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Washing/Drying of ClothingContaminated with a Biological Agent Such as *Bacillus anthracis*

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Washing/Drying of Biological Agent-Contaminated Clothing

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DISCLAIMER

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FOREWORD

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Gregory Sales, Director
Center for Environmental Solutions and Emergency Response

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EXECUTIVE SUMMARY

A large-area accidental or intentional release of *Bacillus anthracis (Ba)* spores may subject emergency response teams as well as the general public to exposure and cross-contamination through personal protective equipment (PPE) or personal clothing. The overall purpose of this study was to assess the use of washers and dryers with a suitable decontamination solution as a potential self-help practice to reduce and/or inactivate biological spores from common clothing material. This approach may be used to lessen potential cross-contamination among emergency first-responders, security personnel, or public citizens exposed to a wide area *B. anthracis* spore release.

The study was conducted in four phases, each using viable spores of *Bacillus atrophaeus* var. *globigii* (*Bg*), a surrogate for *Ba*. Phase I and II were part of a bench-scale study using a Lab Line Incubator-Orbital Shaker to simulate a washing machine. The experiment was conducted using 300-ml liquid inoculated wash solutions in 500-ml Nalgene® bottles placed on the orbital shaker platform. In Phase I, potential decontamination solutions were identified, then inoculated with *Bg* spores and quantitatively assessed for inactivation efficacy. In Phase II, experimental cloth coupons (samples) were inoculated with *Bg* spores, then exposed to the most efficacious decontamination solutions identified in Phase I. Phase III and IV were part of a pilot-scale study in which an off-the-shelf compact washer and dryer was used. Phase III experiments were conducted using a portable off-the-shelf compact twin-tub washer, with the second tub housing a spinner that extracts water from laundry. Phase IV was conducted using the previous compact washer/spinner along with a standalone front load compact laundry dryer.

The overall decontamination approach was evaluated as a function of different parameters such as wash temperature, wash cycle time, material clothing type, wash solution type (water versus bleach-type solution), water rinse, and drying. Six material fabrics (cotton, stretch denim, polyester fleece, spandex, cotton/polyester blend, and US Navy camouflage) were evaluated to determine if the clothing material type had any effect on decontamination efficacy. Spore viability (quantified by enumeration of colony forming units [CFU]) was evaluated at each step in the experimental decontamination process to assess the potential for spores on clothing materials to either be removed, inactivated, or re-distributed to the wash/rinse solutions.

Decontamination/removal efficacy was expressed as a log reduction (LR) of the viable *Bg* CFU recovered following a treatment of a coupon material from the average of the inoculated positive control samples recovered CFU. Typically, for laboratory assessments of decontamination efficacy, for a 1×10^6 CFU challenge or greater, a LR ≥ 6 is considered effective, and when no viable spores are recovered after decontamination treatment, the method is considered highly effective.

The main purpose of this study was to evaluate potential decontamination solutions that can effectively remove and/or inactivate bacterial spores from contaminated clothing. The project included bench scale testing to simulate the washing and drying of contaminated clothes with viable spores of *Bacillus atrophaeus* var. *globigii* (*Bg*) spores, a surrogate for *Bacillus anthracis* (*Ba*) spores. The washing/drying decontamination approach was evaluated as a function of different parameters such as wash temperature, wash cycle duration, material clothing type, wash solution type (water versus bleach-type solution), water rinse, and drying. Six materials (cotton, stretch denim, polyester fleece, spandex, cotton/polyester blend, and US Navy camouflage)) were evaluated to determine if the clothing material type influenced the

effectiveness of various decontamination approaches.

All liquids expended by the wash cycle process were collected and analyzed quantitatively to assess the potential for viable spores from the clothing material coupons to be removed and/or inactivated. The CFU in each type of sample was used to quantify the inactivation of spores on the clothing materials, and/or the redistribution of spores from the contaminated clothing to the wash liquid.

In the first phase (liquid/liquid decontamination study), potential decontamination solutions were identified, then inoculated with *Bg* spores and quantitatively assessed for inactivation efficacy. Results from the first phase indicated that oxygen-based diluted (10%) bleach solutions (Clorox® 2, and Tide® Free & Gentle laundry detergent) were not sporicidal. Full decontamination with no-recoverable spores was observed for the Clorox® chlorine-based diluted bleach solution. The effect of temperature on the decontamination efficacy of the wash solutions was found to be negligible for the two temperatures evaluated.

The non-chlorinated bleach solutions were not tested further, and the wash solutions were maintained at ambient laboratory conditions henceforth. Additionally, Phase I experiments demonstrated that the addition of a detergent to the chlorine-based diluted solution allowed a significant decrease in concentration (down to 1%) while still maintaining adequate decontamination efficacy (> 6 LR). The addition of Tide® detergent may have resulted in a reduction in spore clumping and thus better dispersion of spores in the liquid-contaminated solution, hence increasing the effectiveness of the bleach solution.

Phase II experiments used a simulated washing procedure and small cloth coupons (3 inch [in.] × 3 in.). These experiments confirmed results from the Phase I tests that the 1% Clorox® chlorine-based bleach solution with detergent was a highly effective decontamination approach. This solution provided a > 6 LR for all cloth materials (cotton, cotton/polyester blend, spandex, and polyester) except for denim and military fabrics (5.7 and 4.7 LR, respectively). The lower decontamination efficiencies for these two fabrics may have been the result of a denser, more complex weave and a greater number of spores being entrapped within these types of materials compared to the other four materials. It should also be noted that greater decontamination efficacies were observed during this phase of testing compared to the Phase I tests. This effect may have been caused by the aerosol inoculation of material coupons during Phase II tests compared to the liquid inoculation used during Phase I tests.

Phase III experiments used larger cloth coupons, 14 in. × 14 in., and a compact washing machine with the optimal decontamination parameters applied from the previous phases. Results from this phase confirmed results from Phase II that 1% diluted Clorox® chlorine-based bleach solution with the addition of a detergent using an 18-minute wash time was effective in decontaminating material fabric inoculated with ~10^7 spores per square foot. All materials had surface log reductions > 6. The increased surface decontamination efficacies seen during this phase may have been due to the increased agitation dynamic from the washing machines.

In the final phase of this project (Phase IV), a drying sequence was added to the wash sequence. The addition of a drying sequence resulted in no apparent increase in the decontamination efficacy of the diluted bleach solution. A similar number of spores were detected on cotton material coupons following the wash cycles with and without a drying step. The difference between the number of spores recovered between the two procedures is made up

almost entirely of spores recovered from HEPA filters placed on the air outlet of the dryers. Due to the potential for spore re-aerosolization and lack of a significant increase in decontamination efficacy, drying of washed contaminated clothing may not be the best approach for a potential self-help practice to reduce cross-contamination among an affected population.

