon. The three pieces were assembled with no spaces between boards, attached with 2.5 cm (1 in) staples from the backside, using the same fence lumber material (perpendicular to the surface boards) as support.

Figure 2-3. Rough-Cut Barn Wood Coupon Front (Left) and Back (Right) (Note Offset of Wood Orientation)

- **Concrete** (Figure 2-4): Quikrete® Sand/Topping (Product Number 10389, Home Depot, Durham, NC) mix was used to fabricate 2.5 cm (1 in) thick, 35.6 cm x 35.6 cm (14 in by 14 in) coupons. The mix was prepared according to the manufacturer's instructions and poured into forms. Surfaces were smoothed with a hand trowel and the coupons were allowed to dry overnight. Once set, the coupons were

removed and stacked on a pallet where they were wetted and covered with plastic to cure for at least 30 days.

Figure 2-4. Curing Concrete (Left) and Final Concrete Coupon (Right)

For **Task 3**, several tests required grimed coupons. Recently, a study was conducted to determine the effects of surface grime on biological sampling efficiency[10]. The constituents of the grime used in that study were reportedly similar to grime found on urban surfaces. No other documented recipes of urban grime were found in the literature, so this grime formulation and concentration (1 g/ft^2) was utilized for the current study.

The constituents of the standardized grime included:

- 94% Arizona fine dust – National Institute of Standards and Technology (NIST)-traceable
- 3% Soot mixture:
  - 10 g carbon black
  - 1 g diesel particulate matter – NIST-traceable
  - 0.5 g new 10W30 motor oil
  - 0.5 g α-pinene (neat)
- 3% Mixture of biological materials:
  - 4 g Lycopodium powder
  - 4 g Ragweed pollen
  - 4 g Paper mulberry mixture

This standardized grime was dissolved in ethanol. As discussed in Section 3.3, the grime was irradiated (40 kilogray, kGy) to sterilize it before use. A High Volume Low Pressure (HVLP) sprayer (CX-10, Croix Air Products) was charged with an amount of the solution to deliver 1 g of grime to each coupon (See Figure 2-5). All of the solution was sprayed onto the coupon, and the ethanol was allowed to evaporate from the coupons under a hood for 15 minutes. This process was repeated for each coupon.

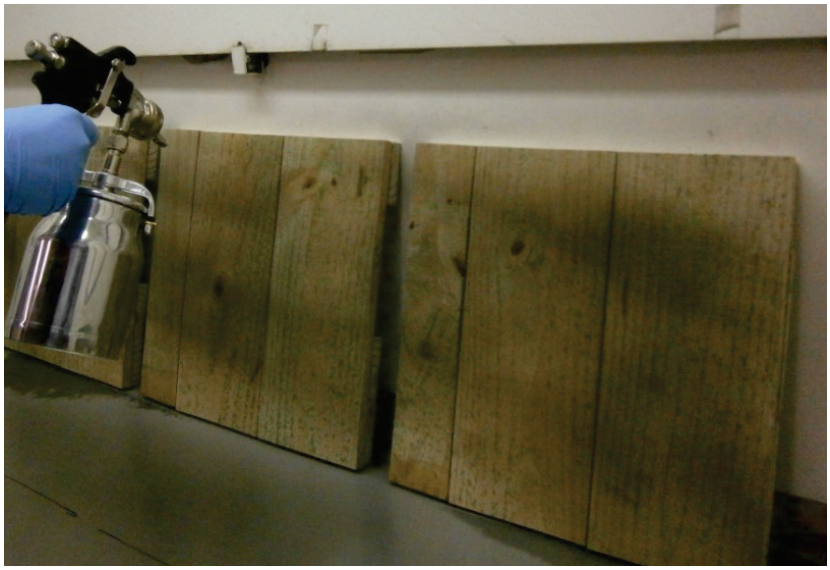


Figure 2-5. Application of Grime to Coupons

2.1.1 Effect of Grime on Surface Recovery.

To determine the effect of grime on recovery of the target organism, preliminary tests were performed on stainless steel coupons. Three sterilized stainless steel coupons were placed in the grime deposition hood (three at a time). Coupons were handled with sterile gloves to minimize contamination. Grime was applied to the coupons according to **MOP 3163** and allowed to dry. Upon drying, coupons were removed from the hood and placed under sterile aerosol deposition apparatus (ADA) pyramids as if being prepared for inoculation. The pyramids were then removed and the coupons were sampled according to **MOP 3144**. The wipe samples were transferred to the NHSRC Biocontaminant Laboratory, along with a field blank sample (handled but unused wipe) and a coupon blank (a wipe sample from a sterile coupon).

The NHSRC Biocontaminant Laboratory extracted the samples according to **MOP 6567**, but then split the samples into two 10 mL aliquots. One aliquot of each sample was then spiked with 1 x 10^4 *Bacillus atrophaeus* spores (total). Three 10 mL aliquots of phosphate-buffered saline with Tween®20 (PBST) were also spiked with the same quantity of spores. Recovery was then determined according to **MOP 6535a**.

2.2 Material Inoculation Procedure

The investigation of the effectiveness of the decontamination procedures requires that a target organism be applied to a “sterile” material surface (i.e., material inoculation) at a precise target loading (e.g., spores per piece of material [or coupon]). This section provides details on the target organism and material inoculation procedures used for this investigation.

Having materials void of all living organisms (i.e., sterile) other than the test organism is necessary in laboratory testing, albeit not realistic of real-world scenarios, because background contamination can

confound the ability to collect and detect the target organism. This condition provides for a laboratory test design which limits potentially confounding, uncontrolled variables, in order to provide a more reliable understand if the impact of the intended parameters.

The inoculation of the coupons using aerosol deposition of viable spores, as opposed to the more traditional use of dispensing precise amounts of liquid spore suspensions onto the material surface (liquid inoculation), was used in this study to more closely represent the nature of the contamination experienced in past "anthrax" incidents. Liquid inoculation has been the more commonly used method of inoculation for studies of decontamination efficacy due to the ease and acceptable precision of the application of the spore suspension.[11,12,13,14] Recently, a highly repeatable (i.e., CV ≤50%) aerosol-based method of coupon inoculation has been developed.[15] This aerosol-based method of inoculation was utilized in this study, consistent with its predecessor studies[3,24], as it more accurately reflects contamination mechanisms experienced during the 2001 letter attacks[16].

2.2.1 *Bacillus* Spore Preparation

The test organism for this work was a powdered spore preparation of *Bacillus atrophaeus* (American Typr Culture Collection [ATCC 9372]) and silicon dioxide particles. This bacterial species was formerly known as *B. subtilis* var. *niger* and subsequently *B. globigii* and is a commonly used surrogate for *Bacillus anthracis*.[14,23] The preparation was obtained from the U.S. Army Dugway Proving Ground (DPG) Life Science Division. The preparation procedure is reported in Brown et al.[17] 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 (Deguss, Frankfurt am Main, Germany). The powdered preparation was loaded into MDIs by the U.S. Army ECBC according to a proprietary protocol. Quality assurance (QA) documentation is provided by ECBC with each batch of MDIs. Control checks for each MDI were included in the batches of coupons contaminated with a single MDI. The low and high inoculation targets used MDIs of differing inoculation doses as described in Section 2.2.2.

2.2.2 Coupon Inoculation Procedure

For **Task 1**, 18 mm diameter (0.71 in) coupons were inoculated (loaded) with spores of *B. atrophaeus* from an MDI using the procedure detailed in **MOP 3113**.

For **Tasks 2 and 3,** 35.6 cm x 35.6 cm coupons (14 in by 14 in) were inoculated with spores of *B. atrophaeus* from an MDI using the procedure detailed in **MOP 6561** and **MOP 3161-LD** for the high and low inoculation levels, respectively. Briefly, each coupon was contaminated independently by being placed into a separate dosing chamber designed to accommodate one 35.6 cm x 35.6 cm (14 in by 14 in) coupon of any thickness. In accordance with **MOP 6561** or **MOP 3161-LD**, the MDI was discharged a single time into the dosing chamber. The spores were allowed to gravitationally settle onto the coupon surfaces for a minimum period of 18 hours. After the minimum 18-hour period, the coupons were then removed from the dosing chamber and moved to an isolated cabinet (Test Coupon Cabinet) which contained all loaded coupons for a single test. Inoculated coupons were handled with care to minimize spore dispersal. One person was tasked with removing the clamps holding the dosing chamber to the coupon and the removal of

the dosing chamber and gasket from the coupon. A second person was then tasked with moving the coupon to the proper location (e.g., test and positive control coupons to the Test Coupon Cabinet and blank coupons to the Blank Coupon Cabinet). The Test Coupon Cabinet is a steel cabinet (1.2 m wide by 0.6 m deep by 2.0 m high) (48 in wide by 24 in deep by 78 in high) with twelve shelves each 15.2 cm (6 inches) apart. Each cabinet could store 36 coupons. Test and positive control coupons were arranged in each cabinet according to material types. Procedural blank coupons of each material/orientation to be used in a single test were contained in a separate isolated cabinet (Blank Coupon Cabinet) of similar construction but with dimensions of 1.2 m wide by 0.6 m deep by 0.9 m high (48 in wide by 24 in deep by 36 in high) with 3 shelves.

There were originally two target contamination ranges: 1 x10^7 CFU (3 x 10^6 to 3 x 10^7 CFU) and 1 x 10^2 CFU (1.7 x 10^2 to 5.6 x 10^1 CFU). **MOP 6561** outlines the higher target range and **MOP 3161-LD** outlines the lower target range. The lower target proved to be problematic: a new lot of MDIs was not as reliable as previous lots, and the 1 x 10^2 CFU target is at the low end of detection. An MDI with a listed concentration of 2 x 10^9 CFU was used for the 1 x 10^7 target recoveries, and an MDI with a listed concentration of 4.5 x 10^6 CFU was used for the low dose target.

The MDIs are claimed to provide 200 discharges per MDI. The number of discharges per MDI was tracked so that use did not exceed this value. Additionally, in accordance with **MOP 6561** and **MOP 3161-LD**, 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 these MOPs, the MDI was retired and a new MDI was used. For quality control of the MDIs, stainless steel 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. These inoculation control coupons (35.6 cm x 35.6 cm or 14 in by 14 in) were contaminated, sampled, and analyzed in the same manner as test coupons.

A log was maintained for each set of coupons that was dosed via the method of **MOP 6561** or **MOP 3161-LD.** Each record in this log contained the unique MDI 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).

2.3 Experimental Approach

All three of the tasks being reported here were conducted in the custom-built test chamber (Figure 2-6) used in predecessors of this study.3[,23,24]. The chamber, located in High Bay Room 130 (H130) at EPA's Research Triangle Park facility, has dimensions of 1.2 m high by 1.2 m wide by 1.2 m deep (4 ft high by 4 ft wide by 4 ft deep) and is designed to accommodate three 0.37 m wide by 0.37 m long (1.2 ft wide by 1.2 ft long) coupons at a time in either orientation (horizontal or vertical, see below). The chamber is of solid stainless steel construction with the exception of the front face and top which are fabricated from clear acrylic plastic. The front face acrylic section is a door allowing full access to the inside of the chamber while standing outside. The back stainless steel wall contains an assembly to hold the vertically oriented coupons (maximum three 0.37 m by 0.37 m [1.2 ft by 1.2 ft] coupons at one time). To minimize cross-contamination, experimentation began with procedural blanks (coupons of each material not contaminated with the target

spore), followed by the test coupons of each material type. Only one material type occupied the test chamber at a time with the exception of the blanks, in which all material types were processed concurrently.

The chamber is fitted with connections allowing HEPA-filtered air to enter and filtered exhaust to exit via a readily accessible connection to the facility's air handling system. The chamber is also designed to be decontaminated easily between runs using either liquids or fumigants, as needed. Decontamination of the chamber is discussed in Appendix A.

The bottom of the chamber is pyramidal in shape with a 7.6 cm (3 in) drain in the center. The drain can be closed or opened either to collect or release the runoff from the coupons during the decontamination procedure. The bottom of the chamber has a 189 L (50 gallon) collection capacity.

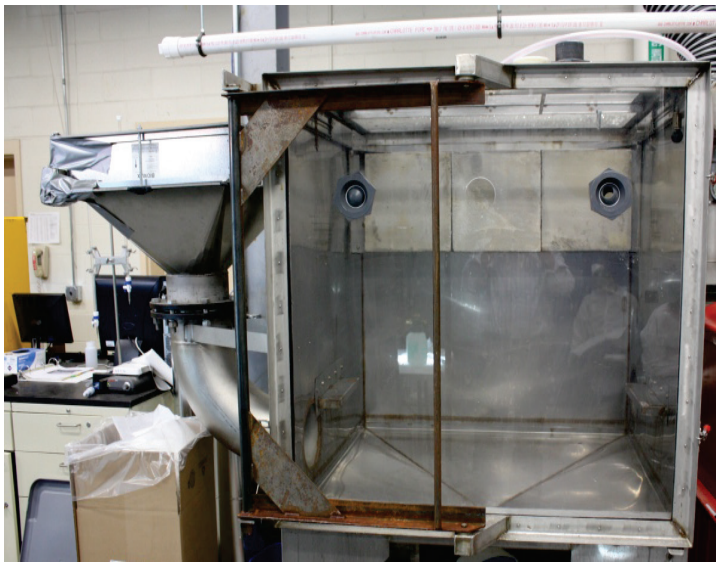


Figure 2-6. Decontamination Chamber

2.4 Decontamination Procedure

It was critical for this project that each step in the decontamination procedure be implemented as uniformly as possible for all sections and tests. Changes in technique during the study could lead to highly variable data and bias the data leading to the drawing of erroneous conclusions. Therefore, the methods for each step were documented in detail in order to provide as much standardization as possible. Staff performing the decontamination procedures practiced each step in advance. Additional details can be found in Appendix D.

For spray-based decontamination procedures, the spray wand was inserted into the center port (see Figure 2-7) and moved in and out as necessary to maintain the correct distance from the three coupons while accomplishing the spray pattern described in Appendix D (decontamination application methods and rinsing

with water). Every effort was made to perform this step consistently and maintain the correct distance from all materials. The port also allowed the chamber door to remain closed during application of the decontamination solutions. During the spraying of the decontamination solutions with the backpack sprayer, the front face door was closed and sealed. The seal was designed to contain any splashed liquid. Maintaining the door in a closed position also prevented exposure of the worker to the toxic fumes from decontamination solutions during application.

The general approach for all spray-based methods was as follows:

1) wet the surface with decontaminant (pAB) using a precise duration and flow rate
2) reapply the decontaminant if prescribed
3) allow the coupons to dry overnight
4) sample surfaces for surviving spores

Figure 2-7. Spraying Through Center-Aligned Port in the Small Chamber Door

Application of the pAB spray on drywall surfaces, though effective against spores, could cause damage to the drywall. Hence, a decontamination method was evaluated using pAB-wetted wipes and/or spritzing (light spraying) of decontaminant. Three tests were conducted to determine the optimal wetted wipe decontamination technique on 35.6 cm x 35.6 cm (14 in by 14 in) drywall coupons in accordance with **MOP-3156**:

- Test 1: Spritzing with pAB, then wiping with pAB-wetted wipe
- Test 2: Wiping with SimWipe (SimChem, Product #: 67F020-01-50, Sarasota, FL)

- Test 3: Combination of wiping with SimWipe, then spritzing with pAB and wiping with pAB-wetted wipe, consecutively

- Test 4: Spritzing with pAB, then wiping with pAB-wetted wipe on coupon with lower inoculum.

The coupons were spritzed with pAB before the pAB wetted wipe procedure only (Test 1 and Test 3). The coupons were allowed to dry overnight, and wipe samples were collected from them one day following the decontamination. Recovery was compared to drywall coupons inoculated the same day as the test coupons that did not undergo any decontamination procedure (positive controls).

2.5 Test Matrix

In **Task 1**, the impact of the age of pAB on sporicidal efficacy was evaluated on small scale (18 mm [0.71 in] diameter; 2.6 cm^2 [0.4 in^2]) coupons of rough-cut barn wood and primed and painted wallboard paper. The materials were inoculated with *Bacillus atrophaeus* (formerly *Bacillus globigii*) spores at 7 log CFU (± 0.5 log CFU). The decontamination solutions were freshly prepared pAB (used within 15 minutes after preparation), and also used at 2, 4, 8, 24, and 32 hours after preparation. The samples collected and analyzed fo viable bacterial spores during this task included whole-coupon extractions from five (5) replicate test coupons of each material type and from two sets of positive controls (one per day of testing) and aliquots of rinsates. Samples were collected directly from the pAB solution for determination of pH and FAC. In addition, aliquots of pAB were collected during spray application, to determine post-spray FAC.

A simplified decontamination procedure was used for **Task 1**. The backpack sprayer was used to spray pAB at 1000 mL/min for 15 seconds at 0 and 15 minutes. The spray pattern, movement, and duration were the same as for the medium-sized coupons (see Appendix D), and based upon results from predecessor tests.[3,24] Coupons were then collected and placed into extraction buffer to quench the sporicidal activity promptly following a 30-minute contact time.

The objective of **Task 2** was to refine application rates and frequency so that a maximum surface area could be fully decontaminated (high efficacy), if achievable, in the least amount of time (low effort). For this effort, **Task 2** the overall effectiveness of decontamination methods were evaluated as a function of application parameters and spore load (surface concentration). Coupons (35.6 cm x 35.6 cm or 14 in x 14 in) of rough-cut barn wood, painted wallboard, and concrete were positioned vertically during testing, but were inoculated horizontally with 1 x 10^7 *Bacillus atrophaeus* spores via aerosol deposition. Decontamination procedures were evaluated for each material type with the decontaminant application process occurring in the spray chamber as described previously.[3,23,24]. Overall decontamination effectiveness was determined as a function of the procedures and material types. Samples included wipes from two coupon materials (painted wallboard and concrete) and from stainless steel inoculation control coupons, vacuum sock samples from rough-cut barn wood coupons, aliquots of runoffs, samples of aerosolized spores, and aliquots of the pAB for pH and FAC measurements. Each test included three replicate test coupons, three replicate positive control coupons, and one procedural blank of each material type, resulting in a total of seven coupons for each material type during each test condition. The backpack sprayer was used for application of the pAB solution to all three test materials.

The decontamination procedure used in the test matrix was based upon results reported previously. [3,23,24]

Results from these studies suggest:

- Unless significant amounts of debris are present on the surface to be decontaminated, no vacuuming step is required before the pAB/TSP solution application;
- Completely cover the surface with pAB solution for the desired 30-minute contact time using the back-pack sprayer for application to all materials (rough-cut barn wood, painted wallboard, and concrete); and
- For comparison of the efficacy of the spray-based method to the efficacy of wetted wipes during drywall surface decontaminations, wipes should be wetted with pAB solution.

.

The sampling strategy included enumeration of viable spores remaining on coupons from decontaminated and non-decontaminated (control) coupons, enumeration of spores in runoffs and aerosols as a result of relocation during decontamination, and evaluation of pAB (pH and FAC). Sampling methods are fully described in Appendix E.

The temperature and pH measurements of the pAB solution and DI water were conducted at the initiation of a test and prior to the start of each test set (i.e., material type) throughout a test. The flow rate from the backpack sprayer was measured at the start and end of each set of three coupons on which the sprayer was used. The spray pattern for the backpack sprayer was confirmed (and adjusted as needed) prior to the start of a test. These methods and the quality control criteria are outlined in the project QAPP entitled, "Quality Assurance Project Plan for the Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Part 4 – Optimization of Method Parameters and Impact of Surface Grime."[18]

For **Task 2**, the test matrix evolved based on the results from previous tests. The matrix as performed is shown in Tables 2-1 and 2-2.

Table 2-1. Test Conditions for High Inoculation Parametric Tests (Task 2)

Test ID	Materials	Decontamination Steps	Prescribed Decontaminant Application Flow Rate
O1	Drywall, Concrete, Wood	1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O2	Drywall, Concrete, Wood	1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse	High (1.5 L/min)
O3	Wood	1 application, 30 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O4	Wood	2 applications (at 0 and 5 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)

O5	Wood	2 applications (at 0 and 15 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)

Table 2-2. Test Conditions for Low Inoculation Parametric Tests (Task 2)

Test ID	Materials	Decontamination Steps	Prescribed Decontaminant Application Flow Rate
O6	Drywall, Concrete, Wood	1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O7	Drywall, Concrete, Wood	1 application, 30 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O8	Wood	1 application, 30 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O9	Wood	2 applications (at 0 and 5 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O10	Wood	2 applications (at 0 and 15 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)

In addition, another set of tests were incorporated into **Task 2** to determine the decontamination effectiveness of wetted wipes at reducing contamination on painted wallboard coupons (Table 2-3). In these tests, pAB solution was spritzed (lightly sprayed) onto the coupon surface with a spray bottle before decontamination was attempted with a wetted wipe.

Table 2-3. Test Conditions for Wetted Wipe Decontamination Tests (Task 2)

Test ID	Materials	Decontamination Steps	Inoculation Level
W1	Drywall	Spritzing with pAB, then wiping with pAB-wetted wipe	High
W2	Drywall	Wiping with SimWipe	High
W3	Drywall	Combination of wiping with SimWipe, then spritzing with pAB and wiping with pAB-wetted	High
W4	Drywall	Spritzing with pAB, then wiping with pAB-wetted wipe	Low

In **Task 3**, the impact of grime on surface decontamination efficacy of rough-cut barn wood and concrete was determined. The coupons were prepared, inoculated, and sampled in accordance with the procedures

described in **Task 2**. In addition, standardized grime (see Section 2.1) was applied to coupons prior to inoculation, as noted in the test matrix (Table 2-4).

Table 2-4. Task 3 Test Matrix, Impact of Grime on Wood and Concrete Surface Decontaminations

Test	Decontaminant	Material	Inoculation	Grime	Scrubbing	Vacuuming[1]
G1	pAB solution	Wood	High	No	No	No
		Concrete				
G2	pAB solution	Wood	High	Yes	No	No
		Concrete				
G3	pAB solution/TSP solution	Wood	High	No	No	No
		Concrete				
G4	pAB solution/TSP solution	Wood	High	Yes	No	No
		Concrete				
G5	pAB solution/TSP solution	Wood	High	No	Yes	No
		Concrete				
G6	pAB solution/TSP solution	Wood	High	Yes	Yes	No
		Concrete				
G7	pAB solution/TSP solution	Wood	High	No	Yes	Yes[1]
		Concrete				
G8	pAB solution/TSP solution	Wood	High	Yes	Yes	Yes[1]
		Concrete				

[1] Vacuum step is the first step performed

Samples included wipes from concrete coupons and from stainless steel inoculation control coupons, vacuum sock samples from rough-cut barn wood coupons, aliquots of runoffs, samples of aerosolized spores, swab samples of vacuum cleaner parts, and aliquots of the pAB for pH and FAC measurements. Three replicate test coupons, three replicate positive control coupons, and one procedural blank of each material type were included in each test during **Task 3**.

The decontamination procedure (see Appendix D) used for **Task 3** was a combination of up to four decontamination steps:

1. Vacuum the surface using the squeegee attachment of a wet/dry vacuum cleaner.
2. Spray the coupon with decontamination solution for 10 seconds at lowest flow rate (1000 mL/min for pAB, 1300 mL/min for pAB/TSP solution).
3. Scrub the coupon with a brush.
4. Allow a 30-minute contact time.

In tests G1 through G6, no vacuum step (step 1) was utilized. In tests G1 through G4, no scrubbing (step 3) was utilized.

Two decontamination solutions were used: pAB and pAB with TSP substitute surfactant (pAB solution/TSP solution). Preparation of the two decontamination solutions is described in **MOP 3128-A** and **3128-B,** respectively.

2.6 Sampling Points

Wipe sample or vacuum sock (Midwest Filtration, Cincinnati OH) samples from test (decontaminated) coupons were collected after a minimum of 18 hours of drying, when coupon surfaces appeared visibly dry. Positive control coupons were sampled at the same time as test coupons. Wipe samples and vacuum sock samples were collected by sampling within a 34.3 cm by 34.3 cm (13.5 in by 13.5 in) sampling template centered on the coupons (Figure 2-8).

