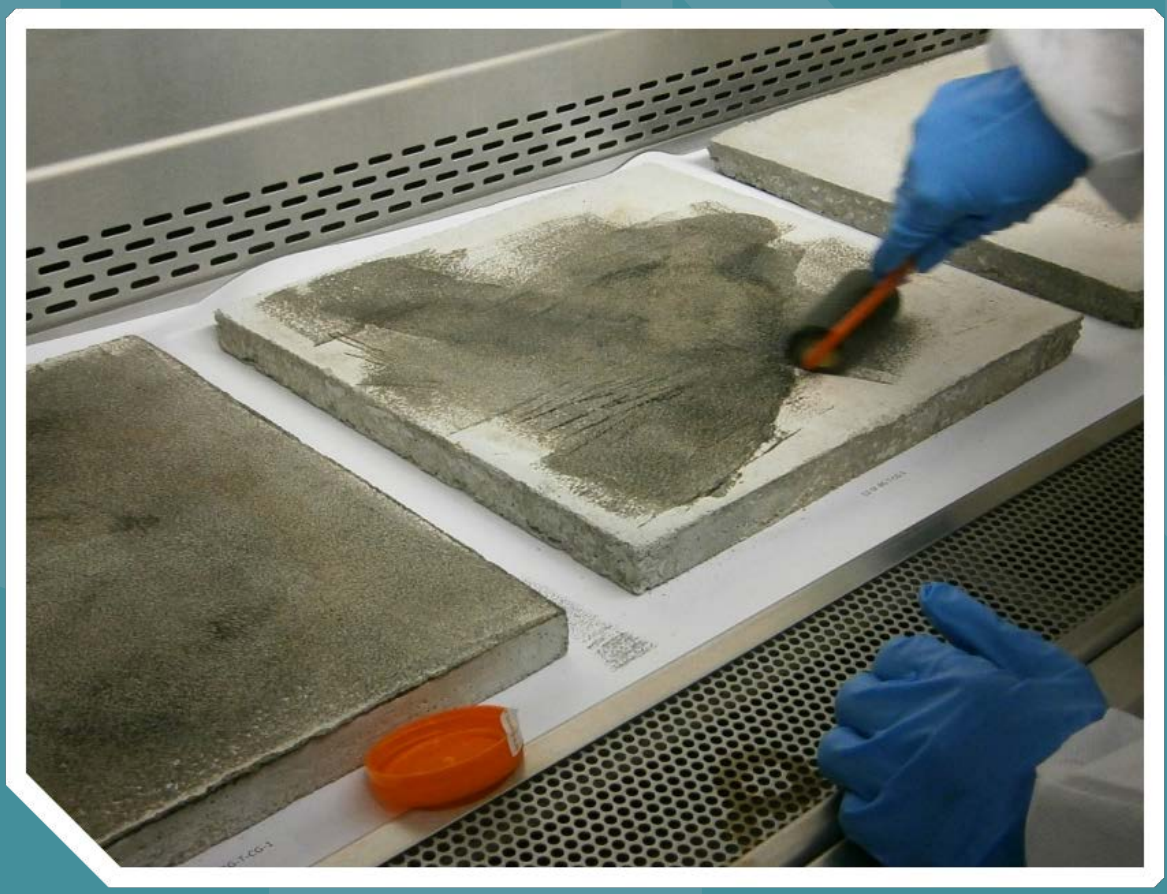
EPA
United States
Environmental Protection
Agency

EPA/600/R-16/162
www.epa.gov/homeland-security-research

Effectiveness of Spray-Based Decontamination Methods for Spores and Viruses on Heavily Soiled Surfaces



Office of Research and Development
Homeland Security Research Program

This page left intentionally blank

EPA 600/R-16/162

Effectiveness of Spray-Based Decontamination Methods for Spores and Viruses on Heavily Soiled Surfaces

Assessment and Evaluation Report

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

Abderrahmane Touati, Ph.D., Snigdha Gayatri Pongur, Timothy McArthur, and Barbara Wyrzykowska-Ceradini, Ph.D.
Jacobs Technology Inc.

Research Triangle Park, NC 27709

This page left intentionally blank

Disclaimer

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development's National Homeland Security Research Center, funded and managed this investigation through Contract No. EP-C-09-027, Work Assignment (4-6)-52 with ARCADIS U.S., Inc. (ARCADIS), and Contract No. EP-C-15-008 with Jacobs Technology, Inc. (Jacobs). 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. This report includes photographs of commercially available products. The photographs are included for purposes of illustration only and are not intended to imply that EPA approves or endorses the product or its manufacturer. EPA does not endorse the purchase or sale of any commercial products or services.

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

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
Telephone No.: (919) 541-7600
Fax No.: (919) 541-0496
E-mail Address: calfee.worth@epa.gov

Acknowledgments

This effort was initiated following the identification of knowledge gaps by US Department of Homeland Security's Agricultural Defense Branch-led subcommittee on Foreign Animal Disease Threats (FADTs), Decon, Disposal, and Depopulation (3D) Working Group, which is co-membered by the US EPA and the US Department of Agriculture (USDA). Emergency response and remediation following a foreign animal disease (FAD) incident will involve numerous federal agencies (particularly those listed), as well as state, local, and private entities. This project addresses closing gaps in our ability to decontaminate and remediate facilities following an agro-terrorism incident. Funding support by the US Department of Homeland Security (DHS) to complete this effort is greatly appreciated.

This effort was directed by the principal investigator from EPA's Office of Research and Development's (ORD's) National Homeland Research Center (NHSRC), utilizing the support of a project team consisting of staff from across the US EPA, DHS, and USDA. The contributions of the following individuals have been a valued asset throughout this effort:

- R. Leroy Mickelsen (US EPA/OLEM/CMAD)
- Joseph P. Wood (US EPA/ORD/NHSRC)
- Michelle Colby (DHS)
- Lori Miller (USDA)

Additionally, the authors would like to thank the following peer reviewers for their significant contributions:

- Nathan Birnbaum (USDA/APHIS)
- Elise Jakabhazy (US EPA/OLEM CMAD)
- Lukas Oudejans (US EPA/ORD/NHSRC)

Contents

Figures

Tables

Acronyms and Abbreviations

%	percent
2 M	2 molar
2 N	2 normal
µL	microliter(s)
µm	micrometer(s)
ACS	American Chemical Society
ADA	aerosol deposition apparatus
APWMC	Animal and Poultry Waste Management Center
ARCADIS	ARCADIS U.S., Inc.
ASTM	American Society for Testing and Materials, now ASTM International
ATCC	American Type Culture Collection
B.	*Bacillus*
BOD	biochemical oxygen demand
BSC	biological safety cabinet
CAS	Chemical Abstract Services
CBRN	Chemical, Biological, Radiological, and Nuclear
CFU	colony-forming unit(s)
cm	centimeter(s)
cm^2	square centimeter(s)
CMAD	Consequence Management Advisory Division
COD	chemical oxygen demand
db	dry basis (% of dry matter)
DE	Dey Engley
DHS	Department of Homeland Security
DI	deionized
DQI	data quality indicator
DQO	data quality objective
DTRL	Decontamination Technologies Research Laboratory
EPA	U.S. Environmental Protection Agency
EtO	ethylene oxide
FAC	free available chlorine
FAD	Foreign Animal Disease
FADT	Foreign Animal Disease Threat
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FMDV	foot and mouth disease virus
g	gram(s)

g/L	gram(s) per liter
Gpm	gallon(s) per minute
GRAS	generally recognized as safe
in	inch(es)
in^2	square inch(es)
IUPAC	International Union of Pure and Applied Chemistry
H_2O_2	hydrogen peroxide
HSRP	Homeland Security Research Program
Jacobs	Jacobs Technology, Inc.
kcal/g	kilocalorie(s) per gram
LB	Luria-Bertani
lb/day	pound(s) per day
lb/day/head	pound(s) per day per head
lb/ft^3	pound(s) per cubic foot
lb/ton	pound(s) per ton
Lowe's	Lowe's Home Improvement
Lpm	liter(s) per minute
LR	log reduction
MDI	metered-dose inhaler
mg	milligram(s)
mg/100 cm^2	milligram(s) per 100 square centimeters
mg/kg	milligram(s) per kilogram
mg/L	milligram per liter
mg/mL	milligram(s) per milliliter
mL	milliliter(s)
mm	millimeter(s)
NCSU	North Carolina State University
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
NPT	National Pipe Taper
NSF	National Sanitation Foundation
obs	observed
OCSPP	Office of Chemical Safety and Pollution Prevention
OLEM	Office of Land and Emergency Management
ORD	Office of Research and Development
PAA	peracetic acid
pAB	pH-adjusted bleach
PAH	polycyclic aromatic hydrocarbon

PARTNER	Program to Align Research and Technology with the Needs of Environmental Response
PBS	phosphate-buffered solution
PBST	phosphate-buffered saline with 0.05% Tween® 20
PFU	plaque-forming unit
PPE	personal protective equipment
psi	pound(s) per square inch
ppm	part(s) per million
ppmv	part(s) per million by volume
PVC	polyvinyl chloride
QA	quality assurance
QAPP	Quality Assurance Project Plan
QC	quality control
RNA	ribonucleic acid
rpm	revolution(s) per minute
RSD	relative standard deviation
RTU	Ready-to-Use
SEM	scanning electron microscopy
SM	magnesium salt
SSL	sebum saturation level
STD	standard deviation
STS	sodium thiosulfate
TKN	total Kjeldahl nitrogen
TSB	tryptic soy broth
USDA	US Department of Agriculture
USP	U.S. Pharmacopeia
VHP	vaporized hydrogen peroxide
wb	wet basis (as is)

Executive Summary

The objective of this project was to assess the effectiveness of spray-based common decontamination methods for inactivating *Bacillus (B.) atrophaeus* (surrogate for *B. anthracis*) spores and bacteriophage MS2 (surrogate for foot and mouth disease virus [FMDV]) on selected neat or heavily soiled (i.e., with a model agricultural grime loaded on the surface) test surfaces (concrete and treated wood). Relocation of viable viruses or spores from the contaminated coupon surfaces into aerosol or liquid fractions during the decontamination methods was investigated. This project was conducted to support jointly held missions of the U.S. Department of Homeland Security (DHS) and the U.S. Environmental Protection Agency (EPA). Within the EPA, the project supports the mission of EPA's Homeland Security Research Program (HSRP) by providing relevant information pertinent to the decontamination of contaminated areas resulting from a biological incident.

The effectiveness of removing target microorganisms from the surfaces of the coupons provided critical information regarding the effectiveness of each decontamination procedure. However, field applicability depends on several other factors, including the ultimate disposition (or fate) of the spores or viruses. This information is required to develop a comprehensive, site-specific remediation strategy. For example, if viable spores or viruses are washed off materials, remediation field strategies may require rinsate collection and treatment. If spores or viruses are detected in air samples, spread of contamination or recontamination of previously decontaminated surfaces must be considered in determining the overall remediation approach. To obtain critical information on the fate of the spores or viruses, several additional samples were collected. To assess the potential for viable spores or viruses to be washed off the surfaces, all liquids used in the decontamination process were collected and quantitatively analyzed (runoff and rinsate samples). To assess the potential for aerosolization of spores or viruses from coupon surfaces during spraying, aerosol samples were collected from the decontamination chamber during spraying activities.

The effectiveness of removing/inactivating two target microorganisms was assessed for three different decontamination solutions. pH-Amended Bleach (pAB) and Spor-Klenz® Ready-to Use (RTU) were evaluated against *B. atrophaeus* spores, and 2 percent (%) weight/volume (w/v) citric acid in sterilized deionized (DI) water and pAB were evaluated against MS2. Three application methods (handheld sprayer, backpack sprayer, and a chemical sprayer) were utilized throughout the testing to deliver decontaminants to the test surfaces. The evaluation was conducted on two test material surfaces (concrete and plywood), with and without agricultural grime. The handheld application method was conducted using a bench-scale test spray apparatus to evaluate the pAB and citric acid spray-based decontamination methods for 18-millimeter (mm) coupons (both grimed and neat) contaminated with MS2. The backpack and the chemical sprayer application methods were conducted to simulate field operations. For all the tests, a wetted surface contact time of 30 minutes was used, followed by a surface rinse with water. The fate of the microorganisms in the runoffs generated during the decontamination procedure and in the subsequent rinse step, as well as their potential re-aerosolization in the air, were also investigated.

Decontamination tests with *B. atrophaeus* spores indicated that higher efficacies were achieved on neat materials than on grimed materials, independent of the type of material or application method. pAB was found more effective than Spor-Klenz® RTU for decontaminating neat concrete materials, while the latter decontaminant was more efficient with neat plywood materials independent of application method

(backpack sprayer versus chemical sprayer). Viable spore levels found in rinsate samples were higher for the backpack sprayer tests than for the chemical sprayer tests, potentially because the chemical sprayer was more effective at physically removing spores before the rinse step. Relatively high aerosolization (greater than 1×10^3 colony forming units [CFU] per test) was observed during some tests with both the backpack and chemical sprayers.

Decontamination tests with MS2 indicated that 2% citric acid was not effective on concrete and plywood. However, pAB was found to be efficacious against MS2, with full decontamination on neat or grimed concrete and limited efficacy for neat or grimed plywood. Further, few viable viruses were detected in the runoff from pAB tests, unlike for the 2% citric acid formulation, which had almost complete wash-off of the viruses from the all coupon types. Finally, no viable MS2 aerosol formation/emission was observed in any of the conducted tests, independent of the type of decontamination solution used. However, it should be noted that the Via-Cell® bio-aerosol cassette sampling method, used in this study, was not validated for MS2 sampling or recovery.

Effectiveness was measured by determining the log reduction (LR) in viable spores or viruses. In this report, data are frequently presented as the average log reduction (LR) for a particular test. In laboratory tests, if a particular set of decontamination conditions achieves ≥ 6 LR (against a 6–7 log challenge), the decontamination is generally considered "effective." This benchmark is consistent with sporicidal efficacy tests used to register sporicides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Achieving complete kill (no viable agent recovered following the decontamination treatment) is considered "highly effective."

Introduction

This report discusses a project that evaluated the effectiveness of spray-based decontamination methods for spores and viruses on heavily soiled surfaces. The project was conducted to support jointly held missions of the U.S. Department of Homeland Security (DHS) and the U.S. Environmental Protection Agency (EPA). Within the EPA, the project supports the mission of EPA's Homeland Security Research Program (HSRP) by providing relevant information pertinent to the decontamination of contaminated areas resulting from a biological incident. The project addresses HSRP strategic goals as described in detail in the Homeland Security Research Multi-year Strategic Plan[1]. Specifically, the project is relevant to Long-Term Goal 2, which states, "The Office of Land and Emergency Management (OLEM) and other clients use homeland security research program products and expertise to improve the capability to respond to terrorist attacks affecting buildings and the outdoor environments." This project addresses a direct need expressed by OLEM's Chemical, Biological, Radiological, and Nuclear (CBRN) Consequence Management Advisory Division (CMAD). In addition the project is relevant to EPA's Office of Chemical Safety and Pollution Prevention (OCSPP) crisis exemption process and the OCSPP's regulatory function under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The following sections discuss the project background and the project description and objectives.

1.1 Background

Contamination incidents may result from intentional or accidental releases of biological materials or human or animal disease outbreaks. All scenarios pose significant challenges with regard to determining the extent of contamination, containing the contaminant spread, and remediating the event so that re-occupancy or reuse can occur. The project that is the subject of this report supports multiagency objectives of better understanding and preparing for the remediation of heavily soiled surfaces after a biological contamination incident.

1.2 Project Description and Objectives

The purpose of this project was to evaluate common decontamination methods for inactivating *Bacillus (B.) atrophaeus* (surrogate for *B. anthracis*) spores and bacteriophage MS2 (surrogate for foot and mouth disease virus [FMDV]) on selected test surfaces (with or without a model agricultural grime). Coupons loaded with the model agricultural grime reflect challenging environments expected during agricultural facility decontamination events. The coupons were then loaded with the target organisms (*B. atrophaeus* and MS2) using an aerosol deposition or liquid inoculation method. The coupons were then treated using the selected decontamination methods, and the effectiveness of each method was measured based on the reduction of viable agent (spores or viruses) achieved. Relocation of viable viruses or spores from the contaminated coupon surfaces into aerosol or liquid fractions during the decontamination methods also was assessed.

Experimental Approach

The general experimental approach used to meet the project objectives is described below.

1. **Preparation of representative coupons of test materials:** Coupons were prepared using two porous materials common to agricultural facilities: treated plywood and unpainted (smooth finish) concrete.
2. **Agricultural grime formulation and optimization of its manufacturing and application methods:** A model agricultural grime formulation and its application method were developed. The grime was applied to two types of common agricultural facility materials (concrete and treated plywood). The grime was added to the test materials to simulate the challenging environments expected during decontamination efforts in agricultural settings and to assess the impact of surface-associated grime on decontamination efficacy. In addition, the compatibility of the grime was tested for the two target organisms: *B. atrophaeus* (surrogate for *B. anthracis*) and bacteriophage MS2 (surrogate for FMDV), prior to testing.
3. **Contamination of coupons using standardized inocula of target organisms:** Coupons were contaminated using an aerosol deposition (*Bacillus* spores) or liquid inoculation (MS2) methods. A known quantity of the surrogate organism (1×10^7 *B. atrophaeus* CFU (colony-forming units) or 1×10^9 PFU (plaque-forming units) bacteriophage MS2) was deposited onto the coupons, followed by quantitative assessment of pre-decontamination loading by sampling positive control (non-decontaminated) coupons (n = 3 per test).
4. **Decontamination of test coupons:** Test coupons (n = 5 coupons per each decontamination procedure tested) were decontaminated using the following decontamination agents: pH-adjusted bleach (pAB), Spor-Klenz® Ready-To-Use (RTU), and 2 percent (%) citric acid solution. Each decontamination agent was applied using either a backpack, chemical, or handheld sprayer, followed by quantitative determination of viable *B. atrophaeus* spores or MS2 particles remaining on the coupons. Recoveries from test coupons subjected to the decontamination treatment were compared to positive control coupons. In addition, quantitative assessment of residual (background) contamination was performed by sampling negative controls (non-inoculated coupons, not subjected to the decontamination process) and procedural blanks (non-inoculated coupons that went through the same decontamination process as the test coupons). The transfer of viable organisms to post-decontamination liquid waste and air was evaluated through quantitative analysis of decontamination procedure residues (such as decontamination solution runoff and rinse water waste) and analysis of air samples collected during the decontamination process. An understanding of the transfer of viable organisms to post-decontamination liquid waste and air is important for determining fugitive emissions, latent infection and health risks, and overall decontamination effectiveness.
5. **Decontamination effectiveness:** Decontamination effectiveness, as a function of the procedure/decontaminant and material type was measured as log reduction (LR) in viable spores/plaques. Typically, for laboratory assessments of sporicidal efficacy a LR ≥ 6 (≥ 99.9999% reduction), when a titer of 1×10^7 challenge organism is used, is considered effective. For virucidal efficacy assessments, a LR ≥ 3 is considered effective against a 1×10^4 challenge. In the current study however, since both spore and virus challenge titers were ≥ 7 log; a 6 LR was

considered “effective” against either organism. Complete kill (no viable agent recovered following the decontamination treatment) was considered “highly effective.”

Post-decontamination results and the physical impact of decontamination on the test materials were assessed through visual inspection and documented in laboratory notebooks and by digital photographs.

This project was conducted in three phases, as summarized below.

1. **Phase I:** The effectiveness and operational parameters for decontamination of *B. atrophaeus* on grimed and neat concrete and treated plywood using pAB or Spor-Klenz® RTU deployed using a backpack or chemical sprayer was determined. For these tests, a wetted surface contact time of 30 minutes was used, followed by a surface rinse with water. The rinse step was used to simulate field operations in which rinsing may be used to minimize collateral damage to facilities resulting from extended contact with harsh decontamination chemicals.
2. **Phase II:** The effectiveness and operational parameters for decontamination of MS2 on grimed and neat concrete and treated plywood using pAB or 2% citric acid deployed using a backpack or chemical sprayer was determined. For these tests, a contact time of 30 minutes for a wetted surface was used, followed by a surface rinse with water.
3. **Phase III:** Due to inconsistencies encountered during the extraction process for MS2 using phosphate-buffered saline with 0.05% Tween® 20 (PBST) in Phase II, an extensive series of method development tests was conducted using smaller coupons (18 millimeters [mm], 0.07 inch [in] diameter) to determine the best buffer solution to maximize recoveries. The buffer solutions investigated were Dey Engley (DE) neutralizing broth, deionized (DI) water, PBST, phosphate-buffered saline (PBS), and tryptic soy broth (TSB). Further, a series of tests was conducted as control testing to evaluate the performance of the pAB and 2% citric acid solutions for decontaminating MS2 on 18-mm round coupons using a handheld sprayer.

Experimental Methods and Materials

This section describes the experimental testing and materials, including the preparation of coupons; grime formulation, preparation, and application; and the test organisms.

3.1 Preparation of Coupons

The representativeness and uniformity of test materials are essential in achieving defensible evaluation results. Materials are considered representative if they are typical of materials currently used in facilities and buildings in terms of quality, surface characteristics, and structural integrity. For this project, representativeness was ensured by: (1) selecting test materials typical of those found in agricultural animal husbandry and farming facilities that meet industry standards and specifications, and (2) obtaining these materials from appropriate suppliers. Material uniformity means that all test materials are equivalent. Uniformity was maintained by obtaining and preparing a quantity of material sufficient to allow the preparation of multiple test samples with presumably uniform characteristics (that is, test coupons were cut from the interior rather than the edge of a large piece of material).

Coupons of two building materials, concrete and treated plywood, were prepared onsite for decontamination testing. Control coupons of stainless steel were also prepared for use as inoculation controls. Table 3.1-1 lists the test coupon materials, suppliers or manufacturers, and preparation methods.

Table 3.1-1. Test Coupon Materials Specifications

Material			
Plywood	ACQ-D pressure-treated plywood ¾ in thick measuring 4 by 8 feet (Catalog No. CCX34T25C)	Lowe's Home Improvement (Lowe's) store	1. Remove wood particles using soft-bristle brush. 2. Sterilize using vaporized hydrogen peroxide (VHP).
Concrete	QUIKRETE® sand/topping mix	QUIKRETE® Companies and Lowe's store	1. Remove particles by power washing. 2. After power washing, allow to air dry in climate-controlled environment for at least five days. 3. Sterilize in an autoclave.
Stainless Steel	Multipurpose stainless steel 0.036 in thick measuring 48 by 48 in, type 304, #2B mill (unpolished)	McMaster-Carr	1. Remove lubricant and grease using acetone, and wipe dry. 2. Remove particles and dust by wiping clean with water and wipe dry. 3. Sterilize in an autoclave.

The coupons were made in two sizes: (1) large coupons measuring 35.6 centimeters (cm) x 35.6 cm (14 in x 14 in) for bench-scale decontamination testing during Phases I and II, and (2) smaller round coupons with a diameter of 18 mm (0.07 in; surface area 1.58 square inches [in^2]) for method development and Phase III testing. The preparation of the large and small coupons is discussed below.

3.1.1 Large Coupon Preparation

This section discusses the preparation methods for the plywood and concrete coupons for decontamination testing and the stainless-steel control coupons.

3.1.1.1 Plywood Large Coupons

The following materials and equipment were used to prepare the large plywood coupons:

- ACQ-D pressure-treated plywood ¾ in thick measuring 4 by 8 feet (ft)
- Table saw
- Appropriate personal protective equipment (PPE, including gloves, safety glasses, hearing protection, and safety footwear and dust masks if needed)

The procedure summarized below was used to prepare the large plywood coupons.

1. Personnel preparing the coupons donned appropriate PPE and put up necessary warning signs around the work area.
2. A table saw was used to cut each 14 by 14-in plywood coupon.

3.1.1.2 Concrete Large Coupons

The following materials and equipment were used to prepare the large concrete coupons:

- QUIKRETE® sand/topping mix
- Water source
- Mixing trough
- Trowel
- Leveling board
- Plastic covering for curing process
- Appropriate PPE (including gloves, safety glasses, and safety footwear)

The procedure summarized below was used to prepare the large concrete coupons.

1. Personnel preparing the coupons donned appropriate PPE and put up necessary warning signs around the work area.
2. Custom 14 by 14-in forms were manufactured for these coupons.
3. The concrete mix was prepared according to instructions on the package using a trough and garden hose for the water supply.
4. The concrete mix was poured into the custom forms.

5. A trowel was used to smooth the coupon surface, and each coupon was allow to dry in the form overnight.
6. After drying, plastic was laid over the coupons, and the coupons were allowed to cure for at least five days (see Figure 3.1-1).

Figure 3.1-1. Curing of Large Concrete Coupons

3.1.1.3 Stainless Steel Large Coupons

The following materials and equipment were used to prepare the large stainless steel coupons:

- Multipurpose stainless steel 0.036 in thick measuring 48 by 48 in, type 304, #2B mill (unpolished)
- Heavy-duty hydraulic shears
- Appropriate PPE (including gloves, safety glasses, and safety footwear)

The procedure summarized below was used to prepare the large coupons.

1. Personnel preparing the coupons donned appropriate PPE and put up necessary warning signs around the work area.
2. Heavy-duty power hydraulic shears were used to cut metal into 14 by 14-in coupons.

3.1.2 Small Coupon Preparation

This section discusses the preparation methods for the plywood and concrete coupons for method development and Phase III decontamination testing.

3.1.2.1 Plywood Small Coupons

The following materials and equipment were used to prepare the small plywood coupons:

- ACQ-D pressure-treated plywood ¾ in thick measuring 4 by 8 ft
- Table saw
- Drill press
- 22-mm hole saw without pilot bit
- Scanning electron microscope (SEM) aluminum stubs with 18-mm diameter and 8-mm pin length (Ted Pella, Inc., Redding, CA, Catalog No. 16119)
- Double-sided adhesive carbon tape (NEM tape, Nisshin Em. Co., Ltd., Tokyo, Japan)
- Parafilm roll (Bemis Company, Inc., Neenah, WI)
- Tweezers
- Arch punch (C.S Osborne & Co., Harrison, NJ, Catalog No. 01236)
- Appropriate PPE (including gloves, safety glasses, and safety footwear)

The procedure summarized below was used to prepare the small plywood coupons.

1. Personnel preparing the coupons donned appropriate PPE and put up necessary warning signs around the work area.
2. Strips of plywood measuring 1.0-in^2 were cut using a table saw.
3. Using a hole saw in the drill press, rounds were drilled to a depth of approximately 0.7 in each. The rounds were not drilled all the way through, so each strip was still in one piece.
4. The table saw guide was set to 1.0 cm.
5. The plywood strip from Step 3 was turned on its edge and cut to make plywood cylinders each measuring 18 mm in diameter, with a height of 1 cm.
6. A 10-in-long strip of NEM tape was cut and laid on a flat surface with the sticky side up. A parafilm strip of the same size as the NEM tape was cut and placed on the NEM strip.
7. Stickers measuring 18 mm in diameter were punched out from the NEM and parafilm strip using the arch punch.
8. The film from the underside of each NEM and parafilm sticker was removed and stuck onto an SEM stub.

9. Using a pair of tweezers, the parafilm was carefully removed from the top of the sticker, and the plywood cylinder was attached to the SEM stub. The plywood cylinder mounted on the SEM stub constituted the small plywood coupon (see Figure 3.1-2).

Figure 3.1-2. Small (18-mm diameter) Plywood Coupon

3.1.2.2 Concrete Small Coupons

The following materials and equipment were used to prepare the small concrete coupons:

- Butterboard measuring 6 by 12 by 2 in. (from McMaster Carr, Atlanta, GA, Catalog No. 86595K1)
- CNC milling machine with 18-mm mill cutter
- SEM stubs with 18-mm diameter and 8-mm pin length (from Ted Pella, Inc., in Redding, CA, Catalog No. 16119)
- QUIKRETE® sand/topping mix
- Suitable plastic container for mixing concrete
- Mixing stick
- DI water
- Appropriate PPE (including gloves, safety glasses, and safety footwear)

The procedure summarized below was used to prepare the small concrete coupons.

1. Personnel preparing the coupons donned appropriate PPE and put up necessary warning signs around the work area.
2. Using the CNC milling machine, the butterboard was drilled to produce a mold as shown in Figure 3.1-3.