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ACRONYMS AND ABBREVIATIONS

ADA	aerosol deposition apparatus
ASTM	ASTM International
Ba	*Bacillus anthracis*
Bg	*Bacillus atrophaeus* var. *globigii*
BioLab	HSMMD RTP Microbiology Laboratory
CESER	Center for Environmental Solutions and Emergency Response
CFU	colony-forming unit
CMAD	Consequence Management Advisory Division
DI	deionized
DTRL	Decontamination Technologies Research Laboratory
EPA	U.S. Environmental Protection Agency
EtO	ethylene oxide
FAC	free available chlorine
H_2O_2	hydrogen peroxide
HSMMD	Homeland Security and Materials Management Division
in.	inch
ISO	International Organization for Standardization
LR	log reduction
MDI	metered-dose inhaler
mg/L	milligram per liter
ml	milliliter
N	normality
NIST	National Institute of Standards and Technology
OLEM	Office of Land and Emergency Management
pAB	pH-adjusted bleach
PBST	phosphate-buffered saline with 0.05% Tween 20
PES	polyethersulfone
PPE	personal protective equipment
ppm	part per million
psi	pound per square inch
QA	quality assurance
QC	quality control
RTP	Research Triangle Park
Stdev	standard deviation
STS	sodium thiosulfate
TSA	tryptic soy agar
VHP	vaporous hydrogen peroxide
V_{pAB}	volume of pH-adjusted bleach

V_{STS} volume of sodium thiosulfate

1.0 INTRODUCTION

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program (HSRP) provides credible information to protect human health and the environment from adverse impacts arising from terrorist threats and other contamination incidents. Within the HSRP, the Center for Environmental Solutions and Emergency Response's (CESER) Homeland Security and Materials Management Division (HSMMD) conducts research to provide expertise and guidance on decontamination methods for wide area remediation efforts. This project addresses HSRP strategic goals as described in detail in the Homeland Security Strategic Research Action Plan (U.S. EPA, 2020).

Purpose of the Study

The purpose of this study was to assess the impact of washing and drying clothes as a potential self-help practice to reduce and/or inactivate a *Bacillus anthracis* (*Ba*) spore surrogate in common clothing material. This approach may be used to lessen potential cross-contamination among people such as emergency first-responders, security personnel, or general public exposed to a wide area *Ba* spore release.

Project Description and Objectives

The objective of this work was to determine the efficacy of washing and drying clothing in the removal and/or inactivation of bacterial spores from contaminated clothing materials. The overall process for data generation involved bench-scale testing to simulate the washing and drying of contaminated clothes. Viable *Bacillus atrophaeus* var. *globigii* (*Bg*) spores, a surrogate for *Ba*, were quantified throughout this process. Tests were designed to evaluate the removal or inactivation efficacy of selected detergents and bleach. The effects of washing temperature and drying time on various clothing materials were also evaluated.

The washing/drying decontamination approach was evaluated in terms of decontamination efficacy (spore inactivation) as a function of different parameters such as wash temperature, wash cycle time, material clothing type, wash solution type (water versus bleach-type solution), and water rinse. To assess the potential for viable spores to be removed and/or inactivated from clothing materials, clothing material coupons, and wash and rinse liquids used in the decontamination process were collected and individually analyzed to determine the number of viable spores (quantified as colony forming units [CFU]) in each type of sample. This analysis strategy provided not only an assessment of spore inactivation on clothing materials, but also of spore re-distribution from contaminated clothing materials to the wash/rinse liquids.

The surface decontamination effectiveness of the washing/drying process was determined by calculating the difference in colony forming units (CFUs) observed for the post-treated material coupon (test sample) and those observed for a similar material coupon that was inoculated but not treated (control sample). Recoveries were expressed on a log-10 scale. The fate and the overall redistribution of the spores were also determined by the CFUs recovered in the wash and rinse solution.

Quality control (QC) samples such as procedural blanks (clothing material samples that underwent the washing/drying treatment process but were not inoculated) and negative controls (clothing material samples that were neither inoculated nor treated) were included to monitor for cross-contamination.

2.0 EXPERIMENTAL APPROACH

The experimental work was performed at the EPA's facilities in Research Triangle Park (RTP), North Carolina. The HSMMD RTP Microbiology Lab (BioLab) quantified the number of viable spores for all samples. This study was performed in four (4) consecutive phases:

- Phase I included scoping tests designed to identify the decontamination solution that would be used in subsequent test phases. These scoping tests were conducted without material coupons using an orbital shaker platform to simulate a washing sequence (see Section 3.7.1). The wash solutions were inoculated with ~ 10^7 *Bg* spores using a liquid inoculation approach.
- In Phase II the experimental design was based on an initial scoping test (Phase I) that determined the testing decontamination parameters that were to be used in Phase II (bench-Scale experiments). Phase II experiments used test coupons of 3 inch (in.) x 3 in. clothing materials inoculated with ~ 10^7 *Bg* spores using an aerosol deposition apparatus (ADA) fitted with an actuator and a metered-dose inhaler (MDI). See Section 3.1.2.1.
- Phase III experiments were conducted using a portable compact twin tub washer spin dryer (see Section 3.7.2) to decontaminate 14-in. x 14-in. inoculated test fabric materials using the ADA dry spore inoculation approach.
- Phase IV was conducted using the previous compact washer along with a standalone front-load compact laundry dryer (see Section 3.7.3) to decontaminate 14-in. x 14-in. inoculated test fabric materials that had been contaminated using the ADA dry spore inoculation approach.

Phase I: Scoping Test (Liquid Decontamination Solution Testing)

This phase was designed to determine the type of decontaminant solution that was to be used in Phase II. The target decontamination solutions were tested at a dilution ratio of 1:10 in deionized (DI) water at two wash temperatures (72 ºF and 120 ºF) to determine if they are sporicidal under these testing conditions. The solutions found to be sporicidal were tested further.

Liquid spore inoculation was used during this scoping phase. The *Bg* liquid spore suspension was purchased from Yakibou, Inc. (Apex, NC) at a population density of ~ 1 x 10^9 CFU per ml. The inoculation was performed by making a 1:10 dilution of the original stock, then aseptically applying 100 µl to the 300-ml volume decontamination solution to reach a target inoculation of 1.0×10^7 spores (see Section 3.4.1). The test parameters evaluated during this Phase I test sequence were:

1. **Type of Decontamination Solutions**
    1. Tide® Free & Gentle detergent (Free and Gentle, Proctor & Gamble, Cincinnati, OH; "detergent" henceforth)
    2. Clorox® concentrated germicidal bleach ("diluted bleach" henceforth)
    3. Diluted Clorox® bleach (Clorox Corp., Oakland, CA), with the addition of detergent (Tide®)
    4. Clorox 2® Free & Clear stain remover and color booster, H_2O_2 bleach (Clorox® 2 henceforth).