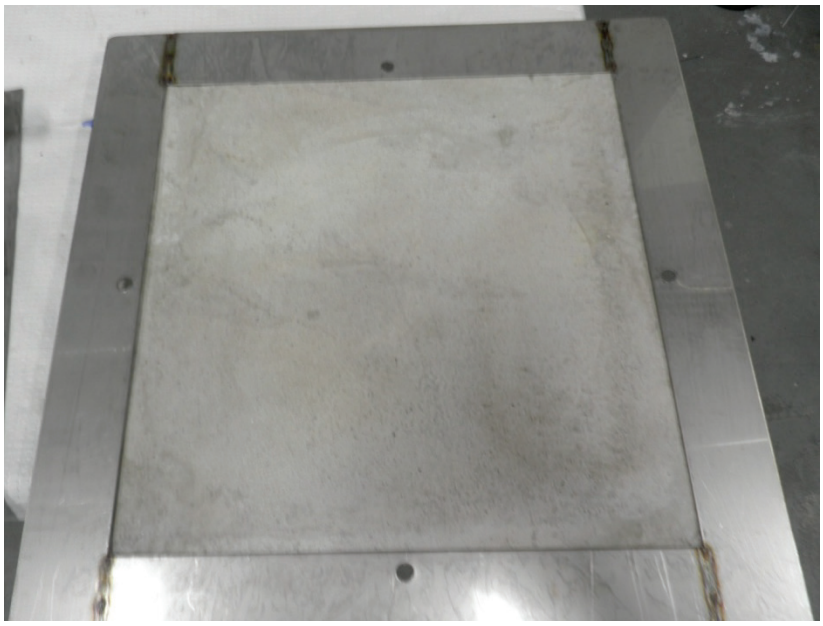


Figure 2-8. Sampling Template Centered on a Representative Concrete Coupon

Extractive samples of **Task 1** coupons were taken immediately after decontamination. The coupons were aseptically transferred to sterile vials while in High Bay Room H130 (the location of the spray chamber). Positive control extractions were conducted after the last test coupons had been extracted.

Runoff was neutralized *in situ* with enough STS to neutralize all bleach sprayed onto the coupons. Aliquots of runoff were collected immediately after all of the runoff from a decontamination procedure had been generated. More details of run-off sample collection and neutralization are provided in Section 2.7.

Aliquots of pAB for FAC and pH were collected and analyzed immediately (within 10 minutes) before use.

Aerosol samples were isokinetically collected during active decontamination activities, from the chamber exhaust duct (Figure 2-9) using a SKC BioSampler® (Model No. 225-9595). The sampling point was eight diameters downstream of and two diameters upstream of any flow disruptions.

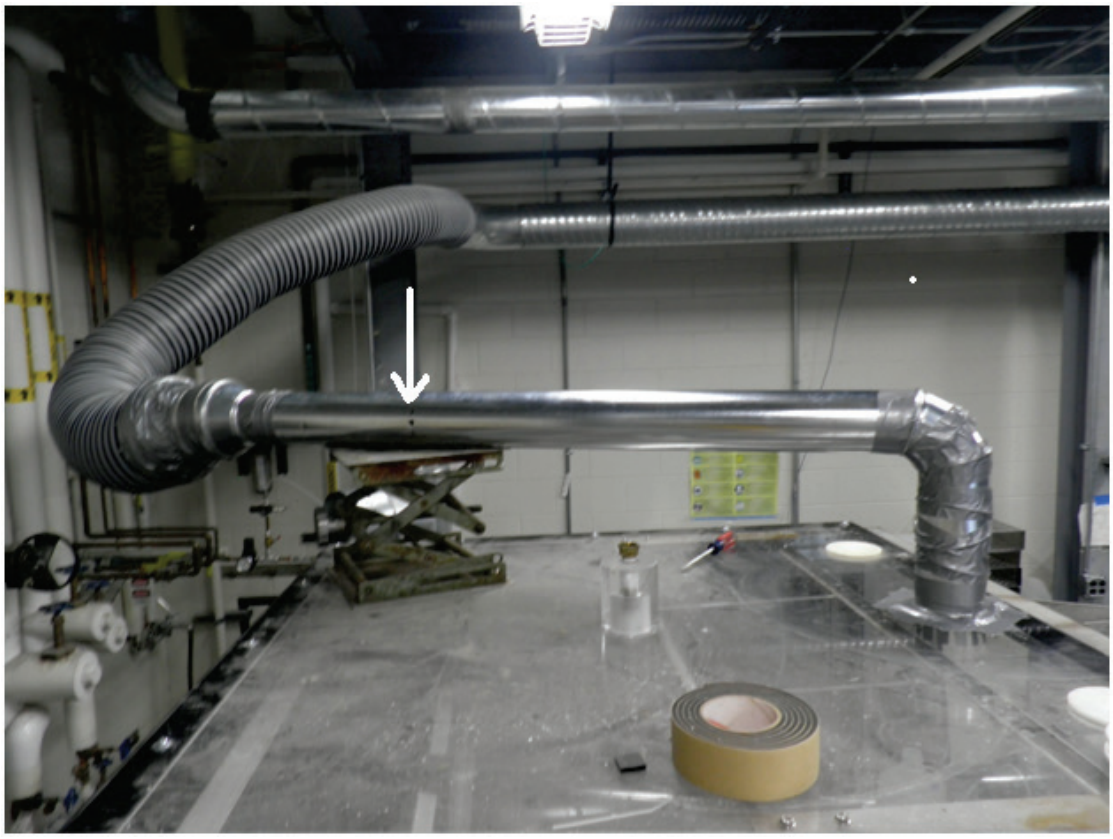


Figure 2-9. Spray Chamber Exhaust Duct with Arrow pointing to Sample Port

Swab samples were collected using **MOP 3135** from the vacuum cleaner nozzle and HEPA filter after use.

- For each coupon set, a swab sample was collected from the HEPA-rated filter within the wet/dry vacuum (if used in the test) and qualitatively analyzed to confirm contamination by the target organism. Such information is relevant to the treatment of the vacuum after use and the potential spread of contamination.

- Two or more exhaust samples were collected, one from the blank wet/dry vacuum (when used) and one or more from the test coupon wet/dry vacuums (when used). These samples were collected because of

the potential for the wet/dry vacuum to spread contamination via the presence of viable spores in the exhaust. This potential was assessed by collecting a composite sample from all vacuuming in a test.

2.7 Sampling and Analytical Procedures

Five types of biological samples were included in this project. These samples are described below and in Table 2-4.

- Extractive samples for quantitative determination of viable spores on coupons were used for **Task 1**.
- Surface sampling procedures were used in **Tasks 2 and 3** to collect samples from the coupon materials. The sampling procedures included wipe sampling or vacuum sampling for quantitative determination of viable spores on coupon surfaces. Additionally, wet swab sampling was done to qualitatively determine the presence of the target organism on wet/dry vacuum filters and for sterility checks of materials and equipment prior to testing.
- The rinsate generated during the decontamination procedure was collected for each material type (**Tasks 2 and 3**).
- HEPA filters from the wet/dry vacuums used for each coupon set were removed and sampled by swabbing (**Task 3**).
- Aerosol samples were collected from the exhaust air flow exiting the decontamination chamber during decontamination operations to quantify spores aerosolized during spray applications (**Tasks 2 and 3**).

Table 2-4. Sample Types

Sample Type	Sample Medium/ Source	Task	Purpose
Extractive sample	18 mm coupons	**Task 1**	Quantify CFU recovery
Wipe Sample	Stainless steel, drywall, and concrete coupons	**Task 2 and 3**	Quantify CFU recovery from material surface
Vacuum Sock Sample	Rough-cut wood coupons	**Task 2 and 3**	Quantify CFU recovery from material surface

Swab samples	Representative Coupons	**Task 2 and 3**	Qualitatively indicate sterility of coupon surface before inoculation
Swab samples	Wet/Dry Vacuum filter	**Task 3**	Qualitatively indicate spore presence
Rinsate Filter	Runoff from coupons during decontamination procedure	**Task 2 and 3**	Quantify CFU in rinsate/runoff
Aerosol	Air from chamber during decontamination procedure	**Task 2 and 3**	Quantify re-aerosolized CFU during decontamination procedure

A sampling event log sheet was maintained for each sampling event (or test). The names of the sampling team members, date, run number, and all sample codes with corresponding coupon codes were recorded on each sheet. The coupon codes were pre-printed on the sampling event log sheet prior to the start of sampling. The materials and equipment used as well as the sampling protocols for sampling are detailed in Table D-2 in Appendix D.

2.7.1 Extraction Sampling

The 18 mm (0.71 in) coupons used in **Task 1** were analyzed by whole-coupon extraction and plating. Following treatment, coupons were transferred aseptically into 50 mL sterile vials containing 10 mL Phosphate Buffered Saline (PBS) + 0.05% Tween®20 (PBST) (P3563, Sigma-Aldrich). This operation was performed in the decontamination chamber in H130. The vials containing the extraction solution with neutralizing STS and coupon were transferred to the NHSRC Biocontaminant Laboratory, where they were sonicated for 20 minutes at 42 kHz and 135 Watts using a Branson 8510 ultrasonic water bath to extract spores from the coupon surface. The solution and coupon were then vortexed 2 minutes to further dislodge any viable spores. Each vial was briefly re-vortexed immediately before any solution was withdrawn. The solution was subjected to five sequential 10-fold serial dilutions (when necessary) following **MOP 6535a**. Each dilution (0.1 mL) was spread-plated in triplicate onto TSA using sterile beads according to **MOP 6555** and incubated at 35°± 2 °C for 18-24 hours. CFU were manually counted.

2.7.1.1 Extraction Method Development

The 18 mm (0.71 in) stubs were placed directly into extraction solution (PBST) using aseptic techniques immediately following decontamination. These stubs were still wet from the pAB application and may have contained enough decontamination liquid to confound enumeration analysis. For this reason, prior to testing described in Section 3.1.2, the following extraction method development test was performed to determine the effects of residual decontaminant on recovery estimates.

Sterilized 18 mm (0.71 in) coupons of rough-cut wood and primed and painted wallboard paper were attached to the center of a 35.6 cm x 35.6 cm (14" x 14") stainless steel mock medium-sized coupon. The coupons were sprayed with fresh pAB in accordance with Appendix D, as if they were a subsection of a medium sized coupon. The backpack sprayer was used to apply pAB at 1000 mL/min for 5 seconds per stainless steel mock medium-sized coupon, with two applications 15 minutes apart, with an additional 15 minute contact time before extraction. The coupons were then placed in extraction solution. A second set of sterilized but unsprayed coupons were placed directly in the extraction solution. Both sets were then spiked with a liquid spore inoculum and allowed to sit for one hour before analysis. Based on the results, discussed in Section 3.1.1, a molar equivalent amount of STS to neutralize 28 ppm FAC was added to the extraction liquid for drywall coupons but not for wood coupons.

2.7.2 Factors Affecting Sampling/Monitoring Procedures

Sampling of coupon surfaces was conducted after coupons that were wetted during the decontamination procedure had become visibly dry. Drying was allowed to occur in the Decontaminated Coupon Cabinet or Procedural Blank Cabinet, facilitated by a slight air flow provided by 5 liters per min (Lpm) of filtered and dried compressed air. All coupons were allowed to dry for at least 18 hours. The actual time that each coupon was allowed to dry was recorded in the laboratory notebook. The biases associated with sampling previously wetted surfaces are unclear, but others have suggested reduced recoveries of viable agent on such surfaces.[19]

2.7.3 Preparation for Sampling/Monitoring

Within a single test, surface sampling of the material sections was completed for all procedural blank coupons first before sampling of any test material sections was performed. This order of operations was followed to minimize the potential for cross-contamination (e.g., sampling progressed from the least contaminated to the most contaminated samples). Surface sampling was conducted either by wipe sampling or vacuum sock sampling in accordance with the protocols included in Appendix E. The surface area for all samples was 0.12 m^2 (1.27 ft^2).

A sampling material bin was stocked with all appropriate items (consistent with the protocols in Appendix E) for each sampling event. The bin contained enough wipe sampling kits to accommodate all required samples for the specific test. An additional kit was also included for backup. Enough gloves and bleach wipes needed to complete the test were available. A sample collection bin was used to transport samples back to the NHSRC Biocontaminant Laboratory for analysis following collection. The exterior of the transport container was decontaminated by wiping all surfaces with a Dispatch® (Clorox) bleach wipe prior to transport from the sampling location to the NHSRC Biocontaminant Laboratory. To ensure the integrity of samples and to maintain a timely and traceable transfer of samples, a proven documented chain of custody (CoC) procedure was followed for each test.

2.7.4 Wipe Sampling

To determine the amount of spores residing on the coupon surface after treatment and assess the effectiveness of the decontamination procedure, wipe sampling was performed for each painted wallboard

and concrete coupon. Wipe sampling is typically used for small sample areas and is effective on nonporous smooth surfaces such as ceramics, vinyl, metals, painted surfaces, and plastics.[17] Wipe samples were also chosen as the preferred surface sampling method for concrete coupons to allow comparisons with results from predecessor tests3, in which wipe sampling was found to perform better with respect to higher number of spores recovered for this type of material and lower standard deviations among repeat tests. Wipe sampling was conducted according to **MOP 3144**. The general approach is that a moistened sterile noncotton pad is used to wipe a specified area to recover bacteria, viruses, and biological toxins.[17] The protocol used in this project is described in **MOP 3144** and has been adapted from that provided by Busher et al.,[20] and Brown et al.[17] Microbiological analysis of the wipe sample was conducted according to **MOP 6567**.

Wipe samples were generally processed on the same day on which they were collected. The concrete wipe samples collected a great deal of debris and fine particles. It is speculated that this confounded filter-plate analyses (conducted in accordance with **MOP 6565),** as some of the samples yielded no viable spores recovered, when spread-plate analyses (conducted in accordance with **MOP** 6535a) indicated viable spores were recovered. Vacuum Sock Sampling

Vacuum sock sampling was conducted on 35.6 cm x 35.6 cm (14 in by 14 in) rough-cut barn wood coupons because of the difficulty implementing the wipe sampling procedure on the rough surface, which snags the wipe fabric. Vacuum sock sampling was conducted according to **MOP 3145**. Microbiological analysis of the vacuum sock sample was conducted according to **MOP 6572**.

Vacuum socks samples were generally processed on the same day on which they were collected. The rough-cut barn wood samples collected a great deal of debris and large wood fragments. This grade of wood is neither planed nor weathered and does have a high capacity to splinter. Vacuum sock extracts were filter-plated in accordance with **MOP 6565**. Some of these samples resulted in no viable spores recovered, which may be due in part to the co-collected debris. Figure 2-10 shows a picture of a 1 mL filter plate (left) and a 14.5 mL filter plate (right). Some samples had to be split among several filters due to the abundant debris clogging the filter.

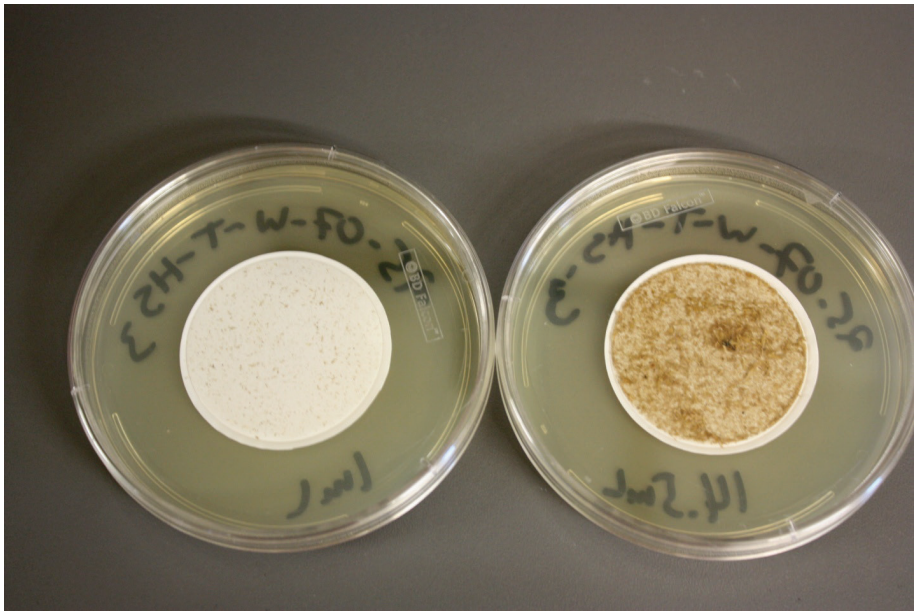


Figure 2-10: Filter Plates of Samples from Rough-Cut Wood.

2.7.5 Swab Sampling

MOP 3135 was followed for collection of swab samples. The general approach is to use a moistened swab to wipe a specified area to recover bacterial spores. Swab samples were collected from the vacuum cleaner HEPA filter during **Task 3**. Swab samples were also used to confirm sterility of materials before use in experimentation. Swab samples were analyzed according to **MOP 6563.**

2.7.6 Run-off Collection and Sampling

In a field application, decontaminant runoff could pool in a location with a high oxidation demand (such as soil). This demand would easily quench remaining sporicidal activity of the decontaminant, resulting in spores evading decontamination and potentially being relocated to uncontaminated areas. To simulate this immediate neutralization, STS, which reacts with and neutralizes chlorine and hypochlorite ions, was added to the runoff collection vessel before use. Prior to decontamination, the runoff collection vessel was charged with enough STS to neutralize all bleach sprayed onto the coupons. Thus, the hypochlorite component of the pAB was neutralized immediately upon collection. The runoff from the coupons throughout the entire decontamination procedure being tested was collected for a given coupon set (material type or all blanks). After all coupons from a single set were moved to the Decontaminated Coupon Cabinet or Procedural Blank Cabinet, the chamber was rinsed with DI water. A pre-weighed, sterile runoff collection carboy collected the runoff. The total mass of liquid collected was recorded by comparison to the tare value. After collection, triplicate 100 mL aliquots were taken using aseptic technique. The aliquot collection procedure was performed as follows:

1. Sampler donned a face mask, pair of examination gloves, disposable lab coat, and bouffant cap.
2. The contents of the carboy were agitated to ensure homogeneity.

3. The carboy cap was removed.
4. Using a new 100 mL sterile serological pipette, sampler aseptically pipetted 100 mL of sample into a sterile 4 oz. container.

Step 4 was repeated until triplicate samples were obtained.

The runoff aliquot containers were triple-contained in sterile bags and transported to the NHSRC Biocontaminant Laboratory for submission and analysis at the conclusion of the entire test. The runoff was stored at 4 ± 2 °C until processed. Processing occurred within 24 hours.

This method of neutralizing the runoff simulates the worst-case scenario for spore survivability in the run-off.

2.7.7 Aerosol Sampling

A 10 cm (4 in) diameter galvanized duct which was 112 cm (44 in) in length was attached to the chamber to allow for precise flow measurements and isokinetic sampling. The duct was attached to the chamber using a coupling and a 90-degree elbow. The sampling port was located 81 cm (32 in)(8 diameters) downstream from the 90-degree elbow which was connected to the chamber and 30.5 cm (12 in)(3 diameters) from the bend in the flexible duct that connects to the main exhaust. This 10.2 cm (4 in) galvanized duct was isokinetically sampled using a standard Method 5-type[21] meter box coupled with a SKC BioSampler® (Model No. 225-9595). Integral to the BioSampler® are critical sonic orifices that require a minimum vacuum of 38 cm (15 in) Hg across the orifices from the sample pump. This vacuum, in turn, provides a constant flow of 12.5 Lpm. The inlet of the BioSampler® was connected to a 0.5 cm (0.185 in) buttonhook nozzle so that the sample gas velocity was isokinetic to the duct velocity. Isokinetic variation was calculated per EPA Method 5[21], Section 12.11. The BioSampler® was filled with 15 mL PBST during operation. As discussed in Section 3, the STS was added to the collection liquid for tests following Test O5. Operation time was limited to a maximum of 1 hour to prevent evaporation of the liquid, but no sample required operation for more than 30 minutes. The aerosol sample was analyzed in two parts per **MOP 6568**. To collect particulate that impinges on or settles in the nozzle and inlet tubing prior to the collection liquid, the nozzle and inlet are rinsed with 50 mL PBST. This rinse is analyzed independently of the collection liquid itself.

2.7.8 pAB Sampling

The pAB was sampled in two ways for two different analyses. During production of the pAB, a 5 mL aliquot from the bulk mixing vessel was collected for FAC analysis. To determine the pH, a probe (Acorn pH5, Oakton, Vernon Hills, IL) was placed in the bulk mixing vessel. During use for decontamination procedures, a 50 mL aliquot was removed from the backpack sprayer tank prior to use, and a 50 mL aliquot was taken from the graduated cylinder following the flow measurement taken immediately before use in a decontamination procedure. The FAC and pH of these aliquots were also determined.

2.7.9 Additional Samples Collected

Samples were collected to describe both the decontamination process itself and the efficacy of its application. An overview of these tasks is described below to provide context for the results described in Section 3.

The objective of the study was to assess the effectiveness of decontamination procedures for reducing surface contamination and to refine the procedures for maximum benefit (low effort and high effectiveness). The effectiveness is measured by the determination of the log reduction calculated per Section 1.3. Hence, surface sampling of the test areas before and after decontamination was required to determine the log reduction after application of the procedure. Since current surface sampling techniques are intrusive (i.e., they remove viable spores from the surface of the section), separate positive control and test coupons had to be used to compare pre- and post-decontamination recoveries. Positive control coupons were inoculated on the same day and analyzed on the same day as test coupons, but were not decontaminated.

The effectiveness of removing contamination from the surface of the sections provides critical information regarding the utility of the procedure. However, field applicability is also dependent upon several other factors including the ultimate disposition (or fate) of the spores. This latter information is required to provide information pertinent to the development of a comprehensive site-specific remediation strategy. For example, if viable spores are washed off materials (e.g., transferred unharmed from surfaces to runoff water), remediation field strategies might require runoff collection and treatment. Hence, it is important to gain a holistic understanding of the fate of the spores during decontamination procedures.

To assess the fate of spores during decontamination, several samples in addition to the surface samples were collected. To assess the maximum potential for viable spores to be washed off the surfaces, all liquids used in the decontamination process were collected in a vessel with enough STS to neutralize all bleach sprayed onto the coupons and quantitatively analyzed. These were composite samples for a set of replicate coupons during a decontamination procedure. Runoff samples were analyzed quantitatively to determine the disposition of viable spores in this medium. The volume of liquid collected in each section set was measured after collection. Aerosol samples were also isokinetically sampled from the chamber duct to assess the potential for re-aerosolization of spores due to the decontamination process itself.

The procedures tested herein were based upon the results from previous studies[3,23,24], andwere originally developed based upon use for the remediation of the wooden shed in Danbury, CT, and via an interagency workgroup on foreign animal disease threats. All materials and equipment utilized during testing, as well as the sampling protocols, are detailed in Appendices D and E.

2.7.10 Split Samples

While no samples were split for separate analyses, replicate aliquots were taken of some samples (replicate rinsate sample aliquots collected to analyze a larger portion of the sample yet contain samples in small vials). In the cases where replicate samples produced results within the acceptable range, these data were averaged. The BioSampler® includes two portions: a rinse of the nozzle and all glassware exposed to the aerosol between the duct and the collection liquid, and the collection liquid itself. These two portions of the

sampler were analyzed independently, yet the data were combined for one estimate of bioaerosol recovery for each sample.

2.7.11 Sample Analyses

Analyses of all biological samples were conducted in the on-site NHSRC Biocontaminant Laboratory. PBST was used as the extraction buffer. After the appropriate extraction procedure, as described in Appendix F, the samples were plated, incubated, and analyzed (CFU enumerated) in accordance with **MOP 6535a**. Appropriate dilutions of the extracted sample (i.e., the initial undiluted sample extraction dilution, and up to a five-stage serial dilution [10^{-1} to 10^{-5}]) would be plated depending on expected CFU concentration. For example, the last three dilutions (10^{-3}, 10^{-4} and 10^{-5}) might not be plated for a decontaminated sample if a low CFU concentration (high decontamination efficacy) was expected.

In addition to the analysis in **MOP 6535a**, supplementary analysis procedures were used for samples resulting in less than 30 CFU/sample in the undiluted sample extract (e.g., wipe in the extraction buffer). These analyses were used to lower the current detection limit associated with **MOP 6535a.** In accordance with **MOP 6565**, Revision 2, samples were filter-plated.