Figure 3.1-3. Mold for Fabricating Small Concrete Coupons

3. A clean SEM stub was placed in each mold hole so that the pin of the SEM stub fit through the smaller hole in the mold.
4. In the plastic container, 1 pound (lb) of QUIKRETE® sand/topping was mixed with 0.1 pint (50 milliliters [mL]) of clean water. The mixture was well-worked using a mixing stick.
5. Additional water was added (not exceeding 0.6 mL or 0.13 pint in total) to obtain a workable, plastic-like consistency.
6. The concrete fabrication mold (Figure 3.1-3) was filled with the concrete mix. The top of the mold was smoothed to ensure a flat surface.
7. The concrete-filled mold was allowed to dry and cure indoors (70 °F or higher) for five days before removal of the coupons from the mold.

Figure 3.1-4 shows a cross section of the final coupon mold, and Figure 3.1-5 shows the final small concrete coupon.

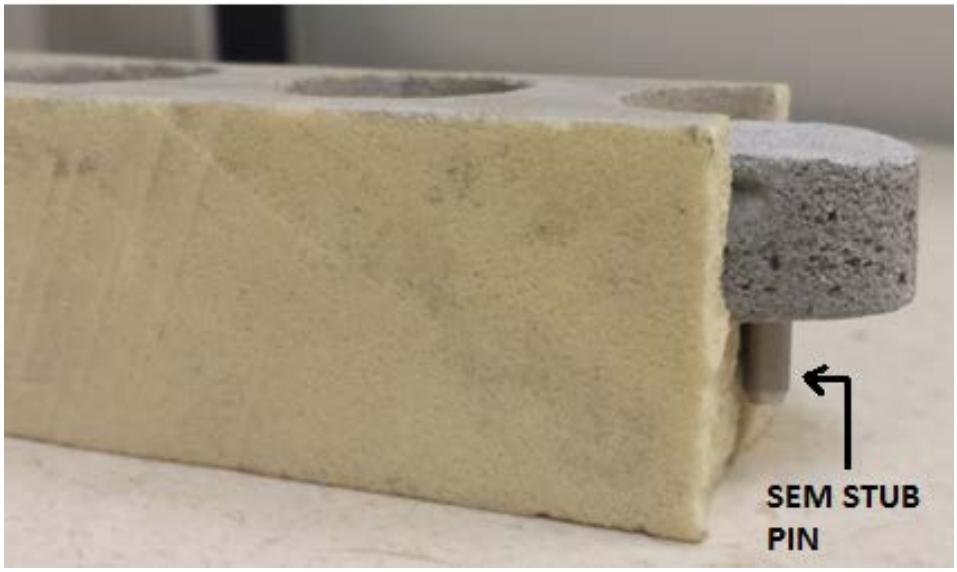


Figure 3.1-4. Cross Section of Final Small Concrete Coupon in Butterboard Mold

Figure 3.1-5. Concrete Small Coupon

3.1.3 Sterilization of Coupons

This section discusses the sterilization of the large and small coupons.

3.1.3.1 Large Coupon Sterilization

The large coupons were individually enclosed in VHP-permeable sterilization bags (General Econopak, Inc., Steam Component Autoclave Bag, white, 20 by 20 in, Item No. 62020TW) before sterilization. The stainless steel coupons were wrapped in aluminum foil, before being placed in the VHP-permeable sterilization bags.

Plywood Coupons-These coupons were sterilized using 250 parts per million (ppm) hydrogen peroxide (H_2O_2) vapor, also referred to as VHP, for four hours using a STERIS VHP ED1000 generator (STERIS Corporation, Mentor, OH). Biological indicators designed for H_2O_2 were included in each fumigation to identify systematic problems. Sterility was evaluated by swab sampling one coupon from each sterilization batch. Prior to use, the coupons treated with VHP were incubated at 30 to 35 °C for two days or at room temperature for 14 days to force off-gassing of H_2O_2 from the coupons, as suggested by Calfee et al.[2] to prevent biocidal activity.

Bagging of the plywood coupons and VHP sterilization was performed after the coupons were deemed sufficiently dry (that is, constant mass was observed for three sample coupons for a period of 48 hours as determined gravimetrically every 12 to 18 hours). The coupons were sterilized in batches. The number of coupons per batch was limited so that all coupons in the chamber were exposed to the VHP without shielding (no physical overlap of coupons) and so that appropriate mixing of the H_2O_2 occurred in the chamber.

After the VHP cycle, plywood coupons were stored in a vertical position using racks or other types of spacers to prevent the formation of mold after sterilization. The coupons were then placed into a sterile container for storage prior to transport to the testing location. The container was marked with the contents, including the sterilization date. The sterility of the coupons was verified through the analysis of laboratory blank control samples.

Aerosol deposition apparatus (ADA) pyramids also were sterilized with 250 parts per million by volume (ppmv) VHP for four hours using a STERIS VHP ED1000 generator.

Concrete and Stainless Steel Coupons- these coupons were sterilized using a large STERIS Amsco Century SV 120 Scientific Pre-vacuum Sterilizer using a one-hour 121°C gravity cycle.

3.1.3.2 Small Coupon Sterilization

The small coupons were sterilized using an Andersen ethylene oxide (EtO) sterilizer system (PN: 333 EOGas®, Haw River, NC, USA). The sterilization procedure is summarized below.

1. The coupons were loaded into stainless-steel stages (see Figure 3.1.6).

Figure 3.1-6. Stainless Steel Stage

2. The stage loaded with the coupons was placed in a glass Petri dish and loosely covered with a crystallization dish (see Figure 3.1-6).
3. Each Petri dish was placed into an appropriate sterilization bag.
4. The sterilization bags were loaded into a cabinet for sterilization using EtO.
5. The sterilization bags were removed from the EtO cabinet with the crystallization dishes still covering the Petri dishes to maintain coupon sterility.

3.2 Grime Formulation, Preparation, and Application

No universal grime substrate is representative of the many types of grime present at various animal production and farming facilities. For this project, the composition of grime substrate was based on scientific literature review and its applicability for use in evaluating the performance of decontamination methods. The grime formulation was intended to challenge decontamination methods for heavily soiled surfaces. Other formulations of agricultural grime may yield different results. This section discusses grime formulation, preparation, and application.

3.2.1 Agricultural Grime Formulation

The agricultural grime surrogate was designed using the following criteria:

- Constituent representativeness criteria:
  - Must include the general grime component (also known as “particulate soil”), a mixture of general outdoor dusts, soils, oils, soot, etc.;
  - Must include a surrogate of agricultural grime-specific components such as animal sebum or animal fat;
  - Must include a surrogate of agricultural grime-specific impurities that are potentially chemically and biologically active (such as nutrient-rich manure)

Note: Impurities that are not chemically or biologically active (such as animal hair) were not considered essential for grime composition.

- Functional and operational applicability criteria:
  - All components must be easily homogenized;
  - All components must be suitable for sterilization, either by steam autoclave, heating or boiling, irradiation, or application of EtO; and
  - At least one component must be a carrier of the other constituents. For example, if the carrier is liquefied, constituents must be mixed into the liquid carrier, and then the complete grime formulation is spread onto coupon surfaces

Table 3.2-1 shows the composition of the agricultural grime.

Table 3.2-1. Synthetic Agricultural Grime Composition

Component			
Particulate soil	Natural humus Paraffin oil Used crankcase motor oil Portland cement Iron oxide Silica Kaolin clay Carbon black Stearic acid Oleic acid	Synthetic particulate surrogate soil for testing cleaning performance of products intended for use on resilient flooring and washable walls	[3]
Animal sebum	Lanolin	Wool grease secreted by sheep sebaceous glands; surrogate for animal sebum	[4]
Animal impurities	Standardized manure	Dry, homogenized cow manure; surrogate for animal impurities	[5]

The following sections discuss each component, its significance and function, and information on sources of standardized individual constituents.

3.2.1.1 Particulate Soil

The particulate soil grime component was prepared using a modified recipe for particulate soil preparation adapted from the American Society for Testing and Materials (ASTM) International Method D4488–95(2001)e1, "Standard Guide for Testing Cleaning Performance of Products Intended for Use on Resilient Flooring and Washable Walls."[3] This ASTM International method provides techniques for soiling, cleaning, and evaluating performance of detergent systems under controlled but practical hard-surface cleaning conditions, where soil is defined as foreign matter on a hard surface, and the soiled surface being cleaned is defined as a substrate.

The types of soils in this method were used for evaluating the cleaning performance of solutions of soluble powdered detergent, dilutions of concentrated liquid detergent, or products intended for full-strength use (such as foams, sprays, liquids, or pastes for cleaning hard surfaces). The method emphasizes that the soils recommended for evaluating general cleaning performance are not a universal soil/substrate combination representative of the many soil removal tasks required for a given type of cleaner under actual use conditions. Choice of soil/substrate and cleaning conditions should be by agreement between the testing laboratory and those using the data to evaluate cleaning performance relative to user experience[3]. The particulate soil recipe adopted for use in this project from ASTM Method D4488–95(2001)e1 is summarized in Table 3.2-2.

Table 3.2-2. Particulate Soil Composition

Constituent	% Weight
Natural humus	38.0
Paraffin oil	1.0
Used crankcase motor oil	1.5
Portland cement	17.7
Silica	18.0
Carbon black	1.5
Iron oxide	0.3
Kaolin clay*	18.0
Stearic acid	2
Oleic acid	2
*Kaolin clay replaces bandy black clay from the ASTM International Method D4488–95(2001) particulate soil recipe. Mineral composition is similar for both clays, but an intense pigmentation typical for bandy black clay is not necessary for biological contamination testing.	

This soil formula has numerous components typical of natural soils (top organic layer of soil, numerous common earth minerals, carbon black, oils, fats, etc.). These components are likely to be found in soils typical of agricultural farming and animal facilities. The organic top layer of soil (natural humus) and the soil mineral components (silica, iron oxide, and kaolin clay) can easily be tracked by humans or cloven-hoofed animals, and Portland cement can be expected in dust from concrete flooring. Impurities from agricultural equipment (used crankcase motor oil and carbon black), mineral oil (paraffin oil), and fatty acids (stearic acid and oleic acid) common in animal and plant fats also are expected to be present in agricultural grime. More information on each soil constituent is given below.

- **Natural humus** – Humus is an organic layer of soil formed during the decomposition of plant litter. Humus has a characteristic black or dark-brown color, is organic due to an accumulation of organic carbon, and may act as a carbon source for microorganisms that subsequently produce acids and contribute to weathering. Soil nutrients (nitrogen, phosphorus, potassium, calcium, magnesium, manganese, iron, zinc, cadmium, and copper) are also present in humus at levels from micrograms (µg) to milligrams (mg) per gram (g) soil.[6]

- **Paraffin oil (mineral oil)** – Paraffin oil, often referred to as mineral oil, is a mixture of liquid hydrocarbons from petroleum. It does not have an exact chemical composition but is a mixture of alkanes with the general formula C_xH_{2x+2}, with the value of "x" typically between 10 and 18. Mineral oils are used to produce animal feeds. Premixing micronutrients with mineral oil, a suitable carrier, is common to ensure the proper distribution of nutrients in the final feed. The carrier's purpose is to physically accommodate finely powdered micro-ingredients and provide uniform distribution in the process. Mineral oils are chemically and biologically stable and do not support bacterial growth.[7]

- **Used crankcase motor oil** – Used mineral-based crankcase motor oil is another name for used motor oil or used engine oil. It is similar to unused oil except that it contains additional chemicals produced or that build up in the oil when it is used as an engine lubricant. Used mineral-based crankcase motor oil has many of the characteristics of unused oil. It smells similar to unused oil and contains the chemicals found in unused oil, including straight-chain (aliphatic) hydrocarbons, aromatic or polycyclic aromatic hydrocarbons (PAH) distilled from crude oil, and various additives that improve the performance of the oil in the engine.

  In addition to the chemicals found in unused oil, used mineral-based crankcase motor oil also contains chemicals formed when the oil is exposed to the high temperatures and pressures inside an engine. It also contains metals such as aluminum, chromium, copper, iron, lead, manganese, nickel, silicon, and tin from engine parts as they wear down. In addition, used mineral-based crankcase motor oil contains small amounts of water, gasoline, antifreeze, and chemicals from gasoline when it burns inside the engine. These stay in the environment for a long time, and can build up in plants, animals, soil, sediments, and non-flowing surface water.[8]

- **Portland cement** (often referred to as "OPC," from "ordinary Portland cement") – OPC is the most common type of cement in general use around the world.[9]. It is a basic mixture of ingredients of concrete, mortar, stucco, and most non-specialty grout. It usually originates from limestone. It is a fine powder produced by grinding. ASTM C150 defines Portland cement as "hydraulic cement (cement that not only hardens by reacting with water, but also forms a water-resistant product) produced by pulverizing clinkers consisting essentially of hydraulic calcium silicates, usually containing one or more of the forms of calcium sulfate as an inter-ground addition".

- **Silica (silicon oxide)** – Silicon and oxygen are the earth's two most abundant elements, and together, they make silica, one of the earth's three most common rock-forming minerals. Silica occurs in three main crystalline forms. The principal occurrence is as the mineral quartz, but silica also occurs in other rarer mineral forms known as tridymite and cristobalite.

It is a very durable mineral resistant to heat and chemical attack, and these properties have made it industrially interesting to man.[10]

- **Carbon black** – Carbon black is a material produced by the incomplete combustion of heavy petroleum products. It is a fine black powder consisting of nearly pure elemental carbon. Carbon black is a form of Para-crystalline carbon that has a high surface area-to-volume ratio, although lower than that of activated carbon. Unlike soot, carbon black has a much higher surface area-to-volume ratio and significantly lower (negligible and non-bioavailable) PAH content. Still, it is widely used as a model compound for diesel soot for diesel oxidation experiments. Carbon black is used mainly as a reinforcing agent in vehicle tires and rubber automotive products. Other common everyday products also often contain carbon black, including inks, paints, plastics, and coatings.[11]
- **Iron oxide** – Iron oxides and oxide-hydroxides are widespread in nature, play an important role in many geological and biological processes, and are widely used by humans (for example, as iron ores, pigments, and catalysts). Common rust is a form of iron (III) oxide. Iron oxides are widely used as inexpensive, durable pigments in paints, coatings, and colored concretes.[12]
- **Kaolin clay** (aluminum silicate hydroxide, bolus, and hydrated aluminum silicate) – Kaolin is a type of rock rich in kaolinite, a common layered silicate clay mineral, part of the group of industrial minerals with the chemical composition $Al_2Si_2O_5(OH)_4$. Kaolin clay occurs in abundance in soils formed from the chemical weathering of rocks in hot, moist climates. Kaolin is the most common mineral in clays. Kaolin is important in the production of ceramics and porcelain. It also is used as a filler for paint, rubber, and plastics because it is relatively inert and long lasting. But the greatest demand for kaolin is in the paper industry for producing glossy papers such as those used in most magazines.
- **Stearic acid** (octadecanoic acid) – Stearic acid is a saturated fatty acid with an 18-carbon chain. The International Union of Pure and Applied Chemistry (IUPAC) gives stearic acid the name “octadecanoic acid.” Stearic acid is a waxy solid occurring in many animal and vegetable fats and oils, but it is more abundant in animal fat (up to 30%) than vegetable fat (typically <5%).
- **Oleic acid** – Oleic acid is a monounsaturated omega-9 fatty acid abbreviated with a lipid number of 18:1 cis-9. Oleic acid occurs naturally in various animal and vegetable fats and oils. It is an odorless, colorless oil, although commercial samples may be yellowish. The term "oleic" means related to or derived from oil or olive oil, an oil predominantly composed of oleic acid.

3.2.1.2 Animal Sebum

Animal sebum was the major component (95% by weight in the final product) of the synthetic animal grime used in this project. It also served as a liquid carrier for grime applications onto coupon surfaces.

In most animals, main wax production is associated with the sebaceous glands of the skin. Sebaceous glands usually are associated with hair follicles, but there are also related structures on the eyelids called “Meibomian glands.” Sebaceous glands secrete mainly non-polar lipids in the form of sebum onto the skin

surface. The rate of sebum excretion varies between species and often is measured using the sebum saturation level (SSL), which represents the maximum amount of lipid that can accumulate on the skin surface. Sebum production may also be affected by metabolism, environmental factors, and gender. For example, in one study, sebum saturation levels for two breeds of cattle were 16.4 and 13.5 mg per 100 square centimeters (mg/100 cm^2) at a thermo-neutral temperature (24 ºC) and 31.2 and 67.2 mg/cm^2 at a constant high environmental temperature (32 °C).[13] An animal with a skin surface area of 1 to 4 square meters ($m^{2)}$ and a sebum excretion rate of approximately 20 mg/100 cm^2 per day would excrete 200 mg to 8 g of sebum a day).[13]

The composition of animal sebum varies between species. Although relatively few species have been studied in detail, it is apparent that a wide range of lipid classes are present in the sebum of different animal species. There also may be variation with age. The composition of human sebum differs appreciably from that of other species, especially in the high content of triacylglycerols and in fatty acid composition. Human sebum is unique in containing cis-6-hexadecenoic acid (6-16:1 or “sapienic” acid), accompanied by an elongation and desaturation product, 5,8-octadecadienoic acid (“sebaleic” acid). Sapienic acid is formed in the sebaceous glands by a distinctive Δ6 desaturase and has powerful antibacterial properties. The skin of mammals also contains a wide range of more polar lipids based on the ceramide backbone.[14]

Table 3.2-3 lists the relative composition (as a percentage of weight) of the non-polar lipids on the skin surfaces of various species.

Table 3.2-3. Relative Composition of Non-Polar Lipids on the Skin Surfaces of Various Species[14]

Total % Weight									
Species	**Squalene**	**Sterols**	**Sterol Esters**	**Wax Esters**	**Diesters**	**Glyceryl Ethers**	**Triacyl-glycerols**	**Free Acids**	**Free Alcohols**
Human	12	1	3	25			41	16	
Sheep		12	46	10	21				11
Rat	1	6	27	17	21	8		1	
Mouse		13	10	5	65		6		

For this project, lanolin was selected as an animal sebum surrogate. Lanolin is the purified secretory product of the sheep sebaceous gland. The raw material is referred to as “Adeps lanae,” “wool wax,” “wool fat,” or “wool grease.” Raw lanolin comprises 10 to 25% of the weight of sheared wool.[14]

Lanolin is a complex mix of fatty acids and alcohols, sterols (including cholesterol and lanosterol), hydroxy acids, diols, and aliphatic and steryl esters.[4] Because lanolin predominantly is composed of high-molecular-weight esters, it is classified chemically as a wax, not as a fat.

Pure anhydrous lanolin is a semi-solid, clear to very slightly hazy, waxy substance. According to the U.S. Pharmacopeia, lanolin is insoluble in water but mixes without separation with approximately twice its weight of water.

For this project, pure pharmaceutical-grade lanolin was purchased from Sigma-Aldrich USA (see Section 3.2.2.1 for details). In a series of preliminary tests, the lanolin was confirmed to be free of the surrogate

test organisms (*B. atrophaeus* and MS2) chosen for this study. In a series of additional solution-based tests, pure lanolin was also shown to be mildly bacteriostatic and to affect the growth of MS2 negatively. However, its overall compatibility with the surrogate test organisms did not hinder the pursuit of further testing with a lanolin-based grime.

3.2.1.3 Animal Impurities

Manure is organic matter used as fertilizer in agriculture. There are two classes of manures in soil management: green manures and animal manures. Green manures are used for crops grown for the express purpose of plowing them under to increase soil fertility through the nutrients and organic matter returned to the soil. Animal manure is the animal excreta (feces or excrement) of plant-eating mammals (herbivores) and plant material (often straw) that has been used as bedding for animals and thus is heavily contaminated with feces and urine (see Table 3.2-4).

Livestock manure has a variable composition, with solid and liquid portions as well as organic and inorganic components. The composition of animal manure varies with livestock type, age, size, nutrition, housing, and bedding as well as the nature and amount of materials (such as bedding and wastewater) added to it.[15] Table 3.2-4 lists manure components and their possible composition.

Table 3.2-4. Manure Components and Their Composition

Manure Component	Possible Composition
Feces	Undigested feed Other bodily wastes Pathogens Pharmaceuticals Organic forms of nutrients and organic acids Inorganic forms of nutrients and salts
Urine	Water Acids and salts Nutrients (such as nitrates)
Bedding	Straw and wood fiber Wasted solid feed
Water	Drinking water Leaking or spilled water Water from eaves, troughs, precipitation, and snow melt
Wash water and runoff	Facility wash water Milking parlor wash water Runoff from yards, stored feed, and manure

Animal manures are rich in nutrients and macro-elements (such as phosphorus, potassium, calcium, magnesium, sodium) and contain some trace elements (such as iron, cobalt, selenium, manganese, aluminum, arsenic, zinc, copper, chromium, and cadmium). Fresh manure is also a habitat for bacteria, fungi, protozoa, nematodes, earthworms, insects (such as springtails and dung beetles), and other arthropods (such as centipedes, millipedes, and pill bugs). Cow manure is rich in humus, the bulky and fibrous material from undigested plant matter.

For this project, the animal manure used was 50/50% dried homogenized swine/cattle manure prepared by The Animal and Poultry Waste Management Center (APWMC) at North Carolina State University (NCSU) in Raleigh, NC, USA.

Tables 3.2-5 and 3.2-6 summarize the comprehensive physical, biological, and chemical characteristics of typical raw swine and cattle manure, respectively. The analytical data are from the Biological & Agricultural Engineering Department of NCSU's Agronomic Division, North Carolina Department of Agriculture & Consumer Services.[5]

Table 3.2-5. Swine Fresh Manure Characteristics

Parameter	Unit	Total No. Obs.	Parameter Concentration					Parameter Mass	
			Min	Max	Median	Mean	STD	lb/day/ head	STD
Manure (total feces and urine)	lb/135 lb/day	81	4.0	23	11	11	3.3	11	82
Urine (total urine expressed as part of manure)	% manure	10	37	68	52	50	8.5	5.6	42
Density	lb/ft^3	23	59	65	62	62	1.4	-	-
Total solids (dry matter)	% weight	78	2.9	28	10	10	4.7	1.1	8.5
Total suspended solids	%db	6	57	80	76	72	8.8	0.83	6.1
Volatile solids	%db	53	53	93	82	80	7.1	0.92	6.8
Volatile suspended solids	%db	2	66	68	67	67	0.76	0.77	5.7
Total alkalinity	mg/kg	1	250	250	250	250	-	0.0028	0.021
BOD	mg/kg	34	23480	48736	32868	37134	6020	0.41	3.1
COD	mg/kg	50	45500	243011	90528	102710	39307	1.1	8.4
Inorganic carbon	mg/kg	2	4970	7120	6045	6045	1075	0.067	0.50
Total organic carbon	mg/kg	7	9460	120866	24800	38699	35144	0.43	3.2
Volatile acids	mg/kg	4	1870	4270	4205	3638	1022	0.040	0.30
pH		2	7.0	8.1	7.5	7.5	0.57	-	-
TKN (as N)	lb/ton	61	3.2	27	12	12	4.6	0.068	0.50
NH_3N (ammoniacal nitrogen as N)	%TKN	15	35	93	58	62	19	0.042	0.31
NO_3N (nitrate nitrogen as N)	lb/ton	1	0.057	0.057	0.057	0.057	-	0.00032	0.0024
P_2O_5 (total phosphate)	lb/ton	56	2.4	24	8.0	9.3	4.4	0.052	0.38
PO_4 (orthophosphate)	$\%P_2O_5$	1	69	69	69	69	-	0.036	0.27
K_2O (potash)	lb/ton	55	2.4	19	8.9	8.8	4.1	0.049	0.36
Aluminum	lb/ton	3	0.074	0.097	0.097	0.089	0.011	0.00050	0.0037
Arsenic	lb/ton	1	0.017	0.017	0.017	0.017	-	0.000093	0.00069
Boron	lb/ton	6	0.060	0.096	0.084	0.082	0.011	0.00046	0.0034
Calcium	lb/ton	25	4.1	18	5.7	8.1	4.3	0.045	0.33
Cadmium	lb/ton	3	0.00018	0.0016	0.00018	0.00065	0.00067	0.0000036	0.000027
Chloride	lb/ton	2	5.0	7.5	6.2	6.2	1.2	0.035	0.26

Parameter	Unit	Total No. Obs.	Parameter Concentration					Parameter Mass	
			Min	Max	Median	Mean	STD	lb/day/ head	STD
Cobalt	lb/ton	4	0.00040	0.0011	0.0011	0.00090	0.00029	0.0000050	0.000037
Copper	lb/ton	23	0.010	0.089	0.023	0.029	0.020	0.00016	0.0012
Iron	lb/ton	16	0.17	1.0	0.46	0.44	0.21	0.0024	0.018
Magnesium	lb/ton	25	1.0	4.4	1.6	1.8	0.77	0.010	0.074
Manganese	lb/ton	12	0.018	0.079	0.038	0.043	0.018	0.00024	0.0018
Molybdenum	lb/ton	5	0.000053	0.0020	0.00040	0.00066	0.00072	0.0000037	0.000027
Sodium	lb/ton	11	0.32	3.9	1.1	1.6	1.3	0.0089	0.066
Nickel	lb/ton	Estimated	-	-	-	0.0019	-	0.000011	0.000079
Lead	lb/ton	3	0.0016	0.0022	0.0022	0.0020	0.00028	0.000011	0.000082
Sulfur	lb/ton	14	0.24	2.9	1.7	1.8	0.96	0.010057	0.074
Zinc	lb/ton	24	0.074	0.31	0.11	0.12	0.056	0.00069	0.0051
Acid detergent fiber	%db	1	18	18	19	19	-	0.21	1.6
Crude fiber	%db	8	15	24	15	18	3.8	0.20	1.5
Crude protein	%db	9	20	35	24	25	3.9	0.28	2.1
Crude fat (ether extract)	%db	9	6.6	11	8.0	8.3	1.5	0.095	0.71
Nitrogen-free extract	%db	7	38	48	38	40	3.5	0.46	3.4
Total digestible nutrients	%db	2	48	68	58	58	10	0.67	4.9
Total protein	%db	2	16	16	16	16	0.18	0.18	1.3
Gross energy	kcal/g db	5	2.9	4.6	4.3	4.2	0.64	2158	15988
Enterococcus bacteria	col/100 g	2	5.50E+08	8.40E+08	6.95E+08	6.95E+08	1.45E+08	3.50E+10	2.59E+11
Escherichia coliform bacteria	col/100 g	1	1.00E+07	1.00E+07	1.00E+07	1.00E+07	-	5.03E+08	3.73E+09
Fecal coliform bacteria	col/100 g	6	6.50E+07	3.40E+08	3.30E+08	2.46E+08	1.23E+08	1.24E+10	9.17E+10
Fecal streptococcus bacteria	col/100 g	4	3.40E+08	8.40E+09	8.40E+09	6.39E+09	1.49E+09	3.21E+11	2.38E+12
Streptococcus bacteria	col/100 g	2	3.00E+06	8.50E+07	4.40E+07	4.40E+07	4.10E+07	2.22E+09	1.64E+10
Total coliform bacteria	col/100 g	3	2.00E+08	1.10E+09	3.30E+08	5.43E+08	1.97E+08	2.74E+10	2.03E+11

Source: [5]
% – Percentage
BOD – Biochemical oxygen demand
COD – Chemical oxygen demand
col/100 g – Colonies per 100 grams
db – Dry basis (% of dry matter)
kcal/g – Kilocalorie per gram
lb/day – Pounds per day
lb/day/head – Pound per day per head
lb/ft^3 – Pound per cubic foot
lb/ton – Pound per ton
mg/kg – Milligrams per kilogram
Obs – Observations
STD – Standard deviation
TKN – Total Kjeldahl nitrogen