2. **Bleach Solution Dilution Ratio**
    1. 15:100
    2. 10:100
    3. 5:100
    4. 3:100
    5. 1:100
    6. 5:1000
    7. 1:1000
    8. 1:10000
3. **Wash Cycle Time**
    1. 9-min
    2. 18-min
    3. 27-min

Phase II: (Bench-Scale Testing with Material Coupons)

A series of tests were conducted with chlorinated bleach solution with and without the addition of a surfactant (Tide® liquid detergent) to determine the decontamination efficacy of chlorinated bleach formulations on 3 in. × 3 in. fabric materials inoculated with ~ 10^7 *Bg* spores. The fabric materials were inoculated using an aerosol deposition apparatus (ADA) fitted with an actuator and a metered dose inhaler (MDI) (see Section 3.4.2). The experiments were conducted using 300-ml volume decontamination solutions in 500-ml Nalgene® bottles (Nalge Nunc International Corporation, Rochester, NY) at ambient temperatures. The test parameters evaluated during this test sequence were:

1. **Type of Decontamination Solutions**
    1. Clorox® diluted bleach
    2. Clorox® diluted bleach with the addition of a liquid Tide® detergent
2. **Material Coupons**
    1. 100% Cotton material
    2. 100% Polyester fabric
    3. Cotton/nylon military fabric
    4. Cotton/polyester blend fabric
    5. Spandex fabric
    6. Stretch denim cotton
3. **Bleach Solution Dilution Ratio**
    1. 1:100
    2. 1:1000
4. **Wash Cycle Time**
    1. 18-min

Phase III: (Decontamination Testing using a Compact Washing Machine)

This series of tests was conducted with chlorinated bleach solution with and without the addition of a surfactant (Tide® detergent) using a portable compact twin tub washer to confirm the results obtained in Phase I and II testing sequences. This testing sequence included the inoculation of larger 14-in. x 14-in. fabric material coupons (see Section 3.4.2.2) in triplicate followed by typical wash procedures.

The concentration of treatment (ratio of bleach to water) was based on results from Phase II. A limited number of tests without the addition of a detergent were performed at a high dilution (1:1000) ratio to determine if the sustained agitation dynamic of the washing machines has any positive impact on the decontamination efficacy when compared to similar chlorinated bleach solutions with added detergents. The test parameters evaluated during this test sequence were:

1. **Type of Decontamination Solutions**

   A. Clorox® diluted bleach
   B. Clorox® diluted bleach with the addition of liquid Tide® detergent

2. **Bleach Solution Dilution Ratio**

   A. 1:100
   B. 1:1000

3. **Wash Cycle Time**

   A. 18-min

4. **Material Coupons**

   A. 100% cotton material
   B. Stretch denim cotton
   C. 100% polyester fabric
   D. Spandex fabric
   E. Cotton/polyester blend fabric
   F. Cotton/nylon military fabric

Phase IV: Decontamination Testing using a Portable Washing Machine and Dryer

This series of tests was designed to evaluate the effect of adding a drying step to the decontamination efficacy following a wash cycle. The chlorinated diluted bleach solution (1:1000 dilution ratio) with the addition of a liquid detergent was not found to be fully effective during the previous testing phases. This diluted bleach solution, which left residual contamination in the test material, was chosen as the target decontamination solution to evaluate if adding a drying sequence would increase the overall decontamination efficacy. The test parameters evaluated during this test sequence were:

A. Clorox® diluted bleach (1:1000) with the addition of Tide liquid detergent

B. 18-min wash cycle

C. 30-min drying cycle

D. 100% cotton material

3.0 TESTING AND MEASUREMENT PROTOCOLS

This section describes the test materials, material coupon preparation, material coupon and equipment sterilization, test organism and spore inoculation, decontamination solution preparation, neutralizing agent for test material coupon, decontamination equipment, decontamination procedures, and neutralization procedures.

Test Materials

The fabrics selected for coupon fabrication were purchased from JOANN stores (Durham, NC) and from Amazon (Seattle, WA). The six (6) clothing materials evaluated during this project are shown in Figure 3-1 and listed in Table 3-1.

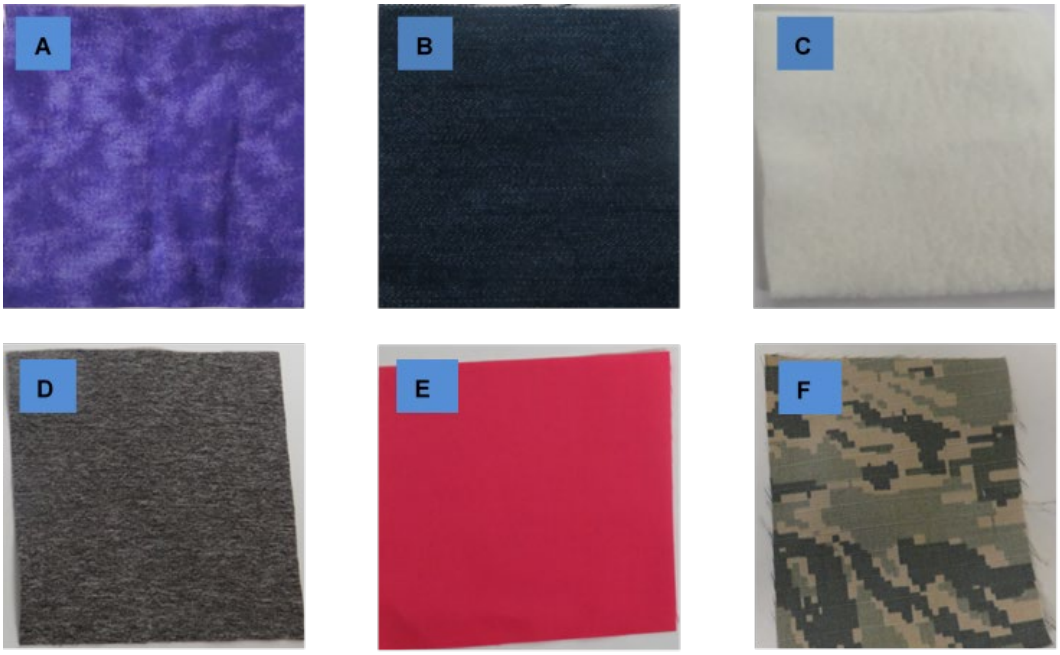

Figure 3-1. Material coupons tested.

Table 3-1. Tested Material Coupons

Material ID	Description	Item Number
A: Cotton	Keepsake Calico 100% Cotton Fabric Purple Tonal	JOANN # 1560390
B: Denim	Stretch Denim Cotton (70% Cotton, 28% Poly/ & 2% Spandex)	JOANN # 1617661
C: Polyester	Blizzard Fleece Fabric (100% Polyester)	JOANN # 3604352
D: Spandex	Performance Apparel Poly Spandex (92% Poly/8% Spandex)	JOANN # 12505251
E: Blend	Sew Classics Knits Interlock Solid Blend (40% Poly/60% Cotton)	JOANN # 12505251
F: Military	US Navy NWU Nyco Camouflage (50% Nylon/50% Cotton)	Amazon (ASIN #B00XUYNBKE)

Material Coupon Preparation

Test material (fabrics) and stainless-steel (for inoculation support) controls were used in this study. The stainless-steel coupons (14-in by 14-in) were made from 16-gauge type 304 mill-finished steel sheet (McMaster-Carr, Elmhurst, IL) using a hydraulic sheet metal shear, and carbide de-burring tool for the edges. These stainless-steel coupons were used as a support for the test material coupons (fabrics) during the inoculation process and as independent inoculation controls.