The PBST was prepared according to the manufacturer's directions and in accordance with **MOP 6562**, dissolving one packet in one liter of sterile water. The solution was then vacuum-filtered through a sterile 0.22 µm filter unit to sterilize.

The extraction procedure used to recover spores varied depending upon the different matrices (e.g., wipes, vacuum socks). The procedures are described in Appendix F.

2.7.12 Coupon, Material, and Equipment Cleaning and Sterilization

Several management controls were administered in order to prevent cross-contamination. This project was labor intensive and required that many activities be performed on coupons that were intentionally contaminated (test coupons and positive controls) and not contaminated (procedural blanks). The treatment of these three groups of coupons (positive control, test, and procedural blank) varied for each group. Hence, specific procedures were put in place in an effort to prevent cross-contamination among the groups.

Each test in the experimental matrix included four primary activities. These activities were preparation of the coupons, execution of the decontamination process (including sample recovery), sampling, and analysis. Specific management controls for each of these activities, as well as cleaning methods put in place to prevent cross-contamination, are shown in Table 2-5. Appendix A details the coupon, test chamber and equipment cleaning and sterilization procedures.

Table 2-5. Cleaning Methods and Frequency for Common Test Materials/Equipment

Material/Equipment	Use	Cleaning Method	Frequency

Material/Equipment	Use	Cleaning Method	Frequency
Decontamination Procedure Chamber	Contain coupons during the application of the decontamination procedure being tested	Washing with pH-adjusted bleach solution, or wiping with Dispatch® Bleach Wipes, rinse with DI water	Before/after each test and between test materials
Coupon Cabinets	Store coupons prior to testing and/or sampling	pH-Adjusted bleach solution or wiping with Dispatch® Bleach Wipes, rinse with DI water followed by EtOH	Before/after each test
Backpack Sprayer	Used to apply decontamination solution	Purge with decontamination solution before use	Before each test
Distilled Water Tanks (Reservoir)	Utilized during chamber decontamination (reset) procedure	Bleach solution, soak overnight	Treated before each test (within 48 hours of the test start)
Wet/Dry Vacuums	Part of the decontamination procedure	Fumigation with hydrogen peroxide	Before each use
Heads of Wet/Dry Vacuums	Part of the decontamination procedure	Fumigation with hydrogen peroxide	Before each use
Other Bulk Equipment (Deposition Housing and Gaskets, Templates, etc.)	Various	Fumigation with hydrogen peroxide or washing with pAB solution in accordance with Appendix A.	Varied
All Work Surfaces	Throughout each test	Cover with new bench liner	Before/after each use (cleaning of surfaces between handling of replicate coupons during sampling)

Due to the amount of waste and reusable items (requiring decontamination after use) generated during this testing (e.g., sterilization bags, sampling templates, etc.), creation of a rigid plan to segregate such items was imperative. Reusable items were clearly distinguished and separated from waste items after use and put in distinct segregated locations within the testing area.

During the decontamination procedure for **Task 2**, one person (sample handler) was tasked with moving the coupons to the decontamination chamber. A different person was tasked with moving the treated coupon to the drying cabinets. Disposable laboratory coats were worn by the sample handler (tasked with moving the

coupons) to further minimize the potential of cross-contamination. The sample handler donned new gloves and a new disposable laboratory coat after moving a complete set (i.e., three of test samples) from the test coupon cabinet to the decontamination chamber.

All bins, buckets, and containers remained closed or covered unless in use (e.g., material being placed into or extracted from the bin, bucket, or container). Adequate cleaning of all common materials and equipment was critical in preventing cross-contamination.

3. Results and Discussion

The test matrix (modification of remediation activity parameters) evolved (i.e., adaptive management approach) as results were obtained during the testing campaign. Tests were progressive in that details of one test were used to inform and design subsequent tests in attempts to optimize the decontamination process (speeding up slow steps without reducing efficacy and eliminating ineffective or counterproductive steps). Therefore, the ultimate goal was to achieve maximum benefit (low effort and high effectiveness) with off-the-shelf products.

In addition to reduction of contamination from material surfaces, determination of the ultimate fate of the spores was also a critical measurement objective. Combined, this information can inform selection or further development of appropriate, situation-specific decontamination procedures. Following discussion of the individual decontamination procedure results, the ultimate fate of the spores and decontamination worker exposure due to the procedures was explored, when possible.

All p-values reported are based on single factor, two-sided analysis of variance (ANOVA) of log-transformed CFU recovered or log reductions with an alpha value of 0.05.

3.1 Task 1: Impact on Efficacy of the Degradation of the pAB Solution over Time

3.1.1 Extraction Method Development

The results of the extraction method testing described in Section 2.7.1 are shown in Table 3-1. To test the effect of residual pAB on CFU recovery, extractions of sprayed and unsprayed coupons were spiked with a liquid spore inoculum as shown in Table 3-1. Five replicate coupons of each type (sprayed, and unsprayed) were tested.

The residual hypochlorite concentration (FAC) from sprayed coupons may interfere with spore recovery; thus the neutralization of FAC by STS may be important. A series of tests was completed to determine the amount of STS solution needed to neutralize the FAC in the buffer solution. Table 3-2 shows the data for the two sprayed materials tested (six 18-mm wallboard paper coupons and six 18-mm wood coupons) titrated with 0.000375N STS solution. The coupons were subjected to two 15-second pAB applications using a starting 6630 ppm FAC bleach solution. When pAB-sprayed drywall coupons were added to the extraction fluid, the FAC in the buffer rose to between 4 and 28 ppm. A molar equivalent amount of STS was added to the extraction fluid to neutralize 28 ppm FAC during Tests STS 2 and STS 3, which were conducted with drywall only, because wood coupons did not show any rise of the FAC in the extraction buffer.

Table 3-1. Recovery from Neutralized (Sprayed) and Control (Not Sprayed) Test Samples (n = 5).

				Sprayed			Not sprayed			
Test ID	Description	Material	Inoculation Titer	Avg CFU/ Sample	Mean of Logs	RSD[1]	Avg CFU/ Sample	Mean of Logs	RSD	T-test (p-value)
STS 1	No STS	Drywall	3×10^7	1.2×10^7	6.13	28%	5.2×10^7	7.71	1%	0.095
		Wood		4.7×10^7	7.66	1%	6.1×10^7	7.78	1%	0.243
STS 2	Extraction fluid with STS	Drywall	6×10^7	1.2×10^8	8.06	0.4%	8.5×10^7	7.93	0.3%	0.0015
STS 3	Extraction fluid with STS	Drywall	4×10^2	5.9×10^2	2.76	4%	8.1×10^2	2.91	2%	0.068
STS 4	No STS	Drywall	5×10^2	1.3×10^2	1.56	57%	1.6×10^3	3.06	12%	0.033
		Wood		9.9×10^2	2.90	11%	5.9×10^2	2.77	2%	0.43

[1] RSD = Relative standard deviation of Log CFU.

Table 3-2. FAC in Extracts of Wallboard Paper and Wood Coupons

Sample Number	Coupon Material	Volume of Titrant	FAC ppm
FAC2-D-1	wallboard paper	5.2	6.9
FAC2-D-2		20.8	27.7
FAC2-D-3		10	13.3
FAC2-D-4		10	13.3
FAC2-D-5		3.8	5.1
FAC2-D-6		3.4	4.5
FAC2-W-1	Wood	0	0
FAC2-W-2		0	0
FAC2-W-3		0	0
FAC2-W-4		0	0
FAC2-W-5		0	0
FAC2-W-6		0	0

The Student's t-test was used to compare recoveries between the two treatments (sprayed and nonsprayed coupons) of the same material type. The first test (STS 1) was conducted to determine the FAC in the extract buffer of pAB-sprayed coupons following extraction and to determine recovery if no neutralizer (STS) was added. These data suggest that residual bleach on sprayed drywall coupons likely reduced recovery. For instance, recovery from pAB-sprayed drywall coupons was less than recovery from control coupons ($p = 0.095$), however this difference was not significantly different.

Analysis of the data with Student's t-test suggested that the sprayed and unsprayed results were significantly different ($p \leq 0.05$) for Test STS 2; however, recovery from the pAB-sprayed dry wall coupons was higher than recovery from unsprayed samples. These data suggest that the presence of neutralized bleach does not have a negative bias on sample recovery.

Tests STS 3 and STS 4 were subsequently conducted with lower inocula to demonstrate the method would be reliable at lower concentrations. Extraction of drywall coupons with STS was demonstrated to introduce no negative bias in recovery due to residual pAB. Samples extracted without STS during STS 4 (low inoculum) had 10-fold lower recoveries when sprayed with pAB versus the control samples (not sprayed with pAB). This difference was not apparent in STS 3, when STS was included in the extraction buffer. These data support the notion that STS is necessary in extraction buffers to reduce downstream effects of the decontaminant during efficacy testing. STS was not used for extraction of wood coupons, as there was no FAC from residual pAB detected in STS 1, likely due to beach demand by the wood. Recoveries from wood were also unaffected by bleach residual in the low inoculum test (STS 4).

3.1.2 Efficacy Testing

FAC was measured from aliquots collected from the bulk container and from the sprayer nozzle. The sprayed pAB was expected to have lost FAC during the act of spraying. Surprisingly, the opposite was observed with the FAC of the sprayed fresh pAB nearly twice that of the bulk container. The increase in FAC of the sprayed pAB was a function of age (or FAC of the original solution). The cause of the increase has not yet been determined. The results are shown in Tables 3-3 and Figure 3-1.

Table 3-3. FAC and pH of pAB in the Bulk Container or Following Spraying, over Time (n=1)

Time	Bulk FAC ppm	Sprayed FAC ppm	Bulk pH	Sprayed pAB pH
0	6530	12459	6.8	7.83
2	5348	5789	6.37	7.1
4	4287	5168	6.06	6.75
8	4487	4647	5.72	6.13
24	3866	3686	5.32	5.48
32	3405	3485	5.24	5.37

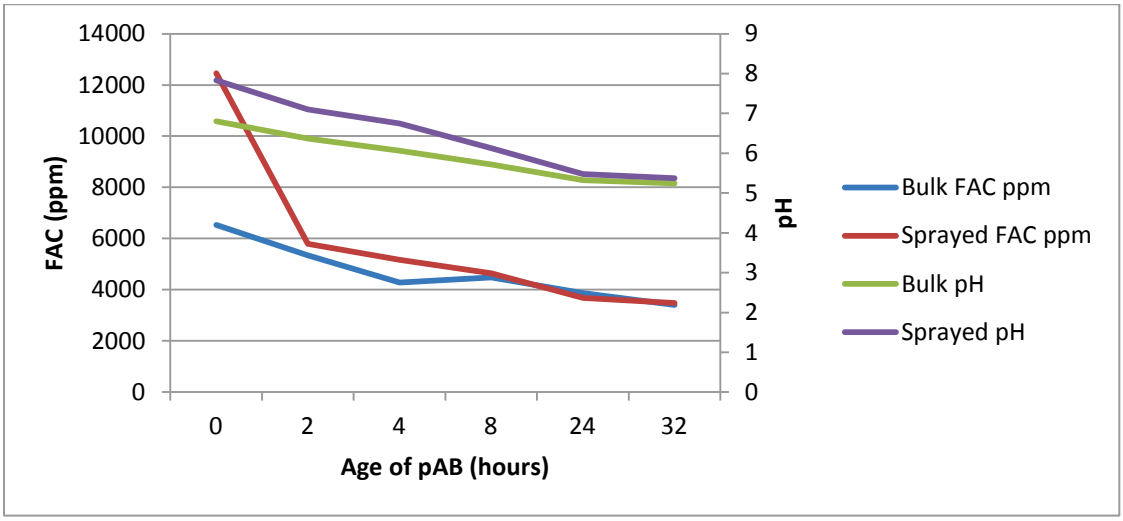


Figure 3-1. FAC and pH of pAB over Time (Task 1)(n=1)

Table 3-4 shows the results for the pAB decontamination on rough-cut barn wood, and Table 3-5 shows the results for the decontamination on drywall coupons. Figure 3-2 graphically shows the effect of age on pAB efficacy. Age of the pAB had little to no effect on the ability of pAB to decontaminate the nonporous drywall coupons. Wood coupons did offer more protection to the spores, and older pAB did not perform as well as fresh pAB. These results suggest that it may be more critical to use freshly prepared pAB on difficult to decontaminate materials, such as those with a higher organic content like wood or grimed materials. One option is to use backpack sprayers modified with a "soap bottle attachment" filled with an acid of suitable strength, so that bleach is acidified just prior to spraying. Acidifying bleach as it is sprayed, rather than by the entire batch, eliminates the problems associated with decreasing FAC over time.

Table 3-4. Task 1 Results - Effect of pAB Age on Wood Surface Decontamination (n=5)

Wood				
Time Elapsed Since pAB Solution Preparation (hours)	**Recovery (Mean CFU/Sample)**	**Mean of Logs**	**RSD**	**LR**
Positive	9.83×10^{6}	6.99	21%	n/a
Fresh (15 min)	4.47×10^{2}	2.50	90%	4.48
2 hours	3.97×10^{3}	2.98	178%	4.00
4 hours	2.26×10^{3}	2.97	168%	4.02
8 hours	9.09×10^{3}	3.79	104%	3.19
24 Hours	4.67×10^{4}	4.34	117%	2.65
32 Hours	3.84×10^{4}	4.33	69%	2.65

n/a = not applicable; LR = log reduction

Table 3-5. Task 1 Results - Effect of pAB Age on Drywall Surface Decontamination (n=5)

Drywall				
Time Elapsed Since pAB Solution Preparation (Hours)	**Recovery (Mean CFU/Sample)**	**Mean of Logs**	**RSD**	**LR**
Positive	9.22×10^{6}	6.96	4%	n/a
Fresh (15 min)	1.42×10^{2}	1.95	130%	5.02
2 hours	8.02×10^{1}	1.83	67%	5.14
4 hours	7.34×10^{1}	1.77	79%	5.22
8 hours	1.27×10^{3}	2.55	182%	4.43
24 Hours	3.93×10^{2}	2.26	128%	4.72
32 Hours	3.59×10^{2}	2.28	110%	4.71

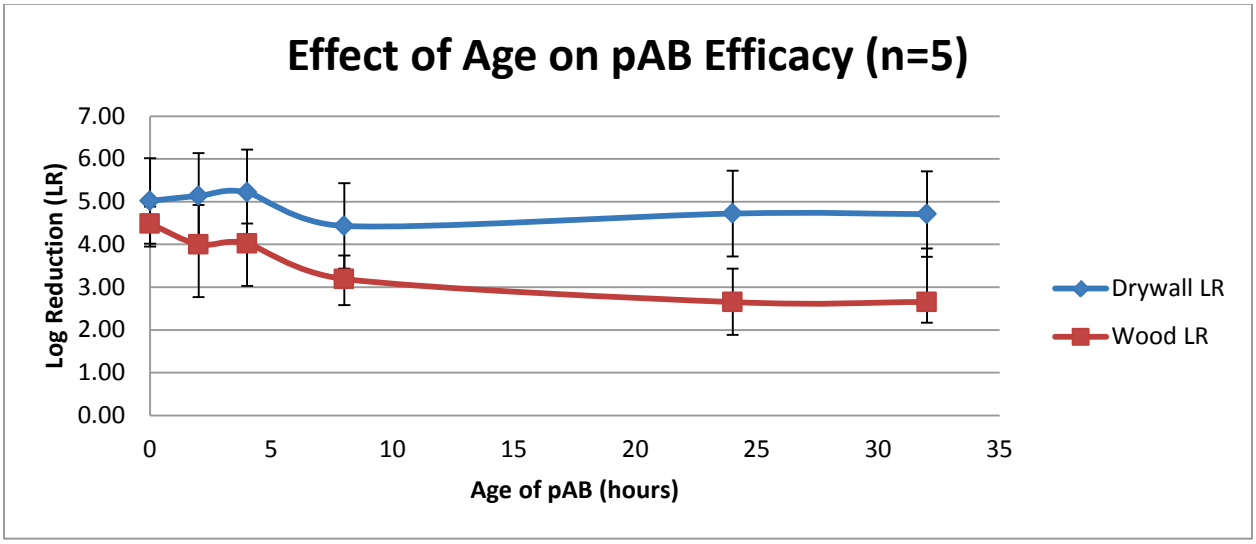


Figure 3-2. Effect of Age on pAB Surface Decontamination Efficacy (Task 1). Data are presented as mean Log Reduction (surfaces) from five replicate samples, error bars indicate standard deviation.

3.2 Task 2: Parametric Evaluation of the Decontaminant Application Procedures

3.2.1 High Inoculation Tests

Tests O1 through O5 were conducted with a spore load (inoculum) that resulted in 1×10^{7} CFU recovered from positive control samples (i.e., high inoculation tests). These tests were targeted at demonstrating a greater than 6 log reduction in recovered spores, a benchmark for determining efficacy of a decontamination procedure.[22]

3.2.1.1 Surface Decontamination

Table 3-6 gives a summary of test conditions for the high inoculation optimization tests (1 x 10^7 CFU/sample). During Test O1, a shortened application procedure was conducted as a starting point for the parametric tests. The conditions (1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse) were chosen as a low efficacy starting point based upon results from previous testing.[3,23,24] Results from Test O1 indicate efficacy was near 6 LR for concrete and drywall, but low (<3 LR) for wood.

Test O2 was performed in an attempt to improve the efficacy with no additional application time. The flow rate used was the maximum flow of the backpack sprayer (1350 mL/min), using the same spray pattern used in Test O1. This simple one-step decontamination procedure provided an increased efficacy over Test O1 procedure and greater than 6 LR for two of the three tested materials (drywall and concrete). However, the sample size was not large enough to demonstrate that the higher flow rate provided a statistically higher efficacy.

Table 3-6. Test Conditions for High Inoculation Parametric Tests (Task 2)

Test ID	Materials	Decontamination Steps	Decontaminant Application Flow Rate Prescribed
O1	Drywall, Concrete, Wood	1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O2	Drywall, Concrete, Wood	1 application, 15 seconds spray per 3 coupons, no reapplication, no rinse	High (1.5 L/min)
O3,	Wood	1 application, 30 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O4	Wood	2 applications (at 0 and 5 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)
O5	Wood	2 applications (at 0 and 15 min), 15 seconds spray per 3 coupons, no reapplication, no rinse	Low (1 L/min)

Further improvements on the efficacy of decontamination could not be detected on drywall or concrete surfaces (e.g., near complete inactivation of these surfaces was achieved with one application), so these materials were not included in Tests O3, O4, and O5, which focused on the most challenging material (i.e., wood).

Tests O3, O4, and O5 were performed to determine whether variations in application times could produce a measured effect in decontamination efficacy. Each test had a total spray time of 30 seconds, but Test O4 and Test O5 had two 15-second applications, each at different times following the first application, while Test O3 used a single 30-second spray. None of the application methods provided a statistically improved efficacy in surface decontamination, nor were they significantly different in their effectiveness at wood surface decontamination (Figure 3-3).

These data suggest that application methods should be designed with the surface material in mind. Less rigorous application procedures were highly effective for concrete and drywall. The higher flow rate in Test O2 may have slightly improved efficacy on wood; however, the marginal benefit in efficacy should be weighed against the higher volume of solution required. These data are consistent with previous studies, and support the notion that rough wood surfaces are difficult to decontaminate.

3.2.2 Fate of Spores

Reduction of the number of spores from the surface of decontaminated materials could be due to three effects:

1. Deactivation of the spores due to the decontamination method;
2. Removal of active spores due to the decontamination method; or
3. Reduction of recovery of active spores due to the decontamination method.

The reduction in recovery (Effect 3) has been suggested by other researchers,[19] but was not investigated in this study.

In order to quantify Effect 2, removal of active spores, samples were collected from the runoff/rinsate and aerosol generated during spraying. As previously mentioned, the runoff collection vessel was charged with enough STS to neutralize all bleach sprayed onto the coupons, which provides conditions for the maximum possible spore survival. This fate of spores in the rinsate, thus, serves as the worst case scenario. In a field application where there was no oxidative demand in the rinsate, continued exposure to pAB in the rinsate is expected to reduce spore survivability.

The results are shown in Figure 3-4 and Table 3-7. Rinsate was not collected from Test O1. Figure 3-4 shows the recovery of viable spores in rinsate liquids. The CFU counts /mL derived from 100 mL aliquots were multiplied by the total volume of rinsate collected. The striking result is the absence of spores in the rinsate of Test O3. Without further investigation, we are unsure whether the presence of spores in rinsates during Tests O4 and O5 was due to the two 15-second pAB applications being less effective at killing spores in the rinsate than the 30-second pAB application, although no more effective at surface decontamination; or if the spores were present due to cross-contamination. Alternately, the longer spraying time (30 sec) certainly generated more runoff, as more of the surfaces were saturated for longer periods of the spray, even though the total spray duration was equal for O3, O4, and O5. More pAB was expected to “runoff” coupon surfaces during Test O3, resulting in more pAB collected in the rinsate sample and therefore a higher potential for insufficient neutralization. Active pAB in the rinsate sample could have lowered the number of viable spores recovered and could explain the unexpectedly low abundance of rinsate spores observed during this test (O3).

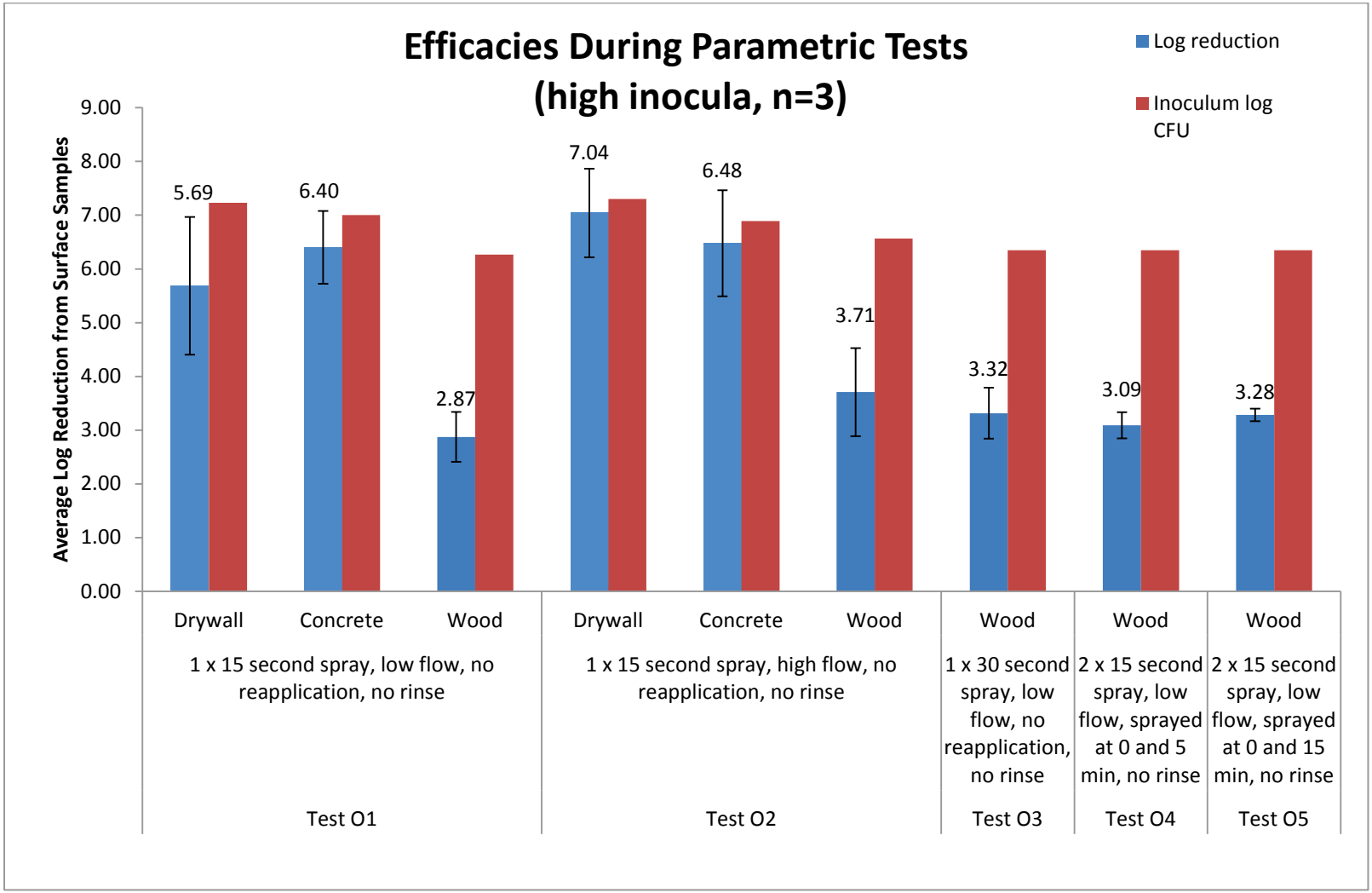


Figure 3-3. Surface Decontamination Efficacy (Log Reductions, LR) by Material Type for the Five Parametric Tests (Task 2).
Error bars indicate standard deviation.