Table 3.2-6. Cattle Fresh Manure Characteristics

Parameter	Unit	Total No. Obs	Parameter Concentration					Parameter Mass	
			Min	Max	Median	Mean	STD	lb/day/head	STD
Total solids (dry matter)	%wb	57	8.2	24	15	15	3.9	7.1	8.9
Total suspended solids	%db	3	73	83	73	76	4.9	5.5	6.8
Volatile solids	%db	36	53	99	85	82	10	5.8	7.3
Volatile suspended solids	%db	1	58	58	58	58	-	4.1	5.2
BOD	mg/kg	21	12750	49085	25004	28082	10180	1.4	1.7
COD	mg/kg	42	72917	239000	127095	130232	38382	6.3	7.9
Total organic carbon	mg/kg	3	40000	81496	58200	59899	16983	2.9	3.6
pH		4	6.5	7.3	7.0	7.0	0.34	-	-
Total Kjeldahl nitrogen (as N)	lb/ton	50	8.2	19	11	12	2.4	0.29	0.36
NH_3N (ammoniacal nitrogen as N)	%TKN	3	22	42	33	33	8.3	0.094	0.12
NO_3N (nitrate nitrogen as N)	lb/ton	2	1.3	1.6	1.4	1.4	0.13	0.035	0.043
P_2O_5 (total phosphate)	lb/ton	53	2.7	12	7.1	7.3	2.1	0.18	0.22
PO_4 (orthophosphate)	$\%P_2O_5$	1	32	32	32	32	-	0.057	0.072
K_2O (potash)	lb/ton	51	3.8	17	8.8	8.9	2.3	0.22	0.27
Boron	lb/ton	2	0.029	0.033	0.031	0.031	0.0022	0.00076	0.00095
Calcium	lb/ton	14	1.7	12	3.3	4.4	2.7	0.11	0.13
Cadmium	lb/ton	Estimated	-	-	-	0.00050	-	0.000012	0.000015
Chloride	lb/ton	Estimated	-	-	-	3.9	-	0.094	0.12
Copper	lb/ton	7	0.0045	0.018	0.0097	0.011	0.0044	0.00027	0.00033
Iron	lb/ton	9	0.077	0.67	0.24	0.29	0.21	0.0070	0.0088
Magnesium	lb/ton	16	0.85	3.0	1.7	1.7	0.53	0.041	0.051
Manganese	lb/ton	7	0.015	0.070	0.032	0.040	0.018	0.00097	0.0012
Molybdenum	lb/ton	1	0.0015	0.0015	0.0015	0.0015	-	0.000035	0.000044
Sodium	lb/ton	6	0.26	2.6	0.86	1.1	0.81	0.025	0.032
Nickel	lb/ton	Estimated	-	-	-	0.0069	-	0.00017	0.00021
Lead	lb/ton	Estimated	-	-	-	0.00056	-	0.000014	0.000017
Selenium	lb/ton	5	1.3	1.7	1.6	1.5	0.18	0.037	0.046
Zinc	lb/ton	8	0.027	0.060	0.030	0.034	0.010	0.00083	0.0010
Specific conductance	umhos/cm	1	3067	3067	3067	3067	-	-	-
Acid detergent fiber;	%db	6	31	47	42	41	5.2	2.9	3.7
Crude fiber	%db	7	17	38	31	27	8.0	1.9	2.4
Crude protein	%db	10	12	20	15	16	3.0	1.1	1.4
Crude fat (ether extract)	%db	6	2.3	6.5	2.8	3.4	1.4	0.24	0.31
Nitrogen-free extract	%db	4	33	53	49	46	17	3.3	4.1
Total digestible nutrients	%db	4	42	48	47	46	2.5	3.3	4.1
Gross energy	kcal/g db	3	4.1	4.8	4.7	4.5	0.30	14676	18345
Total anaerobic bacteria	col/100 g	1	2.40E+10	2.40E+10	2.40E+10	2.40E+10	-	5.28E+12	6.60E+12
Escherichia coliform bacteria	col/100 g	1	2.95E+11	2.95E+11	2.95E+11	2.95E+11	-	6.49E+13	8.11E+13
Enterococcus bacteria	col/100 g	4	1.10E+08	1.00E+09	4.23E+08	4.89E+08	3.70E+08	1.08E+11	1.34E+11
Fecal coliform bacteria	col/100 g	5	2.30E+07	1.10E+09	2.70E+08	4.83E+08	4.73E+08	1.06E+11	1.33E+11
Fecal streptococcus bacteria	col/100 g	4	1.00E+07	1.90E+09	1.30E+08	5.43E+08	7.85E+08	1.19E+11	1.49E+11
Total bacteria	col/100 g	3	1.50E+09	6.54E+12	1.00E+11	2.22E+12	3.06E+12	4.87E+14	6.09E+14
Total coliform bacteria	col/100 g	6	2.89E+07	2.50E+09	7.90E+08	1.09E+09	1.01E+09	2.41E+11	3.01E+11

Source: [5]
% – Percentage
µmhos/cm – Micromhos per centimeter
BOD – Biochemical oxygen demand
COD – Chemical oxygen demand
col/100 g – Colonies per 100 grams
db – Dry basis (% of dry matter)
kcal/g – Kilocalorie per gram
lb/day/head – Pound per day per head
lb/ton – Pound per ton
mg/kg – Milligram per kilogram
Obs – Observations
STD – Standard deviation
TKN – Total Kieldahl nitrogen
umhos/cm – Micromhos per centimeter
wb - wet basis (as is)

3.2.2 Grime Preparation

This section discusses the raw materials of the grime, preparation of individual components of the grime, and grime mixing.

3.2.2.1 Raw Materials

Raw materials for grime preparation either were obtained in their standardized or pure form from national suppliers or prepared by trained personnel using standardized methods. Table 3.2-7 lists the raw materials for grime preparation, trade names, manufacturers, and other information.

Table 3.2-7. Raw Materials for Grime Preparation

Raw Material	Trade Name or Composition	Manufacturer	CAS No.	Product No.
Natural humus	Ancient Forest 0.5 CF Humus Soil Amendment	General Organics, USA	Not available	GH3200
Paraffin oil	Paraffin oil; puris	Sigma-Aldrich USA	8012-95-1	18512-1L
Used crankcase motor oil	Not applicable	Local automobile service station	Not available	Not available
Portland cement	QUIKRETE® Portland Cement	QUIKRETE®, USA	Not available	1124
Silica	About 99% silicon dioxide, 0.5 to 10 micrometers (µm)	Sigma-Aldrich USA	14808-60-7	S5631-1KG
Carbon black	Raven 401	Powder Technology Inc., PTI, USA	Not available	Not available
Oleic acid	Oleic acid technical grade, 90%	Sigma-Aldrich USA	112-80-1	364525-1L
Kaolin clay	Kaolin	Sigma-Aldrich USA	1332-58-7	18672-2.5KG
Iron oxide	Ferric oxide	Sigma-Aldrich USA	1309-37-1	310050-500G
Stearic acid	Stearic acid ≥95%	Sigma-Aldrich USA	57-11-4	W303518-1KG-K
Lanolin	Lanolin	Sigma-Aldrich USA	8006-54-0	L7387-1KG
Manure	Dried homogenized 50/50% swine/cattle manure	APWMC at NCSU	Not available	Not available

3.2.2.2 Preparation of Individual Components

As summarized in Table 3.2-1, the grime was composed of particulate soil, lanolin, and manure. This section discusses the preparation of each component.

Particulate Soil Preparation

Natural humus was dried and homogenized before use as a particulate soil component. The humus was placed in a shallow tray and dried at 40 °C until a constant mass was achieved. After drying, the material was sieved through a 3/8-in (9.5-mm) screen to extract large pieces (such as wood sticks and stones). Then, the material was mixed and placed in a 150-mL container with three plastic balls and mixed in a ball mill (SPEX SamplePrep dual mixer/mill, Metuchen, NJ, USA) for five to six minutes. The final product was sieved through a 35-mesh (0.5-mm) screen. All other components were used as purchased.

In a plastic 250-mL Nalgene bottle, particulate soil was prepared by adding the ingredients listed in Table 3.2-2 in the following order:

- Natural humus (after sieving through 35-mesh screen): 38.0% by weight
- Paraffin oil: 1.0% by weight
- Used crankcase motor oil: 1.5% by weight
- Portland cement: 17.7% by weight
- Silica: 18.0% by weight
- Carbon black: 1.5% by weight
- Iron oxide: 0.3% by weight
- Kaolin clay: 18.0% by weight
- Stearic acid: 2.0% by weight
- Oleic acid: 2.0% by weight

After weighing, one part water of the mix volume was added, with a final volume of slurry not exceeding approximately 60% of the container volume. Five plastic balls were added to the 250-mL container. The slurry was then mixed in a ball mill (SPEX SamplePrep) for 30 minutes. After mixing, the slurry was transferred to a shallow tray and dried overnight at 40 °C until a constant mass was achieved. The material was turned over occasionally. After drying, the material was pulverized using a mortar and pestle and then milled to pass a 35-mesh (0.5-mm) screen.

Lanolin Preparation

Immediately before preparation of the grime, neat lanolin was placed on a laboratory hot plate and liquefied at 50 °C in its original amber glass container.

Manure Preparation

Standardized manure was prepared by the APWMC at NCSU from a 50/50% mix of fresh swine/cattle manure. A representative sample of fresh manure was dried at 40 °C to constant mass and milled to pass a 0.5-mm sieve. Figure 3.2-1a shows a sample of the raw dried manure mix, and Figure 3.2-1b shows the final sample of dried homogenized manure.

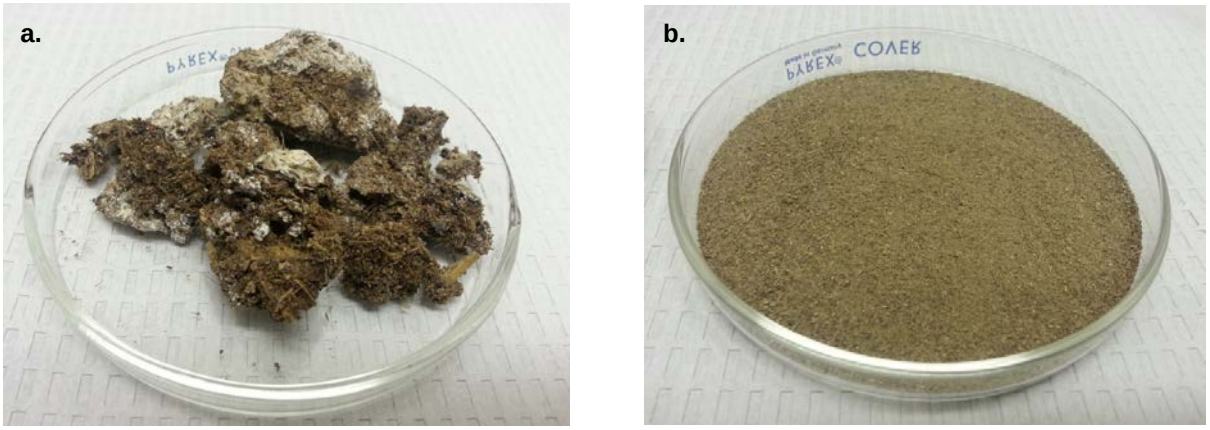


Figure 3.2-1. Raw Dried Manure (a), and Homogenized Sample of Manure (b)

3.2.2.3 Mixing of Grime

Grime was prepared in a commercial paint shaker (Red Devil 5400, Red Devil Equipment, Plymouth, MN, USA) using a two-step process: (1) preparation of concentrated grime, and then (2) preparation of final grime. The two-step process simplified the process by reducing the amount of time required for working with the liquefied lanolin.

First, the required amount of liquefied lanolin (50% by weight), particulate soil component (40% by weight), and standardized manure (10% by weight) was added to a one-pint, paint-shaker-compatible can. The can was capped loosely and placed in a 50 °C water bath for one hour. After one hour, the can was closed tightly and mixed using the paint shaker for one hour. To keep the lanolin warm (in liquid form), the primary container was placed in another bigger container filled with heated sand. After mixing the concentrated grime solution, the cans were transferred to a chemical hood. The final grime was prepared in a one-gallon can by mixing one part of the liquid concentrated grime (10% by weight) with nine parts of liquid lanolin carrier (90% by weight) in the paint shaker for 30 minutes. The final grime solution then was sterilized on a hot plate by gentle boiling at 106 ±,2 °C for 30 minutes (Figure 3.2-2a). Then, the final grime solution was allowed to cool until solid (Figure 3.2-2b). The can then was tightly closed and refrigerated.

a.

b.

Figure 3.2-2. Final Grime Solution Sterilization (a), and Final Batch of Solidified Grime (b)

3.2.3 Grime Application

This section discusses grime application on the large and small coupons.

3.2.3.1 Application of Grime on Large Coupons

Grime was applied onto building materials coupons using the liquefaction-solidification procedure described below.

First, a large batch of sterile grime was liquefied at 80 to 100 °C. The grime was allowed to cool to approximately 50 to 60 °C, and then individual 50-mL aliquots of liquid grime were aseptically transferred to 50-mL, pre-weighed sterile conical tubes (Figure 3.2-3).

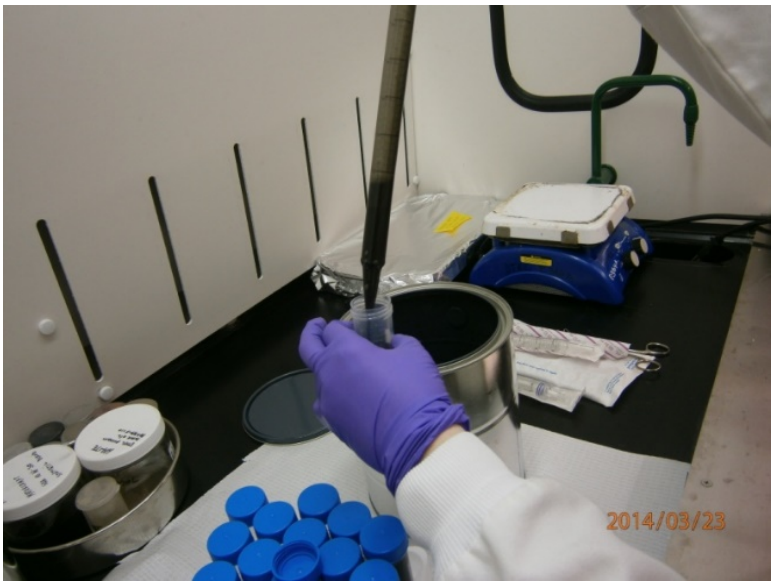


Figure 3.2-3. Grime Aliquot Preparation

Each conical tube was allowed to cool, and its weight was recorded to establish the amount of grime (in grams) in each tube. After weighing, the grime aliquots were refrigerated until used in grime application onto the coupons. Immediately before grime application, each batch of grime was subjected to a 10-minute-long heat shock in a hot water bath at 100 °C to ensure sterility.

Grime was applied onto the coupons in a pre-cleaned, Type II biological safety cabinet (BSC). Sterile coupons of building materials were aseptically assembled in the BSC (Figure 3.2-4).

Figure 3.2-4. Coupons Readied for Grime Application

Grime was applied to each coupon using a sterile paint roller. Rollers were labeled, pre-weighed, and sterilized with EtO before use.

Prior to application, a batch of grime aliquots (in 50-mL conical tubes) was again liquefied and then kept in a warm (50 to 60 °C) water bath to prevent the grime from solidifying. This temperature achieved the optimal grime viscosity to allow even spreading but prevent runoff from the coupon edges.

Each coupon received the contents of two 50-mL conical tubes of grime. First, the entire content of one conical tube was gently poured in the central part of each coupon and immediately spread using a paint roller until the entire surface of the coupon was covered with grime (Figure 3.2-5). This step was performed quickly to prevent premature solidification of the grime.

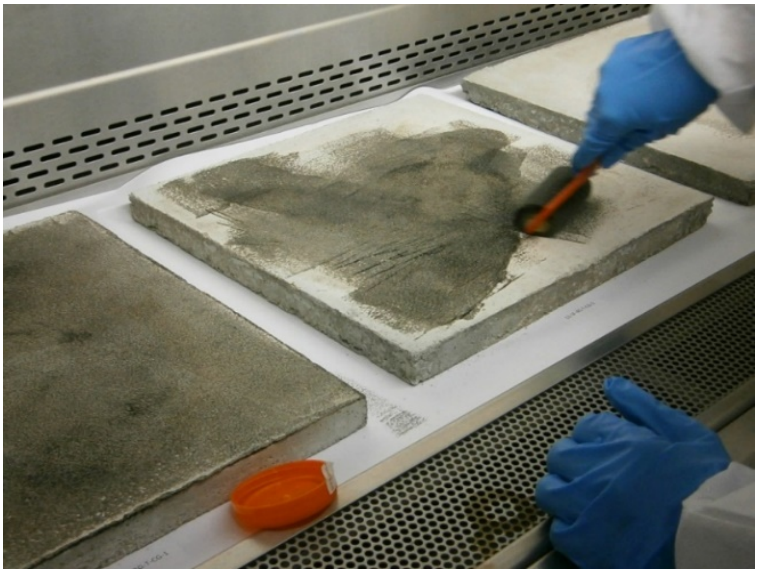


Figure 3.2-5. Grime Application Procedure (First Application)

After application of the first tube of grime, the roller was placed in an empty, sterile specimen cup next to the grimed coupon. The weight of the empty conical tube was recorded on a Grime Application Tracker Form. This procedure was repeated for each coupon. The second aliquot (50 mL) of grime was applied 30 minutes after the first application onto each coupon using the coupon-specific roller used for the first application on that coupon. The weight of the second empty conical tube also was recorded. In addition, the final weight of the roller after the second application was also recorded. Figure 3.2-6 shows a heavily grimed concrete coupon after the second application of grime.

Figure 3.2-6. Heavily Grimed Large Coupon Surface after Second Grime Application

The average amount of grime applied onto concrete and plywood coupons using the procedure discussed above was 54.98 g per 14- by 14-in coupon. The application method had high repeatability of grime delivery for both materials (relative standard deviation [RSD] 8 and 13% for concrete and plywood coupons, respectively).

3.2.3.2 Application of Grime on Small Coupons

Grime was applied on small coupons using the same liquefaction-solidification procedure described in Section 3.2.3.1. Because of the small area of the coupon, 0.4 mL of the grime was poured into the central area of the coupon using a sterile, 1-mL Finntip™ Flex Filter Pipette Tip (or equivalent). The grime was allowed to spread over the entire surface of the coupon as shown in Figure 3.2-7.

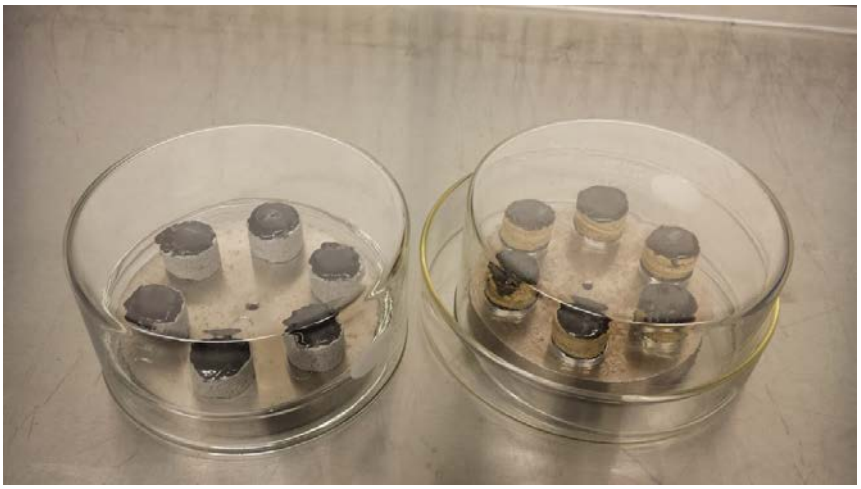


Figure 3.2-7. Heavily Grimed Small Coupon Surface after Second Grime Application

3.3 Test Organisms

Two types of test microorganisms were used for this project.

- *B. atrophaeus*, a surrogate for spore-forming bacterial agent *B. anthracis*
- Bacteriophage MS2, a surrogate for small, non-enveloped viral agents such as FMDV.

3.3.1 B. atrophaeus Surrogate for B. anthracis

B. atrophaeus is a soil-dwelling, non-pathogenic, aerobic, gram-positive spore-forming *Bacillus* species related to *B. subtilis*. This bacterial species was formerly known as *B. subtilis* var. *niger* and subsequently *B. globigii.* For more than six decades, this organism has played an integral role in the biodefense community as a simulant for biological warfare and bioterrorism events. *B. atrophaeus* is commonly referred to by its military two-letter designation “Bg.” The taxonomic placement of *B. atrophaeus* has changed dramatically over the years. Originally isolated as *B. globigii* in 1900 by Migula as a variant of *B. subtilis*, it was at first distinguished from *B. subtilis* by the formation of a black-tinted pigment on nutrient agar and by low rates of heterologous gene transfer from *B. subtilis*. Other than the formation of the dark pigment, it is virtually indistinguishable from *B. subtilis* by conventional phenotypic analysis, and the lack of distinguishing metabolic or phenotypic features has contributed to the confusion in the taxonomy of this organism. Low interspecies DNA transfer frequencies suggest substantial divergence. Based on analysis of comparative DNA hybridization, phenotypic tests, and biochemical tests, Gibbons et al.[16] advocate that pigment-producing *B. subtilis*-like isolates should be classified as a distinct species termed *B. atrophaeus*. Recently, more sensitive typing methods such as amplified fragment length polymorphism analysis show that *B. atrophaeus* strains could be classified into two major biovars: var. *globigii* encompassing the classical commonly used Bg isolates and var. *atrophaeus* encompassing other closely related yet genetically distinct strains.[16]

The *B. atrophaeus* used for this project was a powdered spore preparation of *B. atrophaeus* 9372 (American Type Culture Collection [ATCC], product ATCC® 9372) and silicon dioxide particles purchased from the U.S. Army's Dugway Proving Ground Life Sciences Division in Dugway, UT, USA. *B. atrophaeus* spore preparation and inoculations are discussed below.

3.3.1.1 *B. atrophaeus* Spore Preparation

After 80 to 90% sporulation, the *B. atrophaeus* suspension was centrifuged to generate a preparation of approximately 20% solids. A preparation resulting in a powdered matrix containing approximately 1×10^{11} viable spores per gram was prepared by dry blending and jet milling the dried spores with fumed silica particles (Degussa, Frankfurt am Main, Germany). The powdered spore preparation was loaded into metered-dose inhalers (MDIs) in accordance with an EPA proprietary protocol. The initial weight of each MDI was verified using an Ohaus Adventurer Pro balance ARC120 (Ohaus Corporation, Parsippany, NJ, USA). Ongoing control checks for each MDI were included in the batches of coupons contaminated using a single MDI. The ongoing checks during use were performed using a Mettler-Toledo PL303 balance (Mettler-Toledo, Inc., Columbus, OH, USA).

3.3.1.2 *B. atrophaeus* Spore Inoculations

Coupons were inoculated (loaded) with spores of *B. atrophaeus* using MDIs. Each coupon was contaminated independently by placing it into a separate dosing chamber (ADA)[17] designed to fit one 1.17- by 1.17-foot coupon of any thickness. The MDI was discharged once into the dosing chamber. The spores were allowed to settle onto the coupon surfaces for a minimum of 18 hours.

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, each MDI was weighed after completion of the contamination of each coupon. If an MDI weighed less than 10.5 g at the start of the contamination procedure, it was retired and a new MDI was used. For quality control (QC) of the MDIs, an inoculation control coupon was included as the first, middle, and last coupon inoculated using a single MDI in a single test. The contamination control coupon was a stainless steel coupon measuring 1.17 by 1.17 feet and inoculated, sampled, and analyzed.

3.3.2 Bacteriophage MS2 - Surrogate for Viral Agents

MS2 is an icosahedral ribonucleic acid (RNA) bacteriophage with triangulation number of T = 3 whose capsid is formed by 180 copies of the coat protein, folded as seven antiparallel β-strands and two helices[18]. Each face of the icosahedron is formed by trimers of coat protein. The virus shell has one copy of an additional protein, A, associated with it. Similar to FMDV, MS2 is a small (25 to 30 nanometers), non-enveloped, single-strand RNA virus.

MS2 was purchased from ATCC (product ATCC® 15597-B1™ in ATCC® Medium 271: *Escherichia* medium). ATCC® 15597-B1™ uses ATCC® 15597™ *Escherichia coli* strain C3000 as the host. When grown in presence of the *E. coli*, MS2 forms very hazy plaques with large halos in the Luria-Bertani (LB) agar. Plaques vary in size. The phage was inoculated on LB agar before the *E. coli* and LB agar overlay.

The host range of this phage has not been tested. It has been reported that the titer of the MS2 rapidly drops if the MS2 is kept at refrigerator temperatures. The MS2 stock was stored in a refrigerator at 2 to 8 ºC for short-term storage and -20 ºC for long-term storage. The titer was tested before each use.

MS2 preparation and inoculations are discussed below.

3.3.2.1 MS2 Preparation

E.coli and MS2 preparation and plating are discussed below.

Preparation of E. coli (ATCC 15597) Cell Stock

In a BSC, a vial of *E. coli* was aseptically removed from its packaging. A micropipette was used to aseptically add 0.4 mL of LB broth to the vial containing the freeze-dried pellet. The pellet was suspended by swirling or flicking the vial. Once mixed, the entire volume of the mixture was transferred to a sterile tube containing 5 to 6 mL of LB broth. The tube containing the LB broth and *E. coli* mixture was incubated at 35 ± 2 ºC for 24 hours.

After incubation, two to three sterile tubes containing 10 mL of LB broth were inoculated with 100 microliters (µL) of the *E. coli* overnight culture. These inoculated tubes were then placed in an incubator overnight at 35 ± 2 ºC. Once the incubation period was complete, a serological pipette was used to aseptically combine the contents of the 10-mL tubes into a sterile 50-mL centrifuge tube.

The 50-mL centrifuge tube was placed in the centrifuge at 2,500 revolutions per minute (rpm) for 15 minutes. (The rpm speed and centrifugation time depend on the centrifuge and size of centrifuge tube used to prepare the stock.). The supernatant from the centrifuge was decanted and then discarded. The remaining cells in the tube were resuspended with 15 mL 10-millimolar magnesium sulfate and stored at 2 to 8 ºC.

Preparation of MS2 (ATCC 15597-B1) Stock

An active growing broth culture of *E. coli* (ATCC 15597-B1) was prepared in a BSC by inoculating a sterile tube containing 5 mL of LB broth with 100 µL of the *E. coli* bacterial cell stock prepared as discussed in the paragraph above. The active growing broth culture of *E. coli* was incubated for 4 to 6 hours at 35 ± 2 ºC.

After incubation, 100 µL of the active growing broth culture of *E. coli* was inoculated into a tube containing 5 mL of LB top agar. The LB top agar and inoculum were quickly mixed, and care was taken not to introduce air and create bubbles in the agar. After mixing, the contents of the tube were immediately poured onto an LB agar plate. The LB top agar was spread evenly by gently swirling the plate. The vial containing the freeze-dried pellet of MS2 was carefully and aseptically opened, and 0.5 mL of LB broth was aseptically added to the freeze-dried pellet. The LB broth then was mixed with the pellet by carefully swirling or flicking the vial until pellet was suspended. Then the surface of the LB agar was covered with 0.5 mL of the phage suspension. The plate with the phage suspension was incubated at 35 ± 2 ºC for 24 hours.

After incubation, the soft agar layer was scraped into a 50-mL centrifuge tube, and 15 mL of SM buffer was added to the centrifuge tube. The tube containing the soft agar and magnesium salt (SM) buffer was centrifuged at 7,000 rpm for 15 minutes. (The rpm speed and centrifugation time depend on the centrifuge and size of centrifuge tube used to prepare the stock.) After centrifugation, the supernatant was removed using a micropipette. The supernatant then was filtered through a 0.2-µm filter.

Plating of Samples Containing MS2 (ATCC 15597-B1)

All sample dilutions were prepared in the same manner used for the *B. atrophaeus* samples. Three LB agar plates were labeled for each dilution (plated in triplicate) and labeled using the same sample identification number. Before the plating of the dilutions, the dilution tubes were vortex-mixed for 10 seconds, and then a portion of the dilution (100 µL to 500 µL, depending on the target dilution) was immediately inoculated onto the surface of an LB agar plate. A new pipette tip was used for each set of replicate dilutions.