Material Coupon and Equipment Sterilization

This project required that many activities be performed on material sections or coupons intentionally contaminated (test samples and positive controls) and not contaminated (procedural and control blanks). The treatment of test samples, positive controls, and procedural blanks varied for each group. Hence, specific procedures were used to mitigate cross-contamination among test samples, controls, and coupon inoculation equipment, such as the aerosol deposition apparatus (ADA) for dry spore inoculation. Adequate cleaning of all common materials and equipment was critical to prevent cross-contamination. The fabric coupon materials were used as-is and were not sterilized.

The common materials (templates and actuators) were sterilized using an ethylene oxide (EtO) sterilizer system (PN 333 EOGas®, Andersen Products, Haw River, NC). All the items that were sterilized were packed in appropriate self-sealed EtO pouches, and placed in appropriate large sterilization bags, along with a dosimeter, a Humidichip® relative humidity stabilizer (Andersen Products), and an EtO cartridge. The sterilization bags were vacuum-sealed and loaded into the EtO sterilizer for an 18-hour sterilization cycle.

Stainless-steel coupons and Nalgene bottles were sterilized using a 121 °C gravity cycle autoclave (SV 120 scientific pre-vacuum sterilizer; STERIS Amsco, Mentor, OH). Sterility was evaluated by swab sampling one coupon from each sterilization batch.

Prior to use, test equipment (washers, dryers, and ADAs) were sterilized using Vaporized

Hydrogen Peroxide® (VHP). The hydrogen peroxide (H_2O_2) vapor was generated using a STERIS VHP 1000ED system loaded with a 35% H_2O_2 Vaprox® cartridge (American Sterilization Company, Mentor, OH). Each sterilization cycle lasted 4 hours at 250 ppm VHP.

Test Organism and Spore Inoculation

Bg, a surrogate for the spore-forming bacterial agent *Ba*, was used for this project. Like *Ba*, *Bg* is a soil dwelling, gram positive, spore forming, aerobic microorganism, but, unlike *Ba*, it is non-pathogenic. *Bg* forms an orange pigment when grown on nutrient agar, a desirable characteristic when there is a need to detect viable spores in environmental samples. *Bg* has a long history of use in the biodefense community as a simulant for anthrax associated biowarfare and bioterrorism events (Gibbons et al., 2011).

Bg spores were used at a concentration of about 10^7 spores per test. Liquid spore inoculation was used during the scoping test (Phase I), while dry spore inoculation on test coupons was used for Phase II (Bench-Scale Testing with Coupons), Phase III (Wash Cycle with Coupons), and Phase IV (Wash Cycle and Drying with Coupons).

3.1.1 Liquid Spore Inoculation

Liquid spore inoculation was used during the scoping phase. The *Bg* liquid spore suspension was purchased from Yakibou, Inc. (Apex, NC) at a population density of ~ 1×10^9 CFU per ml. The titer of the stock was confirmed at the start of each testing event by the BioLab. The inoculation was performed during Phase 1 testing by making a 1:10 dilution of the original stock, then aseptically applying 100 µl to the 300-ml volume of distilled water in a 500-mL Nalgene bottle (see Section 3.8.1) to reach a target inoculation of 1.0×10^7 spores per sample.

3.1.2 Aerosol Deposition Apparatus (ADA) Assisted Inoculation of Surfaces

The dry spore test organism for this study was a powdered spore preparation of *Bg* (Gibbons et al., 2011) mixed with silicon dioxide particles. This preparation was obtained from the U.S. Army Dugway Proving Ground Life Sciences Division. The powdered stock preparation procedure is described in Brown et al. (2007). After 80 to 90% sporulation, the suspension is 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).

Prior to inoculation, an ADA of appropriate size was situated directly on top of a flat test surface. The MDI + actuator assembly was placed in the actuator fitting on top of the ADA, then the lid was opened, and the MDI was activated one time to deliver approximately 10^7 *Bg* spores to the test surface under the ADA. Following inoculation, the lid was closed, and the MDI assembly was removed. The ADA was left in place for at least 18 hours to allow time for the spores to settle. This process was repeated for each inoculated surface (inoculation controls, positive controls, and test materials).

3.1.2.1 Phase II Dry Spore Inoculation

Test and positive control coupons (3 in. × 3 in.) were inoculated with ~ 10^7 *Bg* spores using an aerosol deposition apparatus (ADA) fitted with an actuator and an MDI. Each material coupon was aligned and placed on top of a sterile stainless-steel coupon (3 in. × 3 in.) as shown in Figure 3-2. An ADA designed to inoculate a 1 ½ inch diameter area in the center of each coupon was situated on top of the coupon, clamped into place, then coupled with the actuator holding the MDI, as shown in Figure 3-3A. Once the actuator coupling and MDI were fitted into place, the ADA sliding stainless steel cover was opened (as shown in Figure 3-3B) and the MDI was actuated one time to make a single discharge onto the coupon. A 0.22 µm filter covered the ADA exhaust, as shown in Figure 3-2, to prevent spore escape into the test area. Spores were allowed to settle on the test coupon for 18 hours before use.

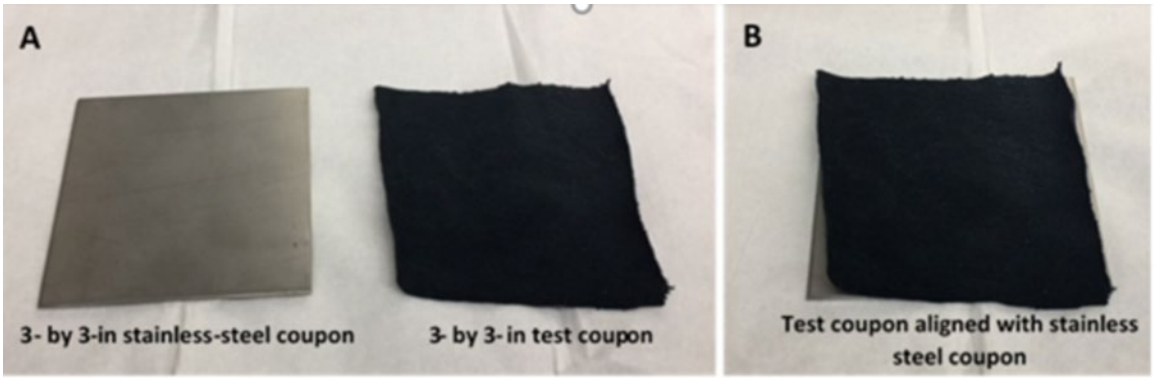

Figure 3-2. Test Coupons (3- x 3-in. stainless-steel and fabric material).