Table 3-7. Task 2 – Surface Decontamination Parametric (high inoculation) Test Results

Test ID	Material	Positive Controls (n=3)			Test Coupons (n=3)			LR	RSD (%)	pAB Decontamination Conditions Achieved	
		Avg. CFU/ Sample	Mean of Logs	RSD (%)	Avg. CFU/ Sample	Mean of Logs	RSD (%)			Decontamination Steps	Flow Rate (ml/min)
Test O1	Drywall	1.72×10^7	7.23	22%	351	1.54	170%	5.69	23%	1 x 15 second spray, low flow, no reapplication, no rinse	1030
	Concrete	1.02×10^7	7.00	22%	7	0.60	83%	6.40	11%		1030
	Wood	1.95×10^6	6.27	41%	3520	3.39	95%	2.87	16%		1040
Test O2	Drywall	2.05×10^7	7.30	25%	6	0.26	155%	7.04	12%	1 x 15 second spray, high flow, no reapplication, no rinse	1340
	Concrete	8.16×10^6	6.89	38%	12	0.41	163%	6.48	15%		1340
	Wood	3.93×10^6	6.57	47%	2236	2.86	154%	3.71	22%		1360
Test O3	Wood	2.31×10^6	6.35	30%	1600	3.03	105%	3.32	14%	1 x 30 second spray, low flow, no reapplication,	1030
Test O4	Wood	2.31×10^6	6.35	30%	1984	3.26	46%	3.09	8%	2 x 15 second spray, low flow, sprayed at 0 and 5	1060
Test O5	Wood	2.31×10^6	6.35	30%	1188	3.06	27%	3.28	4%	1 x 15 second spray, low flow, no reapplication,	1030

RSD = Relative Standard Deviation of CFU/sample

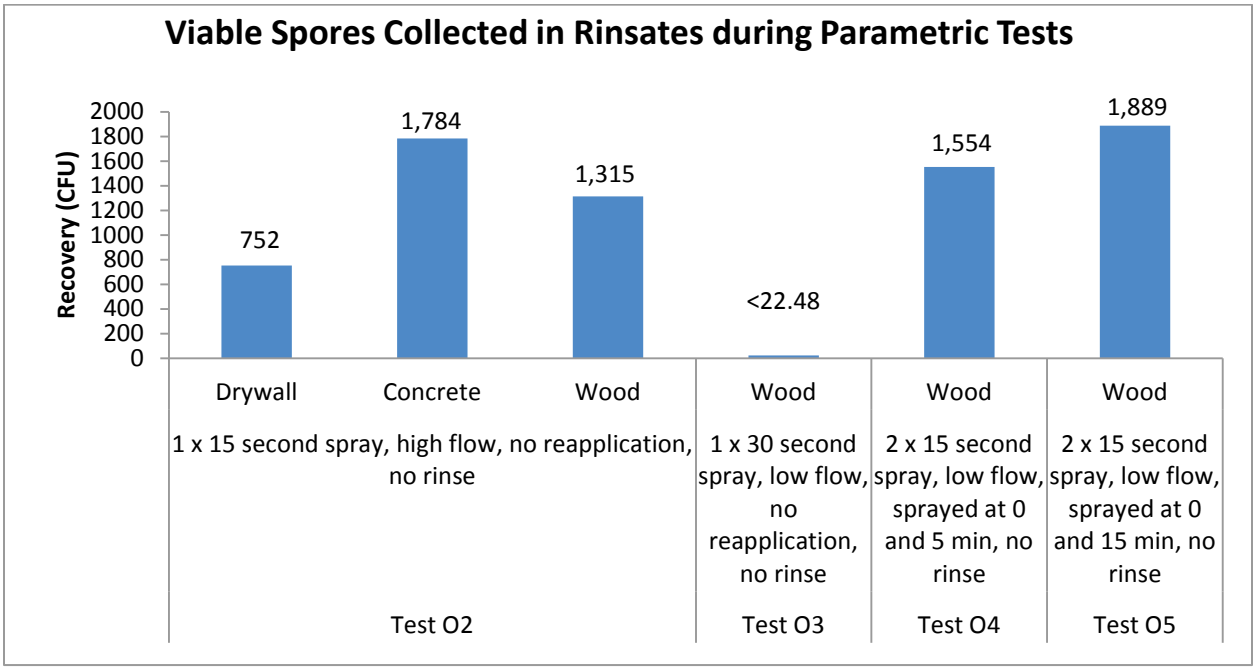


Figure 3-4. Recovery of Viable Spores in Rinsates (Task 2). Data are reported as the total CFU recovered from rinsate samples; this value is printed above the bars in the figure.

During the first round of testing (high inoculation – Test O1 through O5), aerosolized spores were detected. As described in Section 2.6.8, the aerosol sampling equipment consists of a nozzle and the inlet to the collection liquid, and the collection liquid itself. Spores were detected only in the rinse of the SKC BioSampler® nozzle, not in the collection liquid. This observation would be consistent with two scenarios:

1. Spores were in very large clumps and were settling or impacting on the nozzle.
2. Residual droplets of aerosolized bleach were decontaminating or preventing spore recovery from the SKC collection liquid.

The FAC of the collection liquid in the SKC BioSampler® was measured following Tests O3, O4, and O5 and was found to be 36.7 ppm, 38.2 ppm, and 32.3 ppm, respectively (mean of 35 ppm).

To determine if this low concentration could account for the absence of spores in the liquid, a test was designed to measure recovery from spiked extraction fluids. 10 mL of PBST (simulating the BioSampler® collection liquid) was brought to 35 ppm FAC with pAB, and then spiked with spores. The results are shown in Table 3-8 .

Table 3-8. Results from 35 ppm FAC Extraction Efficacy Test (n = 5)

Collection Liquid	Positive Controls			Samples Spiked with 35 ppm FAC pAB			p-value (t-test)
	Avg. CFU/ Sample	Mean of Logs	RSD (%)	Avg. CFU/ Sample	Mean of Logs	RSD (%)	
PBST	3.57E+02	2.55	18%	5.70E-01	-0.24	1%	0.0003
PBST with STS	3.49E+02	2.53	32%	3.57E+02	2.55	18%	0.68

RSD = Relative Standard Deviation of CFU/sample

No spores were collected from the 35 ppm FAC liquid, while the recovery from PBST with STS was the same as the positive controls. Residual FAC in the BioSampler® collection liquid for Tests O3, O4, and O5 (PBST only) could have prevented detection of the spores in the aerosol phase. The BioSampler® collection liquid for subsequent tests (Tests O6 through O10, and all **Task 3** tests) was PBST spiked with STS to neutralize entrained pAB.

3.2.3 Low Inoculation Decontaminations

Tests O6 through Test O10 were designed to evaluate the efficacy of the spray-based procedure on coupons with lower spore inoculations. Log reductions are typically non-linear reactions, with resilient spores resisting decontamination. The results are tabulated in Table 3-8 and displayed graphically in Figure 3-5.

Low-level inoculations proved difficult to perform in a repeatable manner, so inocula for Tests O6 through O10 ranged from ~1 x 10^4 to 1 x 10^6 per coupon (0.09 m^2 or 1 ft^2). Coupons for Tests O8, O9, and O10 − including positive control coupons common to all tests − were all inoculated on the same day to reduce test variation. The MDIs used for these inoculations were manufactured with a propellant of untested shelf-life, and many were unable to perform reliably. While this factor makes it difficult to compare log reductions across tests, the low level inoculations do provide valuable information on the difficulty of full decontamination.

As shown in Table 3-9, no spores were detected on Test O6 decontaminated concrete or drywall coupons (detection limit values of 0.5 CFU are listed). Spores were detected on Test O6 decontaminated wood coupons, however. For Test O7 (wood only), the pAB application time was doubled, and, again, the decontamination procedure failed to remove or inactivate all spores. For Tests O8, O9, and O10, the inoculation level was relatively low. During these tests, some surface samples from decontaminated coupons had more recovered spores than from the positive controls. These results were surprising and somewhat unexplainable yet do illustrate the notion that low levels of contamination should not be assumed to be decontaminated easily. None of the decontamination methods tested proved effective on deactivating spores on wood coupons, even at low contamination levels.

Table 3-9. Task 2 – Surface Decontamination Parametric (low inoculation) Test Results

Test ID	Material	Positive Controls (n=3)			Test Coupons (n=3)					pAB Decontamination Conditions Achieved	
		Avg. CFU/ Sample	Mean of Logs	RSD (%)	Avg. CFU/ Sample	Mean of Logs	RSD (%)	LR	RSD (%)	Decontamination Steps	Flow Rate (ml/min)
Test O6	Drywall	4.36×10^5	5.64	16%	0.5	-0.20	3%	5.84	0.00	1 x 15 second spray, no reapplication, no rinse	1000
	Concrete	6.42×10^4	4.78	42%	0.5	-0.15	1%	4.93	0.00		1000
	Wood	4.33×10^4	4.62	34%	45	1.11	143%	3.51	0.29		1000
Test O7	Wood	3.23×10^2	2.44	55%	47	1.36	136%	1.09		1 x 30 second spray, no reapplication, no rinse	1100
Test O8	Wood	1.73×10^2	2.12	93%	107	1.99	47%	0.13	0.00	1 x 30 second spray, no reapplication, no rinse	1100
Test O9	Wood	1.73×10^2	2.12	93%	160	1.84	141%	0.28	0.00	2 x 15 second spray, sprayed at 0 and 5 min, no rinse	1100
Test O10	Wood	1.73×10^2	2.12	93%	40	1.50	87%	0.62	0.00	2 x 15 second spray, sprayed at 0 and 15 min, no rinse	1000

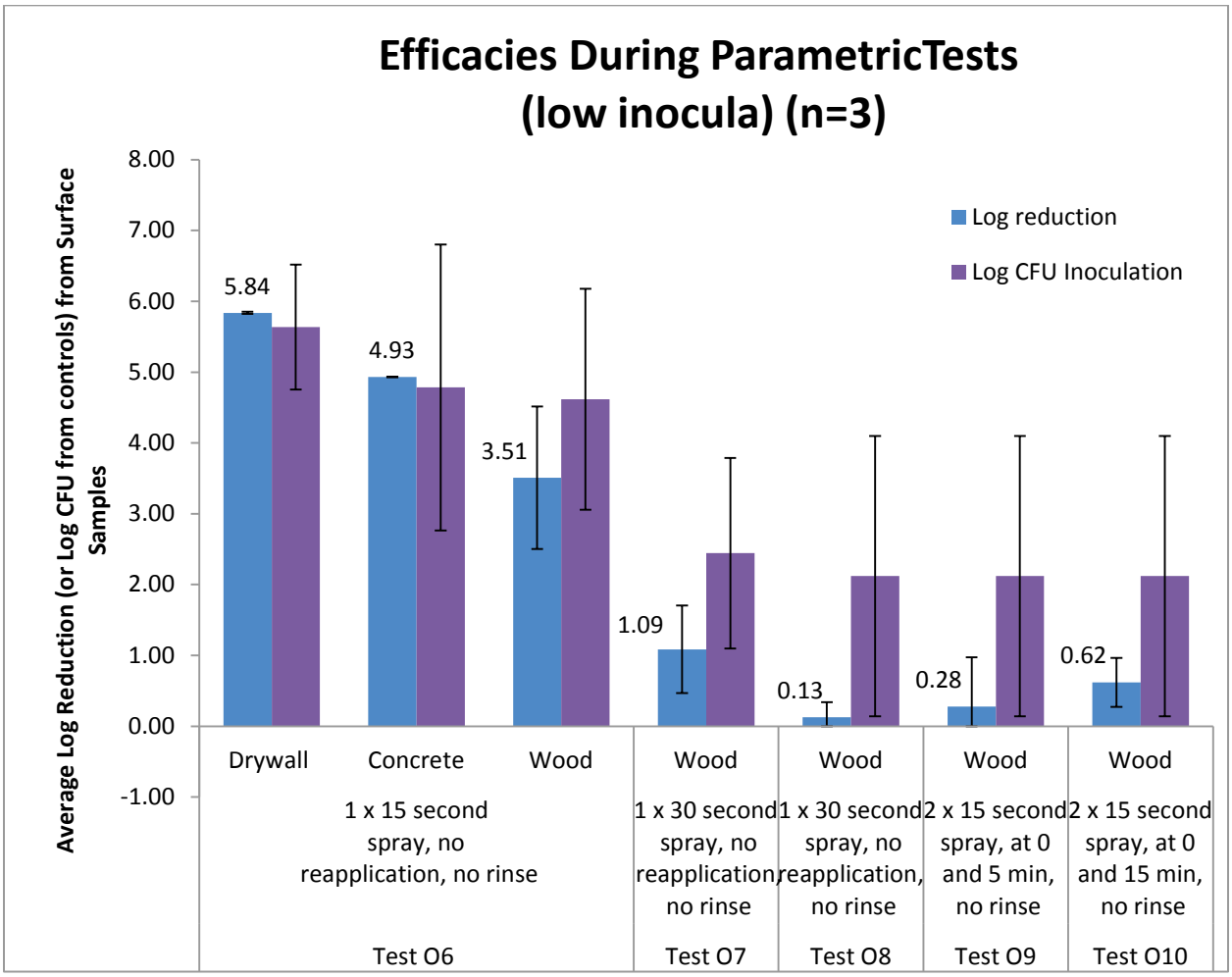


Figure 3-5. Decontamination Efficacy (LR) of Five Decontamination Procedures on Low-Level Contamination (Task 2). Average LR value listed above bar in Figure. Error bars indicate standard deviation.

The positive control samples often required filter-plate analysis, as undiluted spread-plating of these samples often resulted in less than 30 CFU. The low-level inoculation tests were complicated by the presence of wood fibers on the filter following filter-plating, as copious amounts of wood fiber debris covered the filter and prevented enumeration of CFU (Figure 3-6). For example, 1 mL of a sample may contain 18 CFU on the filter-plate, but no CFU were detected on a filter in which16 mL of the same sample was analyzed. The higher volume contained more wood debris and prevented discrimination of CFU. This lack of discrimination resulted in surface samples from wood having a higher detection limit (20 CFU/sample) than samples from some other materials, which had detection limits closer to 0.5 CFU/sample.

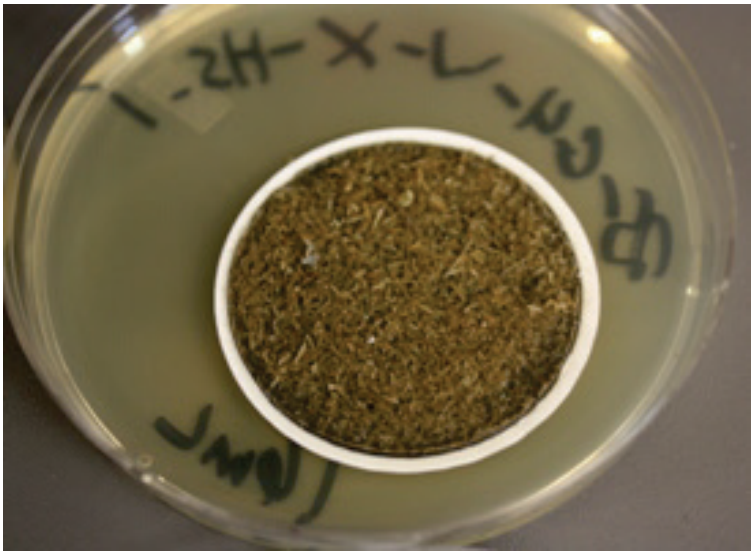


Figure 3-6. Photograph of Wood Fibers on a Filter-plate. The high debris in these samples confounded the filter-plate assay and resulted in higher detection limits for surface samples collected from wood.

For future tests with heavy debris, the extraction and plating procedures should be modified to take into consideration the obscuring effect of the debris. One suggestion is to let the sample sit for two minutes after it is removed from the orbital shaker to let foam subside and the debris settle, then remove the liquid sample near the surface. This procedure may help eliminate interference by debris, but may also result in low recovery estimates as an unknown number of spores may be bound to the settled wood fibers.

The difficulty with recovery, combined with the difficulty in decontamination, point to a major weakness in confirming adequate decontamination of challenging surfaces (i.e., wood) following a *Bacillus* spore release. Spores that are difficult to destroy and detect could pose a significant threat. Further research is warranted to improve both detection and destruction of spores on such materials.

No spores were detected in either the aerosol phase or rinsate of these low-level inoculation tests. However, the detection limit for the aerosol samples was very high (400 CFU) due to the small portion of the flow sampled. The detection limit of the rinsate sample was 5 CFU.

3.2.4 Wetted Wipe Decontamination

The results of the wetted wipe decontamination tests are presented in Table 3-10.

Table 3-10. Wetted-Wipe Test Procedure Results (Drywall Coupons) (n=3)

Test	Decon Method	Positive Controls (n=3)			Test Coupons (n=3)			LR	RSD (%)
		Avg. CFU/ Sample	Mean of Logs	RSD (%)	Avg. CFU/ Sample	Mean of Logs	RSD (%)		
W1	pAB Wetted Wipe	1.70×10^7	7.23	6.7%	3.58×10^5	5.38	95%	1.86	28%
W2	SimWipe	1.70×10^7	7.23	6.7%	4.62×10^6	6.66	3%	0.57	2%
W3	SimWipe + pAB Wetted Wipe	1.70×10^7	7.23	6.7%	2.08×10^4	3.92	109%	3.31	28%
W4	pAB Wetted Wipe (3 wipes/coupon)	4.36×10^5	5.64	16%	1.72×10^3	3.15	67%	2.49	15%

Figure 3-7 shows the range of CFU recovered from the decontaminated 35.6 cm x 35.6 cm (14 in by 14 in) drywall surface. ANOVA reveals that the SimWipe-only decontamination represents a statistically distinct decontamination method (lowest efficacy). While the combination of the SimWipe and the pAB wetted wipe seems to be more efficacious than the pAB wetted wipe alone, the Student's t-test two-tailed homoscedastic value (p-value) is 0.075, suggesting that the variance is not statistically significant. A fourth test (shown in Table 3-10) was evaluated against a lower spore inoculation on drywall coupons, using three wetted wipes, one for each direction of wiping as described in **MOP 3156**. Recovery cannot be compared directly because of the difference in spore loading before decontamination. However, a comparison of the efficacies suggests no statistically significant difference between the efficacies of any wipe decontamination procedure which incorporates pAB (i.e., all instances except the SimWipes alone test). When comparing these results to those of the efficacy of the two spray-based procedures on drywall it is apparent that even the shortest spray procedure (one, 15-second decontaminant application, Test O1) yielded much higher efficacies (5.69 LR) than any of the wetted-wipe procedures (Table 3-7 and 3-10). For a more thorough understanding of the efficacy of wetted wipe decontaminations, these tests should be repeated with larger sample sizes.

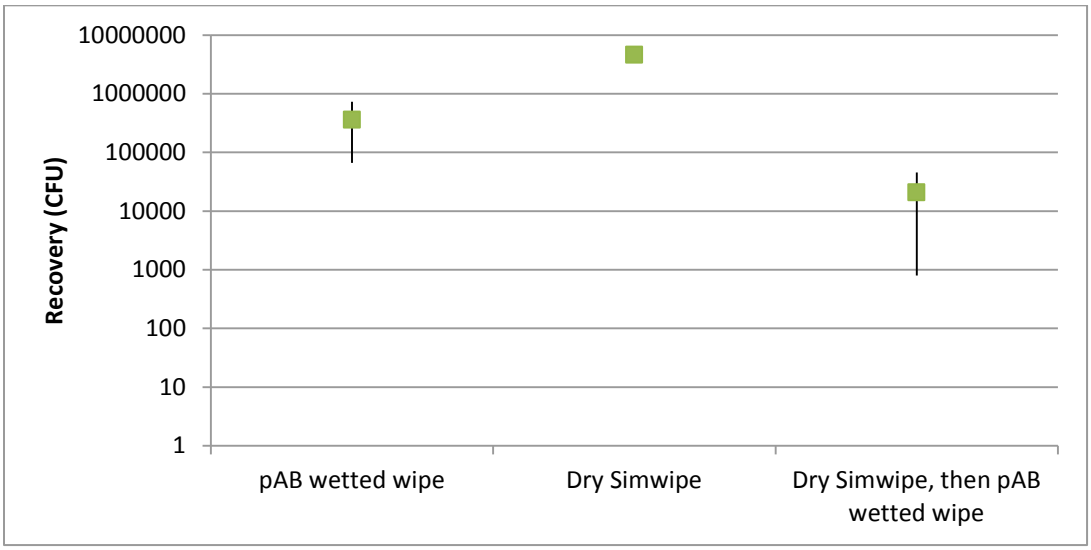


Figure 3-7. Recovery from Coupons after Three Methods of Wipe Decontamination (n=3). Error bars represent maximum and minimum values.

3.3 Task 3: Impact of Soiled Surfaces (Grime) on Decon Efficacy

3.3.1 Preliminary Tests on Effect of Grime on Recovery

Initial results showed that the grime contained substantial contamination. To reduce the background contamination co-collected during sampling, the grime was sterilized by exposure to 40 KGy of gamma irradiation. Verification tests were then conducted to determine if background contamination was eliminated by the sterilization procedure and to determine if recovery was affected by the addition of the grime.

No contamination was recovered from wipe samples of sterilized grime (Table 3-11). The value listed for the unspiked wipe aliquot indicates the detection limit. Recoveries were nearly identical for spiked grime wipes and spiked PBST extraction buffer alone (p-value = 0.87). These results demonstrated that there was no recovery bias, either positive or negative, from the presence of irradiated grime.

Table 3-11. Task 3 - Recovery from Wipe Samples of Grimed Coupons (n = 3)

	Avg. CFU/ Sample	Mean of Logs	RSD (%)
Spiked Grime Wipe	9.82×10^4	4.99	8.8%
Spiked PBST	9.73×10^4	4.99	1.8%
Unspiked Wipe	$<1.00 \times 10^1$	<1.00	0.0%
PBST	<1.05	<0.02	0.0%

3.3.2 Surface Sampling Results

Figure 3-8 shows the efficacy of the four decontamination procedures on grimed and clean coupons. Note that some of the differences in LR are due to differences in recovery from the positive control coupons. Figure 3-9 and Table 3-12 present spore recovery from the surface samples, suggesting no particular advantage to the use of pAB vs. pAB with surfactant (i.e., no difference in observed efficacy). Results from these tests are in agreement with results of **Task 2**, and suggest a single 30-second spray application is effective for concrete, yet yields approximate a 3 LR for wood coupons (with a 7 Log inoculum), regardless of the presence of grime. All four decontamination methods yielded equivalent recoveries from coupons, grimed or clean. This observation suggests no impact of grime at this loading. Tests with higher loadings are required to extend this finding to more heavily soiled surfaces.