The sample inoculated on the LB agar was spread over the surface of the LB agar plate using a cell spreader and a circular motion starting from the center of the plate and working outwards to the edge of the plate. A tube containing 5 mL of LB top agar and 100 µL of active growing *E. coli* solution was removed from the water bath, swirled gently to homogenize it, and immediately poured onto the LB agar plate. The top agar was evenly distributed by gently swirling the plate. The plates were allowed to sit undisturbed on a level surface for a few minutes until the top agar layer solidified. All samples were incubated at 35 ± 2 ºC for 18–24 hours.

After incubation, all PFU were enumerated manually.

3.3.2.2 MS2 Inoculations

The MS2 was propagated as described in Section 3.3.2.1. Before use for experimental testing purposes, MS2 concentrations were confirmed through plating.

After confirmation of the MS2 concentration, the MS2 stock was used to inoculate the coupons. The sterile coupons, either in a grimed or neat state, were carefully and aseptically placed in a BSC. The MS2 inoculum was homogenized using a vortex mixer immediately prior to inoculation and again for every 10-second time lapse that occurred during coupon inoculation (i.e., after each row of droplets was dispensed). Using a standard 20- to 250-µL positive-displacement micropipette and starting at the top right of the coupon, twenty x 100 µL droplets were applied onto each large coupon surface in accordance with the pattern shown in Figure 3.3-1. For the small coupons, one 100 µL droplet was applied at the center of the surface of the using a positive displacement pipette (100-µL droplet). The target surface concentration for MS2 experiments was 1×10^8 PFU.

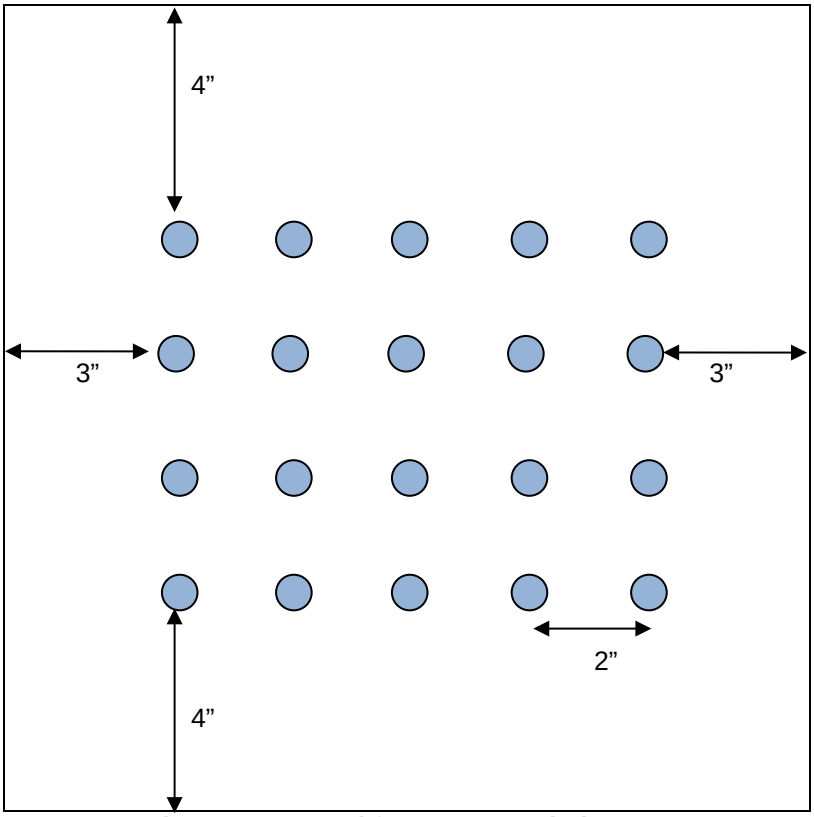


Figure 3.3-1. Droplet Pattern Used for MS2 Inoculations on Large Coupons

For QC purposes, one stainless steel control coupon was inoculated in addition to test material coupons. The time of inoculation was recorded. Sampling of the stainless-steel inoculation control was performed within 10 minutes after inoculation (± 2 minutes).

Decontamination Approach

This section discusses the decontamination materials and equipment, decontamination agents, decontamination testing approach, large coupon decontamination testing, and small coupon decontamination testing.

4.1 Decontamination Materials and Equipment

Changes in technique during the project could introduce variability and bias, thereby leading to erroneous conclusions. Therefore, the decontamination materials and equipment summarized in Table 4.1-1 were used in an attempt to provide as much standardization as possible.

Table 4.1-1. Decontamination Materials and Equipment

Material or Equipment	Description
Backpack sprayer	SHURflo ProPack™ SR600 Rechargeable Electric Backpack Sprayer, Cypress, CA
Chemical sprayer	Model UAG-1003HU, Pro-Chlorine Gas Powered Chemical Sprayer, Ultimate Washer Inc., Jupiter, FL
Handheld sprayer	RL FloMaster Model No. 56HD, Lowell, MI
Bleach	Ultra Clorox® Concentrated Germicidal Bleach (EPA Reg. No. 67619-8); 8.3% sodium hypochlorite; <1% sodium hydroxide
Vinegar	5% v/v technical grade acetic acid
Citric acid	Sigma-Aldrich USA No. 251275, American Chemical Society (ACS) grade, ≥99.5% pure, CAS No. 77-92-9
Spor-Klenz®	STERIS Spor-Klenz® RTU liquid decontaminant (EPA Reg. No. 1043-119); 1% H_2O_2, 0.08% peracetic acid (PAA), <10% acetic acid
Nozzle	Standard brass, adjustable-flow garden hose nozzle 4 in long
Garden hose	75 feet long, 5/8-in diameter heavy duty rubber hose
Pressure regulator	Bronze pressure regulator, plumbing code-rated, standard, ¾-in National Pipe Taper (NPT) female, 25 to 75pounds per square inch (psi)
Bucket of DI water	3 gallons in a 5-gallon plastic bucket
Carboy container	5.25-Gallon (20-L) heavy-duty Nalgene plastic polypropylene carboy container, autoclavable, leak proof, for full vacuum applications up to eight hours, U.S. Pharmacopeia Convention (USP) class VI, vacuum-rated for intermittent vacuum use only, 83B closure size
Pump	National Sanitation Foundation (NSF)-certified rotary vane pump for water with motor, brass, maximum capacity 4.3 gallons per minute (gpm), 3/4 horsepower

4.2 Decontamination Agents

This project focused on providing operationally feasible decontamination methods for the cleaning of farming and animal facilities after contamination with a bacterial or viral agent. The study concentrated on the following two classes of commonly used disinfectants:

- Oxidizers including pAB and Spor-Klenz® RTU; and
- Acids including 2% citric acid.

The following sections discuss each solution of decontamination agent used in this project.

4.2.1 pAB Solution

Sodium hypochlorite (bleach) is a registered antimicrobial pesticide under the authority of FIFRA for use as a sanitizer or disinfectant to kill bacteria, fungi, and viruses in households, food-processing plants, agricultural settings, animal facilities, hospitals, and human drinking water supplies. However, bleach is not a registered sterilant under FIFRA and does not have a registration claim to inactivate bacterial spores (including *B. anthracis*). Published scientific data demonstrate that pAB reduced bacterial spore populations under specific conditions related to concentration, pH, and contact time. Therefore, EPA has issued crisis exemptions permitting the limited sale, distribution, and use of EPA-registered bleach products against *B. anthracis* spores at a number of facilities and locations, including Capitol Hill, the U.S. Postal Service Processing and Distribution Centers at Brentwood (Washington, DC, USA) and Hamilton (Trenton, NJ, USA), the Department of State, the General Services Administration, and Broken Sound Boulevard, Boca Raton, FL, USA. The application of bleach under crisis exemptions was limited to specific buildings or treatment sites, and the specific conditions summarized below applied.

- Only hard, nonporous surfaces could be treated.
- A bleach solution close to but not above pH 7 (neutral), verified using a paper test strip at a concentration of 5,000 to 6,000 ppm was prepared by mixing the following:
    - One part bleach (with a 5.25 to 6% sodium hypochlorite concentration)
    - One part white vinegar
    - Eight parts water.
- Bleach and vinegar were not combined together directly. Water was first added to the bleach (two cups water to one cup of bleach), then vinegar (one cup), and then the remaining water (six cups).
- Treated surfaces had to remain in contact with the bleach solution for 30 minutes.
- Repeated applications were necessary to keep the surfaces wet.
- Treated PPE and containers removed from a treatment area required only 10 minutes contact time with the bleach solution.

Although the chlorine content of the solution affects the time required for inactivation or overall effectiveness, the pH of the solution has a much greater impact. Therefore, the comparative effectiveness of alternative formulations (such as Clorox® Outdoor bleach having a higher sodium hypochlorite concentration) is not easy to predict. The bleach formulation used in this study is the one that EPA used previously under the crisis exemptions.

The concentration of household bleach and the strength of white vinegar can vary by batch and storage time. Therefore, the formulation listed above can vary in pH and chlorine concentration depending on the starting reagents. This source of variation can complicate a laboratory study such as this project by skewing data, potentially leading to erroneous conclusions. To reduce the impact of “natural” variations in the bleach solution for this project, the pH and chlorine content were measured at the start and monitored throughout each test. The frequency of pH measurement was at a minimum at the start of testing of each

coupon set. Data quality indicators (DQI) for the bleach solution are discussed in Section 9.2. The solution had a mean pH close to but not above neutral (>6.5 and <7.0) and a mean total chlorine content of 6,000 to 6,700 ppm. The temperature of the solution was 18 to 24 ºC (64 to 75 ºF). Any solution having a pH, chlorine content, or temperature falling outside these ranges at any time was discarded and a fresh pAB solution was prepared.

The chlorine content was measured by titrating 5 mL of solution with 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 before the initiation of testing on each day and was used within three hours from the time of preparation. After three hours, the bleach solution was discarded and a fresh pAB solution was prepared. However, a single preparation was used within a single coupon set. The pAB solution was prepared as summarized below.

1. The concentration of the concentrated germicidal bleach (~ 8.3% sodium hypochlorite) was measured using a Hach test kit titration approach. If the calculated value was below 7.0%, the feedstock was discarded and replaced with new bleach.
2. The pH-adjusted bleach consisted of 80% DI water, 10% germicidal bleach (prepared in Step 1), and 10% acetic acid. For example, 10 L of solution consisted of 1 L of prepared regular germicidal bleach, 2 L of DI water, 1 L of acetic acid, and 6 L of DI water (in the order listed). The solution was prepared in a container that accommodated the total volume of solution using a funnel if necessary. The total volume was recorded as "V_{start}" in the laboratory notebook.
3. The mixing container was sealed and gently agitated for mixing. The pH probe was placed into the solution, and the pH was measured (target pH = 6.8). If the pH was above 7.0, small increments of acetic acid were added. If the pH was below 6.5, germicidal bleach was added. The Quality Assurance Project Plan (QAPP) discusses permitted adjustments. The volume required for adjustment was recorded as "V_{add}," and "V_{total}" was calculated as $V_{start} + V_{add}$ in the laboratory notebook.
4. The free available chlorine (FAC) was measured. The target FAC was 6,350 milligrams per liter (mg/L). The acceptable range was 6,000 to 6,700 mg/L.
    a. If the FAC exceeded the acceptable range, the total volume was diluted with DI water by the percent difference between the target FAC and the actual FAC.

       Dilution volume = [(actual - target) ÷ target] x (V_{total})

    b. If the FAC was less than the acceptable range, bleach was added according to the following equations:

       Additional volume of bleach = (target – actual)/ target x V_{total}

    V_{total} was recalculated for all the additions, and Steps 3 and 4 were repeated until both parameters were met.

4.2.2 Spor-Klenz® RTU Solution

Spor-Klenz® RTU is a broad-spectrum liquid disinfectant and sporicide registered with the EPA under FIFRA (Registration No. 1043-119). Spor-Klenz® is a mixture of 1.0% H_2O_2, 0.08% PAA, and 98.92% inert proprietary ingredients. The RTU variety of Spor-Klenz® was used for this study instead of the concentrate (Registration No. 1043-120) to reduce the variation between experiments. Preparation of diluted Spor-Klenz® from the concentrate for each day of testing would introduce unwanted variation. Spor-Klenz® RTU requires no dilution before use. A new 3.2 Liter bottle of Spor-Klenz® RTU was used for each day of testing. Unused Spor-Klenz® RTU was neutralized with STS, and discarded through the EPA chemical services. Because Spor-Klenz® RTU is produced under manufacturer quality assurance (QA) criteria, only temperature was a critical measurement for this liquid. The concentrations of H_2O_2 and PAA in Spor-Klenz® RTU were verified during preliminary testing to help determine the amount of neutralizer needed for quenching decontamination in liquid effluents from decontamination (see Section 5.2.2).

Spor-Klenz® is a sterilant and sporicide for nonporous surfaces when a 5.5-hour contact time (at 20 ºC) is used. This contact time far exceeds the planned contact times for this project because the study was conducted to evaluate technologies under conditions realistic for their use in homeland security-related remediation events. Maintaining a 5.5-hour contact time in a farming or animal facility would likely be unrealistic for the amount of surface area requiring decontamination. Consistent with a previous study of Spor-Klenz® RTU, a contact time of 30 minutes was therefore used. Spor-Klenz® RTU was applied using a backpack sprayer and a chemical sprayer.

4.2.3 2% Citric Acid Solution

Citric acid is not sporicidal and therefore was used only for decontamination tests with MS2. Citric acid occurs naturally in plants and in animal tissues and fluids and can be extracted from citrus fruit and pineapple waste. Citric acid contains three carboxylic acid functional groups and has a molecular formula of $H_3C_6H_5O_7$. Citric acid is an active ingredient in pesticide products registered for residential and commercial use as disinfectants, sanitizers, and fungicides. These products, which contain citric acid in combination with other active ingredients, are used to kill odor-causing bacteria, mildew, pathogenic fungi, certain bacteria, and some viruses, and to remove dirt, soap scum, rust, slime, and calcium deposits. Citric acid products are used in bathrooms and on dairy and food processing equipment. It can be produced on an industrial scale by mold-based fermentation of carbohydrates such as molasses.

The first pesticide products containing citric acid as an active ingredient were registered in the early 1970s. Currently, three products containing citric acid and other active ingredients are registered for use as fungicides and bactericides as described above. Citric acid is "generally recognized as safe," or GRAS (see Section 21 of the *Code of Federal Regulations,* 182.1033). Acidic disinfectants function by destroying the bonds of nucleic acids and by precipitating proteins.

Acids also change the pH of the environment, making it detrimental to many microorganisms. Numerous studies have demonstrated the antimicrobial efficacy of acetic acid, citric acid, and sodium bicarbonate using suspensions of bacteria and recovery from treated hard surfaces, and in meat-rinsing and produce-washing operations. In studies, the efficacy of citric acid as demonstrated in suspensions (in the absence

or presence of organic matter) was drastically different from its efficacy demonstrated on produce[20]. Study results suggest that vinegar (acetic acid) exhibits the most antimicrobial efficacy, followed by lemon juice (citric acid) and baking soda (sodium bicarbonate)[21]. Typically, gram-negative bacteria such as *Shigella sonnei*, *Salmonella* spp., *E. coli*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitia* are more susceptible to organic acids (such as acetic acid and citric acid) than gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*. The highly cross-linked cell walls of gram-positive bacteria are believed to impair the diffusion of the organic acids into the cell, preventing antimicrobial action.

Acids are generally highly virucidal. With the correct choice of acid or acid mixture, they can be used under a wide variety of conditions, including residential cleanup. Citric acid is a milder acid available in solid form that is active against acid-sensitive viruses and can be used safely for personnel and clothing decontamination. It is particularly useful when added to detergents for the inactivation of the FMDV. Solutions of 0.2% citric acid at 30 minutes contact time are suggested as a safe decontamination option for clothes and the body, especially for FMDV decontamination. Previous studies on birch wood carriers have shown that 2% citric acid is an effective disinfectant for FMDV.[22]

For this project, citric acid was purchased from Sigma-Aldrich USA and its 2% (weight per volume) solution was prepared using sterilized DI water. The concentration of citric acid was confirmed before each experiment through titration with sodium hydroxide. A total contact time (visibly wetted surface) of 30 minutes was used for citric acid.

4.3 Decontamination Testing Approach

Key operating parameters of the decontamination procedure included the following:

- Type of decontaminant
- Presence of grime or organic matter
- Application mode of decontaminant diluted pAB solution using a pressurized (chemical or backpack) sprayer
- Contact time of 30 minutes, followed by a rinse step.

Each test included five test coupons for each material type, three positive control coupons, one procedural blank coupon, and one negative control coupon. Therefore, a total of 10 coupons were required for each material type per test. The additional stainless steel coupons were used during the inoculation phase as inoculation controls. Procedural blanks (coupons of each material not contaminated with the test organisms) were run first, followed by the test coupons. The test chamber was cleaned both before and after the procedural blank test.

After the decontamination steps, the coupon surfaces were sampled to determine the efficacy of the combination of operational parameters and decontamination approaches. Moistened sterile non-cotton wetted wipes were used to conduct surface sampling of the coupons, as described in Section 6.2.1. Liquid effluent (runoff) samples, rinse water samples, and air samples collected during the decontamination process also were analyzed to determine the fate of the test organisms (See Section 6.2.2).

4.4 Large Coupon Decontamination Testing

Table 4.4-1 below shows the test matrix for *B. atrophaeus* and MS2 testing of the large coupons.

Table 4.4-1. *B. atrophaeus* and MS2 Decontamination Test Matrix for Large Coupons

Test	Decontamination Application Method	Decontamination Liquid	Micro-organism	Material Type	Coupon Condition	Total No. of Coupons*
1	Backpack Sprayer	pAB	*B. atrophaeus*	Concrete	Neat	10
2					Grimed	10
3				Treated Plywood	Neat	10
4					Grimed	10
5		Spor-Klenz® RTU		Concrete	Neat	10
6					Grimed	10
7				Treated Plywood	Neat	10
8					Grimed	10
9	Chemical Sprayer	pAB		Concrete	Neat	10
10					Grimed	10
11				Treated Plywood	Neat	10
12					Grimed	10
13		Spor-Klenz® RTU		Concrete	Neat	10
14					Grimed	10
15				Treated Plywood	Neat	10
16					Grimed	10
17	Backpack Sprayer	pAB	MS2	Concrete	Neat	10
18					Grimed	10
19				Treated Plywood	Neat	10
20					Grimed	10
21		2% (v/v) citric acid		Concrete	Neat	10
22					Grimed	10
23				Treated Plywood	Neat	10
24					Grimed	10

*Five test coupons: three positive control coupons, one procedural blank coupon, and one negative control coupon.

This section discusses the test chamber, application of decontaminants using sprayers, post-decontamination rinse, and decontamination chronology for the large coupon tests.

4.4.1 Test Chamber for Large Coupons

Decontamination testing for large coupons was conducted in a spray chamber located at EPA's Research Triangle Park facility in North Carolina. Briefly, sets of the building material coupons were inserted in a vertical position in the test coupon holders of the spray test chamber as shown in Figure 4.4-1.

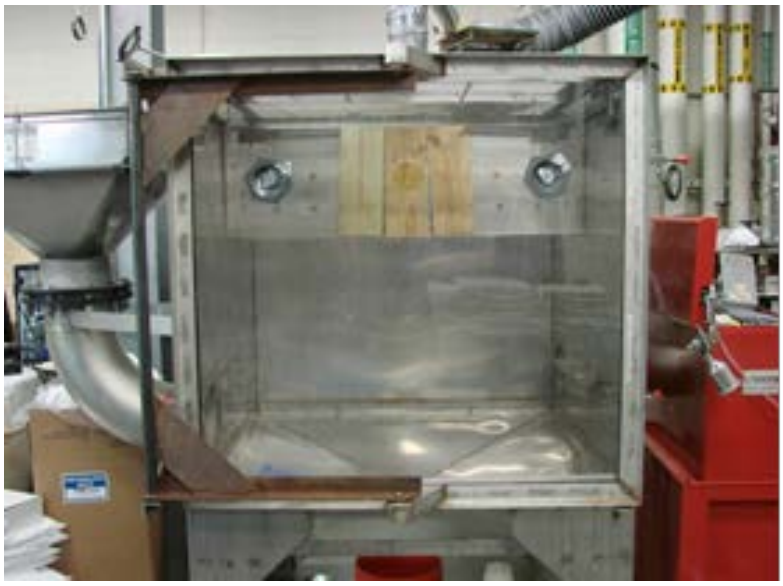


Figure 4.4-1. Decontamination Test Chamber for Large Coupons

The test chamber measured 4 feet high by 4 feet wide by 4 feet deep and was designed to accommodate three large coupons at a time in a horizontal or vertical position. For this project, only the vertical assembly was used. The chamber was constructed of solid stainless steel except for the front face and top, which were constructed of clear acrylic plastic.

The reverse-pyramid design of the chamber bottom allowed the collection of runoff from the coupons during the decontamination procedure through a central drain 3 in in diameter. The bottom of the chamber had a collection capacity of 189 L (50 gallons).

The chamber was fitted with connections allowing air to exit through a readily accessible connection to the facility's air handling system. Aerosol samples were collected from the chamber exhaust duct using Via-Cell® bioaerosol cassettes (Zefon International; Ocala, FL). The sampling points were eight diameters downstream and two diameters upstream of any flow disruptions.

4.4.2 Application of Decontaminants Using Sprayers for Large Coupons

Decontaminant was applied using a backpack sprayer (SHURflo ProPack™ SR600 rechargeable electric backpack sprayer, SHURflo Inc., Cypress, CA, USA) or chemical sprayer (Model UAG-1003HU, Pro-Chlorine gas powered chemical sprayer, Ultimate Washer, Inc., Jupiter, FL, USA). The acrylic door of the test chamber was fitted with three ports, one per coupon, allowing insertion of the sprayer wand into the central area in front of each vertical coupon, as shown in Figure 4.4-2. Each sprayer type and its use for applying decontaminants is discussed below.

Figure 4.4-2. Spraying Through Center-Aligned Port in the Test Chamber Door

4.4.2.1 Backpack Sprayer

The decontamination solution was prepared inside a four-gallon electric backpack sprayer (see Figure 4.4-3).

Figure 4.4-3. Electric Backpack Sprayer

The sprayer was rinsed with DI water and filled with the decontamination solution. The flow rate of each sprayer was verified before each test using a 500-mL graduated cylinder and a stopwatch (liquid collected and volume recorded from 10 seconds of spraying time). The spray pattern was tested by spraying at the appropriate distance of 1 foot onto a piece of 14- by 14-in blue construction paper mounted in the test

chamber in the vertical orientation corresponding to a test coupon. The spray was discharged onto the center of the paper, and the pattern was visually assessed for consistency.

To apply the decontamination solution to the coupons, a set of three replicate coupons was installed vertically in the test chamber. After confirmation that all QC requirements were within specifications, the coupons were sprayed through the center port of the chamber door. The sprayer wand was inserted through the port, keeping the doors closed to minimize the exposure of the worker to toxic fumes from the decontamination solution during application. The spray nozzle was kept a distance of approximately 1 foot (± 2 in) from the coupon surface for the backpack sprayer tests. A spray pressure of 35psi was maintained by the backpack sprayer. At this constant pressure, the target flow rate of the decontamination solution was set to 1.2 liters per minute (Lpm), with a cone spray pattern having a 14-in diameter at 1 foot from the surface of the coupon.

The flow and spray pattern were checked at the start and end of each set of spray applications. Figure 4.4-4 shows the spray pattern and pass order. Each pass was set to five seconds, for a total spray time of 15 seconds per application. The spray pattern shown in Figure 4.4-4 was performed in one continuous application, starting in the top left corner and ending in the bottom right corner. The passes are shown separately in Figure 4.4-4 only for illustration purposes and to show total coverage.

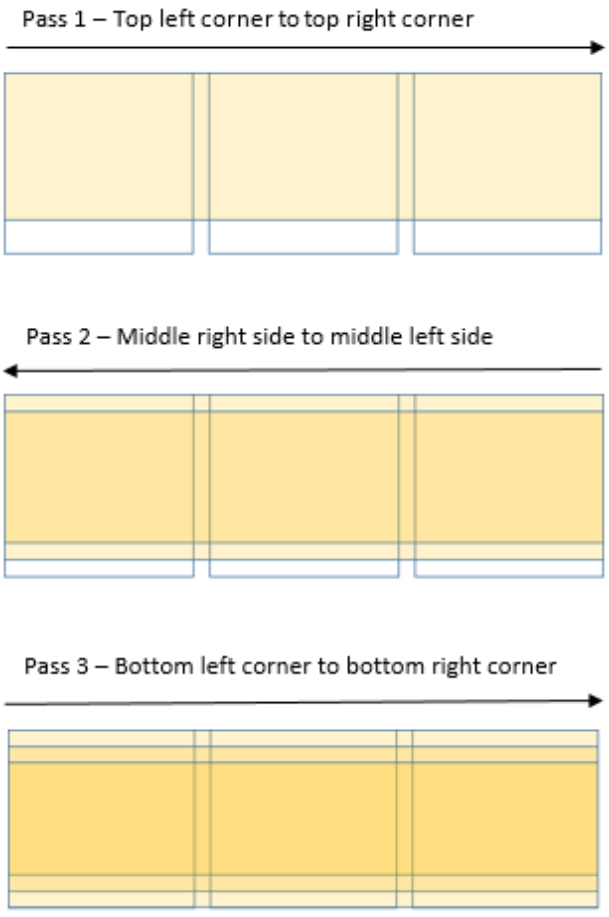


Figure 4.4-4. Spray Pattern for Backpack Sprayer

The spray wand was moved back and forth as evenly as possible to cover the surface of all three coupons completely. This step was repeated as often as necessary to satisfy the required spray duration time of 15 seconds per application. After a 15-minute exposure time, the decontaminant was re-sprayed using the method described above (15-second spray time). After the second application of decontamination solution, another 15-minute exposure time was provided, for a total exposure time of 30 minutes.

4.4.2.2 Chemical Sprayer

The chemical sprayer uses a low-pressure diaphragm pump for spraying corrosive solutions such as those containing sodium hypochlorite. The gasoline-operated pump requires an external source of the chemical to be sprayed. The decontamination solution was prepared inside a source container. During operation, chemical not being sprayed was returned to the source container.

The source container was rinsed with DI water and filled with the decontamination solution. The flow rate of each sprayer was verified before each test using a 500-mL graduated cylinder and a stopwatch (to verify 10 seconds of spraying time). The spray pattern was tested by spraying at the appropriate distance of three feet onto a piece of 14- by 14-in blue construction paper mounted in the test chamber in the vertical orientation corresponding to a test coupon. The spray was discharged onto the center of the paper, and the pattern was visually assessed for consistency.

To apply the decontamination solution to the coupons, a set of three replicate coupons was installed vertically in the test chamber. After confirmation that all QC requirements were within specifications, the coupons were sprayed through the center port of the chamber door. The sprayer wand was inserted through the port, keeping the doors closed to minimize the exposure of the worker to toxic fumes from the decontamination solution during application. The spray nozzle was kept a distance of approximately three feet (± two in) from the coupon surface for the chemical sprayer tests. The chemical sprayer is reported by its manufacturer to achieve a pressure of 200 psi. At this constant pressure, the flow rate was maintained at 11 L/min (2.9 gal/min), with a cone spray pattern having a 14-in-diameter at three feet from the surface of the coupon. The flow and spray pattern were checked at the start and end of each set of spray applications.

A set of three replicate coupons was installed vertically in the test chamber, and the coupons were sprayed using the same procedure, pattern, and exposure time discussed in Section 4.4.2.1 for the backpack sprayer.

4.4.3 Post-Decontamination Rinse for Large Coupons

After the 30-minute exposure time, the large coupons were rinsed with DI water using the standard garden hose nozzle listed in Table 4.4-1. The rinse step was used to simulate field operations in which rinsing may be used to minimize collateral damage to facilities resulting from extended contact with harsh decontamination chemicals. The water was supplied to the nozzle through the garden hose listed in Table 4.4-1 from a 60-gallon tank reservoir by the pump listed in Table 4.4-1 to provide a pressurized stream. The head pressure was constantly maintained at approximately 60 psi using the pressure regulator listed in Table 4.4-1. An Oakton pH probe was maintained in the DI water reservoir to monitor the pH and temperature continually.