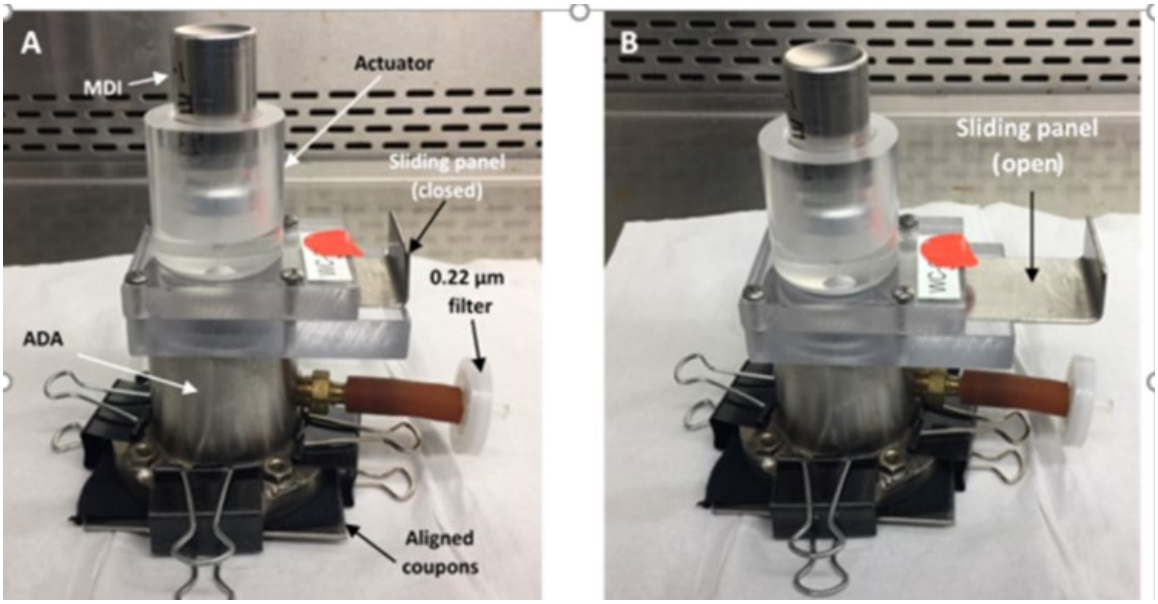

Figure 3-3. ADA with actuator and MDI, clamped onto test coupon, with stainless-steel sliding panel closed (Panel A) and open ready for MDI actuation (Panel B).

3.1.2.2 Phases III and IV: Dry Spore Inoculation

The inoculation of the test material coupons was performed like the dry deposition

inoculation used for Phase 2, with the exception that the ADA was larger to accommodate a 14 in. x 14 in. coupon, as described by Calfee et al., 2013. Briefly, each coupon was contaminated independently with a separate custom-made pyramid-shaped ADA. The ADA was designed to overlay and cover a 12" x 12" surface area of each 14" x 14" coupon. The MDI and the actuator were positioned in the ADA opening, as shown in Figure 3-4, located at the ADA's top center. The MDI was discharged a single time to inoculate the 12" x 12" coupon area.

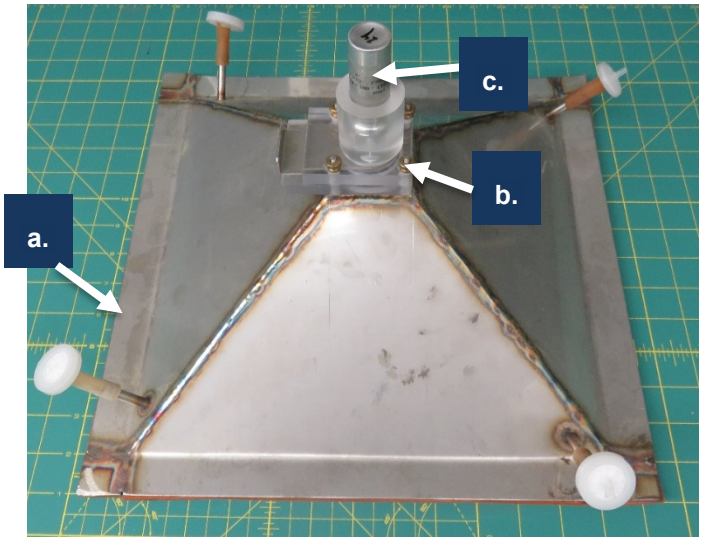

Figure 3-4. ADA (a) with the assembled actuator (b) and MDI (c).

Decontamination Solution Preparation

The decontaminant solutions that were used in this project were Tide®, Clorox® (chlorine bleach), and Clorox® Free and Clear (hydrogen peroxide bleach). The Tide® detergent (Free and Gentle, Proctor & Gamble, Cincinnati, OH) dilution ratio was 1:10 which is more concentrated than the manufacturer's recommendation of 1.56 ounces per load. Clorox® concentrated germicidal bleach (The Clorox® Company, Oakland, CA) was used first at a 1:10 dilution ratio, far greater than the dilution ratio recommended in the manufacturer's instructions (dilute ½ cup [120 mL] of concentrated product in a load of laundry [typically 26 gallons, or 98.4 L]). The optimal dilution ratio was determined during Phase 1 of the study.

Neutralizing Agents for Test Samples

The presence of a bleach-based decontamination solution on the sample surface, or in the liquid effluent following a decontamination event, could negatively bias CFU quantification results. Based on previous studies, sodium thiosulfate (STS) was proven to be an effective neutralizer for bleach on porous and non-porous surfaces (Calfee et al., 2011), and was used during the post-decontamination recovery of the sampling liquids and sample fabric. The volume of STS to be added to the collection media was first determined by measuring free available chlorine (FAC) in the sample using an iodometric method for the determination of chlorine dioxide and chlorite using a HACH® Hypochlorite Test Kit (supplier: Model CN-HRDT, Fisher Scientific, Waltham, MA).

The FAC in the test coupon phosphate-buffered saline with 0.05% Tween 20 (PBST) solution (Tween®, Sigma-Aldrich, St. Louis, MO), along with the volume of diluted bleach

collected in the wash and rinse, was used to calculate the volume of STS (V_{STS}) needed for neutralization using Equation 3-1 adapted from the standard procedure for sample titration using the HACH test kit.

$$V_{STS} = \frac{\text{FAC (mg/L)} \cdot V_{DB}\ (mL)}{N_{STS} \cdot 35453} \tag{3-1}$$

Where:

FAC	=	Free available chlorine (in milligrams per liter [mg/L])
V_{DB}	=	Volume of diluted bleach (mL)
N_{STS}	=	Normality (N) of STS (2.26 N)
35453	=	Equivalent weight (milligrams) per electron

Decontamination Equipment

Various equipment was used to simulate a washing and/or drying decontamination scheme. Phases I and II used an orbital shaker to simulate the liquid/liquid agitation dynamic of a washer, while Phase III and IV used an off-the-shelf compact washer and dryer.

3.1.3 Orbital Shaker

A Lab Line Incubator-Orbital Shaker (Figure 3-5) fitted with an orbital shaking mechanism on a bench-scale platform, designed to hold 500-mL Nalgene bottles, was used to simulate a washing machine.

Figure 3-5. Lab Line Incubator-Orbital Shaker fitted with a platform insert to secure Nalgene bottles.

3.1.4 Compact Washing Machine

A portable compact twin tub washer spin dryer (Model No. KP1040600A, Kuppet, Oslo, Norway), shown in Figure 3-6, was chosen for this study to scale up the tests conducted under Phase 2. The Twin Tub design function allows washing and spinning dry a load of laundry at the same time. Washing is conducted in one tub while the other tub can be used for spin drying. The total capacity for this machine is 8 lbs. for the washer and 5 lbs. for the spin cycle with 5 preset wash times (0, 6, 9, 12, and 15 minutes) and variable spin times between 1 and 5 minutes.