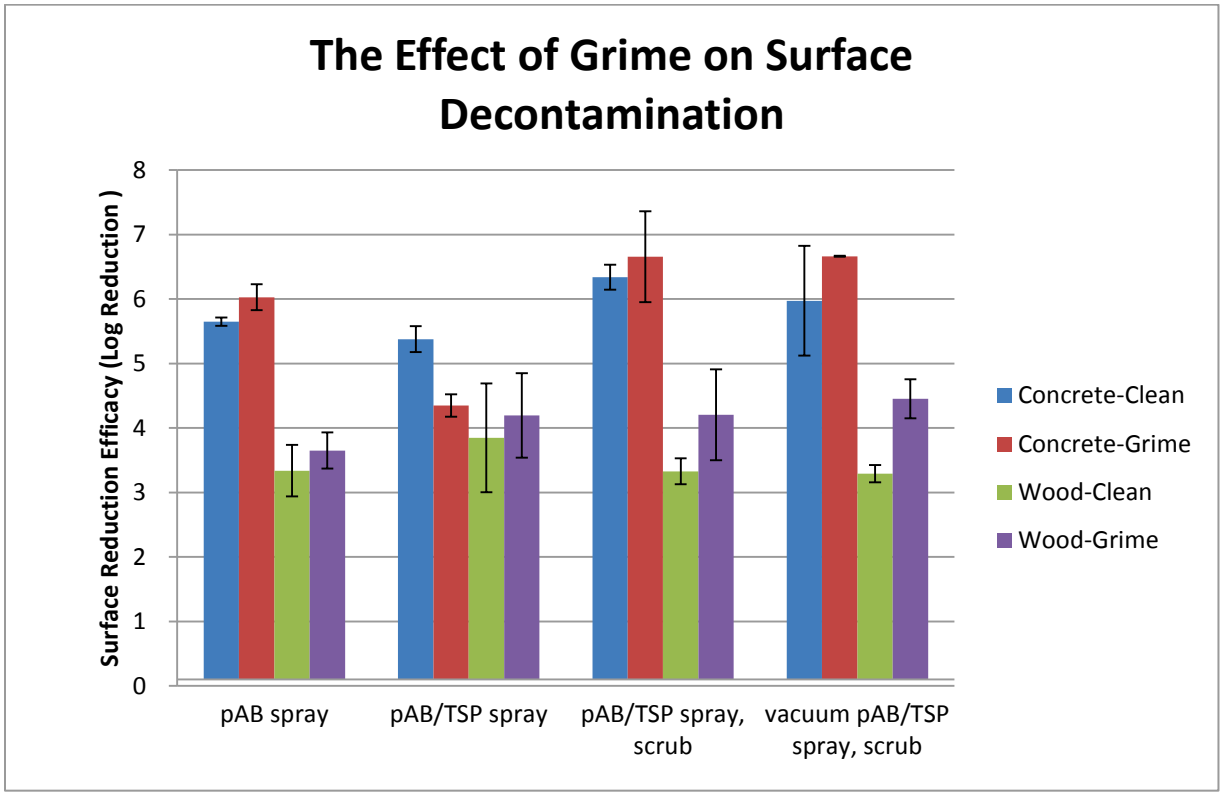


Figure 3-8. Task 3 Results - The Effect of Grime on Surface Decontamination (n = 3). Error bars indicate standard deviation.

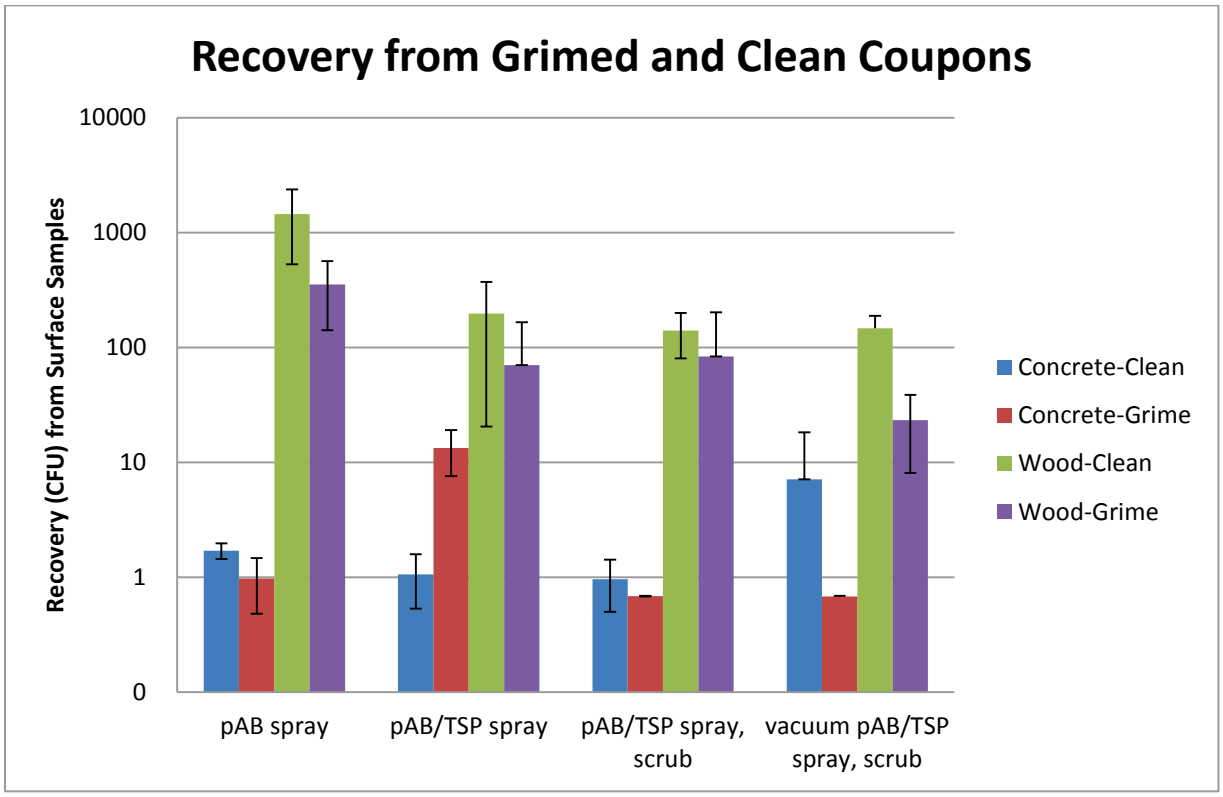


Figure 3-9. Task 3 Results - Recovery from of Grimed vs. Clean Decontaminated Surface Samples (n=3). Error bars indicate standard deviation.

Table 3-12. Task 3 - Results for Grimed and Non-Grimed Decontamination Tests using pAB and pAB/TSP (n = 3)

			Positive Controls			Test Coupons						
Test	**Material**	**Grime**	**Avg. CFU/ Sample**	**Mean of Logs**	**RSD (%)**	**Avg. CFU/ Sample**	**Mean of Logs**	**RSD(%)**	**LR**	**RSD (%)**	**Decontamination Solution**	**Decontamination Conditions (Decontamination Steps)**
G1	Concrete	No	8.80×10^5	5.88	57%	2	0.23	15.6%	5.65	1.2%	pAB	1 x 30 second spray, no reapplication, no rinse
	Wood	No	2.77×10^6	6.40	46%	1450	3.06	63.5%	3.34	12.0%	pAB	
G2	Concrete	Yes	9.89×10^5	5.98	31%	1	-0.05	50.6%	6.03	3.4%	pAB	1 x 30 second spray, no reapplication, no rinse
	Wood	Yes	1.42×10^6	6.14	26%	353	2.49	60.0%	3.65	7.7%	pAB	
G3	Wood	No	6.51×10^5	5.81	14%	197	1.97	89.6%	3.84	21.9%	pAB/TSP	1 x 30 second spray, no reapplication, no rinse
G3b	Concrete	No	2.38×10^5	5.37	20%	1	-0.01	49.7%	5.38	3.7%	pAB/TSP	
G4	Wood	Yes	5.40×10^5	5.71	37%	70	1.52	136.3%	4.19	15.6%	pAB/TSP	1 x 30 second spray, no reapplication, no rinse
G4b	Concrete	Yes	2.98×10^5	5.45	42%	13	1.10	43.3%	4.35	4.0%	pAB/TSP	
G5	Wood	No	2.78×10^5	5.44	5%	140	2.12	42.9%	3.33	6.0%	pAB/TSP	Scrub, then Spray
G5	Concrete	No	2.02×10^6	6.29	30%	1	-0.05	48.0%	6.34	3.0%	pAB/TSP	Scrub, then Spray
G6	Wood	Yes	5.70×10^5	5.75	16%	83	1.55	142.2%	4.20	16.8%	pAB/TSP	Scrub, then Spray
G6	Concrete	Yes	3.50×10^6	6.49	59%	1	-0.16	0.7%	6.66	0.0%	pAB/TSP	Scrub, then Spray
G7	Wood	No	2.78×10^5	5.44	5%	147	2.15	28.4%	3.29	4.1%	pAB/TSP	Vacuum, scrub, and Spray
G7	Concrete	No	2.02×10^6	6.29	30%	7	0.32	156.9%	5.97	14.3%	pAB/TSP	Vacuum, scrub, and Spray
G8	Wood	Yes	5.70×10^5	5.75	16%	23	1.30	65.5%	4.45	6.8%	pAB/TSP	Vacuum, scrub, and Spray
G8	Concrete	Yes	3.50×10^6	6.49	59%	1	-0.17	1.6%	6.66	0.1%	pAB/TSP	Vacuum, scrub, and Spray

3.3.3 Fate of Spores

During **Task 3**, rinsate and aerosol samples were collected and analyzed as they were during **Task 2**. No spores were detected in any rinsate sample except for the concrete rinsate in Test G1. Only one of the triplicate samples tested positive for *Bacillus* spores, suggesting that the presence of these spores may have been due to cross-contamination during collection. The rinsates were homogenized before aliquots were collected, so all three replicate samples should have produced similar results.

Figure 3-10 shows the CFU recovered from aerosol samples during decontamination. Aerosol sampling was conducted during all portions of each procedure including spraying, scrubbing, and vacuuming. Viable spores were detected in all aerosol samples collected during testing with inoculated (test) samples. Aerosol samples collected during decontamination of blank coupons did not yield viable spores. Figure 3-10 shows the combination of both halves of the aerosol sample; the rinse of the nozzle and the collection liquid, which were analyzed separately according to **MOP 6578**.

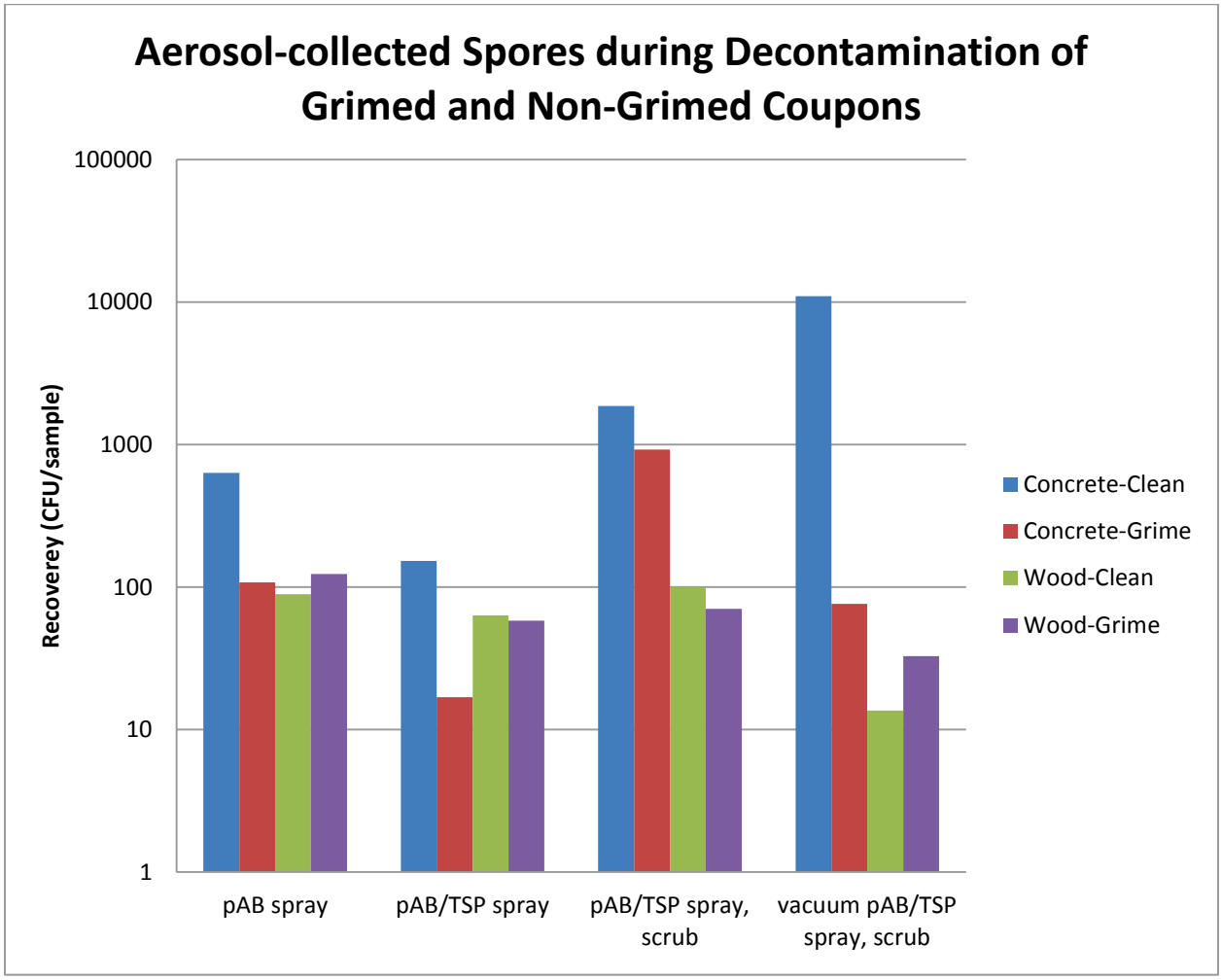


Figure 3-10. Recovery from Aerosol Samples during **Task 3** Testing (n = 1)

The values shown in Figure 3-10 are pooled from all three coupons during a single decontamination. While some are relatively low, these data represent the re-aerosolized spores from only approximately 0.37 m^2 (4 ft^2). Furthermore, these data have not been normalized to the total volume of air sampled, for the simple reason that it is unknown during what portion of the decontamination cycle most of the spores are aerosolized.

Figure 3-10 suggests that the decontamination procedures with more steps are likely to result in a greater magnitude of spore re-aerosolization. Figure 3-10 also suggests that laboratory studies using clean materials may overestimate the actual aerosolization from grimed materials. The concrete coupons used for this study were not polished concrete, so the surface was sometimes powdery. This powdery surface may help explain the higher aerosolization from concrete.

The results from these tests suggest that spray or mist should be the first decontamination step, rather than the use of a vacuum cleaner, as the amount of aerosol-collected spores was highest in tests where vacuuming was conducted before wetting of surfaces.

3.4 Assessment of Operational Parameters

3.4.1 Time

The time required to decontaminate a batch of coupons depended on the decontamination procedure being applied. The methods used in Test O1 and O2 were very effective on drywall and concrete and relatively rapid at 5 seconds/ per 0.09 m^2 (ft^2). A single worker on a four-hour shift may therefore be able to decontaminate 260 m^2 (2800 ft^2). Such an application would require between 61 to 80 L (16 and 21 gallons) of sporicide per hour, depending on the chosen flow rate. This volume would require the backpack sprayer (5 gallon capacity) be refilled every 15 to 20 minutes. Due to safety concerns with fatigue while wearing a National Fire Sprinkler Association (NFSA) Class C suit, cooling vests may be necessary to sustain a 4-hour shift.

The four-step procedure used in **Task 3** would likely require three responders rather than the one responder per area the spray alone procedure required: one to vacuum the area, one to spray the area, and one to scrub the area. As a result, this four-step procedure would triple the required personnel.

Since special care was taken to prevent cross-contamination and produce repeatable and documented decontamination steps, the procedures used in this study may provide an underestimate of field-scale productivity per person. Field-scale personnel, though, may be hampered by Level C suits and supplied air respirators (see Section 3.4.3). The Personal Protective Equipment (PPE) used in the field may perhaps be more restrictive than the PPE used in this study.

3.4.2 Physical Impacts on Materials

The materials used in this study were rugged, and, as expected, did not show any physical changes following decontamination.

3.4.3 Impact on Decontamination Workers

For this study, the decontamination steps were performed by personnel outside the chamber housing the coupons because of the high concentration of chlorine (Cl_2) gas generated by the pAB. Based upon Cl_2 concentrations during a field-scale event, respiratory protection may be required. The measured Cl_2 concentrations in the space may determine whether Occupational Safety and Health Administration (OSHA) Level C air purifying respirators or supplied air (e.g., Self Contained Breathing Apparatus) might be required. These refined procedures were much less onerous than procedures used under previous studies3.

4. Quality Assurance and Quality Control

This project was performed under an approved Category III QAPP entitled, "Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Part 4 – Optimization of Method Parameters and Impact of Surface Grime (June 2011)".[18]

4.1 Calibration of Sampling/Monitoring Equipment

There were standard operating procedures for the maintenance and calibration of all laboratory and NRMRL/NHSRC Biocontaminant Laboratory equipment. All equipment was verified as being certified calibrated or having the calibration validated by EPA's Air Pollution Prevention and Control Division's (APPCD) on-site (RTP, NC) Metrology Laboratory at the time of use. Standard laboratory equipment such as balances, pH meters, biological safety cabinets (BSCs) 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.

Table 4-1. Instrument Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
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
pH meter	Perform a 2-point calibration with standard buffers that bracket the target pH before each use.	± 0.1 pH units
Relative humidity (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
Pressure Guage	Compare to independent NIST Pressure gauge annually.	± 2 psi
Scale	Check calibration with Class 2 weights	± 0.1% weight

4.2 Data Quality Indicator (DQI) Goals

Target acceptance criteria for the critical measurements are shown in Table 4-2 with precision goals.

Table 4-2. Acceptance Criteria and Test Values for Critical Measurements

Measurement Parameter	Target Value	Test Value Task 1/ Task 2/ Task 3	Target Precision RSD (%)	Test Precision RSD (%) Task 1\ Task 2\ Task 3
FAC in Decontamination Solution	6000-6700 parts per million (ppm)	6520/ 6180-6700/ 6000-6620	± 1.6	NA/ 3%/ 3%
pH of Decontamination Solution	6.5 < pH < 7.0	6.8/ 6.6-7.0/ 6.7-7.0	± 2%	NA/ 2%/ 2%
Temperature of Liquids	18 – 24 °C	23 °C/ 22.7-24.5 °C/ 22-23.5 °C	± 1 °C	NA/ ± 1 °C/ ± 1 °C
Pressure of Backpack Sprayer used for Decontamination Solution Spraying	35 psi	40/ 42-72/ 21-43	± 6 %	NA/ 28%/ 23%
Flow Rate of Decontamination Spray	1050 mL/min	1049/ 1140/ 1208	± 5 %	1%/ 13%/ 8%
Positive Control CFU (high inoculation only)	1×10^7 CFU/coupon	8.36×10^4 - 9.6×10^6/ 1.9×10^6 - 2.05×10^7/ 2.38×10^5 - 3.5×10^6	50 %	14%/ 92% 89%
Test Coupon CFU	30-300 CFU per plate	30-300	20 %	≤20 %

4.2.1 Temperature Measurements

The contamination prevention protocol required the DI water reservoir to be filled the day of testing to minimize cross-contamination. Thus, the protocol for the daily filling of the DI reservoir consisted of the following steps: on the morning of testing, the reservoir (1) was filled with a diluted bleach solution, (2) was allowed to sit for one hour, (3) was emptied, (4) was triple-rinsed with DI water, then (5) was refilled with DI water for testing. As a result of this procedure, the water temperature was dependent on the room temperature, and measurements outside the target range were recorded. The temperature of the DI rinse

water is expected to have minimal effect on project results and was therefore allowed to remain outside specification without corrective action.

The temperature of the pAB was measured only when prepared, not when applied.

4.2.2 pH Measurements

The pH measurements listed in Table 4-2 are of the solution as prepared, not as applied to the coupons during decontamination. The pAB degrades over its 3-hour lifetime. The Oakton pH probe was calibrated with pH 7.0 buffer solution per manufacturer's instructions at the start of each test day. All the results were within the specified target range.

4.2.3 Pressure Measurements

Significant variation in the pressure reading of the backpack sprayer was observed. Some of the variation in **Task 2** was due to the targeted higher flow rate from the sprayer. The higher pressure at this flow rate was intentional (though the exact value was not known at the writing of the QAPP amendment). The low values during **Task 3** testing were due to a malfunctioning gauge. The flow rates for these tests were within specification.

4.2.4 FAC Measurements

The HACH High Range Bleach Test Kit was used to titrate a standard solution of 1000 ppm $NaClO_2$. The HACH test kit returned a value within 10 percent of the standard. The FAC measurements listed in Table 4-2 are of the solution as prepared, not as applied to the coupons during decontamination. The pAB degrades over its 3-hour lifetime. The FAC of the bulk solution was measured periodically during testing and was expected to be lower than the FAC of the fresh, as-prepared solution.

4.2.5 Flow Measurements

The target flow rates listed in the QAPP for the backpack sprayer were based on pAB; this study also used a pAB/TSP solution, which had higher flow rates at the same sprayer setting. The sprayer was set to its lowest (or highest for Test O2) setting to provide a spray pattern of 16-in diameter from a distance of 3 ft.

4.2.6 Positive Control CFU

The target concentration was not always met, and, as expected, there was much variation between material types and material condition (grimed vs. non-grimed). Most inoculation levels did provide sufficient loading to allow for a 6 log dynamic range.

4.2.7 CFU Counts

Twenty-five percent (25%) of all plates containing CFU within the acceptable range (30-300) were counted by a second technician. The second enumeration was required to fall within 10 percent of the initial count.

The positive control enumerations (i.e., inocula) were occasionally above or below the target value due to malfunctioning MDIs, but the inoculations were deemed acceptable by the EPA Principal Investigator (PI)..

4.3 Data Quality Audit

At least 10 percent of the data acquired during the investigation were audited. The QA Manager traced the data from the initial acquisition through reduction to final reporting to ensure the integrity of the reported results. All data treatment calculations were checked before inclusion in this report.

4.4 QA/QC Reporting

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

4.5 Amendments to and Deviations from the Original QAPP

4.5.1 Formal Amendments

During the course of the projects, six amendments were added to the QAPP by the EPA PI in response to data results or equipment failures. These amendments, listed in Appendix G, were submitted by e-mail to the EPA QA officer for formal approval.

4.5.2 Other QAPP Deviations

The QAPP states that, following coupon inoculation, a second person, wearing new gloves for each coupon, is tasked with moving the coupon to the proper location (e.g., test and positive control coupons to the Test Coupon Cabinet and blank coupons to the Blank Coupon Cabinet). This person did not wear new gloves for each sample. Any cross-contamination that occurred between similarly inoculated coupons is not expected to have biased the results.

Rather than transfer the 18 mm coupons in **Task 1** in the BSC as listed in the QAPP, the operation was performed inside the chamber where the decontamination took place. Transfer of the holding plate from the chamber to the BSC would expose the coupons to much more risk of contamination.

The QAPP states that the time required for the grimed coupons to appear dry would be recorded in a laboratory notebook. However the coupons never appeared wet. Instead, a drying time of 15 minutes (based on application of the grime to paper) was chosen, as listed in the **MOP 3163**.

Amendment 3 states that all coupons will be spritzed with pAB before wiping. Spritzing was not done with the SimWipe due to a misinterpretation of the wiping **MOP 3156** at the time of testing. The SimWipe method is expected to have been more effective with the addition of pAB.

The BioSamplers® were not filled with peptone-buffered water as stated in the QAPP due to the unavailability of peptone-buffered water for the first test. Instead, PBST was used as the collection liquid. There was no excessive foaming due to the TWEEN®-20 in the PBST.

The flow rate of 1000 mL/min is listed for **Task 3** decontamination. However, the flow rate at the lowest setting of the sprayer is 1300 mL/min when the pAB/TSP solution is used.

Although rinsate samples were to be processed on the same day as they were taken, due to the length of time required to collect the samples, they were placed in the refrigerator at 4 ± 2 °C overnight and processed the next day.

The glassware accompanying the BioSamplers® was to be rinsed with 100 mL of sterile PBST. However, because the glassware was shorter in length than originally anticipated, only 50 mL of PBST was used to rinse the glass.