The three coupons were rinsed in the same manner as the decontamination solution was applied, with side-to-side strokes using the pattern shown in Figure 4.4-4 from the central port of the test chamber. The spray was controlled using the nozzle to have a one-foot diameter measured at threex feet from the nozzle. Each rinse was conducted for 10 seconds. The start time and duration of rinsing were recorded and performed as consistently as possible across all coupons.

After rinsing, the coupon positions were recorded on a Coupon Tracker form. Then each coupon was removed from its position in the test chamber and placed on a drying pan. Each drying pan containing a coupon then was moved to a designated storage cabinet and allowed to dry overnight. Negative control coupons were transferred to the blank coupon cabinet, and decontaminated coupons were transferred to the decontaminated coupon cabinet.

The volume, FAC, pH, and temperature of the rinsate were recorded, and rinsate samples were collected for microbiological analyses as discussed in Section 6.4.2.

4.4.4 Decontamination Chronology for Decontamination Testing (Large Coupons)

The backpack and chemical sprayers were used to apply the pAB, Spor-Klenz® RTU, and 2% (v/v) citric acid solutions to the large coupons. The decontamination chronology for each microorganism testing for the large coupons is summarized below.

Day 0

Coupon Inoculation

- Five test and three positive control coupons were inoculated with spores of *B. atrophaeus* through aerosol deposition using an MDI or with MS2 through liquid inoculation.
- Coupons were then moved to coupon storage cabinets. Non-contaminated control coupons were transferred to a blank coupon cabinet, and contaminated coupons were transferred to a positive coupon cabinet. Positive control coupons and material sterility blank coupons were not used for decontamination testing and remained in their respective cabinets until sampling.

Day 1

Test Chamber Preparation and Air Sampling Cassette Assembly

- The test chamber was sterilized using pAB solution prepared as a 1:10 dilution of bleach in DI water, pH-adjusted to ~6.8 using glacial acetic acid.
- The gaskets used during the inoculation procedure were cleaned via fumigation with a STERIS VHP sterilization cycle, which maintained a constant H_2O_2 concentration of 250 parts per million by volume (ppmv) in a decontamination chamber for four hours.
- Via-Cell® bioaerosol cassettes for air sampling were assembled prior to testing.

Coupon Assembly in the Test Chamber

- Three coupons at a time were inserted into the test chamber. All coupons were tested in the vertical orientation.
- Coupons were inserted into the test chamber using sterile gloves, taking care not to touch the coupon surface.

Liquid Decontaminant Solution Preparation

- The procedure for preparing pAB solution is discussed in Section 4.2.1. Critical operational parameters consisted of FAC, pH, and temperature measurements.
- As discussed in Section 4.2.2, the Spor-Klenz® RTU formulation was used as is without dilution. The temperature and pH of the Spor-Klenz® RTU solution were recorded, but only temperature was considered a critical measurement.

Runoff and Rinse Water Collection

A clean, sterile carboy (Carboy No. 1) loaded with neutralizer was placed under the drain of the test chamber to collect liquid effluent type 1 (runoff of liquid decontaminant) during the decontamination testing. A second clean sterile carboy (Carboy No. 2) loaded with neutralizer was placed under the drain of the test chamber to collect liquid effluent type 2 (water from rinsing of the liquid decontaminant). Approximate volumes of material-specific runoff and rinsate were assessed during liquid effluent characterization tests (Section 5.2.2). The amount of neutralizer needed for each material type was determined in preliminary testing (see Section 5.2. for details).

Decontaminant Solution Application

- Application of liquid decontaminant was performed using either a backpack or chemical sprayer as discussed in Section 4.4.2.

Post-Decontamination Coupon Rinse

- The post-decontamination rinse of the large coupons was conducted as discussed in Section 4.4.3.

Assessment of Surface Damage on Coupons

- The surfaces of the procedural blank and positive control coupons were visually inspected side-by-side before and after decontamination of the procedural blanks. Differences in color, reflectivity, and roughness were qualitatively assessed, and observations were documented in the laboratory notebook. Time- and date-stamped digital photographs were taken to document any observed surface change.

Day 2

Sampling of Test Coupons

- After a minimum of 18 hours and when all coupons surfaces were visibly dry, the coupons were sampled using wipe sampling as discussed in Section 6.4.1.

Transfer of Samples for Microbiological Analysis

- Samples were transferred in sterile, primary, independent packaging within sterile, secondary containment in logical groups for analysis. All samples were accompanied by a completed chain of custody form.

4.5 Small Coupon Decontamination Testing

Table 4.5-1, below, shows the test matrix for MS2 testing of the small coupons.

Table 4.5-1. MS2 Decontamination Test Matrix for Small Coupons

Decontamination Application Method	Decontamination Liquid	Exposure Time	Material Type	Coupon Condition	Total No. of Coupons*	Total No. of Effluents**
Handheld sprayer	2% citric acid	30 minutes	Concrete	Neat	13	8
				Grimed	13	8
	pAB	30 minutes	Treated Plywood	Neat	13	8
				Grimed	13	8
*Five test coupons: three procedural positive controls, one procedural blank, and one negative control. **Eight effluents: Five test rinsate samples and three procedural positive rinsate samples.						

This section discusses the spray apparatus, decontamination procedure, and control testing for MS2 testing for the small coupons.

4.5.1 Spray Apparatus for Small Coupons

A bench-scale test spray apparatus was used to evaluate the pAB and citric acid spray-based decontamination methods for the small coupons (both grimed and neat) contaminated with MS2. Figures 4.5-1 through 4.5-3 show the apparatus used.

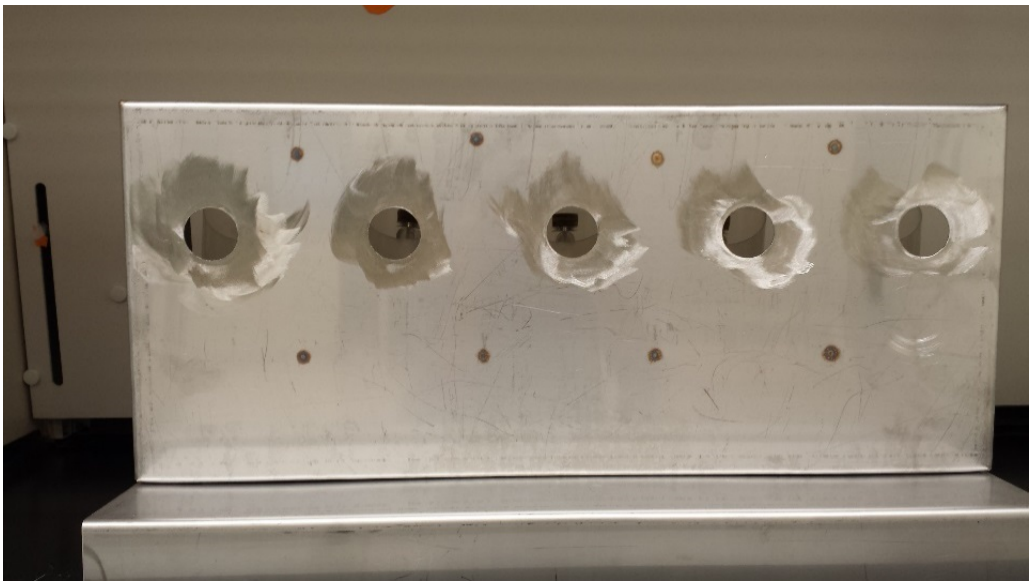


Figure 4.5-1. Front View of Spray Apparatus with Orifice Plate

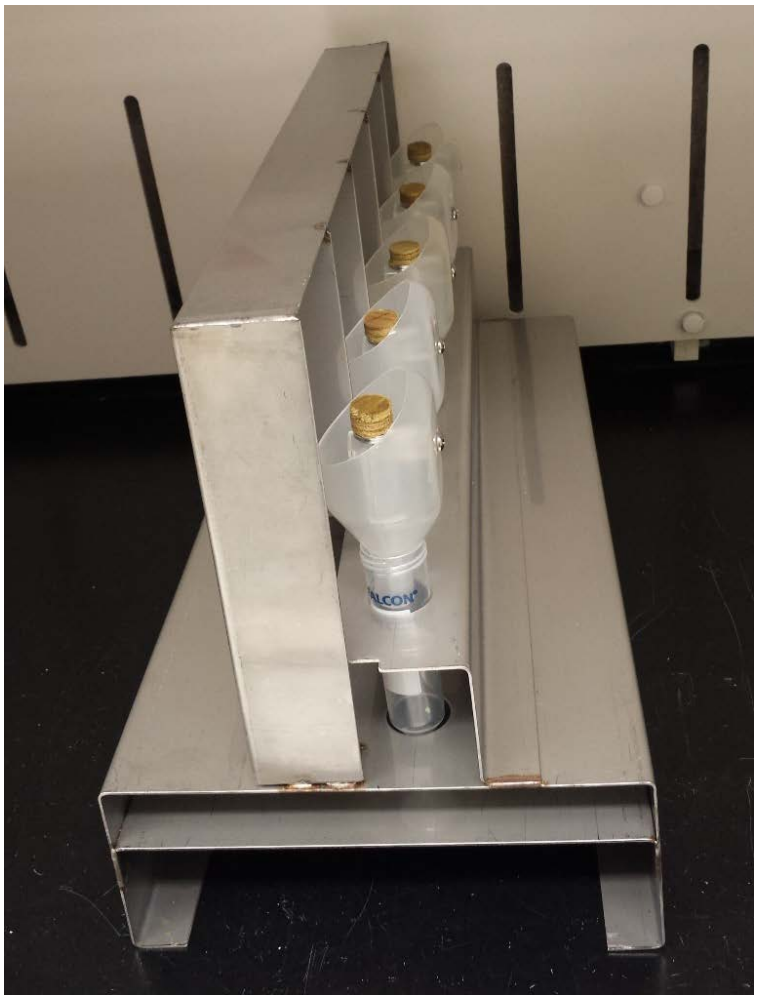


Figure 4.5-2. Side View of Spray Apparatus with Orifice Plate

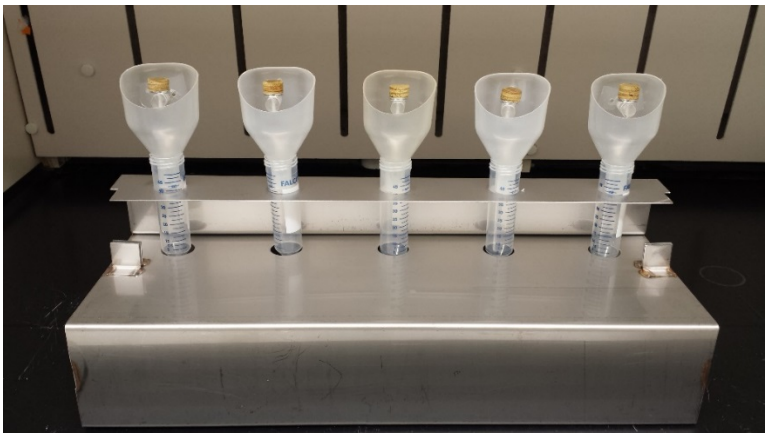


Figure 4.5-3. Front View of Spray Apparatus without Orifice Plate

In this apparatus, each test coupon was attached to a specially designed funnel connected to a conical 50-mL collection vial (Fisher Scientifics, BD Falcon, Catalog No. 352098) to retain runoff generated during spraying with the target decontaminant fluid. The coupon was attached horizontally though a custom-made connector.

The decontamination solution was applied using a 1.75-L chemical- and break-resistant, adjustable, commercial handheld sprayer (RL FloMaster Model No. 56HD) made of high-density polyethylene with a Viton seal. The handheld sprayer includes a pump trigger that can provide controlled delivery and was adjusted to deliver a fine mist to minimize runoff and dripping of the decontaminant. The sprayer also was fitted with a pressure gauge (≤30 psi). The entire spray bottle was disinfected by rinsing and purging with decontaminant solution three times before the bottle was filled with the final decontaminant solution of the target formulation.

The spray pattern was controlled by placing an orifice between the spray bottle and the sample to confine the spray as tightly as possible to the coupon. The spray nozzle was maintained at a distance of six in from the surface of the coupon. Pre-test method development trials were conducted to determine the spray duration required to fully wet the coupon surface yet minimize decontaminant solution runoff and overspray. These spray conditions and resulting decontaminant volumes were then used to determine the volumes of neutralizer required for pre-loading in the collection vials to fully quench decontaminant activity and achieve a precise contact time.

4.5.2 Decontamination Procedure for MS2 Testing (Small Coupons)

The small coupon efficacy testing for MS2 included 2% citric acid as well as pAB. Each coupon was inoculated with 1×10^8 PFU of MS2. Test samples were tested in quintuplicate, with exposure contact time of 30 minutes. Four material types (grimed concrete, grimed plywood, neat concrete, and neat plywood) were tested during this phase. The decontamination procedure for MS2 on small coupons is summarized below.

1. The commercial handheld sprayer was rinsed with decontamination solution three times to sterilize and disinfect the sprayer.
2. After triple-rinsing of the sprayer, the decontamination solution was discarded, and then the sprayer was filled with at least 500 mL of decontamination solution (the minimum volume required in the sprayer to ensure consistent spraying).
3. The sprayer was pressurized by pumping to 20 psi.
4. The conical tube was pre-loaded with the required amount of DE broth and PBST neutralizer solution.
5. The runoff collection vials were preloaded with the required amount of neutralizer solution.
6. The inoculated coupons were aseptically installed in the decontamination spray apparatus with 50-mL conical tube runoff collection vials.
7. The sprayer was held six in from the spray apparatus orifice plate, and each coupon was sprayed for 10 seconds. The five test coupons and one procedural control coupon were sprayed. The resulting volume of decontaminant used on each coupon was recorded.
8. The coupons were allowed to sit for the required 15-minute initial exposure time, and were then sprayed again for five seconds. The coupons were then allowed to sit for an additional 15-minute exposure time.
9. Each decontaminated coupon was aseptically extracted into its respective coupon collection vial after a total exposure time of 30 minutes.
10. The funnels were rinsed with sterile DI water, and this rinsate was collected in the rinsate collection vials. The total volume in each runoff collection did not exceed 35 mL.
11. Samples were transferred into sterile, primary, independent packaging within sterile, secondary containment in logical groups for analysis. All samples were accompanied by a completed chain of custody form.

Neutralizing Agents for Extracted Samples

A series of tests was conducted to identify the optimal neutralizing agent, if any, for each decontaminant and to determine its effectiveness in neutralizing (quenching decontaminant activity) and maintaining the integrity of the samples potentially containing viable *B. atrophaeus* spores or MS2. For *B. atrophaeus*, sodium thiosulfate (STS) was used as the neutralizing agent for pAB and potassium carbonate was used as the neutralizing agent for Spor-Klenz® RTU. For MS2, DE broth was used as the neutralizing agent for 2% citric acid and pAB.

This section discusses neutralization agent preparation, neutralization for large coupons, and neutralization for small coupons.

5.1 Neutralization Agent Preparation

STS was prepared in 1 L batches, in a 1-L volumetric flask. The solution was prepared at a 2 Normal (2 N) strength as described below.

1. STS pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$, 496.4 g) crystals were added to 1 L of DI water in an Erlenmeyer flask.
2. The solution was stirred until all the crystals dissolved completely.
3. Once the crystals dissolved completely in water, the solution was transferred to a 1-L volumetric flask. Additional DI water was added if needed until the lower meniscus of the solution aligned with the gradation line of the volumetric flask.
4. The 2 N STS solution was then sterilized using a bottle-top filter (150-mL Corning Bottle Top Filter, 0.22-µm cellulose acetate (CA), 33-mm neck, sterile, Catalog No. EK-680516, Corning, NY, USA). The liquid was poured 150 mL at a time into the top part of the filtration unit, which was connected to a sterile bottle to collect the filtrate. Vacuum was used to pull the liquid through the filter.
5. Each batch of STS solution prepared in-house was used within six months of preparation.

Potassium carbonate was prepared in 1 L batches, in a 1-L volumetric flask. The solution was prepared at a 2 Molar (2 M) strength as described below.

1. Potassium carbonate (K_2CO_3, 276.41 g) crystals were added to 1 L of DI water in an Erlenmeyer flask.
2. The solution was stirred until all the crystals dissolved completely.
3. Once the crystals dissolved completely in water, the solution was transferred to a 1-L volumetric flask. Additional DI water was added if needed until the lower meniscus of the solution aligned with the gradation line of the volumetric flask.
4. The 2 M K_2CO_3 solution then was sterilized using a bottle-top filter (150-mL Corning Bottle Top Filter, 0.22-µm CA, 33-mm neck, Sterile, Catalog No. EK-680516, Corning, NY, USA). The liquid was poured 150 mL at a time into the top part of the filtration unit, which was connected to a sterile bottle to collect the filtrate. Vacuum was used to pull the liquid through the filter.

5. Each batch of potassium carbonate solution prepared in-house was used within six months after preparation.

DE broth was prepared in 1 L batches, in a 1-L volumetric flask. The solution was prepared at a 1X (100%) strength as described below.

1. Dehydrated DE broth media granules (1,000 g) were added to 1,000 mL of DI water in an Erlenmeyer flask.
2. The flask was placed on a heated stir plate and heated gently to dissolve the broth granules completely.
3. The solution was carefully transferred to an autoclave-safe glass bottle and autoclaved at 121 °C for 15 minutes.
4. Each batch of DE broth prepared in-house was stored below 8 °C, protected from direct light.

5.2 Neutralization for Large Coupons

Neutralization of the sample extraction buffer for the large coupons required surface neutralization tests and neutralization tests for liquid effluents as discussed below.

5.2.1 Surface Neutralization Tests

Table 5.2-1 summarizes the tests conducted on large coupons necessary to investigate the need for neutralizer within sample extraction buffers after post-decontamination sampling during pAB or Spor-Klenz® RTU testing with *B. atrophaeus*.

Table 5.2-1. Neutralization Tests for Extractive Samples

Test	Decontaminant*	Material Type	Total No. of Coupons
A1	pAB	Concrete	3
A2	Spor-Klenz® RTU	Concrete	3
A3	None (water only)	Concrete	3
A4	pAB	Plywood	3
A5	Spor-Klenz® RTU	Plywood	3
A6	None (water only)	Plywood	3
*Decontaminant applied using sprayer			

These tests were performed before the testing of each decontamination solution using representative sets of concrete and plywood coupons. Completion of the test matrix was expected to provide information on whether or not neutralization of samples after collection was necessary.

Representative non-inoculated coupons were subjected to decontamination procedures and allowed to dry for approximately 18 hours before undergoing surface sampling. After sampling, the sample extracts were spiked with 1×10^7 CFU of *B. atrophaeus* spores. Recoveries for samples collected from decontaminated coupons were compared with recoveries for samples collected from extracted blank (not decontaminated) coupons (Tests A3 and A6). If a statistically significant difference existed for each coupon type between the two populations (CFU for decontaminated coupons vs. CFU from blanks

sprayed with water only), then it was determined that the samples required neutralization after collection. If needed, the various neutralizing solutions discussed in Section 5.2.1 were tested to confirm that the presence of the neutralizer did not bias recovery negatively. The spore inocula were enumerated in triplicate at the beginning, middle, and end of spiking.

5.2.2 Neutralization Tests for Liquid Effluents

The continued action of the decontamination agents (pAB, Spor-Klenz® RTU [H_2O_2 and PAA], and citric acid) in liquid effluents collected during the decontamination process (in runoff and rinsate water) may bias recovery. Therefore, as discussed in Section 4.4.4, carboys used to collect liquid effluents were pre-loaded with liquid neutralizer solution, to prevent loss of viable spores/plaques after the prescribed contact time.

Neutralization tests were performed before decontamination testing using concentrations of active ingredients determined in runoff and rinsate water during characterization tests of process-specific liquid effluents as described below.

Liquid Effluent Type 1

1. Blank coupons were assembled in the test chamber.
2. Three coupons of concrete or plywood material sprayed using a backpack or chemical sprayer were evaluated to account for inter-material differences of the decontamination procedure (material demand for sporicide and amount of runoff for each material, depending on porosity).
3. Clean Carboy No. 1 triple-rinsed with DI water was placed under the drain of the test chamber to collect liquid effluent type 1 (runoff of liquid decontaminant).
4. Liquid decontaminant was applied using a sprayer as described in Section 4.4.2.
5. A clean, sterile carboy (Carboy No. 1) loaded with neutralizer was placed under the drain of the test chamber to collect liquid effluent type 1 (runoff of liquid decontaminant) during the decontamination testing. A second clean sterile (Carboy No. 2) loaded with neutralizer was placed under the drain of the test chamber to collect liquid effluent type 2 (water from rinsing of the liquid decontaminant). Approximate volumes of material-specific runoff and rinsate were assessed during liquid effluent characterization tests (Section 5.2.2). The amount of neutralizer needed for each material type was determined in preliminary testing (see Section 4.9.2. for details).

Liquid Effluent Type 1

1. To characterize liquid effluent type 2 (water from rinsing of the liquid decontaminant), Steps 1 through 4 above were repeated using a clean Carboy No. 2 triple-rinsed with DI water placed under the drain of the chamber to collect liquid effluent type 2 (rinsate of liquid sporicide).
2. The volumes of the runoff or the rinsate were collected as composite samples from the spraying of the three coupons and determined using a graduated bucket of appropriate size.

3. The concentration of the decontamination agent (pAB, Spor-Klenz® RTU [H_2O_2 and PAA], and citric acid) was determined through titration using the appropriate methods. The pH and temperature measurements of runoff solution were recorded.

5.3 Neutralization for Small Coupons

The following series of tests was conducted on the small (18-mm -diameter) coupons:

- **Test I** to determine the DE broth neutralizer effectiveness to quench decontamination activity of the 2% (v/v) citric acid solution in DI water against MS2 inoculated on the small coupons with and without grime on their surfaces.
- **Test II** to determine the neutralizer buffer effectiveness of DE broth for samples that do not receive decontamination solution.
- **Test III** to determine a suitable extraction buffer for the samples and determine the maximum post-inoculation hold time for the coupons.

Each test is discussed below, followed by a discussion of method development test to determine the neutralizer volume.

5.3.1 DE Broth Neutralizer Effectiveness, Test I

Test I was designed to determine the neutralizer effectiveness in quenching the activity of the decontamination solution at a desired contact time on a coupon. Table 5.3-1 summarizes the test matrix.

Table 5.3-1. Test I Matrix

Material					
Concrete (grimed)	Test coupon	Yes	Yes	Yes	5
	Positive control coupon	No	Yes	No	3
	Procedural blank coupon	Yes	No	Yes	1
	Negative control coupon	No	No	No	1
Concrete and plywood	Runoff liquid test coupon tube	Yes	Yes	Yes	Five each for concrete and plywood
	Runoff liquid positive control coupon tube	Yes	Yes	No	Three each for concrete and plywood
	Runoff liquid negative control coupon tube	Yes	No	No	One each for concrete and plywood
	Test runoff collection tube	Yes	No	No	Five total
Plywood (grimed)	Test coupon	Yes	Yes	Yes	5
	Positive control coupon	No	Yes	No	3
	Procedural blank coupon	Yes	No	Yes	1
	Negative control coupon	No	No	No	1

A known quantity of surrogate organism (MS2) was deposited on small coupons of grimed materials: unpainted (smooth finish) concrete and pressure-treated plywood. For each decontamination test sequence set, five test and three positive control coupons were inoculated with MS2 inoculum using a positive displacement pipette (100-µL droplets). The target surface concentration for these experiments was 1×10^8 PFU.

The test coupons and 1 mL of 2% citric acid solution (the decontamination agent) were added simultaneously to 50-mL conical tubes, each containing 1 mL of the sterile DE broth and 10 mL of sterile PBST. The same operation was performed with positive control coupons that were transferred to 50-mL conical tubes containing only 10 mL of PBST. Procedural blank coupons underwent the same process as the test coupons.

Runoff liquids were also tested with and without neutralizer to determine their effectiveness. For these tests, five mL of citric acid decontamination solution (corresponding to the runoff from the spray-down decontamination method) was added to test runoff collection vials (with DE broth) inoculated with 1×10^8 PFU of MS2. The samples were extracted for two hours, and 18 hours, after inoculation, and then plated the same day for Day 0 testing, and re-plated 18 hours later for Day 1 testing. Table 4.5-1 summarizes the matrix for the runoff liquid tests. The procedure summarized below was used for this test.

1. Ten 50-mL conical tubes were prepared, each containing one mL of sterile DE broth and 10 mL of PBST. These tubes were the test coupon collection vials: five for the concrete test coupons and five for the plywood test coupons.
2. Six 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and 10 mL of sterile PBST. These tubes were the positive control coupon collection vials (that is, they were inoculated with MS2): three for the concrete positive control coupons and three for the plywood positive control coupons.
3. Two more 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and 10 mL of sterile PBST. These tubes were not inoculated with MS2 and served as negative control coupon tubes (one each for concrete and plywood).
4. Five 50-mL conical tubes were prepared, each containing five mL of the sterile DE broth and five mL of sterile DI water. These tubes were the test runoff collection vials.
5. To each test coupon collection tube, one mL of the 2% citric acid solution was added to a test coupon collection tube. Simultaneously, a grimed concrete coupon was aseptically transferred to a test coupon collection tube. This step was conducted on all the concrete and plywood test coupon tubes. The neutralizer to decontamination solution ratio was 1:1.[23]
6. A sterile concrete coupon was aseptically transferred to a positive control coupon tube. This step was conducted on all concrete and plywood positive control and negative control coupon tubes.
7. To each of the test runoff collection vials, five mL of citric acid solution was added (corresponding to the runoff from the spray-down decontamination method).
8. The sample tubes were transported to the analytical laboratory with appropriate chain of custody for inoculation and microbial analysis.

9. Each tube was inoculated with MS2 inoculum using a positive displacement pipette (100-µL droplets). The target surface concentration for these experiments was 1 × 108 PFU.

5.3.2 Neutralizer Buffer Effectiveness, Test II

The Test II evaluations were conducted to determine the effectiveness of DE broth as a neutralizing buffer for samples that did not receive decontamination treatment. This test was designed to determine how the reaction between traditionally used PBST buffer and the DE broth (without the presence of citric acid) affects recoveries of MS2. Table 5.3-2 summarizes the test matrix.

Table 5.3-2. Test II Matrix

Material					
Concrete (Grimed)	Test coupon	Yes	Yes	Yes	5
	Positive control coupon	Yes	Yes	No	5
	Negative control coupon	No	No	No	1
Plywood (Grimed)	Test coupon	Yes	Yes	Yes	5
	Positive control coupon	Yes	Yes	No	5
	Negative control coupon	No	No	No	1

The procedure summarized below was used for Test II.

1. Ten 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and 10 mL of sterile PBST. These tubes were the test coupon collection vials.
2. Twelve 50-mL conical tubes were prepared, each containing 10 mL of sterile PBST. Ten of these tubes were the positive control coupon collection vials, and two were the negative control coupon collection vials.
3. A coupon was aseptically transferred to each test and each positive control coupon collection tube. This step was conducted for all concrete and plywood test coupons.
4. The sample tubes were transported to the analytical laboratory with appropriate chain of custody for inoculation and microbial analysis.
5. Each tube was inoculated with MS2 using a positive displacement pipette (100-µL droplets). The target surface concentration for these experiments was 1×10^8 PFU

5.3.3 Suitable Extraction Buffer and Inoculation Hold Time, Test III

The Test III evaluations were conducted to determine the optimal extraction buffer for the samples and determine the post-inoculation hold time for the coupons. The following four extraction buffers were tested and compared:

- DI water

- PBST
- PBS
- TSB.

The following materials were tested:

- Concrete (grimed and neat)
- Pressure-treated plywood (grimed and neat)

The samples were analyzed at two different time points:

- Two hours after inoculation (Day 0)
- One day after inoculation (Day 1)

Table 5.3-3 summarizes the test matrix.

Table 5.3-3. Test III Matrix

Material	Type	Buffer	No. of Day 0 Replicates	No. of Day 1 Replicates
Concrete	Grimed	DI water	2	2
		PBST	2	2
		PBS	2	2
		TSB	2	2
	Neat	DI water	2	2
		PBST	2	2
		PBS	2	2
		TSB	2	2
Plywood	Grimed	DI water	2	2
		PBST	2	2
		PBS	2	2
		TSB	2	2
	Neat	DI water	2	2
		PBST	2	2
		PBS	2	2
		TSB	2	2

The procedure summarized below was used for Test III.