Figure 3-6. Portable Compact Twin Tub Washer Spin Dryer.

3.1.5 Laundry Dryer

A standalone front load compact 1.5 cubic-feet capacity laundry dryer (Model No. MCSDRY15W, Magic Chef, Wood Dale, IL)), shown in Figure 3-7, was used to evaluate the effect of adding a drying step on the decontamination efficacy following a wash cycle. The dryer was operated under a "High Heat" position for optimum drying with a timer set at 30 minutes.

Figure 3-7. Magic Chef compact laundry dryer.

Decontamination Procedures

Decontamination procedures with sampling protocols were devised for each testing phase as described in the next sections. All procedures described in these methods should be conducted under sterile conditions to prevent contamination of the sample with background contaminants and to prevent cross-contamination.

3.1.6 Phase I: Scoping Test

The detailed decontamination testing sequences for Phase I (scoping test without test coupons) is described in the following section:

1. Aseptically pour 270 ml of sterile DI water into each sterile 500-ml Nalgene bottle.
2. Add 30 mL of treatment liquid (detergent or bleach).
3. Use a second Nalgene bottle containing 300 ml of DI water, to be treated in the same water bath, so that the water temperature inside the bottles can be measured.
4. Heat the Nalgene bottles in a water bath until the desired water temperature (either 72 °F or 120 °F) is achieved.
5. Add liquid inoculum to reach a target concentration of 1.0×10^7 spores per bottle (100 µL as per Section 3.4.1).
6. Place bottles in an orbital shaker fitted with a platform designed to hold 500-ml Nalgene bottles.
7. Shake at ambient temperature and 250 rpm for a 9-minute cycle.
8. Add 25 mL of 2N STS, and mix.
9. Analyze the **wash liquid** for the presence of spores.

3.1.7 Phase II: (Bench-Scale Testing with Material Coupons)

The detailed decontamination testing sequence for Phase II is described in the following section:

1. Place coupon (inoculated or control) in a 500-ml Nalgene bottle.
2. Aseptically pour 300 mL of sterile DI water, 10% bleach in water (treatment), or diluted bleach with detergent into each sterile 500-ml Nalgene bottle.
3. Place bottles in an orbital shaker fitted with a platform designed to hold 500-ml Nalgene bottles.
4. Shake at 250 rpm at ambient temperature for a 9-minute cycle.
5. Transfer coupon to labeled 500-mL Nalgene bottle and add 300 mL of DI water at ambient temperature.
6. Add 40 mL 2N STS to the wash liquid solution, and mix.
7. Take sample of the neutralized wash solution (**Wash Sample**).
8. Place bottles with the coupons in an orbital shaker fitted with a platform designed to hold 500-ml Nalgene bottles.
9. Shake at 250 rpm and ambient temperature for a 9-minute cycle.
10. Add 40 mL 2N STS to the rinse solution and mix.
11. Take sample of the rinse (**Rinse sample**).
12. Transfer coupon to labeled Stomacher® bag(P/N BA6141/CLR Seward Ltd., Worthing, West Sussex, United Kingdom) (**Coupon sample**).
13. Analyze the wash liquid, rinse liquid and coupons for the presence of spores.

3.1.8 Phase III: (Decontamination Testing using a Washer)

The detailed decontamination testing sequence for Phase III (washer) with test coupons is as summarized below:

1. **Day 1: Spore Inoculation:**
    1. Dose 3 fabric coupons of each material (3 per test), and 2 steel coupons (**inoculation controls**) with 10^7 CFU Bg spores using ADAs (section 3.4.2.2).
    2. Leave coupons, procedural blank and inoculation controls covered with ADAs for 18 hrs.
2. **Day 2: Decontamination Testing**
    1. Wipe sample the stainless-steel inoculation controls and negative controls.
    2. Place **fabric material negative** and **fabric material positive controls** into individually labeled Stomacher bags.
    3. Collect 100 mL of tap water for **sterility check**.
    4. Prepare 14.0 L of the 1% dilute bleach solution in tap water.
    5. Titrate the 1% dilute bleach solution to verify concentration.
    6. Add 4.2 mL Tide® detergent to the 14 L of 1% dilute bleach solution to be used as wash water.

7. Titrate the solution to determine the amount of 2N STS needed for decontamination solution neutralization.
8. Collect 100 mL of this solution for **sterility check**.
9. Pour 3.25 L of this solution into each of 4 compact washers (3 for testing, one for procedural blank).
10. Place inoculated fabric material coupons into 3 test washers and the procedural blank into the washer designated for it.
11. Set the wash cycle to 9 minutes for each washer. At the end of 9 minutes reset the timer to another 9 minutes for a total wash time of 18 minutes.
12. At the end of 18 minutes, move the coupons to the spin side of the washer and spin for 5 minutes.
13. As the spin cycle proceeds, drain the wash water from each washer into an adequately sized sterile vessel.
14. From each vessel pipette 300 mL of the **wash solution** to a sterile Nalgene bottle that contains 5.0 mL of 2N STS.
15. After the spin cycle, place the coupon back in the wash side and add 3.25 L of tap water only from the remaining sterile pitchers. Set the rinse cycle for 9 minutes.
16. At the end of the rinse cycle, add 50 mL 2N STS to the rinse water containing the cloth coupons. Turn on the washer briefly to ensure good mixing.
17. Drain the rinse water into the same pitcher the tap water was in and transfer the fabric material coupons to the spin basket and spin for 5 minutes.
18. Pour 300 mL of the **rinse water** into sterile Nalgene bottles.
19. At the end of the second spin cycle, transfer the test fabric material coupons into a Stomacher bag for analysis.
20. Analyze all wash solution, rinse water, and material fabric coupons for the presence of spores.

3.1.9 Phase IV: (Decontamination Testing using a Washer & Dryer)

The detailed decontamination testing sequence for Phase IV is similar to the Phase III testing sequence with the inclusion of a drying sequence. The changes in the testing and sampling approaches are shown in italics in the following test sequence.

1. **Day 1: Spore Inoculation:**
    1. Dose 6 cotton material coupons (*2 duplicate sample coupons for each test*), and 2 steel coupons (inoculation controls) with 10^7 CFU Bg spores using ADAs.
    2. Leave coupons, procedural blank and inoculation controls covered with ADAs for 18 hrs.
2. **Day 2: Decontamination Testing**
    1. Wipe sample the stainless **inoculation controls** and **stainless-steel negative controls**.
    2. Place **fabric material negative** and **fabric material positive** control coupons into individual Stomacher bags.
    3. Collect 100 mL of tap water for **sterility check**.
    4. Prepare 14.0 L of 1% dilute bleach solution in tap water.
    5. Add 4.2 mL Tide® detergent to the 14 L of 1% dilute bleach solution.