4.5.3 Data Quality Indicator Assessment

Most of the data quality indicators for the critical measurements were within their specified target ranges as indicated in Table 4-2. However, in some instances, some small deviations were noted such as deionized water temperatures, sprayers flow rates, or CFU counts. These small deviations in the measurements, although critical, were consistent throughout the tests and did not affect the affect the intra-test comparisons.

5. References

[1] Canter, D. A. (2005). "Remediating anthrax-contaminated sites: Learning from the past to protect the future." Chemical Health and Safety 12(4): 13-19.

[2] After Action Report – Danbury Anthrax Incident, U.S. EPA Region 1, September 19, 2008.

[3] U.S. EPA. Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Development and Evaluation of the Decontamination Procedural Steps . U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-12/025, 2012.

[4] Snook, C. P., J. Cardarelli, et al. (2008). "Medical toxicology and public health-update on research and activities at the centers for disease control and prevention and the agency for toxic substances and disease registry." J Med Toxicol 4(4): 289-291.

[5] Canter, D. A. (2005). "Addressing residual risk issues at anthrax cleanups: How clean is safe?" Journal of Toxicology and Environmental Health 68(11-12): 1017-1032.

[6] ASTM International. Standard Test Method E-2414-05. Quantitative sporicidal three-step method (TSM) to determine sporicidal efficacy of liquids, liquid sprays, and vapor or gases on contaminated carrier surfaces. West Conshohocken, PA: American Society for Testing and Materials; 2005.

[7] AOAC International. (2008). Method 2008.05 - Determining efficacy of liquid sporicides against spores of *Bacillus subtilis* on a hard nonporous surface using the quantitative three step method (TSM).

[8] Tomasino, S. F., R. M. Pines, et al. (2008). "Determining the efficacy of liquid sporicides against spores of *Bacillus subtilis* on a hard nonporous surface using the quantitative three step method: Collaborative study." Journal of AOAC International 91(4): 833-852.

[9] Wood JP. Evaluation of liquid and foam technologies for the decontamination of *B. anthracis* and *B. subtilis* spores on building and outdoor materials. Washington, D.C.: U.S. Environmental Protection Agency; 2009 November. Report No.: EPA/600/R-09/150.

[10] Einfeld, W., R. M. Boucher, et al. (2011). Evaluation of Surface Sampling Method Performance for *Bacillus* Spores on Clean and Dirty Outdoor Surfaces. Albuquerque, NM, Sandia National Laboratories: SAND2011-4085.

[11] Rastogi, V. K., L. Wallace, et al. (2009). "Quantitative Method To Determine Sporicidal Decontamination of Building Surfaces by Gaseous Fumigants, and Issues Related to Laboratory-Scale Studies." Appl. Environ. Microbiol. 75(11): 3688-3694.

[12] Rogers, J. V., C. L. K. Sabourin, et al. (2005). "Decontamination assessment of *Bacillus anthracis, Bacillus subtilis,* and *Geobacillus stearothermophilus* spores on indoor surfaces using a hydrogen peroxide gas generator." Journal of Applied Microbiology 99(4): 739-748.

[13] Tomasino, S. F., V. K. Rastogi, et al. (2010). "Use of Alternative Carrier Materials in AOAC Official Method(SM) 2008.05, Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard, Nonporous Surface, Quantitative Three-Step Method." Journal of Aoac International 93(1): 259-276.

[14] Sagripanti, J. L., M. Carrera, et al. (2007). "Virulent spores of Bacillus anthracis and other Bacillus species deposited on solid surfaces have similar sensitivity to chemical decontaminants." Journal of Applied Microbiology 102(1): 11-21.

[15] Lee, S. D., S. P. Ryan, et al. (2011). "Development of an Aerosol Surface Inoculation Method for *Bacillus* Spores." Appl. Environ. Microbiol. 77(5): 1638-1645.

[16] Edmonds, J., P. Clark, et al. (2010). "Multigeneration Cross Contamination of Mail with *Bacillus* Species Spores by Tumbling." Appl. Environ. Microbiol. 76(14): 4797-4804.

[17] Brown, G. S., R. G. Betty, et al. (2007). Evaluation of rayon swab surface sample collection method for Bacillus spores from nonporous surfaces." Journal of Applied Microbiology 103(4): 1074-1080.

[18] ARCADIS U.S., Inc. Quality Assurance Project Plan for the Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Part 4 – Optimization of Method Parameters and Impact of Surface Grime. Prepared under Contract No. EP-C-09-027, Work Assignment No. 2-25. U.S. Environmental Protection Agency, National Homeland Security Research Center, Research Triangle Park, NC. June 2011.

[19] Grand, I., M.-N. Bellon-Fontaine, et al. (2011). "Possible Overestimation of Surface Disinfection Efficiency by Assessment Methods Based on Liquid Sampling Procedures as Demonstrated by In Situ Quantification of Spore Viability." Appl. Environ. Microbiol. 77(17): 6208-6214.

[20] Busher, A.; Noble-Wang; J.; Rose, L. Surface Sampling. In Sampling for Biological Agents in the Environment; Emanual, P., Roos, J.W., Niyogi, K., Eds.; ASM Press: Washington, DC, 2008; pp 95 – 131.

[21] EPA Method 5 – Determination of Particulate Matter Emissions from Stationary Sources. CFR 40, Part 60.

[22] U.S. Environmental Protection Agency (2007) Guidance on test methods for demonstrating the efficacy of antimicrobial products for inactivating Bacillus anthracis spores on environmental surfaces. In FIFRA Scientific Advisory Panel Meeting. Arlington, VA: US Environmental Protection Agency (SAP Minutes No. 2007-05).

[23] Calfee, M. W., S. P. Ryan, et al. (2012). "Laboratory evaluation of large-scale decontamination approaches." Journal of Applied Microbiology 112(5): 874-882.

[24] US Environmental Protection Agency (2011) Effectiveness of Physical and Chemical Cleaning and Disinfection Methods for Removing, Reducing or Inactivating Agricultural Biological Threat Agents. US Environmental Protection Agency, Office of Research and Development, National Homeland Security Research Center. EPA 600/R-11/092, September 2011.

Appendix A: Coupon, Test Chamber and Equipment Cleaning and Sterilization Procedures

The pAB solution used for cleaning surfaces in equipment in both the decontamination and NRMRL/NHSRC Biocontaminant Laboratory was prepared as a 1:10 dilution of bleach in DI water, pH-adjusted to ~6.8 using glacial acetic acid.

The following steps were followed for cleaning the decontamination chamber between each material type and before/after each test:

- Using the back sprayer, the interior surfaces are kept wet with pAB solution for 10 minutes.
- With the drain open, the surfaces are then rinsed with DI water. The run-off is collected in a carboy and ultimately discarded.
- After ensuring all runoff is removed from the chamber, the valve is closed in preparation for the next test.
- A mop assembly with a disposable pad is used to wipe down the interior of the chamber with isopropyl alcohol or ethanol.
- The pad is then removed and placed in a bucket of pAB solution for decontamination prior to disposal.

The following steps are followed for cleaning the wet/dry vacuum (including head assembly) after use in a test:

- Soak the head assembly in pAB for at least 60 minutes.
- Spray the wet/dry vacuum drum with pAB and maintain wetted for at least 60 minutes.
- Soak the hoses in pAB for at least 60 minutes.
- Rinse all parts with DI water.
- Air dry prior to re-use.
- Alternatively, the wet/dry vacuums may be fumigated with a STERIS VHP® sterilization cycle. This cycle entails the use of a STERIS VHP® ARD hydrogen peroxide (H_2O_2) generator and exposure of all components of the wet/dry vacuum to H_2O_2 at 250 ppmv for 4 hours by maintaining this constant concentration in a decontamination chamber.
- Replace HEPA filters.

The following steps are followed for cleaning the buckets after use in a test:

- Fill the buckets with pAB and leave them covered for at least 60 minutes.
- Rinse all buckets five times with DI water.
- Air dry prior to re-use.

The following steps are followed for cleaning the brushes after use in a test:

- Soak the brushes in pAB for at least 60 minutes.
- Rinse with DI water.
- Air dry prior to re-use.

The following steps are followed for cleaning the work surfaces before and after use:

- Wet all surfaces with pAB solution or using Dispatch® bleach wipes.
- Rinse with DI water.
- Wet and wipe surfaces with isopropyl alcohol or ethanol.
- Air dry prior to re-use.
- Alternatively, cover paper can be used and replaced before/after each use.

The sampling templates are autoclaved before/after each use.

The following steps are followed for cleaning the coupon cabinets before and after use:

- Wet and wipe all surfaces with pAB solution or using Dispatch® bleach wipes.
- Rinse with DI water.
- Wet and wipe surfaces with isopropyl alcohol or ethanol.
- Air dry prior to re-use.

The gaskets used in **MOP 6561** during the contamination procedure were cleaned via fumigation with the STERIS VHP® sterilization cycle. This cycle entails the use of a STERIS VHP® ARD hydrogen peroxide (H_2O_2) generator at 250 ppmv for 4 hours by maintaining this constant concentration in a decontamination chamber.

The carboys were autoclaved according to **MOP 6570**.

The BioSampler and front end of the BioSampler train were autoclaved according to **MOP 6570**.

Concrete coupons were autoclaved according to **MOP 6570**. Other types of coupons were cleaned via fumigation with the STERIS VHP® sterilization cycle. This cycle entails the use of a STERIS VHP® ARD H_2O_2 generator at 250 ppmv for 4 hours by maintaining this constant concentration in a decontamination chamber.

Appendix B: Miscellaneous Operating Procedures (MOPs)

MOP 3113: Procedure for Deposition of *Bacillus Subtilis* Spores using a Metered Dose Inhaler

MOP 3128-A Procedure for Preparing pH-Adjusted Bleach Solution

MOP 3128-B Procedure for Preparing pH-Adjusted Bleach Solution with Tri-sodium Phosphate Substitute

MOP 3135: Procedure for Sample Collection using BactiSwab™ Collection and Transport Systems

MOP 3144: Procedure for Wipe Sampling of Coupons

MOP 3145: Procedure for HEPA Vacuum Sampling of Large and Small Coupons

MOP 3150: Procedure for Fabrication of 14" x 14" Material Coupons

MOP 3156: Procedure for Wetted Wipe Decontamination

MOP 3161-LD: Aerosol Deposition of Spores onto Material Coupon Surfaces Using the Aerosol Deposition Apparatus (ADA) – Low Dosing

MOP 3163: Aerosol Application of Grime on Material Coupons

MOP 6535a: Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spores

MOP 6555: Petri Dish Media Inoculation Using Beads

MOP 6561: Aerosol Deposition of Spores onto Material Coupon Surfaces Using the Aerosol Deposition Apparatus

MOP 6562: Preparing Pre-Measured Tubes with Aliquoted 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 6567: Recovery of *Bacillus* Spores from Wipe Samples

MOP 6568: Aseptic Assembly of Wipe Kits

MOP 6570: Use of STERIS Amsco Century SV 120 Scientific Prevacuum Sterilizer

MOP 6571: Recovery of *Bacillus* Spores from Via-cell Aerosol Sampling Cassettes

MOP 6572: Recovery of Spores from HEPA Sock (Vacuum Sock) Samples

MOP 6578: Preparation and Analysis for SKC BioSamplers®

Appendix C: Spore Deposition and Handling Procedures

The handling of the contaminated coupons for Task 1, including movement to minimize or control spore dispersal, was done in accordance with MOP 6561. One person was tasked with removing the clamps holding the dosing chamber to the coupon and the removal of the dosing chamber and gasket from the coupon. A second person was then tasked with moving the coupon to the proper location (e.g., test and positive control coupons to the Test Coupon Cabinet and blank coupons to the Blank Coupon Cabinet).

For Task 2, two personnel were used to move the 40 in by 40 in coupons into their vertical positions in COMMANDER following removal of the deposition devices. This was the only time the coupons were handled, and this occurred a minimum of two days prior to sampling.

For Task 1, the Test Coupon Cabinet was a steel cabinet (48 in wide by 24 in deep by 78 in high) with twelve shelves each 6 in apart. Each cabinet held a total of 36 coupons, hence, two Test Coupon Cabinets were needed for a test. These were labeled as Test Coupon Cabinet 1 and Test Coupon Cabinet 2. Test and positive control coupons were arranged in each cabinet according to material types. A single material type was not split among cabinets. Procedural blank coupons of each material/orientation to be used in a single test were contained in a separate isolated cabinet (Blank Coupon Cabinet) of similar construction, however, with dimensions of 48 in wide by 24 in deep by 36 in high with 3 shelves.

Each MDI was claimed to provide 200 discharges. The number of discharges per MDI was tracked so that use did not exceed this value. Additionally, in accordance with MOP 6561, the weight of each MDI was recorded 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 6561, the MDI was retired and a new MDI used. For quality control of the MDIs, a contamination control coupon was run as the first, middle, and last coupon contaminated with a single MDI in a single test. The contamination control coupon was a stainless steel coupon (1.2 feet by 1.2 feet) that was contaminated in accordance with MOP 6561, sampled, and analyzed.

A log was maintained for each set of coupons that were dosed via the method of MOP 6561. Each record in this log recorded a unique coupon identifier (see Table C-1), 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 contamination (dosing).

Additionally, after a coupon was dosed via the above procedure, the coupon was labeled with the unique identifier using the coding outlined in Table C-1. The label was printed on the side of the coupon using a permanent marker (e.g., black or silver Sharpie®). 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. Each coupon was marked with only the material descriptor and unique code number. Once the coupons were transferred to the NRMRL/NHSRC Biocontaminant Laboratory for plate counts, each sample was additionally identified by replicate number and dilution.

Table C-1. Coupon Sample Coding

Coupon Identification: 25-TN-(X)M-D-(TT)-SS--NN		
Category	**Example Code**	
TN	A1	Test Number (from Table 2-1, Table 2-2, Table 2-3, Table 2-4, or Table 2-5)
(X) M (Material)	X	Procedural Blank
	K	Concrete
	W	Rough-cut barn wood
	D	Painted wallboard
	S	Stainless Steel (for QC purposes)
D (Descriptor)	0	Blank coupon
	P	Positive control sample
	T	Test sample
	C	The sample has had contact with the coupon
	X	The sample has had no contact with the coupon
	FB	Field Blank
TT	TT	Time point: 00,02,04,08,24,32 (A tests only – Table 2-1)
SS (Sample Type)	DE(F/B)	Ambient (duct) exhaust (Front or Back half of sample)
	VE (F/B)	Vacuum cleaner exhaust(Front or Back half of sample)
	FN	FAC and pH of pAB sample from nozzle
	FB	FAC and pH of pAB sample from bulk
	R	Run-off
	VN	Swab sample of vacuum nozzle
	VF	Swab sample of vacuum filter
	W	Wipe Sample
	HS	Vacuum sock sample
	E	Direct extraction
NN (Sample Number)	NN	Sequential numbers
NRMRL/NHSRC Biocontaminant Laboratory Plate Identification: 25-TN-(X)M-D-(TT)-SS-NN-R-d		
25-TN-(X)M-D-SS-(T)-NN	As above	
R (Replicate)	R	A – C

Coupon Identification: 25-TN-(X)M-D-(TT)-SS--NN		
Category	**Example Code**	
d(Dilution)	1	0 to 4, for 10E0 to 10E4

Each material section will be identified by a description of the material and a unique sample number. The sampling team will maintain an explicit laboratory log which will include records of each unique sample number and its associated test number, contamination application, any preconditioning and treatment specifics, and the date treated. Each material section test area sample will be marked with only the material descriptor and unique code number. The wet/dry vacuum samples and exhaust sample from each test will be identified with an associated test number and material section type. The sample codes will ease written identification. Once the coupons are transferred to the NRMRL/NHSRC Biocontaminant Laboratory for plate counts, each sample will additionally be identified by replicate number and dilution. Table 2-7 specifies the sample identification. The NRMRL/NHSRC Biocontaminant Laboratory will also include on each plate the date it was placed in the incubator.

The procedural blank coupons will have a two letter material code, with the first letter being “X”. For runoff samples, however, no material code will be used for the procedural blanks, since both materials will be present. For the STS tests in Table 2-5, the decontaminated blanks are defined as the test coupons, and the blanks that do not undergo decontamination are the controls. Not all the categories may be used for all samples. For instance, a field blank for wipe coupons will be taken every day, but will not be affiliated with any material. Hence, the first wipe field blank for Test G1 would be 25-G1-FB-W-1. The “C” and “X” descriptor codes are used for samples such as the vacuum nozzle swab. A code of 25-G1-C-X-VN-1 designates a swab sample from the vacuum nozzle to be used on concrete coupons of Test G1, but before such use occurred. As such, these “X” descriptors act as background blanks.

Appendix D: Decontamination Procedures

Coupon Storage Cabinets

On the decontamination procedure test day, the procedural blank, test, and positive control coupons were placed into the appropriate cabinets. A total of three cabinets were used to contain the coupons prior to decontamination (one for the procedural blanks, and two containing the contaminated [positive controls and test] coupons). One additional cabinet was used to store test coupons for drying after the decontamination procedure has been applied. The cabinets with their intended purpose are listed in Table D-1.

Table D-1. Coupon Storage Cabinets

Cabinet Name	Description
Test Coupon Cabinet #1 Test Coupon Cabinet #2	For storage of contaminated coupons (both positive control and test coupons); each cabinet can hold 36 coupons, thus, two cabinets will be needed for all tests
Procedural Blank Cabinet	For storage of procedural blank coupons; the cabinet will be under slight positive pressure in order to prevent contamination from the test environment (i.e., laboratory) and allow passive air flow to promote drying.
Decontaminated Coupon Cabinet	For storage of all test coupons after application of the decontamination procedure; the cabinet will be under slight positive pressure in order to prevent contamination from the test environment (i.e., laboratory) and allow passive air flow to promote drying.

Materials and Equipment

The materials and equipment used for the decontamination procedures were standardized as much as possible and are listed in Table D-2. Decontamination steps are described in the subsequent sections of this Appendix.

Table D-2. Material and Equipment Used in the Decontamination Procedural Steps

Material/Equipment	Description
Wet/dry vacuum	RIDGID 14 Gallon Pro Vac WD1450 (http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&catalogId=10053&productId=100081216&N=10000003+90401+524502+1600) Head attachment: RIDGID 2-1/2 In. Wet Nozzle (Squeegee) Accessory (http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&catalogId=10053&productId=100046467&N=10000003+90401+524502+1600) Filter: RIDGID 5-Layer Vacuum HEPA Filter(http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&catalogId=10053&productId=100022800)
Sprayer	12 Volt battery-operated 4 gallon backpack sprayer (http://www.agrisupply.com/product.asp?pn=59540&c2p=ppv&bhcd2=1237402084)
Bleach	Ultra Clorox® Regular Bleach (EPA Reg. No. 67619-8) http://www.clorox.com/products/overview.php?prod_id=clb) 6.15% sodium hypochlorite; <1% sodium hydroxide (http://www.thecloroxcompany.com/products/msds/bleach/cloroxregularbleach0505_.pdf)
Vinegar	5% v/v technical grade acetic acid
Bucket of cleaning solution	3 gallons in a 5-gallon plastic pail
Detergent	Klean-Strip TSP Substitute (http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&cataloged=10053&productId=100259541)
Brush	Rubbermaid Floor Scrubber (http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&catalogId=10053&productId=100644166)
Sponge	QEP Extra Large Grouting Sponge, 7-1/2 x 5-1/2 x 2 In., Rectangle with Rounded Corners (http://www.homedepot.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=10051&langId=-1&catalogId=10053&productId=100173109)
Nozzle	Standard Adjustable-Flow Garden Hose Nozzle Standard Brass, 4" Length (McMaster-Carr, P/N 7484T1)
Garden hose	100 ft; 5/8 inch diameter (McMaster-Carr, P/N 7453T2)
Pressure regulator	Bronze Pressure Regulator-Plumbing-Code Rated Standard, 3/4" NPT Female, 25-75 PS' (McMaster-Carr, P/N 8138K14)

Material/Equipment	Description
Bucket of DI water	3 gallons in a 5-gallon plastic pail
Carboy container	Carboys; Nalgene; Heavy Duty; polypropylene; Autoclavable; Leakproof; For full vacuum applications up to 8 Hours; USP class VI, vacuum rated for intermittent vacuum use only; 83B Closure size; capacity: 5.25 gal. (20L)
Pump	NSF-Certified Rotary Vane Pump for Water with Motor, Brass, 4.3 Max GPM, 3/4 Horsepower

pH-adjusted Bleach (pAB)Solution

To reduce the impact of "natural" variations in the bleach solution in this study, the pH and chlorine content was measured at the start and monitored throughout each test. The solution had to have a mean pH close to, but not above neutral (>6.5 and <7.0) and a mean total chlorine content of 6,000-6,700 ppm. The temperature of the solution had to be between 18 – 24 °C (64 – 75 °F). Any solution having a pH, chlorine content or temperature falling outside of this range at any time was discarded and a fresh pAB solution prepared. The chlorine content was measured using a Hach high range bleach test kit (Method 10100). The pH and temperature were measured using an Oakton pH probe (OKPH502; pH5). DI water was used as the base for all solutions.

The pAB solution was prepared just prior to the initiation of testing on a particular day and was used within a window of three hours from the time of preparation (for Tasks 2 and 3). After three. hours, the bleach solution was tested to see if it still met the QA criteria; if not, it was discarded and a fresh pAB solution prepared. Additionally, technical grade acetic acid (5% v/v) was used instead of off-the-shelf white vinegar. This change was expected to reduce the variability to in the pAB solution for the purpose of this study.

The pAB solution was applied to each coupon using a backpack sprayer (see Tables 2-2, 2-3 and 2-4).

Backpack Sprayer Application of Decontaminant

Prior to the start of testing, the spray pattern from the backpack sprayer was tested by spraying at the appropriate distance (1 ft) onto a piece of 1.2 ft by 1.2 ft blue construction paper mounted in the position of the material section. The spray was discharged into the center of the paper and the pattern was visually assessed for consistency with that shown in Figure D-1. The diameter of the spray was checked to ensure that was within the acceptable limits (12" to 16" diameter at 1 foot for the bleach sprayer, and at 12" to 16" diameter at 3 feet for DI water).

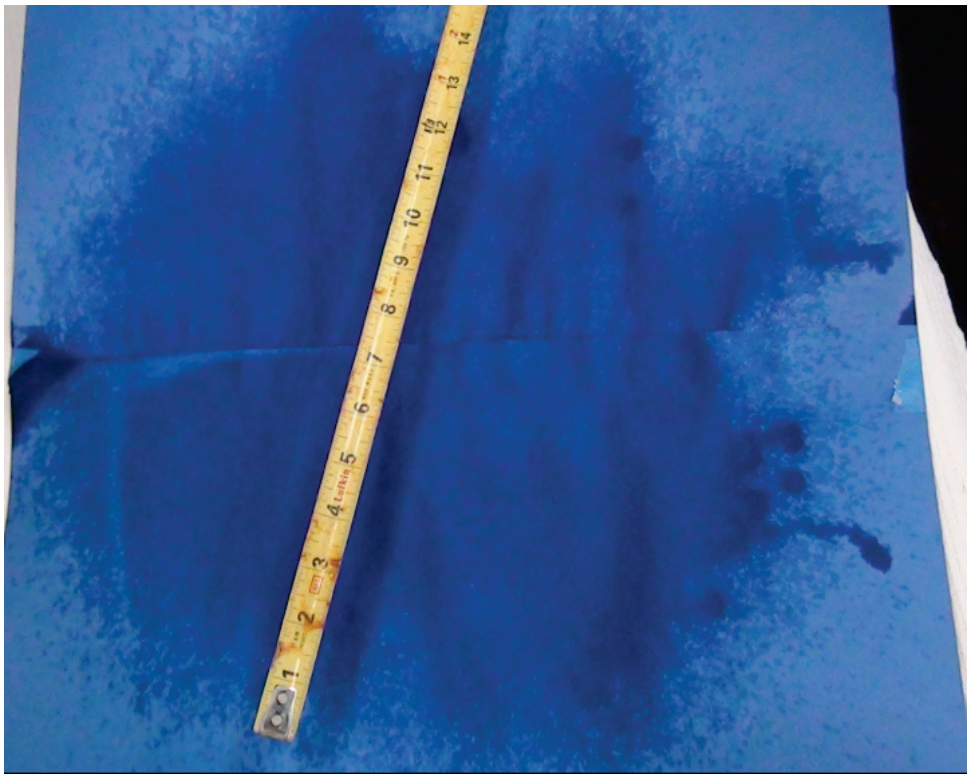


Figure D-1. Bleach Spray Pattern from Nozzle 1 Foot Away

pAB Solution and Application

The pAB solution was applied to each material section using a 4-gallon rechargeable backpack sprayer (ShurFlo ProPack SRS-600), see Figure D-2). The bleach was applied starting at the upper left or far left corner and spraying across the section from left to right, then across the section from right to left, back and forth 5 times for vertical surfaces. A constant spray having a diameter of about 12 inches at the section surface was used; the surface was completely flooded to wet the surface. The spray nozzle was held a constant distance of approx. 1 ft from the section surface. The battery-operated sprayer automatically maintained a constant pressure of 35 psi throughout the application. The pressure was indicated by the pressure gauge that is located on the sprayer. The flow rate of the pAB at 35 psi is 1000 mL/min. The flow rate was measured before and after each application to a section. The pAB solution was reapplied throughout the specified contact time, as outlined by the test plan. The sections were sprayed to completely wet (or flood) the surface of the materials. During the bleach application, the duration of each spray application was recorded. This measurement and the recording of flow rate allowed for a determination of the amount of bleach applied to each material.