Day 0

1. The grimed and neat concrete 18-mm coupons were inoculated with 0.1 mL of the MS2 suspension and allowed to dry for two hours.

2. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile DI water. These tubes were the DI water test coupon collection vials.
3. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile PBST. These tubes were the PBST test coupon collection vials.
4. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile PBS. These tubes were the PBS test coupon collection vials.
5. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile TSB. These tubes were the TSB test coupon collection vials.
6. One coupon was aseptically transferred to each of the test collection vials previously prepared.
7. The sample tubes were transported to the analytical laboratory with appropriate chain of custody for inoculation and microbial analysis.

Day 1

1. The grimed and neat concrete coupons were inoculated with 0.1 mL of the MS2 suspension and allowed to dry overnight (18 hours).
2. After the drying period, eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile DI water. These tubes were the DI water test coupon collection vials.
3. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile PBST. These tubes were the PBST test coupon collection vials.
4. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile PBS. These tubes were the PBS test coupon collection vials.
5. Eight 50-mL conical tubes were prepared, each containing one mL of the sterile DE broth and nine mL of sterile TSB. These tubes were the TSB test coupon collection vials.
6. One coupon was aseptically transferred to each of the test collection vials previously prepared.
7. The sample tubes were transported to the analytical laboratory with appropriate chain of custody for inoculation and microbial analysis

5.3.4 Method Development Test for Neutralizer Volume Determination

A series of tests was performed to determine the required volume of the neutralizer broth and PBST needed to be preloaded into the 50-mL conical coupon collection vials (BD Falcon, Catalog No. 352098) (described in Section 4.3) to achieve complete quenching of the decontaminant. This testing was conducted as summarized below.

1. The spray apparatus was aseptically set up with coupons and rinsate collection vials. The runoff collection vials, coupon collection vials, and coupons were weighed before installation. Three replicates per coupon material were used.
2. Each coupon was sprayed with the decontamination solution and allowed to sit for an initial exposure time of 15 minutes.

3. After the initial 15-minute exposure time, the coupon was again sprayed with the decontamination solution and allowed to sit for an additional 15 minutes.
4. After a total of 30 minutes of exposure time, the coupon was aseptically placed into a coupon collection vial for extraction.
5. The rinsate and coupon collection vials were weighed, and the difference in weight was recorded. The recorded weights were used to calculate the volume of decontamination solution as collected on the coupon and as rinsate.
6. An equivalent portion of neutralization solution (1:1 neutralizer-to-citric-acid volume ratio) was determined for the highest sprayed volume of rinsate collected. For example, if three mL of runoff was collected, three mL of DE neutralizing broth was determined as the volume of the neutralization solution.
7. Similarly, depending on the amount of decontamination solution collected on the coupon, the corresponding neutralizer volume was determined using a 1:1 ratio.

Sampling Approach

A sampling event log sheet was maintained for each sampling event (or test) that included each sampling team member's name, the date, run number, and all sample codes with corresponding coupon codes. The coupon codes were pre-printed on the sampling event log sheet before sampling began. Digital photographs of selected coupons with a noticeable change due to the decontamination procedure were taken after the completion of sampling for all coupons during an event or test. Pre- and post-decontamination photographs were taken for three representative coupons of each material type.

Table 6-1 lists the materials and equipment used for sampling.

Table 6-1. Sampling Materials and Equipment

Material or Equipment	Description
Non-powdered, sterile surgical gloves	KIMTECH PURE* G3 Sterile Nitrile Gloves, Kimberly-Clark (VWR P/N HC61110 for extra-large, VWR P/N HC61190 for large, and VWR P/N HC61180 for medium)
Non-powdered, non-sterile surgical gloves	Examination gloves (Fisherbrand Powder-Free Nitrile Exam Gloves, Fisher P/N 19-130-1597D for large and 19-130-1597C for medium)
Dust masks	3M Particulate Respirator 8271, P95
Disposable laboratory coats	Kimberly-Clark Kleenguard A10 Light Duty Apparel, P/N 40105
PBS	PBS with PBST (Sigma Aldrich USA, P/N: P3563-10PAK)
50-mL conical tubes	BD Falcon® BlueMax Graduated Tubes, 15-mL (Fisher Scientific P/N 14-959-70C)
Sterile sampling bags	Fisherbrand Sterile Sampling Bags (TWIRL'EM) Overpack Size 10- by 14-in Inner bag size: 5.5- by 9-in (wipe) Sample bag size: 5.5- by 9-in
Bleach wipes	Dispatch® Bleach Wipes (Chlorox Co., Oakland, CA)
Wipes	Kendall Curity Versalon absorbent gauze sponge, 2- by 2-in, sterile packed (rayon/polyester blend) (http://www.mfasco.com/ , last accessed June 14, 2016)
Swabs	Bacti Swab® (http://www.remelinc.com/Industrial/CollectionTransport/BactiSwab.aspx , last accessed June 14, 2016)
Carboys (2)	Nalgene autoclavable carboys with tabulation (20 L) (Fisher Catalog No. 02-690-23)
Analytical filter units	150-mL Nalgene analytical filter units (0.2-µm cellulose acetate) (Fisher Catalog No. 130-4020)
Vacuum pump	Gast oil-free vacuum pump with adjustable suction (Fisher Cat# 01-092-25)
Tubing	Fisher polyvinyl chloride (PVC) clear tubing (1/2-in-inside diameter, 1/16-in thick) (Fisher Catalog No. Cat# 14-169-7J) Fisher PVC clear tubing (3/8-in-inside diameter, 1/16-in thick) (Fisher Catalog No. 14-169-7G) Fisher PVC clear tubing (vacuum tubing), (3/8-in-inside diameter, 1/8-in thick) (Fisher Catalog No. 14-169-7H)
Filter cassettes	Via-Cell® Bioaerosol Sampling Cassette (http://www.zefon.com/store/via-cell-bioaerosol-sampling-cassette.html , last accessed June 14, 2016)
Sampling pump	Isokinetic Method 5 Source Sampling Console (http://www.apexinst.com/product/xc-50-method-5-source-sampling-console)

The following sections discuss the sampling strategy, sampling methods, and sample handling.

6.1 Sampling Strategy for Large Coupons

The following sections discuss the sample types, sample quantities, sampling and monitoring points, and frequency of sampling and monitoring events.

6.1.1 Sample Types

The three major sample types are discussed below.

- **Surface samples** include surface wipe samples from each material collected in sets of five, positive control surface samples collected in sets of three, and procedural blank surface samples.
- **Liquid runoff and rinsate samples** were collected to assess the potential for viable microorganisms to be washed off the surfaces. Samples were collected from all liquid runoff and rinsates from the decontamination process and from post-decontamination chamber cleaning. These samples were analyzed quantitatively for each test. In addition, liquid decontamination solution was sampled and verified before each decontamination procedure began.
- **Aerosol samples** were collected using Via-Cell® bioaerosol cassettes during each decontamination and procedural blank test. Results for these samples were used to estimate the occurrence and magnitude of fugitive emissions of viable *B. atrophaeus* or MS2 during the decontamination process.

6.1.2 Sample Quantities

For each coupon, only one wipe sample was taken. The liquid waste samples were composite samples collected from a set of five (5) test coupons (liquid runoff and rinsate samples were collected separately). One aerosol sample per material was collected for the entire test decontamination procedure. Table 6.1-1 lists the total numbers of samples of each type for each test.

Table 6.1-1. Sample Types and Numbers for Each Decontamination Test

Sample Type	
Positive control	3
Inoculation control	3
Test sample (decontaminated)	5
Procedural blank	1
Laboratory blank	1
Wipe field blank	1
Liquid samples	3
Aerosol	1
Swab	2 per item

6.1.3 Sampling and Monitoring Points

The front face of each coupon was the only surface sampled in this study. All coupons were sampled using wipes. The liquid runoff from a coupon set was collected during the application of the decontamination solution. Each runoff sample was from a combination of all five coupons. In addition to runoff samples, an aerosol sample was collected during the active spraying phase for each coupon set. Aerosol samples were collected from the bulk volume of the chamber containing the coupons for each decontamination method.

6.1.4 Frequency of Sampling and Monitoring Events

Table 6.1-2 summarizes the frequency of sampling and monitoring events for each decontamination testing sequence as well as the measurement method or equipment and the sampling ranges.

Table 6.1-2. Sampling Frequencies

Testing Sequence	Measurement Method or Equipment	Range	Frequency
Decontamination Formulation			
2% citric acid solution	Titration	2%	Once before and after testing
	Oakton pH probe	1.98 to 2.02 pH unit	
Decontamination Testing			
For each decontamination test	Decontamination solution negative controls (PFU, CFU)	0 to 200 PFU/CFU per sample per filter	One sample per material type
	Laboratory blank solution (PFU, CFU)		One sample per neutralization test
	Positive controls (PFU, CFU)	0 to 1 x 10^8 PFU or CFU per coupon material, 20 to 200 per plate	Three samples per material type
	Test samples (PFU, CFU)		Five samples per material type

6.2 Sampling Methods

The following sections discuss the methods used for wipe, runoff and rinsate, aerosol, and QA/QC sampling.

6.2.1 Wipe Sampling

6.2.1.1 Wipe Sampling Preparation

For *B. atrophaeus* testing, the large coupons were allowed to dry for 18 hours before sampling. For MS2 testing, the coupons were sampled 15 minutes after decontamination (15 minutes after completion of the water rinse). All coupons were placed horizontally for sampling regardless of their orientation during the decontamination process. Sample volumes, time of day, and observations were recorded in laboratory notebooks.

The general approach for wipe sampling was to use a moistened, sterile, non-cotton gauze pad to wipe the area to recover bacteria or viruses. A three-person team was used, employing an aseptic technique throughout. The team consisted of a sampler, coupon handler, and support person.

The surface area sampled was one square foot. Wipe samples were collected within an area measuring 12- by 12-in using a sampling template centered on the coupon as shown in Figure 6.2-1. The outer 1.0-in around each coupon was not sampled to avoid unrepresentative edge effects.

Figure 6.2-1. Sampling Template Centered on Heavily Grimed Large Plywood Coupon

Before the sampling event, all materials needed for sampling were prepared using aseptic techniques. Table 6-1 lists all the materials used for sampling. Non-powdered surgical gloves were used during sampling. Individually wrapped pre-moistened bleach wipes (Hype-Wipe - current technologies, Indianapolis, Indiana), used for sample bag decontamination, were placed in sterile sampling bags. Alternatively, Dispatch® bleach wipes were used. A sampling material bin was stocked for each sampling event based on the sample quantity. The bin contained enough wipe sampling kits to accommodate all required samples for the specific test. Five additional kits also were on hand for backup. Enough prepared packages of gloves and bleach wipes were included in the bin as well as extra gloves and wipes.

Paper sampling templates each measuring 1.17- by 1.17-feet with an interior opening of 12- by 12-in were prepared, sterilized, and packaged in sterile bags (10 templates per bag). These bags of templates were included in the sampling kits. A sample collection bin was used to transport samples to the NHSRC RTP Microbiology Laboratory. The exterior of the transport container was decontaminated by wiping all surfaces with bleach wipes or towelettes moistened with a solution of pAB before transport to the NHSRC RTP Microbiology Laboratory.

6.2.1.2 Wipe Sampling Procedure

A three-person team was used, employing aseptic technique throughout. The team consisted of a sampler, sample handler, and support person who followed a strict sampling protocol to avoid any potential cross-contamination among coupons, or among samples. Throughout the procedure, the support person logged anything deemed to be significant into the laboratory notebook and handled the sampling kits (pre-moistened all-purpose sponge, conical tube, sampling bags, etc). The sample handler handled the sample coupon and placed it on the sampling area, being careful to handle the coupon only around the edges. The sampler conducted the sampling as follows:

- Wipe the surface of the sample horizontally using S-strokes to cover the entire sample area of the coupon using a consistent amount of pressure.
- Fold the all-purpose sponge concealing the exposed side and then wipe the same surface vertically using the same technique.
- Fold the all-purpose sponge over again and roll up the folded sponge to fit into the conical tube.
- Carefully place the all-purpose sponge into the 50 mL conical tube that the support person is holding, being careful not to touch the surface of the 50 mL conical tube or plastic sterile sampling bag.

For each single test, surface sampling of the materials was completed for all procedural blank coupons ***before*** sampling of any test material. Positive controls were sampled.

6.2.2 Runoff and Rinsate Sampling

Rinsate samples were collected during the decontamination procedure. Liquid effluents from the decontamination process were collected into sterilized carboys. The two types of liquid effluent samples summarized below were collected.

- Runoff is defined as excess liquid decontaminant applied to the coupon surface that flowed from the surface. Runoff samples were collected as one composite sample for each decontamination test (composite of five test coupons subjected to the decontamination procedure).
- Rinsate is defined as water used to remove residual decontaminant from the coupons after active decontamination (post-decontamination rinsate).

Note: Neutralizer was prepared in the collection vessel before collection of liquid samples so that the active (sporicidal) ingredient was neutralized as the sample was being collected, not after collection.

Before decontamination, Carboy No. 1 (used for runoff collection, sterile and pre-weighed) was charged with enough neutralization solution to neutralize all residual decontamination solution collected. After the active decontamination concluded, Carboy No. 2 (pre-weighed carboy) was charged with enough neutralization solution to neutralize all decontamination solution collected from the water rinsing step. Therefore, any residual decontaminant was neutralized upon collection.

The runoff and rinsate from the coupons was collected throughout the entire decontamination procedure for a given coupon set (material type or all blanks).

After collection, 1,000-mL aliquots were collected using the aseptic technique summarized below.

- For each carboy, the total mass of liquid collected was recorded to compare the final versus the initial weight.
- The contents of the carboy were agitated to homogenize.
- The carboy cap was removed.
- Using a new 100-mL sterile serological pipette, 10 x 100 mL (1 L total) aliquots of sample were aseptically withdrawn into a sterile 1-L container.
- Sterile bags were used as secondary and tertiary containment during sample storage and transport to the NHSRC RTP Microbiology Laboratory for analysis at the conclusion of the entire test. Samples were processed immediately. If, due to the test schedule, liquid samples must be stored, they were refrigerated at 4 °C ± 2 °C until processed (within 24 hours).

6.2.3 Aerosol Sampling

The use of high-pressure sprayers was expected to generate aerosols that could contain viable spores or viruses removed from the coupon surfaces. Therefore, aerosol samples were collected during the entire decontamination process. A single composite aerosol sample was collected for all five coupons. Aerosol samples were collected from the chamber exhaust duct using Via-Cell® bio-aerosol cassettes.

A 4-in diameter, 44-foot long, flexible galvanized duct was attached to the test chamber to allow precise flow measurements and sampling. The duct was attached to the chamber using a coupling and a 90-degree elbow. The sampling port was located 32 in (eight diameters) downstream from the 90-degree elbow connected to the chamber and 12 in (three diameters) from the bend in the duct connected to the main exhaust. Figure 6.2-2 shows the sampling point location with a white arrow symbol.

Figure 6.2-2. Test Chamber Exhaust Duct (white arrow shows sampling point location)

The flow through the Via-Cell® bioaerosol cassettes was isolated as much as possible to minimize potential contamination from the laboratory environment.

6.2.4 QA/QC Sampling

The additional QA/QC samples summarized below were collected.

- Swab samples for were used for sterility checks on coupons and equipment before use in the testing. A single, pre-moistened swab sample was collected from each item and coupon.
- Material samples and field samples for biological DQIs were collected. Results from these samples provided information on the level of contamination possibly present during sampling due to contaminated materials. These samples were referred to as unexposed field blank samples. Blank plating of microbiological supplies was conducted to provide controls for testing the sterility of supplies used in dilution plating.
- Grime samples were collected and analyzed for each batch of grimed coupons as a sterility check.
- Decontamination solution samples for chemical DQIs were evaluated before each decontamination event.

6.3 Sampling Strategy for Small Coupons

The following sections discuss the sample types, sample quantities, sampling and monitoring points, and frequency of sampling and monitoring events.

6.3.1 Sample Types

The two major sample types are discussed below.

- **Surface samples** included coupon samples from each material collected in sets of five for surface extraction samples (as discussed in Sections 4.4 and 4.5), positive control surface samples collected in sets of three, and procedural blank surface samples.
- **Liquid rinsate samples** were collected to assess the potential for viable microorganisms to be washed off the surfaces. Samples were collected from all liquid runoff and the funnels were rinsed with sterile deionized water where rinsate was collected in the same vials as runoff collected as a single composite sample. These samples were analyzed quantitatively for each test. In addition, liquid decontamination solution was sampled and verified before each decontamination procedure began.

6.3.2 Sample Quantities

For each coupon, only one wipe sample was taken. The liquid waste samples were composite samples collected from a set of five (5) test coupons (liquid runoff and rinsate samples were collected together as a composite sample). Table 6.1-3 lists the total numbers of samples of each type for each test.

Table 6.1-3. Sample Types and Numbers for Each Decontamination Test

Sample Type	
Positive control	3
Test sample (decontaminated)	5
Procedural blank	1
Laboratory blank	1
Liquid runoff/rinsate	5
Decontamination Solution	1

6.3.3 Sampling and Monitoring Points

All coupons were extracted in a vial containing PBST and DE Broth. The liquid runoff/rinsate from a coupon set was collected during the application of the decontamination solution. The run/rinsate vials were pre-loaded with DE Broth.

6.3.4 Frequency of Sampling and Monitoring Events

Table 6.1-4 summarizes the frequency of sampling and monitoring events for each decontamination testing sequence as well as the measurement method or equipment and the sampling ranges.

Table 6.1-4. Sampling Frequencies

Testing Sequence	Measurement Method or Equipment	Range	Frequency
Decontamination Formulation			
2% citric acid solution	Titration	2%	Once before and after testing
	Oakton pH probe	1.98 to 2.02	
Decontamination Testing			
For each decontamination test	Decontamination solution negative controls (PFU, CFU)	0 to 200 PFU/CFU per sample per filter	One sample per material type
	Laboratory blank solution (PFU, CFU)		One sample per neutralization test
	Positive controls (PFU, CFU)	0 to 1 x 10^8 PFU/CFU per coupon material, 20 to 200 per plate	Three samples per material type
	Test samples (PFU, CFU)		Five samples per material type

6.4 Sample Handling

This section discusses the sample containers and sample preservation.

6.4.1 Sample Containers for Large Coupons

For each wipe sample and grime sample, the primary containment container was an individual sterile 50-mL conical tube. Secondary and tertiary containment consisted of sterile sampling bags.

For aerosol sampling, each Via-Cell® bioaerosol cassette was placed into a sterile foil bag and zipped closed. The red safety seal label was applied over the top of the foil bag opening to ensure sample integrity until analysis. The foil bag containing the cassette was then placed inside a pre-labeled 5.5- by 15-in sterile bag for tertiary containment.

Liquid effluent samples were collected in individual sterile specimen cups or Nalgene bottles placed inside pre-labeled sterile bags for secondary containment.

A large plastic container was used for storage of sampling kits in the decontamination laboratory during testing and for transport of kits post-collection to the NHSRC RTP Microbiology Laboratory.

6.4.2 Sample Containers for Small Coupons

For each coupon, a runoff/rinsate sample was extracted in an individual sterile 50-mL conical tube.

A large plastic container was used for storage of sampling kits in the decontamination laboratory during testing and for transport of kits post-collection to the NHSRC RTP Microbiology Laboratory.

6.4.3 Sample Preservation for Large Coupons

After sample collection for large coupons, sample integrity was maintained by storing the samples in four containers (one sample collection container, one sterile inner bag, one sterile outer bag with the exterior sterilized during the sample packaging process, and one sterile container holding all samples from a test). All individual sample containers remained sealed while in the decontamination laboratory and during transport.

6.4.4 Sample Preservation for Small Coupons

After sample collection for small coupons, the individual sample vials remained sealed while in the decontamination laboratory and during transport to the NHSRC RTP Microbiology Laboratory.

Analytical Procedures

This section discusses analytical procedures for microbiological analyses, filtration and plating of bacteria from liquid extracts, and plating of MS2 from liquid extracts.

7.1 Analytical Procedures for Microbiological Analyses

Table 7.1-1 lists the analytical procedures used for this project.

Table 7.1-1. Analytical Procedures

Matrix						
B. atrophaeus inoculated surfaces	CFU/area	Wipe sampling	Filtration and plating of *B. atrophaeus* from liquid extracts	Triple-bagged	Refrigeration	72 hours
MS2 inoculated surfaces	PFU/area	Wipe sampling	Filtration and plating of MS2 from liquid extracts	Triple-bagged	Refrigeration	1 hour
Surface sterility checks	Growth or no growth	Swab sampling	Filtration and plating of *B. atrophaeus* and MS2 from liquid extracts	Swab container	Refrigeration	1 to 72 hours
Grime	CFU/volume or PFU/volume	Aseptic bulk collection	Filtration and plating of *B. atrophaeus* and MS2 from liquid extracts	Sterile specimen cup and double-bagged	Refrigeration	1 to 24 hours
Solid sample roller	CFU/sample or PFU/volume	Aseptic bulk collection	Filtration and plating of *B. atrophaeus* and MS2 from liquid extracts	Double-bagged	Refrigeration	1 to 24 hours

7.2 Filtration and Plating of Bacteria from Liquid Extracts

The NHSRC RTP Microbiology Laboratory analyzed all samples for presence (sterility check samples) and to quantify the CFU per sample. For all sample types, PBST was used as the extraction buffer. After the extraction procedure, the buffer was subjected to a five-stage serial dilution (10^{-1} to 10^{-5}). The resulting samples were plated in triplicate and incubated overnight (minimum of 18 hours) at 35 °C ± 2 °C. After incubation, CFU were enumerated manually. If the number of CFU on all three plates did not fall between 30 and 300 CFU and/or was less than 30 CFU, filter plating or re-plating procedures were conducted in an attempt to quantify recoveries at the lowest level possible. Figure 7.2-1 shows a dilution and a filter plate with colonies of *B. atrophaeus*.

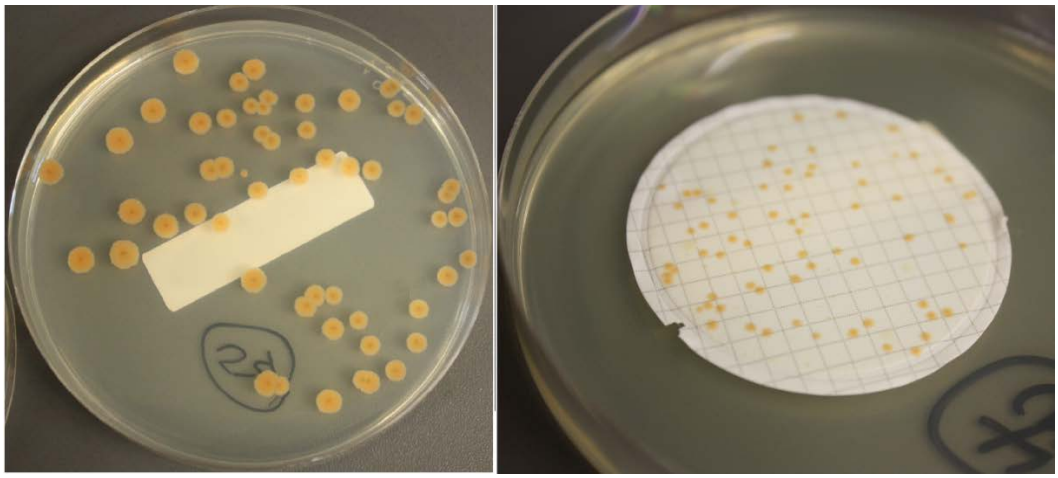


Figure 7.2-1. Dilution Plate (Left) and Filter Plate (Right) Showing Colonies of *B. atrophaeus*

7.3 Plating of MS2 from Liquid Extracts

The NHSRC RTP Microbiology Laboratory analyzed all samples for presence (sterility check samples) and to quantify the PFU per sample. For all sample types, PBST was used as the extraction buffer. After the extraction procedure, the buffer was subjected to a five-stage serial dilution (10^{-1} to 10^{-5}). The resulting samples were plated in triplicate and incubated overnight (minimum of 18 hours) at 35 °C ± 2 °C. After incubation, PFU were enumerated manually. Due to the limited stability of MS2 in sample storage, additional dilutions of samples were plated during initial plating. Figure 7.2-2 shows a dilution plate showing plaques of MS2.

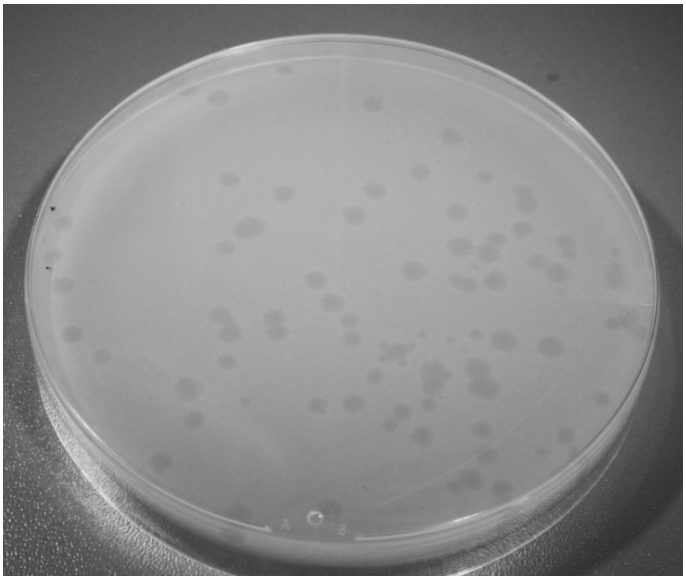


Figure 7.2-2. Dilution Plate Showing PFU of MS2

7.4 Data Reduction

Data reduction was performed on measurements of the total spores (CFU) or virus (PFU) recovered from each replicate coupon; average recovered CFU or PFU and standard deviation (STD) for each group of coupons. The groups of coupons included the following for each combination of material type and extracted sample type:

- Positive control areas (replicates, average, STD)
- Test areas (replicates, average, STD)
- Procedural blank coupons.

Efficacy is defined as the extent (by LR) to which the agent recovered from the surface of the coupons after the decontamination procedure has been reduced from the positive control areas (not exposed to the decontamination procedure). Efficacy was calculated using Equation 7-1, below, for each material within each combination of decontamination procedure (i) and test material (j).

$$LR_{ij} = \sum_{c=1} \log(XFU_{ijc}) / N_{ijc} - \sum_{k=1} \log(XFU_{ijk}) / N_{ijk} \quad [7\text{-}1]$$

where:

LR_i	=	the average log reduction of spores on a specific material surface
$\sum_{c=1} \log(XFU_{cj}) / N_C$	=	the average of the logarithm of the number of viable spores (determined by CFU) or viral particles (PFU) recovered on the control coupons [*C* = control, *j* = coupon number, and N_C = the number of coupons (1, *j*)]
$\sum_{k} \log(XFU_S) / N_t$	=	the average of the logarithm of the number of viable spores (determined by CFU or PFU) remaining on the surface of a decontaminated coupon [*S* = decontaminated coupon, *k* = coupon number, and N_t = the number of coupons tested (1, *k*)]

X = C for colony, and X=P for plaque

When no viable surrogates were detected, the detection limit of the sample was used, and the efficacy was reported as greater than or equal to the value calculated using Equation 9-1.

The cumulative standard deviation for the LR is calculated as follows: Let S_{Un} and S_{Tr} denote the standard deviations of the log reduction values for the untreated carriers (positive controls) and the treated carriers (post-decontamination samples), respectively. Then, the cumulative standard deviation is calculated as follows:

$$S_{LR} = [(S^2_{Un} / n_{Un}) + (S^2_{TR} / n_{Tr})]^{1/2} \qquad 7\text{-}2$$

where n_{Un} and n_{Tr} designate, respectively, the number of control and post-decontamination samples.