6. Titrate a sample of the wash solution to determine the amount of 2N STS needed for wash solution neutralization.
7. Collect 100 mL of the wash solution for **sterility check**.
8. Pour 3.25 L of this solution into each of 4 compact washers (3 for testing, one for procedural blank).
9. Place a set of *duplicate* inoculated fabric material coupons into each of 3 washers, and a set of *duplicate* non-inoculated cloth coupons in the fourth washer (procedural blank).
10. Set the wash cycle for 9 minutes for each. At the end of 9 minutes reset the timer to another 9 minutes for a total wash time of 18 minutes.
11. At the end of 18 minutes, move the duplicate coupons to the spin side of the washer and spin for 5 minutes.
12. As the spin cycle proceeds, drain the wash solution from each washer into an adequately sized sterile vessel.
13. From each vessel pipette 300 mL of the **wash solution** to a sterile Nalgene bottle that contains 5.0 mL of 2N STS.
14. After the spin cycle, place the duplicate coupons back in the wash side and add 3.25 L of tap water only from the remaining sterile pitchers. Set the rinse cycle for 9 minutes.
15. At the end of the rinse cycle, add 50 mL 2N STS to the rinse water with cloth coupons. Turn on the washer briefly to ensure good mixing.
16. Drain the rinse water into the same pitcher the tap water was in and transfer the duplicate cloth coupons to the spin basket and spin for 5 minutes.
17. Pour 300 mL of the **rinse water** into sterile Nalgene bottles.
18. At the end of the second spin cycle transfer one test coupon (**Coupon 1**) per set of duplicates into a labeled Stomacher bag for analysis and transfer the second (Coupon 2) to a separate dryer for a 30-min drying sequence.
19. At the end of 30 minutes drying time, transfer **Coupon 2** into a Stomacher bag for analysis.
20. Remove the **discharge filter** from each dryer and transfer to Stomacher bags for analysis.
21. Analyze all wash water, rinse water, coupons, and *dryer HEPA filter* for the presence of spores.

Sample Neutralization Procedure

Neutralization tests were designed to determine the neutralizer's effectiveness in stopping the activity of the residual decontamination solution in the collected samples (wipe, runoff, and rinse) before microbiological analysis. To test for the efficacy of neutralization at low concentrations of spores, 10% and 1% dilute bleach solutions were neutralized with appropriate amounts of 2N sodium thiosulfate (STS) for the chlorine-based solution and potassium permanganate ($KMnO_4$) and sulfuric acid (H_2S) for the bleach-based solutions, and then inoculated with a known concentration of spores (~5 x 10^2 CFU for low concentration and 10^7 CFU for high concentration). These samples were plated immediately and again after 4 days in order to test for any residual decontamination effects.

To test for the possible toxicity of the STS solution, the amount of 2N STS used to neutralize the respective dilute bleach solutions, was added to PBST, and to a separate set of

samples with the same amount of STS added to DI water. These samples were plated on the same days as the neutralized bleach solutions. All samples were compared to positive controls consisting of inoculated PBST and inoculated DI water. The neutralization procedure for each test sample is summarized below:

Chlorine-based Solution Neutralization

1. **Day 1 Activities**
    1. Perform concentration check of inoculum to be used.
    2. Ensure that enough filter sterilized STS is on hand to be used for the test.
    3. Filter sterilize 3 liters of DI water to be used in the test.
    4. Sterilize 6 Erlenmeyer flasks and 6 stir bars.
2. **Day 2 Activities**
    1. Prepare 2 Erlenmeyer flasks with 200-mL of PBST with sterile stir bar.
    2. Prepare 2 Erlenmeyer flasks with 200-mL of DI water with sterile stir bar.
    3. Titrate initial bleach stock solution to be used. Ensure FAC is within the QC criteria.
    4. Make 200 mL of a 1:10 dilution of stock bleach (10% Dilute Bleach solution used for the 3 test samples and 1 procedural blank sample).
    5. Calculate amount of STS needed to neutralize the total 200-mL stock solution of 10% Dilute Bleach.
    6. Add 2× the amount of STS needed to the stock dilute bleach solution and place on stir plate for 5 minutes to ensure appropriate mixing of STS and bleach.
    7. Add this same amount of STS to one of the flasks containing DI water and one of the flasks containing PBST (solutions used to check for and STS toxicity).
    8. Re-titrate the now neutralized 10% bleach stock solution and ensure that FAC measurement is 0.
    9. Dispense 40 mL aliquots of each of these solutions into four 50-mL conical tubes. (There should be 4 tubes with 40-mL of the neutralized dilute bleach solution, 4 with the PBST and STS, and 4 with the DI water and STS)
    10. Repeat steps 4-9 except using a 1:100 dilute bleach stock and appropriate amount of STS for that solution.
    11. Inoculate all test samples at the target *Bg* spores concentration from a liquid stock solution prepared in PBST.
    12. Plate all samples immediately.
3. **Day 6 Activities**

1. Plate all test samples with same volumes used on initial plating to ensure no residual decontamination occurred.

Hydrogen Peroxide-based Solution Neutralization

1. **Day 1 Activities**
    1. Perform concentration check of inoculum to be used.
    2. Sterilize 6 Erlenmeyer flasks and 6 stir bars.
2. **Day 2 Activities**
    3. Transfer approximately 5 mL of the 1:10 diluted hydrogen peroxide-based solution to a tared 250 mL flask. Record weight of H_2O_2 sample.
    4. Add 40 mL of 5% H_2SO_4, then dilute to about 150 mL with DI water.
    5. Place the flask containing the sample on a stir plate, then titrate to the first permanent pink color with 1N $KMnO_4$. Record required volume of $KMnO_4$.
    6. To determine % H_2O_2 (wt/wt %), use the following equation.

% H_2O_2 (by weight) = (mL $KMnO_4$) / 1000 x N / 2 x (34.0146 g/mol) / (g of sample) x 100,

Where: N = Normality of the standard $KMnO_4$

1. Inoculate all test samples at the target Bg spore concentration from a liquid stock solution prepared in PBST.
2. Plate all samples immediately

3. **Day 6 Activities**
    1. Plate all test samples with same volumes used on initial plating to ensure no residual decontamination occurred.

4.0 SAMPLING AND ANALYSIS APPROACH

The inoculation of the test solutions and fabric coupons, decontamination testing, and sampling took place in the EPA/RTP Decontamination Testing Research Facility (High Bay Room H130A). Care was taken so that the inoculation area and the sampling area were separated to avoid cross-contamination.

The following sections discuss sampling material and equipment, sampling methods, quality assurance/quality control (QA/QC) samples, sample containers, sample preservation, sample quantities, and prevention of cross-contamination of sampling and monitoring equipment.

Sampling Material and Equipment

Before sampling, all materials needed for sampling were prepared using aseptic techniques. In addition, general sampling supplies were sterilized and disinfected for each sampling event. A sampling material bin was stocked for each sampling event. The bin contained enough sampling kits to accommodate all required samples for the specific test. Table 4-1 lists the materials that were used for sampling. In addition to the items listed in the table some other items that are needed include: sterile forceps, conical tubes, Stomacher bags, sampling data sheets, permanent marker or pre-printed sample labels, and PPE.