Figure D-2. Backpack sprayer

pAB with TSP Substitute

The pAB with TSP substitute (surfactant) solution was made according to MOP 3128-B. This MOP describes production of a TSP solution (DAP, SKU 7079860001, Baltimore, MD) made according to the manufacturer's recommendations (1/4 cup per gallon solution). The solution, instead of being water, also includes 6000 ppm bleach, neutralized with acetic acid. The general solution is made of 1/4 cup TSP, 12.5 oz (370 ml) germicidal bleach, and 30 oz (900 mL) 5% acetic acid in 1 gallon solution (3.785 liters).

The pAB + TSP solution was applied to each material section using a backpack sprayer (see Figure E-2). The liquid was applied starting at the upper left or far left corner and spraying across the section from left to right, then across the section from right to left, back and forth 5 times for vertical surfaces and four times for horizontal surfaces. A constant spray having a diameter of about 12 inches at the section surface was used; the surface was completely flooded to wet the surface. The spray nozzle was held a constant distance of approx. 1 ft from the section surface. The battery-operated sprayer automatically maintained a constant pressure of 35 psi throughout the application. The pressure was indicated by the pressure gauge that is located on the sprayer. The flow rate of the pAB with TSP at 35 psi is 1000 mL/min. The flow rate was measured before and after each application to a section. The pAB with TSP solution was reapplied throughout the specified contact time and frequency. During the bleach application, the duration of each spray application was recorded. This measurement and the recording of flow rate allowed for a determination of the amount of bleach applied to each material.

One exception to this application method was for Tests O1, O2, O3, O6, and O7, a separate set of wallboard coupons that had the pAB with TSP applied using a sponge. The sponge was squeezed to

remove excess liquid prior to application to the coupon surface. Sponges were sterilized using VHP® and were not reused.

Wet/Dry Vacuum

For each material type, a single wet/dry vacuum was used and clearly identified with a label indicating the test number and material set (control or material type). The wet/dry vacuum exhaust was routed directly to the air handling system to prevent re-entrainment of particles during this phase. A 6-inch duct was used to exhaust filtered gas to the facility air handling system. The wet/dry vacuum used was a 6.0 HP unit with a 14-gallon capacity. The make and model are shown in Table E-2. The head attachment that was used in this study was the 16-inch wet nozzle (squeegee) (Figure D-3).

Figure D-3. 16 In. Wet Nozzle (Squeegee) Accessory

For vertical surfaces, the vacuuming action started with the center of the squeegee at the upper right corner of the material. Nine downward strokes were used, each overlapping the last by 50 percent.

The filter inside the wet/dry vacuum was a 5-layer HEPA-rated filter (Figure D-4) that can be purchased as an accessory replacement for the standard pleated filter (non-HEPA-rated) provided with the vacuum as received by the consumer. Proper seating of the HEPA filter was confirmed for each assembly. The details of the filter can be found in the link provided in Table D-2.

Figure D-4. 5-Layer HEPA Filter

During application of the procedure, the time required for each material set was recorded. Before and after each use of the vacuum, the vacuum cleaner was disassembled and sterilized using VHP®.

Brushing

The brush type used in this project was a synthetic fiber brush designed for heavy-duty rough floor scrubbing and adsorbing cleaning solution (see Figure D-5). The dimensions of the brush are 10 inches wide by 3 inches deep. The scrubbing action started at the upper right corner of the section. Using a zigzag stroke, the brush was firmly moved down the surface. The time required for the brushing application to each section was recorded.

Figure D-5. Brush (handle not shown)

Used brushes were placed into a bucket filled with the pAB solution. Brushes were left in the solution for a minimum of one hour before being removed, rinsed with DI water, and left to dry. After the brushes were dry, a swab sample of the brushes was taken and plated to ensure sterility of the brushes. If viable target organism was found on any brush, the entire batch was re-treated in the pAB solution, rinsed with DI water, dried and then re-sampled. If a positive sample was returned a second time for a batch of brushes, all brushes were discarded and replaced.

Rinsing with a Garden Hose

Rinsing the decontamination chamber was done using a standard garden hose nozzle as listed in Table D-2. The water was supplied to the nozzle through a 75 ft garden hose of 5/8 inch diameter. The head pressure was maintained constant at approx. 60 psi using a pressure regulator listed in Table D-2. The water was supplied via a closed loop system having a carboy container as the reservoir and a pump to provide a pressurized stream and continual recirculation. An Oakton pH probe was used to monitor the temperature and pH of the water throughout the test. The water used in this study was DI water. Aliquots (100 mL) were collected for filter plating as sterility checks the day each decontamination procedure was run. Via adjustment of the nozzle, the spray pattern was controlled to be 1 ft in diameter measured 3 ft from the nozzle.

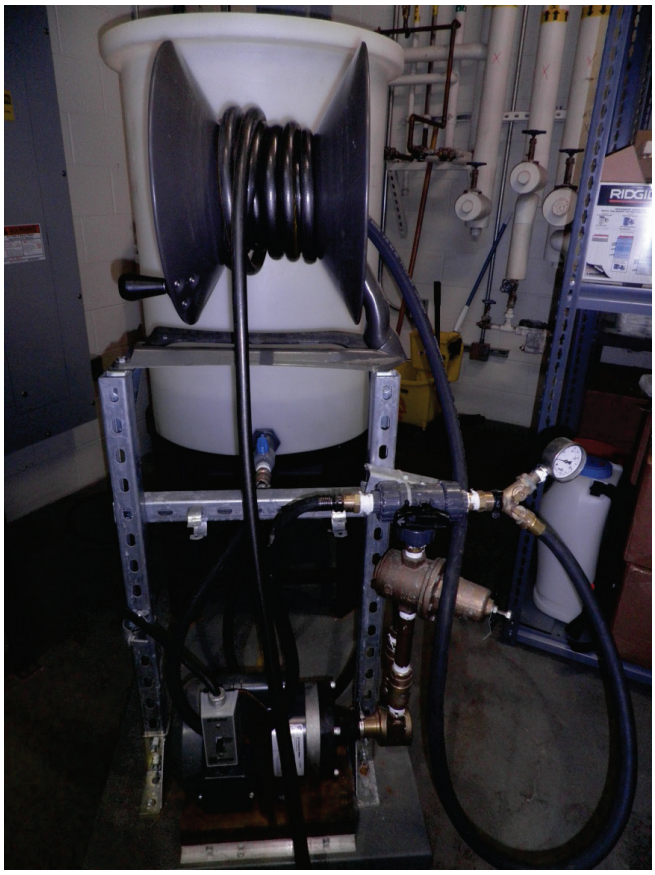


Figure D-7: DI water supply system

Quality Control Measures

Additional measurements prior to or during the decontamination procedure application are also required in order to ensure quality control in the testing. These measurements include quality control checks on the reagents and equipment being used in the decontamination procedure. The pH and chlorine concentration of the pAB solution has been shown to have a significant impact on the inactivation of *Bacillus* species spores. After preparation of the pAB solution, the pH was measured using an Oakton pH probe. Additionally, the pH was measured during the decontamination testing after each material was run within a test. The Cl_2 concentration was measured after preparation of the pAB solution by Hach High Range Bleach Test Kit, Method 10100 (Model CN-HRDT). The temperature was also measured after the mixture was prepared and prior to use on each material section using a NIST-traceable thermometer.

The water pressure at the head of the garden hose (i.e., faucet) was controlled with a pressure regulator. The pressure was confirmed prior to each use of the hose. The flow rate and spray pattern from the hose were checked prior to the start of the decontamination test. The flow rate was measured using an inline flow meter. The spray pattern was visually verified to be nominally a 1 ft. diameter (10 – 14 in) at the coupon surface from a distance of 3 ft. between the nozzle and coupon face.

The time for application of each procedural step and time between procedural steps on each coupon was measured using a NIST-traceable stopwatch and recorded in the laboratory notebook.

Appendix E: Sampling Procedures

E.1 Sampling Material and Equipment

The materials and equipment used for sampling are listed in Table E-1.

Table E-1. Material and Equipment Used in Sampling

Material/Equipment	Description
Nonpowdered, sterile surgical gloves	KIMTECH PURE* G3 Sterile Nitrile Gloves, Kimberly-Clark (VWR P/N HC61110 for extra-large; VWR P/N HC61190 for large; VWR P/N HC61180 for medium)
Nonpowdered, non-sterile surgical gloves	Exam gloves (Fisherbrand Powder-Free Nitrile Exam Gloves, Fisher P/N 19-130-1597D (for large);19-130-1597C (for medium))
Dust Masks	3M Particulate Respirator 8271, P95
Disposable lab coats	Kimberly-Clark Kleenguard A10 Light Duty Apparel, P/N 40105
Disposable Bench Liner	No source provided
Phosphate Buffered Saline	Phosphate Buffered Saline with TWEEN® 20 (Sigma Aldrich, P/N: P3563-10PAK)
50 mL conical tubes	BD Falcon® BlueMax Graduated Tubes, 15mL (Fisher Scientific P/N 14-959-70C)
Sterile sampling bags	Fisherbrand Sterile Sampling Bags (TWIRL'EM) Overpack Size : 10” by 14”, P/N 01-002-53 Inner bag size: 5.5" by 9" (wipe); Sample Bag Size: 5.5” by 9 “
Bleach wipes	Dispatch® Bleach Wipes, P/N 69260
Wipes	Kendall Curity Versalon absorbent gauze sponge 2" by 2" sterile packed (rayon/polyester blend) (http://www.mfasco.com/)
Swabs	Bacti Swab® (http://www.remelinc.com/Industrial/CollectionTransport/BactiSwab.aspx)
Sampling Vacuum	Omega Vac, Atrix International
Vacuum socks (sampling)	Midwest Filtration, Cincinnati OH. x-cell 100 (http://www.midwestfiltration.com/dust-sampling.php)
Carboys (2)	Nalgene autoclavable carboys with tabulation (20 L) (Fisher Cat# 02-690-23)
Analytical Filter Units	150 mL Nalgene Analytical Filter Units (0.2 µm Cellulose Acetate) (Fisher Cat# 130-4020)
Vacuum pump	Gast oil-free vacuum Pump with adjustable suction (Fisher

Material/Equipment	Description
	Cat# 01-092-25)
Tubing	Fisher PVC clear tubing (1/2“ i.d., 1/16” thickness) (Fisher Cat# 14-169-7J) Fisher PVC clear tubing (3/8” i.d., 1/16” thickness) (Fisher Cat# 14-169-7G) Fisher PVC clear tubing (vacuum tubing) (3/8” i.d., 1/8” thickness) (Fisher Cat# 14-169-7H)
Filter cassettes	Via-Cell® Bioaerosol Sampling Cassette P/N VIA010 http://www.zefon.com/store/via-cell-bioaerosol-sampling-cassette.html
Sampling pump	Isokinetic Method 5 Source Sampling Console Model 511E http://www.apexinst.com/products/consoles.htm

E.2 Surface Sampling Procedures

Within a single Task 2 and 3 test, surface sampling of the coupons was completed for all procedural blank coupons first, followed by all test coupons, and then followed by all positive control coupons. . Surface sampling was done by wipe sampling in accordance with the protocol documented below. The surface area for all samples was 1.3 sq. ft. A template was used to cover the exterior 0.25 in. of each Task 2 and 3 coupon leaving a 13.5 in. by 13.5 in. square exposed for sampling. The outer 0.25 in. around each coupon was not sampled in order to avoid unrepresentative edge effects. A large stainless steel template covering the entire coupon was used for Task 2 and 3 sampling. This template also prevented the outer edges from being sampled, and provided a 0.5 in border between samples

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 needed. A sampling material bin was stocked for each sampling event, using the information included in these sampling protocols. The bin contained enough wipe sampling and vacuum sock sampling kits to accommodate all required samples for the specific test. Additional kits of each type were also included for backup. Enough prepared packages of gloves and bleach wipes were included in the bin. Extra gloves and wipes were also included. A sample collection bin was used to transport samples back to the NRMRL/NHSRC Biocontaminant Laboratory. The exterior of the transport container was decontaminated by wiping all surfaces with a bleach wipe or towelette moistened with a solution of pAB prior to transport from the sampling location to the NRMRL/NHSRC Biocontaminant Laboratory.

E.2.1 Coupon Wipe Sampling

Wipe sampling is typically used for small sample areas and is effective on nonporous, smooth surfaces such as ceramics, vinyl, metals, painted surfaces, and plastics.[11] The general approach is that a moistened sterile non-cotton pad is used to wipe a specified area to recover bacteria, viruses, and biological toxins.[11] The protocol that was used in this project is described below and has been adopted from that provided by Busher et al.[11] Brown et al.[12], and documented in the INL 2008 Evaluation Protocols.[13,14] It should be noted that none of these references provide a validated wipe procedure for *Bacillus* spores, as a validated sampling procedure does not currently exist.

The following procedure was used in this study for Task 2 and 3 wipe sampling of each coupon surface:

1. A three person team was used, employing aseptic technique throughout. The team consisted of a sampler, coupon handler, and support person.
2. All materials needed for collection of each sample was prepared in advance using aseptic technique. A sample kit for a single wipe sample was prepared as follows:
    a. Two sterile sampling bags (10" by 14", 5.5" by 9 ") and a 50 mL conical tube, capped, were labeled in accordance with Appendix D. These bags and conical tube had the same label. The 5.5" by 9" labeled sterile sampling bag was referred to as the sample collection sterile sampling bag.
    b. A dry sterile wipe was placed in an unlabeled sterile 50 mL conical tube using sterile forceps and aseptic technique. The wipe was moistened by adding 5 mL of sterile phosphate buffered saline with 0.005% TWEEN®-20. The tube was then sealed.
    c. The labeled 50 mL conical tube, capped, the unlabeled conical tube containing the pre-moistened wipe, and the 5.5" by 9" labeled sampling bag were placed into the 10" by 14" labeled sterile sampling bag. Hence, each labeled sterile sampling bag contained a labeled 50 mL conical tube (capped), an unlabeled capped conical tube containing a pre-moistened wipe, and an empty labeled sterile sampling bag.
    d. Each prepared bag was one sampling kit.
3. All members of the sampling team each donned a pair of sampling gloves (a new pair per sample); the sampler's gloves were sterile sampling gloves. All members wore dust masks to further minimize potential contamination of the samples.
4. The coupon handler removed the coupon from the appropriate cabinet and placed it on the sampling area. The sampling area was covered with a new piece of lab bench cover for each coupon.
5. The support person recorded the coupon code on the sampling log sheet.
6. The support person removed a template from the bag and handed it to the sampler.
7. The sampler placed the template onto the coupon surface.
8. The support person removed a sample kit from the sampling bin and records the sample tube number on the sampling log sheet next to the corresponding coupon code just recorded.
9. The support person:
    a. Opened the outer sterile sampling bag touching the outside of the bag.

b. Touching only the outside of the overpack bag, removed and opened the unlabelled conical tube and poured the pre-moistened wipe onto the sample.

c. Discarded the unlabelled conical tube.

d. Maneuvered the labeled 50 mL conical tube to the end of the outer sterile sampling bag and loosened the cap.

e. Removed the cap from 50 mL conical tube immediately preceding the introduction of the sample into the tube.

10. The sampler:

    a. Wiped the surface of the sample horizontally using S-strokes to cover the entire sample area of the coupon using a consistent amount of pressure.

    b. Folded the wipe concealing the exposed side and then wiped the same surface vertically using the same technique.

    c. Folded over again and rolled up the folded wipe to fit into the conical tube.

    d. Carefully placed the wipe into the 50 mL conical tube that the support person was holding being careful not to touch the surface of the 50 mL conical tube or plastic sterile sampling bag.

11. The support person then immediately closed and tightened the cap to the 50 mL conical tube and slid the tube back into the sample collection sterile sampling bag.

12. The support person then put the 50 mL conical tube into the empty labeled 5.5” by 9” sampling bag and sealed the bag.

13. The support person then sealed the outer sample collection bag now containing the capped 50 mL conical tube (containing the sample wipe) inside a sealed 5.5” by 9” sample collection bag.

14. The support person then decontaminated the outer sample bag by wiping it with a Dispatch® bleach wipe.

15. The support person then placed the triple contained sample into the sample collection bin.

16. If sampling from the coupon was completed, the coupon handler moved the coupon and template to the appropriate location for archival or discarding.

17. All members of the sampling team removed and discarded their gloves.

18. Steps 3 – 17 were repeated for each sample to be collected.

.

E.2.2 Vacuum Sock Sampling

Vacuum sock sampling is typically used for large porous areas. [2] The general approach is that a collection sock is used to trap dust material. [2] The protocol that will be used in this project is depicted below and has been adopted from that provided by Busher et al.[2], Brown et al.[8], and documented in the INL 2008 Evaluation Protocols[12]. None of these references provide a validated vacuuming procedure for *Bacillus* spores.

The following procedure will be used in this study for Vacuum Sock sampling of each coupon surfaces:

1. A three-person team will be used, employing aseptic technique. The team will consist of a sampler, sample handler, and support person.
2. All materials needed for each sample to be collected will be prepared in advance using aseptic technique A sample kit for a single HEPA vacuum sample will contain the following:
    a. Two sampling bags (10" x 14", 5.5" x 9") will be labeled in accordance with Section 4.9. These bags will have the same label. An additional unlabeled bag contains the HEPA sock collection assembly listed in Table 4-1. The label will be clearly distinguishable through the unlabeled bag.
    b. The two sterile, labeled sampling bags and the vacuum sock assembly bag will be placed inside a second 10" x 14" unlabeled bag.
    c. Each prepared bag is one Vacuum Sock sampling kit.
3. All members of the sampling team will each don a pair of sampling gloves (a new pair per sample); the sampler's gloves shall be sterile sampling gloves. All members shall wear dust masks to further minimize potential contamination of the samples.
4. The sampler will plug in the HEPA vacuum power cord and then don his/her sterile gloves.
5. The HEPA vacuum will be maintained on a rolling cart for easy movement into place.
6. The sampler will hold the vacuum nozzle for the support person to place the vacuum sock assembly onto the nozzle.
7. The support person will open the sampling supply bin and remove one vacuum sock sample kit from the bin.
8. The support person will record the sample collection bag number on the sampling log sheet.
9. The sample handler will remove the coupon from the appropriate cabinet and place it on the sampling area.
10. The support person will remove a template from the bag and hand it to the sampler.
11. The sampler will place the template onto the coupon surface.
12. The support person will record the coupon code on the sampling log sheet next to the corresponding vacuum sock collection bag number that was just recorded.
13. The support person will:
    a. Open the vacuum sock sample kit outer bag and remove the unlabelled vacuum sock assembly bag.
    b. Open the small unlabelled sampling bag containing the vacuum sock assembly and push the assembly from the bottom to expose the cardboard applicator tube opening.
    c. Place the vacuum sock assembly onto the nozzle of the vacuum tube, using the bag to handle the sock assembly, while the sampler holds the vacuum nozzle.
14. The sampler will:
    a. Securely hold the outer edge of the sock onto the tube.
    b. Turn on the vacuum with her foot.
    c. Not let go of the filter sock while the vacuum is turned on in order to prevent the sock from being sucked into the vacuum
    d. Vacuum "horizontally" using S-strokes to cover the entire area of the material surface not cover by the template, while keeping the vacuum nozzle perpendicular to the sample surface.
    e. Vacuum the same area "vertically" using the same technique.
    f. Turn off the vacuum when sampling is completed.
15. The support person will remove the sock assembly from the nozzle, using the inner sterile sampling bag.
16. The support person will then seal the inner sterile sampling bag and place it into the outer sterile sampling bag.
17. The support person will then seal the outer sterile sampling bag.
18. The support person will then seal the 10" x 14" overpack sample bag now containing the outer and inner bags, the inner containing the vacuum sock assembly. The 3rd size bag will then be wiped with a bleach wipe and then dispose of the bleach wipe.
19. The sampler will wipe down the nozzle (inside and out) and end of the tubing with bleach wipe; dispose of the bleach wipe.

20. The support person will then place the triple contained sample into the sample collection bin.
21. If sampling from the coupon is completed, the sample handler will move the coupon and template to the appropriate location for archiving or disposal.
22. All members of the sampling team will remove and discard their gloves.

Steps 3 – 21 will be repeated for each sample to be collected.

E.2.3 Swab Sampling

Swab sampling was used for sterility checks on coupons and equipment prior to use in the testing. A single swab sample was collected from each item and coupon. MOP 3135 was followed (see Appendix C), which employs the use of a pre-moistened swab.

E.3 Run-off Collection and Sampling Procedures

During application of the decontamination procedure for each set of Task I coupons, the drain in the decontamination test chamber remained open. The runoff from the coupons throughout the entire decontamination procedure being tested was collected for a given coupon set (material type or all blanks) into a vessel which was pre-dosed with sodium thiosulfate (STS). The volume of sodium thiosulfate needed to neutralize the total volume of decontamination liquid to be applied was determined by titration, and was set to 150% in excess. After all coupons from a single set had been moved to the Decontaminated Coupon Cabinet or Procedural Blank Cabinet, the chamber was rinsed with DI water. For Task II, a run-off collection vessel (trough) was placed under the coupon, and curtains arranged such that splashing run-off drained into the trough. The trough was also dosed with enough STS to neutralize the decontamination liquid.

Analysis of the liquid was accomplished by filter-plating triplicate 100 mL aliquots of each run-off sample. The collection procedure for the 100 mL aliquots was performed as follows:

1. Sampler donned a face mask, pair of examination gloves, disposable lab coat, and bouffant cap.
2. The contents of the carboy were agitated to ensure homogeneous mixing.
3. The carboy cap was removed.
4. Using a new 50 mL sterile pipette tip, 100 mL of sample was aseptically pipetted into a sterile 100 mL container.

Step 4 was repeated until triplicate samples were obtained.

The runoff aliquots are triple-contained and transported to the NRMRL/NHSRC Biocontaminant Laboratory for submission and analysis at the conclusion of the entire test according to MOP 6565 (see Appendix C). Briefly, spores in the runoff sample were collected onto 0.2 µm pore-size analytical filters by vacuum filtration (Figure E-1). The filter was then placed (particulate side up) onto bacterial growth media and incubated 18 ± 2 hours at the optimal growth temperature. After incubation, colonies were enumerated on the filter surface by visual inspection as shown in Figure E-2.