Results and Discussion

This section discusses the results of the tests evaluating common decontamination methods for inactivation of *B. atrophaeus* and MS2 on two test material surfaces (concrete and plywood), with and without agricultural grime. The test materials were loaded with an agricultural grime surrogate that reflects challenging environments expected during agricultural facility decontamination. The decontamination solutions investigated were pAB and Spor-Klenz® RTU for *B. atrophaeus*-contaminated large coupons and a solution of 2% (v/v) citric acid in DI water and pAB against MS2 inoculated on small and large coupons. The following sections discuss the results for *B. atrophaeus* and MS2 testing.

8.1 *B. atrophaeus* Decontamination Testing

This section discusses the testing results for extraction efficacy from neat and heavily grimed surfaces for the large coupons, neutralizing agent testing for extracted samples, and *B. atrophaeus* decontamination testing using Spor-Klenz® RTU and pAB.

8.1.1 Extraction Efficacy from Neat and Heavily Grimed Surfaces (14-in x 14-in Coupons)

During scoping tests, grimed and neat test coupons were inoculated with 1×10^7 spores of *B. atrophaeus* by aerosol deposition. After the settling period, each coupon was wipe-sampled. The recoveries from the grimed and neat test coupons were compared to recoveries from the neat stainless steel inoculation controls. Table 8.1-1 summarizes the results. The results suggest that the addition of the grime affected the recovery of the surrogate spores by almost one order of magnitude. The presence of grime prevented the target recovery of 6 logs from being achieved on wood and concrete.

Table 8.1-1. *B. atrophaeus* Recovery from Grimed and Neat Surfaces

Test Results	Log CFU	CFU	Average	STD	Recovery[1] (%)
Neat Stainless Steel Inoculation Control (inoculated @ E07)					
Control coupon 1	7.4	2.28E+07	2.59E+07	2.95E+06	100
Control coupon 2	7.5	2.87E+07			
Control coupon 3	7.4	2.63E+07			
Neat Concrete Control Coupons					
Test coupon 1	6.1	1.29E+06	3.41E+06	1.85E+06	13.2
Test coupon 2	6.7	4.66E+06			
Test coupon 3	6.6	4.29E+06			
Grimed Concrete Coupons					
Test coupon 1	5.2	1.54E+05	2.02E+05	6.76E+04	0.78
Test coupon 2	5.2	1.73E+05			
Test coupon 3	5.4	2.79E+05			
Concrete Procedural Blanks (not inoculated)					
Procedural blank 1	Non-detects				

Test Results	Log CFU	CFU	Average	STD	Recovery[1] (%)
Neat Wood Control Coupons					
Test coupon 1	6.4	2.77E+06	3.45E+06	1.11E+06	13.3
Test coupon 2	6.7	4.74E+06			
Test coupon 3	6.5	2.85E+06			
Grimed Wood Coupons					
Test coupon 1	5.5	3.30E+05	3.99E+05	6.07E+04	1.54
Test coupon 2	5.6	4.21E+05			
Test coupon 3	5.6	4.45E+05			
Wood Procedural Blanks (not inoculated)					
Procedural blank 1	Non-detects				

[1]Material Recovery was calculated as percent of neat Stainless Steel Recovery (Control Coupons)

8.1.2 Neutralizing Agent Testing for Extracted Samples

A series of tests was conducted to identify if a neutralizing agent is warranted to neutralize residual decontaminant on the surfaces of the coupons after a decontamination event. The coupons were allowed to dry overnight before sampling using wipes. The results from these decontaminated coupons were compared with results for spiked samples from extracted blank (not decontaminated) coupons (Tests A3 and A6).

The results presented in Table 8.1-2, and illustrated in Figure 8.1.1 show no reduced recoveries resulting from residual decontaminant, for both decontaminants (pAB and Spor-Klenz® RTU), suggesting there is no need to neutralize the coupon surfaces after decontamination. However, STS (at 2 N [normal]) and potassium carbonate (at 2 M [molar]) were used as neutralizing agents for pAB and Spor-Klenz® RTU in runoff and rinsate samples.

Table 8.1-2. Neutralization Test Results for *B. atrophaeus* Extracted Samples

Test	Decontaminant*	Material Type	*B. atrophaeus* Recovery (CFU)	
			Average	STD
A1	pAB	Concrete	1.69E+07	2.14E+06
A2	Spor-Klenz® RTU	Concrete	2.12E+07	4.74E+06
A3	None (water only)	Concrete	1.65E+07	1.96E+06
A4	pAB	Plywood	1.66E+07	3.27E+06
A5	Spor-Klenz® RTU	Plywood	2.15E+07	1.69E+06
A6	None (water only)	Plywood	2.07E+07	3.69E+06

* Decontaminant applied using sprayer

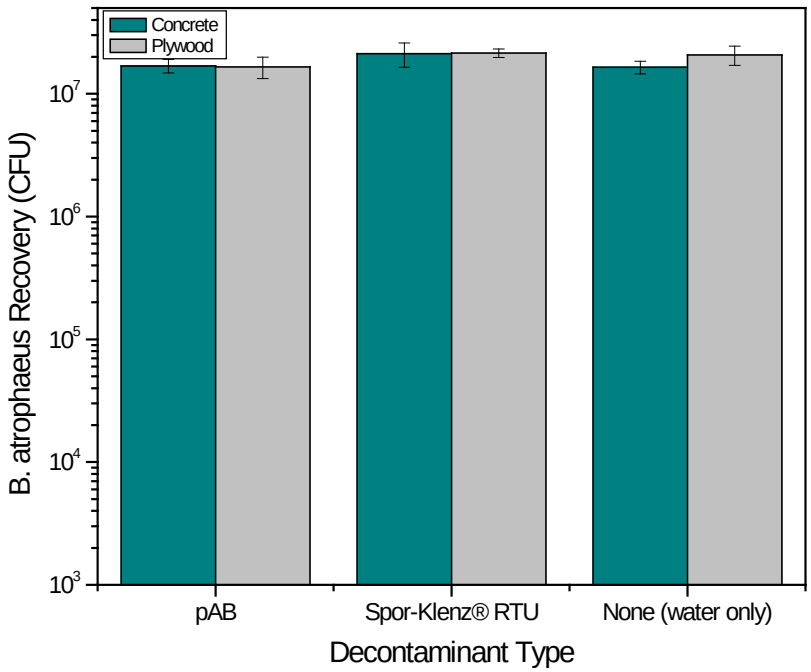


Figure 8.1-1. *Bacillus atrophaeus* Recoveries on Pre-Decontaminated Inoculated Surfaces

8.1.3 B. atrophaeus Decontamination Testing Using pAB and Spor-Klenz® RTU

Testing was conducted to evaluate the decontamination efficacy of pAB and Spor-Klenz® RTU against *B. atrophaeus* spores on selected grimed and neat surfaces (concrete and plywood). Table 8.1-3 summarizes the results for the surface decontamination results, and Figure 8.1-1 illustrates the results. Table 8.1-4 summarizes the results for liquid effluents (runoff and water rinsates) and air samples collected during the decontamination process.

The decontamination efficacies encompassed a wide range of LR values from roughly 2.0 to 7.4 (Table 8.1-3). pAB was found to be more effective than Spor-Klenz® RTU for decontaminating concrete, while the latter decontaminant was more effective on neat plywood, independent of application method (backpack sprayer versus chemical sprayer). Both decontaminants were less effective on grimed materials, compared to neat, with LR values on grimed surfaces ranging from 2.1 to 4.6, independent of the material/application method.

A greater number of viable spores were found in rinsate samples during tests conducted with the backpack sprayer than with the chemical sprayer, potentially because the chemical sprayer was more effective at physically removing spores before the rinse step (during the decontamination step). Also, for the backpack sprayer, rinsates from grimed coupons had higher viable spore levels than neat coupons. Relatively high aerosolization (over 1×10^3 per sample) was observed during some tests with both the backpack and chemical sprayers.

Table 8.1-3. *B. atrophaeus* Decontamination Results

Test	Decontamination Application Method	Material Type	Decontamination Liquid	Coupon Condition	Positive Controls (CFU)		Test Coupons		LR (CFU)	
					Average	STD	Average	STD	Average	STD
1	Backpack sprayer	Concrete	pAB	Neat	1.63E+07	1.67E+06	ND	-	7.3	0.02
2				Grimed	1.02E+06	1.77E+05	1.24E+03	8.78E+02	3.0	0.36
3	Backpack sprayer	Treated plywood	pAB	Neat	2.92E+06	1.08E+06	1.99E+02	3.65E+01	6.6	0.90
4				Grimed	6.46E+05[1]	3.01E+05	6.36E+02	5.99E+02	3.3	0.64
5	Backpack sprayer	Concrete	Spor-Klenz® RTU	Neat	7.21E+06	3.72E+06	2.67E+02	2.03E+02	4.6	0.62
6				Grimed	1.24E+04	1.51E+03	1.01E+02	9.22E+01	2.4	0.66
7	Backpack sprayer	Treated plywood	Spor-Klenz® RTU	Neat	1.59E+07	7.09E+06	ND	-	7.4	0.01
8				Grimed	1.27E+06	5.26E+05	1.88E+03	2.20E+03	3.1	0.53
9	Chemical sprayer	Concrete	pAB	Neat	2.01E+06	1.46E+06	ND	ND	6.4	0.01
10				Grimed	1.66E+05[1,2]	1.44E+05	4.65E+02	4.03E+02	3.5	0.52
11	Chemical sprayer	Treated plywood	pAB	Neat	6.73E+06	2.72E+06	1.27E+00	9.33E-01	6.8	0.27
12				Grimed	4.29E+05[1]	2.05E+05	1.96E+02	3.40E+02	3.9	0.79
13	Chemical sprayer	Concrete	Spor-Klenz® RTU	Neat	4.94E+04[1]	2.39E+04	5.10E+02	3.33E+02	2.5	1.31
14				Grimed	1.51E+06	2.80E+05	3.60E+01	3.78E+01	4.8	0.43
15	Chemical sprayer	Treated plywood	Spor-Klenz® RTU	Neat	9.58E+06	3.09E+05	ND	-	7.1	0.14
16				Grimed	Samples were exposed to exccess heat during heat shock process					

[1]Positive control recoveries below 6 logs, prevent achievement of 6 LR

[2]Some replicates were too contaminated to enumerate.

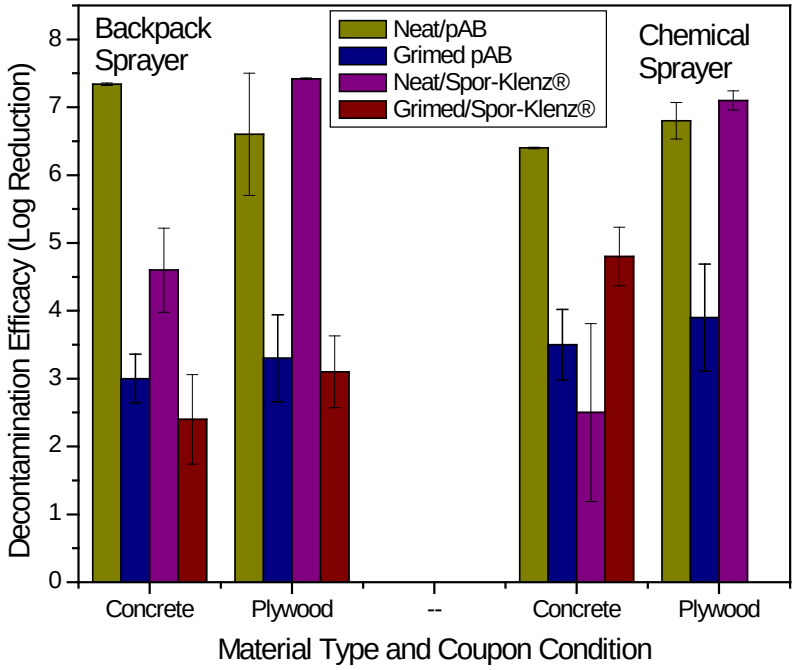


Figure 8.1-1. *Bacillus atrophaeus* Decontamination Efficacy

Table 8.1-4. Fate of *B. atrophaeus* Spores during Decontamination Procedures

Test	Decontamination Application Method	Material Type	Decontamination Liquid	Coupon Condition	Rinsate CFU/ Test	Runoff CFU/ Test	Aerosol CFU/ Test
1	Backpack sprayer	Concrete	pAB	Neat	320	1	6,080
2				Grimed	3,550	3	1
3	Backpack sprayer	Treated plywood	pAB	Neat	310	1	1
4				Grimed	699	3	1
5	Backpack sprayer	Concrete	Spor-Klenz® RTU	Neat	908	16,166	93
6				Grimed	1,500	2	1
7	Backpack sprayer	Treated plywood	Spor-Klenz® RTU	Neat	1,682	3	41
8				Grimed	21,179	4	1
9	Chemical sprayer	Concrete	pAB	Neat	28	45	10
10				Grimed	197	45	1
11	Chemical sprayer	Treated plywood	pAB	Neat	23	42	5
12				Grimed	72	41	1
13	Chemical sprayer	Concrete	Spor-Klenz® RTU	Neat	1,577	42	1180
14				Grimed	15	41	1
15	Chemical sprayer	Treated plywood	Spor-Klenz® RTU	Neat	Not available*		7
16				Grimed			0

***** Spor-klenz effluents could not be neutralized with STS alone, therefore rinsate and runoff data were not of sufficient quality to report. Method development was later performed with potassium carbonate to neutralize SK

8.2 MS2 Decontamination Testing

This section discusses the testing results for extraction efficacy from neat and heavily grimed surfaces for the small and large coupons, neutralizing agent testing for extracted samples, and MS2 decontamination testing using citric acid solution and pAB.

Preliminary scoping experiments performed on large coupons showed inconsistencies during the extraction process for MS2 using PBST. As a result, the following tests were conducted as discussed in Section 5.3:

- **Test I** to determine the neutralizer effectiveness of DE broth for the 2% (v/v) citric acid solution in DI water and MS2 inoculated on small coupons with and without grimed surfaces
- **Test II** to determine the neutralizer buffer effectiveness of DE broth for samples that do not receive decontamination treatment
- **Test III** to determine a suitable extraction buffer for the samples and determine the inoculation hold time for the coupons

The results of these tests are discussed in sections 8.2.1 through 8.2.3

Decontamination testing was conducted on small and large coupons (see Sections 4.4 and 4.5) to evaluate the decontamination efficacy of pAB and 2% (w/v) citric acid against MS2 on selected grimed and neat surfaces (concrete and plywood). The results of these tests are discussed in section 8.2.4 and 8.2.5 for small control coupons and large coupons, respectively.

8.2.1 DE Broth Neutralizer Effectiveness Test I Results

Table 8.2-1 summarizes the Test I results, and Figure 8.2-1 illustrates the results for these tests. The results demonstrate that the DE broth acts not only as a stabilizer but also as a buffer that increases the MS2 extraction efficiency (test coupons with DE broth demonstrated greater recoveries than positive controls with no DE broth) but also maintains the integrity of the sample test coupons (coupon Day 4 versus Day 0), which is not the case for the positive coupons that received PBST alone.

Table 8.2-1. MS2 - DE Neutralizer Broth Effectiveness Test I Results

Material	Sample Type (Number of Coupons)	Received DE Broth	Inoculated	Day Plated	Recovery (Log PFU)	STD (Log PFU)
Concrete (grimed)	Test coupon (5)	Yes	Yes	Day 0	8.1	0.02
				Day 4	7.5	0.12
	Positive control coupon (3)	No	Yes	Day 0	3.5	5.4
				Day 4	ND	-
	Negative control coupon (1)	No	No	Day 0	ND	-
				Day 4	ND	-
Concrete and plywood	Runoff liquid (5)	Yes	Yes	Day 0	7.7	0.04
				Day 4	0.3	0
Plywood (grimed)	Test coupon (5)	Yes	Yes	Day 0	7.9	0.18
				Day 4	7.6	0.16
	Positive control coupon (3)	No	Yes	Day 0	7.2	1.0
				Day 4	1.6	3.4
	Negative control coupon (1)	No	No	Day 0	ND	-
				Day 4	ND	-

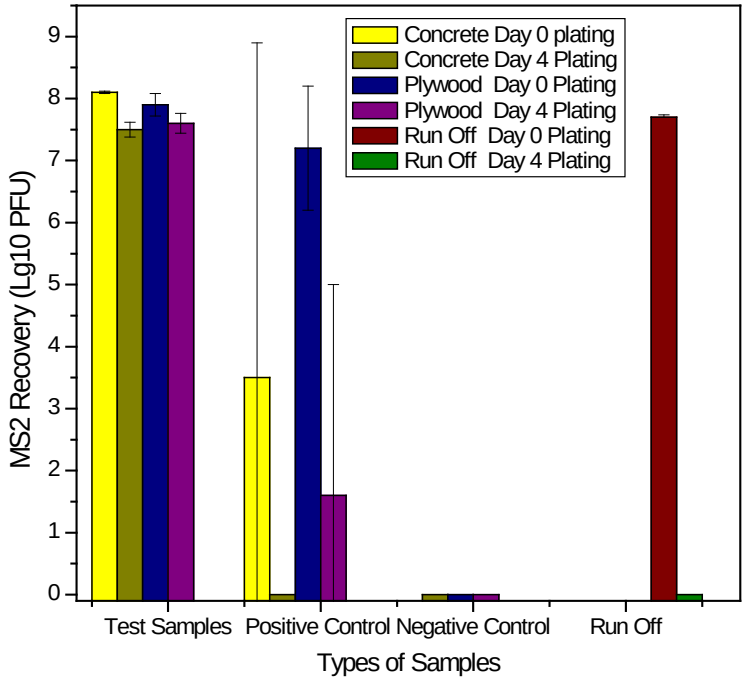


Figure 8.2-1. MS2 Recoveries Using DE Broth as an Extraction Buffer

8.2.2 DE Broth Neutralizer Effectiveness Test II Results

Table 8.2-2 summarizes the Test II results, and Figure 8.2-2 illustrates the results for these tests. The results suggest that the addition of DE broth to the grimed positive controls (concrete and plywood) increased the extraction efficiency of MS2 compared to the positive controls without DE broth. Further, the results suggest that delaying the plating process negatively affected recoveries for samples plated using PBST alone and, to a lesser extent plating process negatively affected recoveries for samples containing PBST alone, and to a lesser extent samples in PBST/DE broth solutions.

Table 8.2-2. MS2 - DE Broth Neutralizer Effectiveness Test II

Sample Type					
Concrete test coupon	1 mL of sterile DE broth and 10 mL of sterile PBST	Yes	Day 0	4.47E+07	7.90E+06
			Day 4	1.07E+07	3.16E+06
Concrete positive control coupon	10 mL of sterile PBST	Yes	Day 0	1.7E+04	4.5E+03
			Day 4	8.8E+02	2.27E+03
Concrete negative control coupon	None	No	Day 0	ND	-
			Day 4	ND	-
Plywood test coupon	1 mL of sterile DE broth and 10 mL of sterile PBST	Yes	Day 0	3.0E+07	1.1E+07
			Day 4	4.39E+06	1.36E+06
Plywood positive control coupon	10 mL of sterile PBST	Yes	Day 0	2.4E+04	3.1E+04
			Day 4	2.84E+03	6.21E+04
Plywood negative control coupon	None	No	Day 0	Not detected	Not detected
			Day 4	Not detected	Not detected
*Four out of five test results were non-detects					

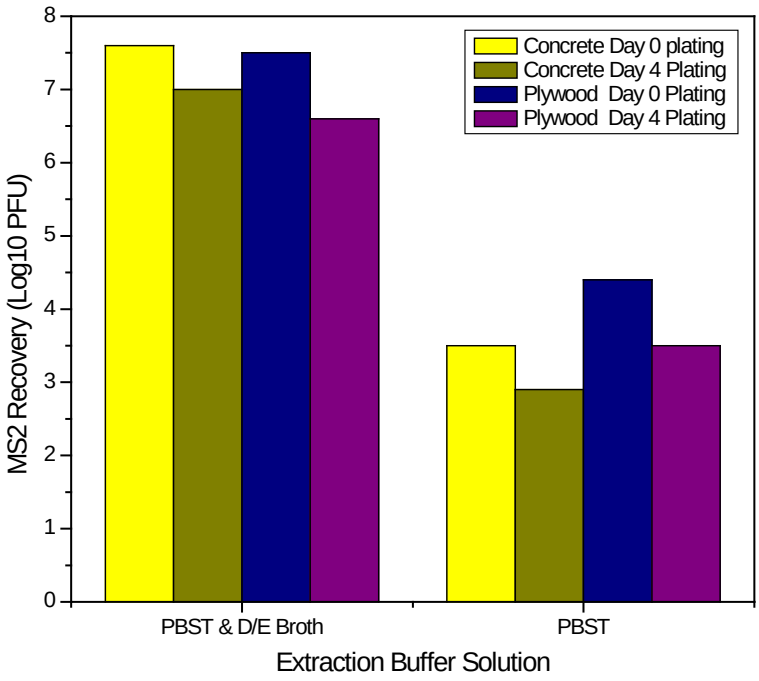


Figure 8.2-2. MS2 Extraction Efficacy with and without DE Broth, after 0 and 4 Days

8.2.3 Suitable Extraction Buffer and Inoculation Hold Time Test III Results

Table 8.2-3 summarizes the Test III results, using DE broth as a neutralizer with different extraction buffers. The results suggest the following:

- DE broth increased recovery of MS2,
- The PBST/DE broth neutralization/extraction buffer achieved the highest recoveries, although DI water/DE broth was comparable as an extraction combination,
- Grimed samples provided better recovery compared to neat samples,
- Same-day inoculation (10-minute hold time for neat samples and 2-hour hold time for grimed samples) resulted in higher MS2 recoveries compared to overnight inoculation (18- to 24-hour hold time), and
- PBST with DE broth was the most effective neutralizer/extraction buffer (except for grimed concrete samples where DI water was more effective).

Each sample was plated on the day of extraction (Day 0) and one day after extraction (Day 1). Although samples from Day 1 had lower recoveries than samples from Day 0, the average log difference between Day 0 and Day 1 recoveries for PBST/DE broth buffer was approximately 0.5 log.

The overall results suggest that neat inoculated coupons should be tested within minutes after inoculation and that grimed coupons should be tested within one day after inoculation. Neutralized test samples and positive samples should be extracted in a DE broth-PBST neutralizer/buffer solution to achieve sufficient recoveries and maintain the viability of MS2. The sample extracts can be then plated within four days without any substantial reduction in recovery

Table 8.2-3. Suitable Extraction Buffer and Inoculation Hold Time Test III Results for MS2

Test Type	Coupon Material	Buffer Used	Day Plated	Recovery (Log PFU)
Neat – 2-hour inoculation hold	Concrete	DI Water	Day 0	6.9
			Day 1	7.1
		PBST	Day 0	6.9
			Day 1	7.3
		PBS	Day 0	3.6
			Day 1	0.7
		TSB	Day 0	6.9
			Day 1	6.7
	Plywood	DI Water	Day 0	5
			Day 1	ND
		PBST	Day 0	5.9
			Day 1	5.9
		PBS	Day 0	0.7
			Day 1	ND
		TSB	Day 0	5.6
			Day 1	5.3
Neat – 1-day inoculation hold	Concrete	DI Water	Day 0	0.7
			Day 1	ND
		PBST	Day 0	0.7
			Day 1	ND
		PBS	Day 0	0.7
			Day 1	ND
		TSB	Day 0	0.7
			Day 1	ND
	Plywood	DI Water	Day 0	0.7
			Day 1	ND
		PBST	Day 0	0.7
			Day 1	ND
		PBS	Day 0	0.7
			Day 1	ND
		TSB	Day 0	0.7
			Day 1	ND
Grimed – 2-hour inoculation hold	Concrete	DI Water	Day 0	7.7
			Day 1	7.6
		PBST	Day 0	7.9
			Day 1	6.3
		PBS	Day 0	7.6
			Day 1	0.7
		TSB	Day 0	7.2
			Day 1	7.2
	Plywood	DI Water	Day 0	7
			Day 1	6.8

Test Type	Coupon Material	Buffer Used	Day Plated	Recovery (Log PFU)
		PBST	Day 0	8.2
			Day 1	8.1
		PBS	Day 0	8
			Day 1	0.7
		TSB	Day 0	7.5
			Day 1	6.8
Grimed – 1-day inoculation hold	Concrete	DI Water	Day 0	7.8
			Day 1	7.4
		PBST	Day 0	6.6
			Day 1	6.2
		PBS	Day 0	4.2
			Day 1	0.7
		TSB	Day 0	5.8
			Day 1	3.5
	Plywood	DI Water	Day 0	7.7
			Day 1	7.3
		PBST	Day 0	7.3
			Day 1	6.7
		PBS	Day 0	0.7
			Day 1	ND
		TSB	Day 0	6.6
			Day 1	6.3

8.2.4 MS2 Decontamination Testing on Small Coupons Using pAB and 2% Citric Acid Formulation

The decontamination solutions were tested for effectiveness in quintuplicate for an exposure time of 30 minutes for all material types using 18-mm coupons. Control testing was conducted to determine the physical removal of MS2 due to the liquid spraying. To do this, a separate set of coupons were sprayed with DI water using the same methods used with pAB and citric acid. These test coupons were designated as procedural positive control coupons. For each test, there was one negative test control (non-inoculated coupon) that underwent the same approach as the test coupons and three positive test controls. All the coupons were extracted in DE broth/PBST solutions.

Coupons of four material types (grimed concrete, grimed plywood, neat concrete, and neat plywood) were tested, each inoculated with 1×10^8 PFU MS2.

The results for MS2 recovery (PFU) on the surfaces of all materials tested are illustrated in Figure 8.2-3 and summarized in Table 8.2-4. The results show that the liquid spraying with water had low efficacy (determined by comparing results for the procedural positive controls to the non-sprayed positive controls), suggesting that physical removal during spraying is minimal.

Table 8.2-4 shows the decontamination efficacy (positive controls PFU compared to log PFU remaining after decontamination) for all the materials. The results of these tests suggest that the 2% (v/v) citric acid formulation is not effective against MS2 on the concrete and plywood test materials. However, pAB was found to be efficacious against MS2 for concrete, with full decontamination on neat concrete material and

near full decontamination on grimed concrete material (four out of five samples with non-detects). However, limited efficacy was observed for neat or grimed plywood materials.

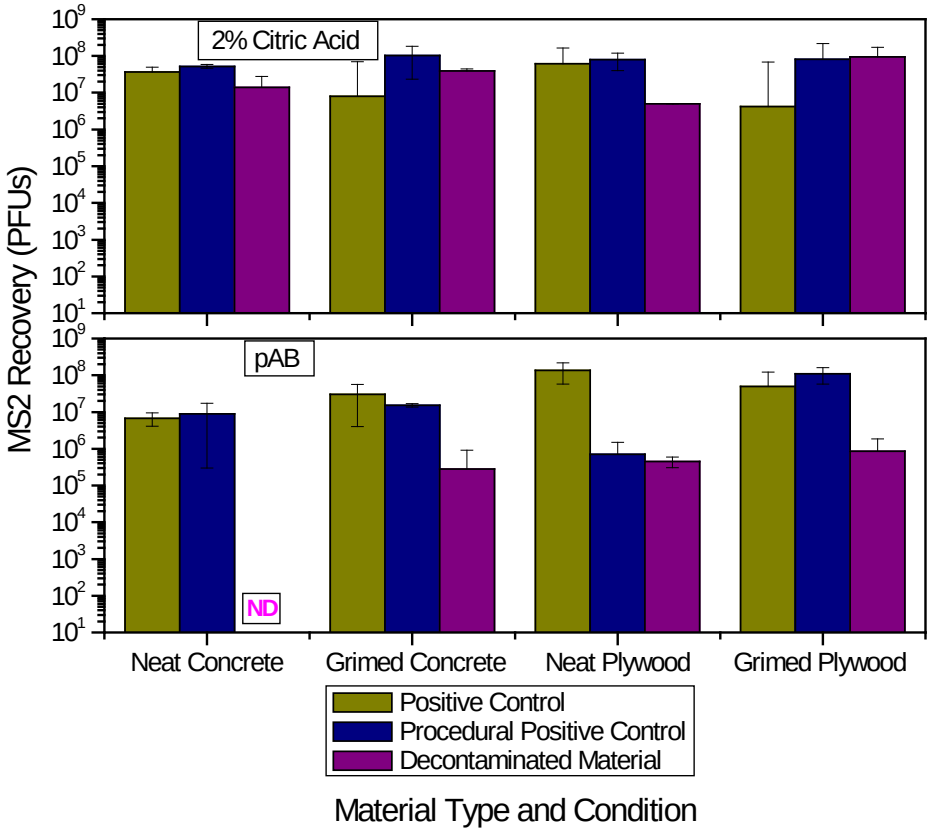


Figure 8.2-3. MS2 Recoveries from Positive Control, Procedural Control, and Decontaminated Test Coupons

Table 8.2-4. MS2 Recoveries from Positive Control, Procedural Control, and Decontaminated Test Coupons

Decon Agent	Material	Positive Coupon PFU		Procedural Positive Control Coupon		Test Coupon PFU		Surface Log Reduction (LR)	
		Average	STD	Average	STD	Average	STD	Average	Cumulative STD
pAB	Neat concrete	6.77E+06	2.68E+06	8.84E+06	8.54E+06	ND	-	7.1	0.12
	Grimed concrete	2.99E+07	2.59E+07	1.53E+07	1.75E+06	2.83E+05	6.34E+05	6.4	1.3
	Neat plywood	1.37E+08	7.97E+07	7.01E+05	8.05E+05	4.54E+05	1.46E+05	2.4	0.19
	Grimed plywood	4.91E+07	7.36E+07	1.10E+08	5.22E+07	8.57E+05	9.86E+05	3.7	1.7
2% Citric acid	Neat concrete	3.68E+07	1.24E+07	5.22E+07	6.00E+06	1.39E+07	7.93E+06	0.46	0.15
	Grimed concrete	6.17E+07	1.03E+08	7.96E+07	3.95E+07	4.99E+06	4.21E+06	1.1	1.1
	Neat plywood	6.21E+07	1.12E+07	3.10E+05	3.93E+05	3.52E+04	3.83E+04	3.5	0.25
	Grimed plywood	6.35E+07	8.05E+07	1.37E+08	9.44E+07	7.88E+07	6.96E+07	0.08	0.56

The fate of MS2 was assessed by collecting and analyzing runoff samples to address the potential physical removal of the virus from the surface of the coupons during the decontamination procedure. These results are shown in Table 8.2-5 and demonstrate that most of the runoff from pAB effluent had no detectable MS2, which was not the case for the 2% citric acid formulation, which showed almost complete wash off of viable viruses from the coupons independent of type of materials exposed was observed. This result confirms that the citric acid formulation does not have the desired biocidal effect on the MS2.

Table 8.2-5. MS2 Recoveries from Runoff Samples from Small Coupons

Decon Agent	Material	Positive Coupon (PFU)		Runoff (PFU)	
		Average	STD	Average	STD
pAB	Neat concrete	6.77E+06	2.68E+06	ND	-
	Grimed concrete	2.99E+07	2.59E+07	ND	-
	Neat plywood	1.37E+08	7.97E+07	6.05E+01	8.17E+01
	Grimed plywood	4.91E+07	7.36E+07	ND	-
2% Citric acid	Neat concrete	3.68E+07	1.24E+07	4.82E+03	2.87E+03
	Grimed concrete	6.17E+07	1.03E+08	1.44E+07	1.50E+07
	Neat plywood	6.21E+07	1.12E+07	1.32E+04	1.62E+04
	Grimed plywood	6.35E+07	8.05E+07	5.89E+04	9.72E+04

8.2.5 MS2 Decontamination Testing Using pAB and 2% Citric Acid Formulation on Large Coupons

Testing was conducted to evaluate the decontamination efficacy of pAB and citric acid against MS2 on selected grimed and neat surfaces (concrete and plywood). A backpack sprayer was used to spray the

decontamination solution on large 14-in x 14-in coupon materials. Table 8.2-6 summarizes the results for the surface decontamination results, and Figure 8.2-4 illustrates these results.

During these large coupon tests, pAB was found to be effective at inactivation of MS2 on grimed materials (complete kill, and > 6 LR for grimed concrete and grimed plywood, respectively). The lower decontamination efficacies on grimed surfaces observed during tests with *B. atrophaeus* were not observed during the tests with MS2. Complete kill of MS2 was also achieved with pAB on neat concrete. Viable MS2 was recovered following pAB treatment from neat wood. A greater occurrence of non-detects (complete kill) following decontamination during large coupon tests as compared to small coupon tests could be explained by the lower efficiency of wipe sampling (large coupons) as compared to extraction-based sampling (small coupons). This disparity is evident in the higher recoveries from positive control coupons during small coupon testing. Consistent with the small coupon tests, the 2% citric acid formulation was found to be ineffective for neat concrete materials (LR $\underset{\sim}{}$ 0.2), and more effective with the grimed concrete material (LR $\underset{\sim}{}$ 4.3). However, complete kill was not achieved for either neat or grimed concrete, when 2% citric acid was the decontaminant. Testing of 2% citric acid against MS2 on neat and grimed plywood have not been completed at the time of report preparation.

Table 8.2-6. MS2 Recoveries during Large Coupon Testing

Decon Agent	Material	Positive Coupon (PFU)		Test Coupon (PFU)		Surface Log Reduction (LR)	
		Average	STD	Average	STD	Average	Cumulative STD
pAB	Neat Concrete	2.46E+04	6.61E+03	ND	-	4.7	0.06
	Grimed Concrete	1.54E+06	2.65E+05	ND	-	6.2	0.04
	Neat Plywood	3.64E+06	-	9.78E+01	4.44E+01	4.8	0.33
	Grimed Plywood	4.70E+06	4.71E+04	ND	-	7.0	0.00
2% Citric Acid	Neat Concrete	6.20E+03	6.74E+03	2.89E+03	1.98E+03	0.20	0.36
	Grimed Concrete	8.36E+05	3.26E+05	1.15E+02	1.06E+02	4.3	0.35

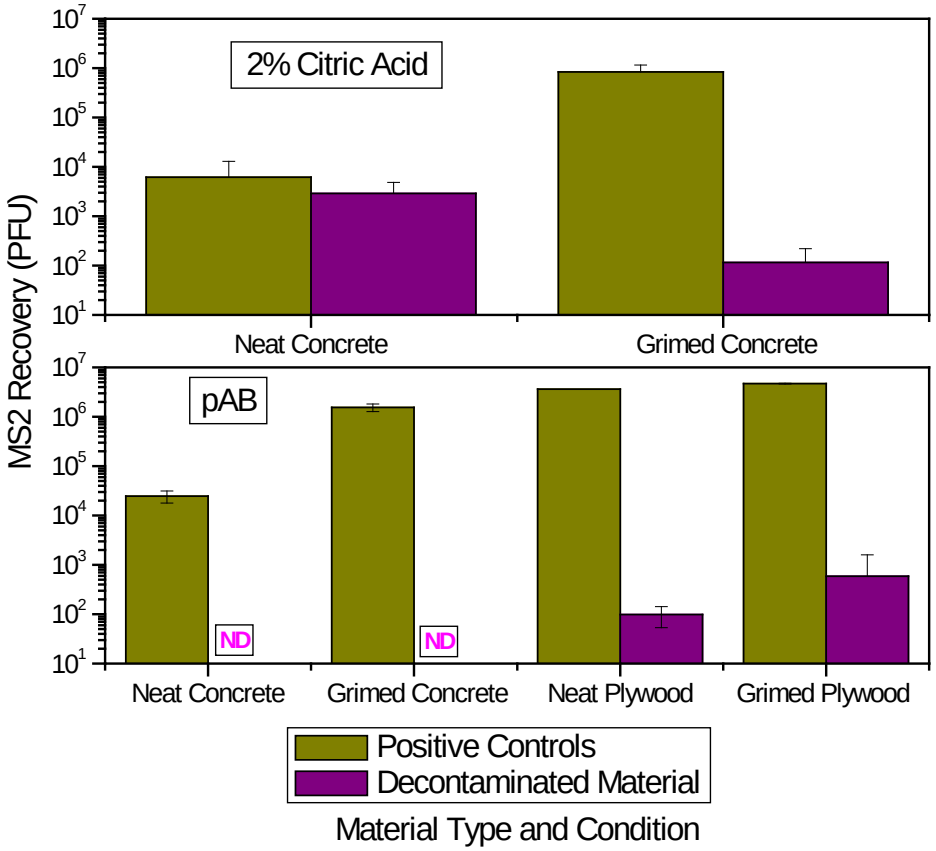


Figure 8.2-4. MS2 Recoveries from Large Coupon Tests

Liquid effluent (runoff) samples, rinse water samples, and air samples collected during the decontamination process also were analyzed to determine the fate of the test organisms. The results of these tests are shown in Table 8.2-7. The effluents from the pAB experiments show no-detects of MS2 independent of material type or condition (neat or grimed), while runoffs from the 2% citric acid formulation tests resulted in substantive amounts of the MS2. These results confirm the ineffectiveness of the 2% citric acid formulation, observed during the small coupon testing. No viable MS2 was observed in any of the aerosol samples (Table 8.2-7). No impact of grime on MS2 recoveries was observed in runoff or rinsate samples.

Table 8.2-7. Fate of MS2 during Decontamination Procedures

Test	Material Type	Decontamination Liquid	Coupon Condition	Runoff CFU/ Sample	Rinsate CFU/ Sample	Aerosol CFU/Sample
1	Concrete	pAB	Neat	ND	ND	ND
2			Grimed	ND	ND	ND
3	Treated plywood		Neat	ND	ND	ND
4			Grimed	ND	ND	ND
5	Concrete	2% citric acid formulation	Neat	2.20E+03	2.42E+03	ND
6			Grimed	ND	4.50E+02	ND

Quality Assurance and Quality Control

All test activities were documented via narratives in laboratory notebooks and the use of digital photography. The documentation included, but was not limited to, a record for each decontamination procedure, any deviations from the QAPP, and physical impacts on materials. All tests were conducted in accordance with established EPA Decontamination Technologies Research Laboratory (DTRL) and NHSRC RTP Microbiology Laboratory procedures to ensure repeatability and adherence to the data quality validation criteria set for this project

9.1 Criteria for Critical Measurements/Parameters

The data quality objectives (DQOs) are used to determine the critical measurements needed to address the stated objectives and specify tolerable levels of potential errors associated with simulating the prescribed decontamination environments. The following measurements were deemed critical to accomplish part or all of the project objectives:

- pH and temperature of the pAB solution
- Sodium hypochlorite concentration (FAC) of the pAB decontamination solution
- Citric acid concentration of the 2% citric acid decontamination solution
- Temperature of incubation
- CFU or PFU abundance per plate
- Neutralizer volume
- Mass of grime applied onto test coupons
- Backpack sprayer spray diameter at one foot
- Chemical sprayer spray diameter at three feet
- Flow rate of backpack sprayer, chemical sprayer, and water hose
- Pressure of backpack sprayer and garden hose.

The following measurements were non-critical, but were monitored and recorded throughout the entire testing schedule:

- Temperature and pH of the Spor-Klenz® RTU and citric acid liquid sporicide solutions and of the rinse water
- Head pressure for the rinse water.

9.2 Data Quality Indicators

The data quality indicators (DQIs) for the critical measurements listed in Table 9.2-1 were used to determine if the collected data met the quality assurance objectives. If a measurement method or device resulted in data that did not meet these goals, the data derived from the critical measurement were rejected. Decisions to accept or reject test results were based on engineering judgment used to assess the likely impact of the failed criterion on the conclusions drawn from the data. The acceptance criteria were set at the most stringent levels that can routinely be achieved. All the DQIs were within the target acceptance criteria set for this project as shown in Table - 9.2-1.

Table 9.2-1. DQIs for Critical Measurements

Measurement Parameter	Analysis Method	Accuracy	Acceptance Criteria	Mean Value / Pass or Fail Test
Mass of grime	Gravimetric	0.1 g	± 10% of target value 30% RSD between test set	56.5 g (Pass)
FAC and pH in pAB solution	$Na_2S_2O_3$/KI titration pH meter/NIST-traceable buffer solutions	±0.06 g/L ±0.01 pH units	6,000 to 6,700 mg/mL 6.5<pH<7	6,169 mg/mL 6.74 pH (Pass)
H_2O_2/PAA concentration* and pH* in Spor-Klenz® RTU**	$Ce(SO_4)2/Na_2S_2O_3$/KI titration pH meter/NIST-traceable buffer solutions	± 0.01 g/L ± 0.01 pH units	± 10% of target value	0.93 % H_2O_2 0.09 % PAA (Pass)
Citric acid concentration and pH** in citric acid decontamination solution***	NaOH titration pH meter/NIST-traceable buffer solutions	± 0.03 g/L ± 0.01 pH units	± 10% of target value	2.04 % Citric Acid 2.14 pH (Pass)
Time	NIST-calibrated stopwatch	± 1 minute per hour	± 2 min (2 x ± 1 min)	Pass
Volumes	Serological pipette tips	0.1 mL	± 10% of target value	Pass
Pressure of backpack and chemical sprayer	Class B pressure gauge	± 2 psi	± 20% of target value	Pass
Flow rate of backpack and chemical sprayer and water hose	Volume collected in a graduated cylinder per time	± 50 mL	± 20% of target value	Pass
Chemical sprayer spray diameter at three feet	Tape measure	1/8 in	± 20% of target value	Pass
Backpack sprayer spray diameter at one foot	Tape measure	1/8 in	± 20% of target value	Pass
Counts of CFU or PFU per plate	Manual counting	± 10% CFU/ plate between 1st and 2nd count	100% RSD between triplicates	Pass
Plated volume	Pipette	2%	± 1%	Pass
Temperature of incubation chamber	NIST-traceable thermometer (daily)	± 2 °C	Not applicable	Pass

Mg/mL = milligrams/milliliter
NIST = National Institute of Standards and Technology
g/L = grams per liter
* Performed only for neutralization testing (see Section 5 for details).
** pH of Spor-Klenz® RTU and citric acid decontamination solutions were established experimentally before testing in a series of preliminary experiments through triplicate measurements of this parameter performed for each liquid decontaminant. The averages from these measurements were then established as baseline or threshold pH for Spor-Klenz® RTU and citric acid decontamination formulations.

9.3 Quality Control Checks

Many QA/QC checks were used in this project to ensure that the data collected met all the critical measurements listed in Table 9.2-1. The measurement/parameter criteria were set at the most stringent level that can routinely be achieved. The integrity of the sample during collection and analysis was evaluated. Control samples and procedural blanks were included along with the test samples so that well-controlled quantitative values were obtained. Background checks for the presence of bacterial spores were included as part of the standard protocol. Replicate coupons were included for each set of test conditions. Specific quality control checks that were performed in this project are described in the following sections.

9.3.1 Integrity of Samples and Supplies

Samples were carefully maintained and preserved to ensure their integrity. Samples were stored away from standards or other samples that could possibly cross-contaminate them.

Project personnel carefully checked supplies and consumables prior to use to verify that they met specified project quality objectives. All pipettes were calibrated yearly by an outside contractor (Calibrate, Inc.), incubation temperature was monitored using NIST-traceable thermometers, and balances were calibrated yearly by the EPA Metrology Laboratory.

9.3.2 NHRSC Biolab Control Checks

Quantitative standards do not exist for biological agents. Quantitative determinations of organisms in this investigation did not involve the use of analytical measurement devices. Rather, the CFU were enumerated manually and recorded. If the CFU count for bacterial growth did not fall within the target range, the sample was either filtered or re-plated. For each set of results (per test), a second count was performed on 25 percent of the plates within the quantification range (plates with 30 - 300 CFU). All second counts were found to be within 10 percent of the original count.

9.4 QA/QC Sample Acceptance Criteria

The acceptance criteria for the critical CFU measurements were set at the most stringent level that could be achieved routinely. Positive controls and procedural blanks were included along with the test samples in the experiments so that well-controlled quantitative values were obtained. Background checks were also included as part of the standard protocol. Replicate coupons were included for each set of test conditions. Further QC samples were collected and analyzed to check the ability of the NHSRC Biolab to culture the test organism, as well as to demonstrate that materials used in this effort did not themselves contain spores. The checks included the following:

- Negative control coupons: sterile coupons that underwent the same sampling process without spore deposition.

- Field blank coupons: sterile coupons carried to the decontamination location but not decontaminated.
- Laboratory blank coupons: sterile coupons not removed from NHSRC Biolab.
- Laboratory material coupons: includes all materials, individually, used by the NHSRC Biolab in sample analysis.
- Stainless steel positive control coupons: coupons inoculated but not decontaminated.

QA/QC acceptance criteria are shown in Table 9.4-1. These criteria provide assurances against cross-contamination and other biases of microbiological samples.

Table 9.4-1. Additional DQIs Specific to Microbiological Data

Coupon or Sample Type				
Positive control coupons sample from material coupon contaminated with biological agent and sampled using the wipe method	1 x 10^7 for *B. atrophaeus* 1 x 10^8 for MS2 30% RSD between coupons in each test set	Shows viability of wipe sampling technique and plate's ability to support growth of *B. atrophaeus* and MS2	Identify and remove source of variability if possible	Pass
Procedural blank coupon without biological agent that underwent the sampling procedure	Non-detect	Controls for sterility of materials and methods used in the procedure	Analyze data with procedural blank results as test minimum; identify and remove source of contamination if possible	Pass
Material blank grime, roller, and sterilized coupon of each material	Non-detect	Controls for sterility of materials and methods used in the procedure	Analyze data with procedural blank results as test minimum; identify and remove source of contamination if possible	Pass
Blank plating of microbiological supplies	No observed growth after incubation	Controls for sterility of supplies used in dilution plating	Sterilize or dispose of source of contamination; replate samples.	Pass
Blank tryptic soy agar sterility control Plate incubated but not inoculated	No observed growth after incubation	Controls for sterility of plates	All plates incubated before use, so contaminated plates discarded before use	Pass
Exposed field blank samples; a wipe kit will be handled	Non-detect	Level of contamination present during sampling	Clean up environment; sterilize sampling materials before use	Pass
Unexposed field blank samples; a wipe kit will be transferred without handling	Non-detect	Level of contamination present during sampling	Clean up environment; sterilize sampling materials before use	Pass

Summary

The objective of this study was to assess the effectiveness of spray-based common decontamination methods for inactivating *Bacillus* (B.) *atrophaeus* (surrogate for *B. anthracis*) spores and bacteriophage MS2 (surrogate for foot and mouth disease virus [FMDV]) on selected test surfaces (with or without a model agricultural grime). Relocation of viable viruses or spores from the contaminated coupon surfaces into aerosol or liquid fractions during the decontamination methods was also investigated.

The effectiveness of removing/inactivating two target microorganisms was assessed for three different decontamination solutions. pH-Amended Bleach (pAB) and Spor-Klenz® Ready-to Use [RTU] were evaluated for their effectiveness against *B. atrophaeus* spores, and 2% (w/v) citric acid in sterilized DI water and pAB were evaluated against the bacteriophage MS2. Three application methods (handheld sprayer, backpack sprayer, and a chemical sprayer) were utilized throughout the testing to deliver decontaminants to the test surfaces. The evaluation was conducted on two test material surfaces (concrete and plywood), with and without a model agricultural grime on the surface. The handheld application method was conducted using a bench-scale test spray apparatus to evaluate the pAB and citric acid spray-based decontamination methods for 18-mm coupons (both grimed and neat) contaminated with MS2. The backpack and the chemical sprayer application methods were performed to simulate field operations. For all the tests, a wetted surface contact time of 30 minutes was used, followed by a surface rinse with water. Method developments were conducted to determine the most effective extraction buffer, if any, for each decontaminant and to determine its effectiveness in neutralizing (quenching decontaminant activity) and maintaining the integrity of the samples potentially containing viable *B. atrophaeus* spores or MS2

Testing conducted to evaluate the decontamination efficacy against *B. atrophaeus* spores on selected grimed and neat surfaces (concrete and plywood) using pAB and Spor-Klenz® RTU indicated that higher efficacies were achieved on neat materials than on grimed materials, independent of material type (concrete or wood) or decontaminant application method (backpack sprayer versus chemical sprayer for large coupons and handheld sprayer for small coupons). pAB was found to be more effective than Spor-Klenz® RTU for decontaminating neat concrete materials, while the latter decontaminant was more efficient at decontamination of neat plywood materials, independent of application method. Viable spore levels found in rinsate samples were higher for the backpack sprayer tests than the chemical sprayer tests, potentially because the chemical sprayer was more effective at physically removing spores before the rinse step (during the decontaminant application step). Relatively high aerosolization (greater than 1×10^3 CFU per test) was observed during some tests with both the backpack and chemical sprayers.

Tests conducted to evaluate decontamination efficacy against MS2 on selected grimed and neat surfaces (concrete and plywood) using pAB and 2% (v/v) citric acid formulation indicated that 2% citric acid was not effective against MS2 on these test materials. Conversely, pAB was found to be efficacious against MS2, with full decontamination (complete kill) on neat or grimed concrete and limited efficacy for neat or grimed plywood. No apparent effects of grime on decontamination efficacy were observed during MS2 tests. Further, it was demonstrated that few viable viruses were detected in the runoff from pAB tests, unlike for the 2% citric acid tests, which had almost complete wash off of viable viruses from all coupon types. Finally, no viable MS2 aerosol formation/emission was observed in any of the conducted tests, independent of the type of decontamination solution used. However, the Via-Cell® bio-aerosol cassette

sampling method used in this study was not validated for MS2 collection and subsequent analytical methods. Lack of recovery could be indicative of low or no viral aerosol formation during tests, poor collection efficiency of the method, or loss of viability of viral particles due to desiccation following collection but prior to analysis.

From the neutralizer optimization tests, sodium thiosulfate (STS) at 2 N (normal) and potassium carbonate at 2 M (molar) were found to be suitable neutralizing agents for pAB and Spor-Klenz® RTU. PBST was also found to be a very effective extraction buffer for *B. atrophaeus* spores, but not for MS2. For testing involving MS2, a phosphate-buffered saline with 0.05% Tween® 20 (PBST)/Dey Engley (DE) broth combination was found to act not only as a stabilizer but also as a neutralizer/extraction buffer that increased the efficiency of MS2 recovery.

The results of this study may help emergency responders select decontamination chemicals and application methods that are effective yet feasible.

References

1. USEPA, Homeland Security, Strategic Research Action Plan 2012-2016. **2012**. https://www.epa.gov/research/homeland-security-strategic-research-action-plan-2012-2016 , last accessed June 15, 2016.
2. Baron, P. A., Estill, C.F., Beard, J.K., Hein, M.J., Larsen, L. Bacterial endospore inactivation caused by outgassing of vapourous hydrogen peroxide from polymethyl methacrylate (Plexiglas). *Letters in Applied Microbiology* **2007,** *45*(5), 485-90.
3. ASTM, Standard Guide for Testing Cleaning Performance of Products Intended for Use on Resilient Flooring and Washable Walls. ASTM International: PA, United States, 2001.
4. Jover, E., Moldovan, Z., Bayona, J. M. Complete characterisation of lanolin steryl esters by sub-ambient pressure gas chromatography–mass spectrometry in the electron impact and chemical ionisation modes. *Journal of Chromatography A* **2002,** *970*, 249-258.
5. (a) NCSU Swine Fresh Manure Characteristics. http://www.bae.ncsu.edu/topic/animal-waste-mgmt/program/land-ap/barker/a&pmp&c/sfm.htm; (b) NCSU Beef Fresh Manure Characteristics. http://www.bae.ncsu.edu/topic/animal-waste-mgmt/program/land-ap/barker/a&pmp&c/bfm.htm, last accessed June 15, 2016.
6. Berg, B., McClaugherty, C. *Plant Litter Decomposition, Humus Formation, Carbon Sequestration*. Third Edition. Springer - Verlag Berlin Heidelberg: 2014. ISBN 978-3--642-38821-7.
7. Sonneborn Mineral Oil for Agriculture. http://www.sonneborn.com/markets/agriculture, last accessed June 15, 2016.
8. ATSDR *Toxicological Profile For Used Mineral-Based Crankcase Oil*; Agency for Toxic Substances and Disease Registry: 1997. https://www.atsdr.cdc.gov/toxprofiles/tp102.pdf, last accessed June 15, 2016.
9. PCA Cement & Concrete Basics. http://www.cement.org/cement-concrete-basics, last accessed June 15, 2016..
10. Association, M. P. Silica sand. http://www.mineralproducts.org/prod_silica01.htm, last accessed June 15, 2016.
11. Long, C.M., and Valberg, P.A. Carbon black vs. black carbon and other airborne materials containing elemental carbon: Physical and chemical distinctions. *Environmental Pollution* **2013,** *181*, 271-286.
12. Cornell, R. M. and Schwertmann, U. *The iron oxides: structure, properties, reactions, occurrences and uses.* Wiley VCH Verlag GMBH + Co., KGaA Weinheim: 2003. ISBN 3-527-30274-3,
13. O'Kelly, J.C., and Reich, H.P. Sebum output and water metabolism in different genotypes of cattle in hot environments. *Journal of Thermal Biology.* **1981,** *6(2)*, 97-101.
14. AOCS Waxes. Structure, Composition, Occurrence And Analysis. http://aocs.files.cms-plus.com/LipidsLibrary/images/Importedfiles/lipidlibrary/Lipids/waxes/file.pdf.
15. Alberta Agriculture and Rural Development Environmental Stewardship Division Beneficial Management Practices. Environmental Manual for Livestock Producers in Alberta. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex13088/$file/400_28-2.pdf?OpenElement, last accessed June 15, 2016.
16. Gibbons, G.S., Broomall, S.M.,, McNew, L.A., Daligault, H., Chapman, C., Bruce, D., Karavis, M., Krepps, M., McGregor, P.A., Hong, C., Park, K.H., Akmal, A., Feldman, A., Lin, J.S., Chang, W.E., Higgs, B.W., Demirev, P., Lindquist, J., Liem, A., Fochler, E., Read, T.D., Tapla, R., Johnson, A., Bishop-Lilly, K.A., Detter, C., Han, C., Sozhamannan, S., Rosenzweig, C.N., Skowronski, E.W. Genomic signatures of strain selection and enhancement in Bacillus atrophaeus var. globigii, a historical biowarfare simulant. *PLoS ONE* **2011,** *6* (3), e17836. doi:10.1371/journal.pone.0017836.
17. Lee, S. D,; Ryan, S. P., Snyder, E. G. Development of an aerosol surface inoculation method for Bacillus spores. *Applied and Environmental Microbiology* **2011,** *77*(5), 1638-1645.
18. Anobom, C.D., Albuquerque, S. C., Albernaz, F.P., Oliveira, A.C.,. Silva, J.L., Peabody, D.S., Valente, A.P. Almeida, F.C. Structural studies of MS2 bacteriophage virus particle disassembly

by nuclear magnetic resonance relaxation measurements. *Biophysical Journal* **2003,** *84*(6), 3894-3903.

19. EPA *Effectiveness of Physical and Chemical Cleaning and Disinfection Methods for Removing, Reducing or Inactivating Agricultural Biological Threat Agents.* EPA/600/R-11/092, 2011; Washington, DC, 2011.
20. Hilgren, J., Swanson, K. M. J., Diez-Gonzalez, F., Cords, B., Inactivation of Bacillus anthracis spores by liquid biocides in the presence of food residue. *Applied and Environmental Microbiology* **2007,** *73* (20), 6370-6377.
21. Fong, D., Mê-Linh Lê, C.G., Shum, M. Effectiveness of alternative antimicrobial agents for disinfection of hard surfaces. National Collaborating Centre for Environmental Health, Vancouver, B.C. **2011**.
ISBN: 978-1-926933-23-8.
22. Krug, P.W., Larson, C.R., AEslami, A.C., Rodriguez, L.L. Disinfection of foot-and-mouth disease and African swine fever viruses with citric acid and sodium hypochlorite on birch wood carriers. *Veterinary Microbiology* **2012,** *156(1-2)*, 96-101.
23. Nerandzic, M. M., Sunkesula, V. C. K., Thriveen Sankar C., Setlow, P., Donskey, C. J., Unlocking the sporicidal potential of ethanol: Induced sporicidal activity of ethanol against *Clostridiumdifficile* and *Bacillus* spores under altered physical and chemical conditions. *PloS one* **2015,** *10* (7). http://dx.doi.org/10.1371/journal.pone.0132805.

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