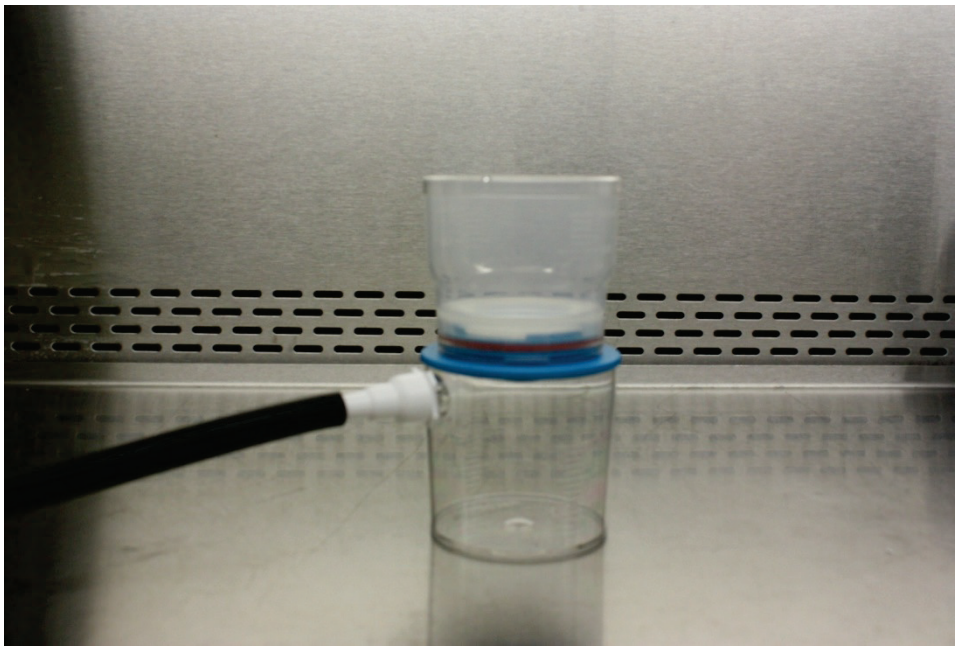


Figure E-1. Nalgene Analytical Filter Unit connected to a Filter Unit.

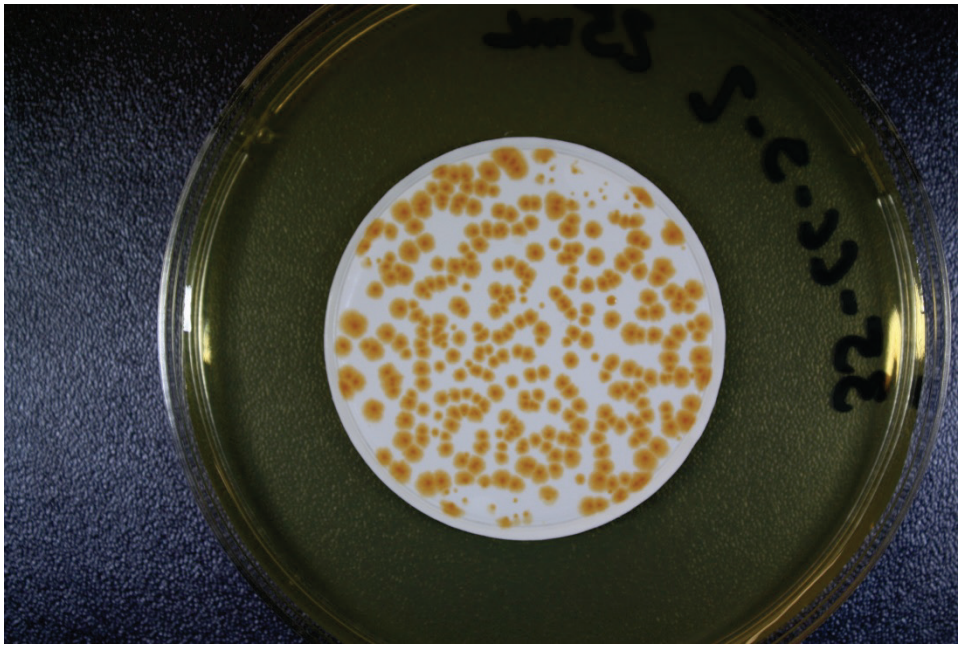


Figure E-2. *B. atrophaeus* CFU on a Filter Unit.

E.4 Aerosol Sampling Procedures

The use of high-pressure hoses and pressure washers is expected to generate aerosols. There is potential for generated aerosols to contain viable spores removed from the coupon surfaces. Bioaerosol samples were collected from the decontamination chamber during all spraying activities. Zefon Via-Cell® Bioaerosol Sampling Cassettes (Figure E-3) were used to collect aerosol samples. During Via-Cell® sample collection,

the air concentration of chlorine gas (during pH-amended bleach application) or hydrogen peroxide vapor (during Spor-Klenz® application) was also monitored.

The Via-Cell® sampler was operated and analyzed according to the manufacturer's recommendations. (http://www.zefon.com/analytical/download/Via-Cell_Lab_Manual_Booklet.pdf). During Task I, separate Via-Cell® samples were collected during the liquid decontamination application and the DI water rinse application. During Task II, separate Via-Cell® samples were collected before each decontamination step, two samples during the decontamination step, and after the decontamination procedureto provide some baseline data similar to the procedural blank during Task I. The Via-Cell® samples were analyzed according to MOP 6571 (see Appendix C).

Figure E-3 Via-Cell BioAerosol Cassette

Filters are analyzed to determine viable CFUs collected per volume of air sampled.

The following sampling procedure was used to collect the Via-Cell® samples:

1. With a clean pair of gloves, the Via-Cell® was removed from the foil pouch. The cassette and the pouch were labeled with the sample ID.
2. The small blue plug was removed and the cassette connected to the dry gas meter pump.
3. A leak-check was performed by turning on the pump with the inlet to the Via-Cell® closed capped off. The flow of air should have stopped. If not, all connections were checked.
4. The cap of the Via-Cell® was removed and affixed in the ambient air around the coupon to be decontaminated.
5. The starting volume on the dry gas meter (DGM) was recorded and the timer reset.
6. When time to collect a sample, the two switches on the meter box for the pump and the timer were simultaneously turned on. The sample ID, the time of day and the meter temperature were recorded.

7. The valve settings on the meter box were adjusted so that the ΔH pressure reading was 1.1" water.
8. At the end of sample collection, the two switches on the meter box for the pump and the timer were simultaneously turned off. The final reading on the DGM, the meter temperature, and the elapsed time were recorded.
9. The cap of the Via-Cell® was replaced and the pump disconnected. The outlet plug was reinserted.
10. The Via-Cell® was placed in the foil pouch. The exterior of the pouch was wiped with a Dispatch® wipe and placed in secondary containment.

E.5 Sample Preservation

After sample collection, sample integrity was maintained by storage of samples in quadruple containers (1 – sample collection container, 2 – sterile bag, 3 – sterile bag with exterior sterilized during sample packaging process, 4 – sterile container holding all samples from a test). All individual sample containers remained sealed while in the decontamination laboratory or in transport after the introduction of the sample. The locking lid on the container holding all samples remained closed except for the brief period it is opened for sample introduction by the support person of the sampling team. The sampling person did not handle any samples after they were relinquished to the support person during placement into the primary sample container.

After sample collection for a single test was complete, all samples were transported to the NRMRL/NHSRC Biocontaminant Laboratory immediately, with appropriate chain of custody form(s).

In the NRMRL/NHSRC Biocontaminant Laboratory, all samples were stored in the refrigerator at 4 ± 2 °C until they were analyzed. All samples were allowed to stabilize at room temperature prior to analysis.

E.6 Sample Holding Times

All samples were stored in accordance with Section F.5. 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. A typical holding time, prior to analyses, for most biological samples was two days.

During the analysis procedure, samples could be stored in the refrigerator overnight after extraction and prior to the dilution plating. All samples were allowed to equilibrate to room temperature and were vortexed for 10 seconds prior to plating.

Because pAB is not shelf-stable, it was analyzed immediately upon collection.

Appendix F: Sample Analyses

F.1 Sample Analyses

The APPCD Biocontaminant Laboratory located in E-288 of the RTP, NC, campus facility analyzed all samples to quantify the number of viable spores per sample. For all sample types except those from the BioSampler, phosphate buffered saline with 0.05% TWEEN®-20 (PBST) was used as the extraction buffer. After the appropriate extraction procedure, as described in the sections to follow, the buffer was subjected to a five stage serial dilution (10^{-1} to 10^{-5}), plated in triplicate and incubated overnight at 35 ± 2 °C in accordance with MOP 6535a (see Appendix B). Following incubation, CFUs were enumerated according to MOP 6567.

The PBST was prepared according to the manufacturer's directions and in accordance with MOP 6562 (see Appendix C), dissolving one packet in one liter of sterile water. The solution was then vacuum-filtered through a sterile 0.22 µm filter unit to sterilize.[9]

The extraction procedure used to recover spores was varied depending upon the different matrices (wipes, filter socks, wet/dry vacuum filter, liquid, filter cassette) and can be found in MOP 6572 for extraction of HEPA socks (vacuum socks), MOP 6567 for extraction of wipe samples, MOP 6563 for analyses of swab samples, MOP 6571 for air sample cassettes. The procedures are described in the following subsections.

F.1.1 Recovery from Wipe Samples

The procedure for the recovery of spores from wipe samples (MOP 6567, Appendix B) was adapted from the INL 2008 Evaluation Protocols[8], and was performed as follows:

1. The analyst donned a fresh pair of gloves. Gloves were changed periodically (at least between batches) or after direct contact with a sample to reduce contamination.
2. The 50 mL conical tube containing the sample wipe was removed from the double sterile bag and wiped with a bleach wipe. The analysts changed gloves after the wipe step.
3. PBST (20 mL) was added to each 50 mL conical tube by aseptically pouring a pre-measured volume.
4. The sample was then vortexed for 2 minutes in 10 second bursts, leaving the wipe in the same tube.
5. If the sample sat for more than one minute after Step 4, the sample was re-vortexed individually to homogenize prior to dilution plating. To complete dilution plating, the conical tube was uncapped and the cap placed underside up on the Bio Safety Cabinet surface while the aliquot was removed from the tube. Immediately after the aliquot was removed, the cap was aseptically replaced.
6. Each sample was processed individually. Steps 1-5 were repeated for each sample in the batch. Dilution plating occurred as described in Section G.1.

F.1.2 Recovery from HEPA Socks (Vacuum socks)

The extraction from HEPA sock (vacuum sock) samples is described in MOP 6572.

F.1.3 Recovery from Swabs

Swab sampling procedures were used to confirm sterility of coupons and equipment prior to their use in testing. The protocol that was used in this project is described in MOP 6563. Each item to be used in the decontamination process was sampled before use. The randomly selected procedural blank coupon (from the same sterilization batch as the rest of the coupons of the same type) was also swab sampled before the inoculation operations began.

F.1.4 Recovery from Liquid

The abundance of viable spores in the runoff samples was determined by filtration of runoff aliquots (MOP 6565). Filter samples were cultured on bacterial growth media, and recovery was determined by enumerating colony forming units (CFU). The abundance of spores in the original runoff water was determined by multiplying the calculated abundance of spores per milliliter of aliquot by the total runoff volume.

F.1.5 Recovery from Wet/Dry Vacuums

The purpose of sampling the wet/dry vacuum after use was to confirm contamination of the unit with the target organism. The most logical place to sample to confirm contamination was the HEPA filter. The filter was sampled using the swab protocol described in MOP 3135. Each pleat of the filter was sampled with a single swab. The vacuum nozzle (squeegee) was also sampled before and after use.

F.1.6 Recovery from 18 mm Coupons

The extraction of the spores from 18 mm coupons was accomplished as follows: (1) the coupons were transferred into 50 mL sterile vials containing 10 mL PBST; (2) the vials containing the solution and coupon were sonicated for 20 minutes at 42 kHz and 135 Watts (Branson 8510 ultrasonic waterbath), then vortexed 2 minutes to further dislodge any viable spores; and (3) each vial was briefly re-vortexed immediately before any solution was withdrawn and subjected to a five stage serial dilution following MOP 6535a.

F.1.7 Recovery from Aerosol Samplers

Aerosol samples collected in the BioSampler were processed according to MOP 6575. The total flow in the duct was measured using an S-type pitot tube as described in EPA Method 2. This measurement was done at the beginning and end of the day of decontaminations.

Appendix G: QAPP Amendments

1.1.1.1 Amendment 1 (6/16/2011)

The conditions of this first test "O1" were based upon preliminary testing conducted by the WAM and Leroy Mickelsen with the testing equipment. Conditions for the remaining tests were to be decided once data were collected and analyzed for test O1.

Table 2-2. Task 2 (Optimization) Test Matrix (*Table and numbering directly from original amendment.*)

Test	Decontaminant	Inoculation	Application Rate	Application frequency
O1	pAB solution	High[1]	850 – 900 mL/min flow rate, 1 second/ft^2, (SHURflo sprayer set to low pressure setting with nozzle at the most restricting position,, i.e,, mist)	At 0 minutes (no reapplication) No Rinse, Coupons dried overnight, sampled the next day.

1.1.1.2 Amendment 2 (6/17/2011)

Amendment 2 detailed tests that were outside the scope of this report.

1.1.1.3 Amendment 3 (9/23/2011)

Amendment to Task 2

The overall objective of Task 2 is to determine the effectiveness of the decontamination methods on larger coupons (14 in. x 14 in) as a function of application parameters and spore loading. These parameters include, but are not limited to: initial contamination level, decontamination procedure (including decontaminant), decontaminant application rate, decontaminant reapplication frequency, and time.

This amendment includes a specific decontamination procedure applied to drywall coupons using wipes wetted with sporicide or disinfectant.

MOP-3156: Procedure for Wetted Wipe Decontamination

The testing procedures will be conducted according to **MOP-3156** included in Appendix A. The purpose of this MOP is to ensure a consistent and representative procedure for using wetted wipe decontamination.

Test Matrix

Three tests will be conducted to determine the optimal wetted wipe decontamination technique on 14 in. x 14 in drywall coupons in accordance with **MOP-3156**. All three tests will include moistening of the coupon surface with a freshly-made pAB solution as stated in **MOP-3156**, before applying the wiping procedure:

Test 1: pH-Adjusted Bleach (pAB) Wetted Wipe

Test 2: SimWipe or ready-made “tack cloth”

Task 3: Combination of SimWipe and pAB wetted wipe procedures consecutively

Wipe samples from each test will be collected after 18 hours of coupons drying, and appearing visibly dry. Positive control coupons will be sampled at the same time. Wipe samples are collected by sampling within a 13.5” x 13.5” sampling template centered on the coupons. Each test will require three test, one procedural blank, and three positive control coupons of drywall coupons, with a total of 7 coupons per test.

Sample ID, Tracking, and Chain of Custody

Each material section will be identified by a description of the material and a unique sample number. The sampling team will maintain a detailed laboratory log which will include records of each unique sample number and its associated test number, contamination application, any preconditioning and treatment specifics, and the date treated. Each material section test area sample will be marked with only the material descriptor and unique code number. The wet/dry vacuum samples and exhaust sample from each test will be identified with an associated test number and material section type. The sample codes will ease written

identification. Once the coupons are transferred to the NRMRL/NHSRC Biocontaminant Laboratory for plate counts, each sample will additionally be identified by replicate number and dilution. Table 4-1 specifies the sample identification. The NRMRL/NHSRC Biocontaminant Laboratory will also include on each plate the date it was placed in the incubator.

Table 4-1. Sample Coding (*Table and numbering directly from original amendment.*)

Coupon Identification: 25-WA-(X)M-DD-NN		
Category	**Example Code**	
WA	W1	Wipe Procedure test A(1=pAB, 2=SimWipe, 3= Combined)
(X) M (Material)	X	Procedural Blank
	D	Painted wallboard
	S	Stainless Steel (for QC purposes)
DD (Descriptor)	B	Blank coupon
	P	Positive control sample
	T	Test sample
	FB	Field Blank
NN (Sample Number)	NN	Sequential numbers
NRMRL/NHSRC Biocontaminant Laboratory Plate Identification: 25-WA-(X)M-DD-NN-R-d		
25-WA-(X)M-DD-NN	As above	
R (Replicate)	R	A – C
d(Dilution)	1	0 to 4, for 10 to 10 x 10^4

1.1.1.4 Amendment 4 (10/3/2011)

Amendment to Task 2

The overall objective of Task 2 is to determine the decontamination method effectiveness on larger coupons (14 in. x 14 in) as a function of application parameters and spore loading. These parameters include, but are not limited to: initial contamination level, decontamination procedure (including decontaminant), decontaminant application rate, decontaminant re-application frequency, and time.

This amendment includes a test matrix that will determine the efficacy of specific decontamination procedures for coupon materials loaded with a relatively low inoculum of spore.

1. Coupons Material List

The coupons material list is shown in Table 1.

Table 1. Material Coupon List (*Table and numbering directly from original amendment.*)

Coupon Material Type	Number of Coupons	
Coupon loading (CFU)	1×10^2	Blank
Drywall	9	4
Concrete	6	3
Wood	6	3

Sample ID, Tracking, and Chain of Custody

Each material section will be identified by a description of the material and a unique sample number. The sampling team will maintain a detailed laboratory log which will include records of each unique sample number and its associated test number, contamination application, any preconditioning and treatment specifics, and the date treated. Each material section test area sample will be marked with only the material descriptor and unique code number. The sample codes will ease written identification. Once the coupons are transferred to the NRMRL/NHSRC Biocontaminant Laboratory for plate counts, each sample will additionally be identified by replicate number and dilution. Table 2 specifies the sample identification. The NRMRL/NHSRC Biocontaminant Laboratory will also include on each plate the date it was placed in the incubator.

Table 2. Sample Coding (*Table and numbering directly from original amendment.*)

Coupon Identification: 25-O7-(X)M-D-BB-SS-NN		
Category	**Example Code**	
(X) M (Material)	X	Procedural Blank
	K	Concrete
	W	Rough-cut barn wood
	D	Painted wallboard
D (Descriptor)	0	Blank coupon
	P	Positive control sample
	T	Test sample
	FB	Field Blank
BB (decontamination procedure)	O1	BB=O1 For Test procedure , and W1 for Wetted wipe with Amended Bleach (for Painted Wall board only)
SS (Sample Type)	WS	Wipe Sample
	HS	Vacuum sock sample
NN (Sample Number)	NN	Sequential numbers (1 to 3)
NRMRL/NHSRC Biocontaminant Laboratory Plate Identification: 25-O7-(X)M--D-BB-SS-NN-R-d		
25-O7-(X)M-A-D-B-SS-NN	As above	
R (Replicate)	R	A – C
d(Dilution)	1	0 to 4, for 10 to 10 x 10^4

2. Decontamination Testing Protocol

Decontamination Methods

Repeat of Test O1

Spray once with pAB for 15 seconds, no reapplication, and no rinse (identical to Test O1). Allow about 18 hours of spore drying before the sampling phase.

Repeat of Test W1 for Painted Dry Wall Only

One test will be conducted to determine the efficacy of the wetted wipe decontamination technique on 1 x 10^2 contaminated 14 in. x 14 in drywall coupons in accordance with **MOP-3156**. This test will be similar to Test W1 that involved moistening of the coupon surface with a freshly-made pAB solution as stated in **MOP-3156**, before applying the wetted wiping procedure:

3. Testing Protocol

Testing schedule

Day 1:

- Inoculate 1 x 10^2 coupons based on earlier results. Leave 1 coupon of each concrete and wood and 2 coupons of drywall as procedural blanks, and 1 coupon of each material as field blanks. An additional set of sterilized coupons will be required as blanks on Day 3.

Day 2:

- Perform Test O1 decontamination procedure on 1 blank coupon of each type and place them in the Blank cabinet. Perform W1 procedure on an additional drywall coupon.
  Clean chamber.

- Perform decontamination procedure(s) for coupons of each type, and place them Test cabinet 2.

Day 3:

Sample coupons in the following order:

- procedural blanks

- sample coupons

- 1 x 10^2 positive control,

Analysis

Due to the various levels of spores in the coupons, the following analysis procedure will be followed:

- 1 x 10^2 positive controls zero dilution plate, 1 mL filter plates

- All other samples 1 mL and remainder filter plates

1.1.1.5 Amendment 5 (10/12/2011)

Recovery from Grimed Coupons

To determine the effect of grime on recovery of our target organism, an additional test is necessary. This test will be performed on stainless steel coupons. Three sterilized stainless steel coupons will be placed in the grime deposition hood (three at a time). Coupons will be handled with sterile gloves to minimize contamination. The coupons will undergo the grime application procedure (details to follow) and be allowed to dry. As soon as the coupons are dry, they will be removed from the hood and placed under sterile ADA pyramids as if being prepared for inoculation, but no inoculation will take place. Once all three coupons have been through this procedure, the pyramids will be removed and the coupons will be sampled according to **MOP 3144**. The wipe samples will then be sent to the NRMRL/NHSRC Biocontaminant Laboratory, along with a field blank sample (a handled but unused wipe) and a coupon blank (a wipe sample from a sterile coupon).

The NRMRL/NHSRC Biocontaminant Laboratory will extract the samples according to **MOP 6567**, but will then split the samples into two 10 mL aliquots. One aliquot of each sample will then be spiked with 1×10^4 *Bacillus atrophaeus* spores (total). Three 10 mL aliquots of PBST will also be spiked with the same quantity of spores. All samples will be plated at the 0 and -1 dilution, with subsequent plating at higher dilutions pending results.

Sample IDs will be as follows:

25-GR1-WN-{1, 2, and 3}:	Replicate samples of unspiked portion of wipe sample from grimy coupon
25-GR1-WS-{1, 2, and 3}:	Replicate samples of spiked portion of wipe sample from grimy coupon
25-GR1-FB-1:	Field blank wipe
25-GR1-WX-1:	Sterile coupon wipe
25-GR1-P-{1,2, and 3}:	Spiked PBST used to verify inoculum

The N and S portions of the first two sample IDs will be appended by the NRMRL/NHSRC Biocontaminant Laboratory following extraction and splitting.

1.1.1.6 Amendment 6 (1/11/2012)

The overall objective of **Task 2** is to determine the effectiveness of the decontamination method on larger coupons (14 in. x 14 in) as a function of application parameters and spore loading. These parameters include, but are not limited to: initial contamination level, decontamination, decontaminant application rate, decontaminant re-application frequency, and time. The test matrix for Task 2 was changed in response to Test O1 Test results; the decontaminant for this testing sequence is pAB solution.

(*Table directly from original amendment.*)

Test	Inoculation	Material	Spraying Time (sec)	Sprayed at Time (min)	Spray Flow Rate (mL/min)
01	High	Concrete	15 sec	0	1000
		Dry Wall			
		Rough-cut wood			
02	High	Concrete	15 sec	0	1500
		Dry Wall			
		Rough-cut wood			
O3	High	Rough-cut wood	30 sec	0	1000
O4			15 sec	At 0 and 5 min	
O5			15 sec	At 0 and 15 min	
O6	Low	Concrete	15 sec	0	1000
		Dry Wall			
		Rough-cut wood			
O7[1]	Very low	Rough-cut wood	30 sec		1000
O8	Low	Rough-cut wood	30 sec	0	1000
O9			15 sec	At 0 and 5 min	
O10			15 sec	At 0 and 15 min	

[1]Test O7 was added for a very low inoculation level

In **Task 3**, sections (14 in x 14 in) of grimed or neat materials positioned as walls will be inoculated with *Bacillus atrophaeus* (formerly *Bacillus globigii*) via aerosol deposition for a chosen decontamination procedure developed in Task 3 as a function of the procedures and material types used. The test matrix for Task 3 is presented in this amendment:

Test Procedures: The test procedure will be the same as the one applied for Test O3 (1 x 30 second spray (1000 mL/ min, mist).

The test matrix for Task 3 shall be completed as follows: (*Table directly from original amendment.*)

Test	Decontaminant	Inoculation	Grime (yes/no)	Scrubbing	Vacuuming
G1	pAB solution	High	No	No	No
G2	pAB solution	High	Yes	No	No
G3	pAB solution/TSP solution	High	No	No	No
G4	pAB solution/TSP solution	High	Yes	No	No
G5	pAB solution/TSP solution	High	No	Yes	No
G6	pAB solution/TSP solution	High	Yes	Yes	No
G7	pAB solution/TSP solution	High	No	Yes	Yes[1]
G8	pAB solution/TSP solution	High	Yes	Yes	Yes[1]
G9[2]	pAB solution/TSP solution	Low	No	No	No
G10[2]	pAB solution/TSP solution	Low	Yes	No	No

[1]This step will occur first; [2]these tests will be optional upon approval by the WAM and if funding and time permit.

United States
Environmental Protection
Agency

PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development (8101R)
Washington, DC 20460

Official Business
Penalty for Private Use
$300