Table 4-1. Sampling Materials and Equipment

Material or Equipment	Description
Disposable laboratory coats	Kimberly-Clark Kleenguard™ A10 light duty apparel (P/N 40105)
PBST	PBS with 0.05% Tween 20 (Sigma Aldrich USA, P/N: P3563-10PAK)
Sterile sampling bags	TWIRL'EM® sterile sampling bags (Labplas Inc. Corporation Canada, Quebec, Canada) Overpack Size 10 by 14 inches Inner bag size: 5.5 by 9 inches (wipe) Sample bag size: 5.5 by 9 inches
1 L or 500 ml Nalgene bottles	Nalgene bottle (Part No. 2187-0032, Thermo Fisher Scientific, Waltham, Massachusetts [supplier])
Bleach wipes	Dispatch® bleach wipes (Clorox Co., Oakland, CA) or Hype-Wipe® towelettes (Current Technologies, Indianapolis, IN)
Polyester-rayon blend wipes for sampling	Kendall Curity™ Versalon all-purpose, absorbent gauze sponges, 2 by 2 inches, sterile packed, #8042, four-ply (Covidien PLC, Dublin, Ireland)
Swabs	BactiSwab® swab (Remel Inc., San Diego, CA) (http://www.remelinc.com/Industrial/CollectionTransport/BactiSwab.aspx; last accessed July 2, 2020)
1.5 cubic ft. electric dryer	Magic Chef® - Compact 1.5 cu. ft. Electric Dryer in White. Model # MCSDRY15W (MC Appliance Corp., Wood Dale, IL)
Washing machine, portable with spin dryer	Kuppet washing machine, portable compact twin tub washer spin dryer; 13 lb capacity; Model # KP1040600A (Kuppet, Oslo, Norway)
Clorox® concentrated germicidal bleach	Clorox® 121 oz. Concentrated Germicidal Bleach, UPC: 4460030798
Clorox 2®	Clorox 2® - Free & Clear Stain Remover & Color Booster, UPC: 44600300481
Tide® detergent	Tide® Original Liquid Laundry Detergent, UPC: 037000138822 (Proctor & Gamble, Cincinnati, OH)

Additional measurements prior to or during the decontamination procedure application were required to ensure quality control during testing. These measurements include quality control checks on the reagents and equipment being used in the decontamination procedure. A sampling data log sheet was maintained for each sampling event (or test) that included each sampling event, the date, test name, sample IDs, and other test details such as test temperature, sample volumes, and sample extraction time. The following sections discuss the sampling protocol, sample types, and frequency of sampling and monitoring events.

Sampling Methods

The major classes of operational samples for this study are summarized below:

1. Cloth material coupon
2. Liquid (wash solution and rinse)
3. Surface wipe samples

4.1.1 Cloth Material Coupon, and Liquid Samples

The cloth material coupons, and liquid samples (wash solution and rinse) were collected as described in the testing sequence (see Section 3.8.). Within each sampling event, each sampling coupon was placed in a Stomacher bag with 100 ml of sterile PBST, the wash and rinse solutions were pipetted into sterile 500-ml Nalgene bottles.

4.1.2 Wipe Sampling

Wipe sampling was performed on stainless-steel coupons: three (3) inoculation controls to verify the consistency of spore application associated with each MDI, and one (1) negative control to monitor for cross-contamination. The general approach was that a moistened sterile polyester-rayon blend wipe (Curity™ all-purpose sponges #8042, 2-in x 2-in, 4-ply, Covidien PLC, Dublin, Ireland) was used to wipe a specified area to recover the target organism. Sampling was conducted on one coupon at a time. All coupons were placed horizontally for sampling.

The wipe sampling kits were processed in the BioLab by aseptically removing the wipes from their packing and placing them into an unlabeled sterile 50-mL conical tube (Cat. No. 14-959-49A, Fisher Scientific, Waltham, MA) using sterile forceps (Part No. 7190 Busse Hospital Disposables, Hauppauge, NY). Each transferred wipe was then moistened by adding 2.5 mL of sterile PBST and capped. The bags and conical tubes need to be labelled with the correct sample information. The general sampling approach for the MDI inoculation controls is summarized below:

1. Don a fresh pair of sterile boot covers and personal protective equipment (PPE) (laboratory coat, nitrile gloves, P-95 respirator, and safety glasses).
2. Remove the clamps from the Blank MDI control coupon, and then lift the ADA off the coupon.
3. Wipe the surface of the coupon horizontally, using a consistent amount of pressure and using S-strokes to cover the designated sample area of the coupon.
4. Fold the wipe in half, concealing the exposed side and then wipe the same surface vertically using the same S-stroke technique.
5. Fold the wipe again and roll it so that it fits into a conical tube.
6. Place the folded wipe into the conical tube.
7. Place the conical tube in a sterile Twirl‘Em® bag (Labplas Inc. Corporation Canada, Quebec, Canada).
8. Sterilize the outside of the Twirl‘Em bags using bleach wipes.

9. Place the sample in the collection bin.

Repeat this process for the 3 MDI control coupons and the 1 MDI negative control.

4.1.3 Quality Assurance/Quality Control (QA/QC) Samples

QA/QC samples were collected as indicators of sterility of sample materials and equipment before and after a sampling event. The QA/QC samples including swab and material samples are discussed next.

4.1.3.1 Swab Samples

Swab samples for biological data quality indicators were used for sterility checks on coupons, materials, and equipment before use in the testing. A single, pre-moistened swab sample was collected from each item and coupon.

The general approach for swab sampling was to use a moistened swab (BactiSwab® collection and transport system, R12100, Remel, Thermo Fisher Scientific, Waltham, MA) to wipe a specified area to recover bacterial spores. The swab sampling procedure is described below:

1. Through the sleeve, crush the moistening ampule of the BactiSwab™ swab at midpoint.
2. Hold BactiSwab™ tip end up for at least five seconds to allow the medium to wet the swab.
3. Open the package and remove the BactiSwab™ swab.
4. Label the plastic tube.
5. Remove the cap-swab from the plastic tube.
6. Swab the surface while spinning the cap-swab between the thumb and index fingers.
7. Return cap-swab to tube.
8. Date and initial each sample tube.

4.1.3.2 Material Samples

QA/QC material samples were collected to provide information on the level of contamination possibly present during sampling due to contaminated materials. These samples were the unexposed field blank (sterility) samples. Also, a blank plating of microbiological supplies served as the control for sterility checks of supplies used in dilution plating.

Sampling Containers

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

Cloth and filter coupons were placed into Stomacher closure bags (P/N BA6141/CLR closure bags, Seward Laboratory System Inc., Islandia, NY), with 100 ml of PBST. Secondary and tertiary containment consisted of a second and third Stomacher bag.

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

Samples were transferred to the BioLab for microbiological analysis in sterile, primary, independent packaging within sterile, secondary containment containing the collected samples for analysis.

Sample Preservation

After sample collection, sample integrity was maintained by storing samples in quadruple containers: first, a sample container; second, a sterile bag; third, a sterile bag with its exterior sterilized during sample packaging; and fourth, a sterile container holding all samples from a testing event. All individual sample containers remained sealed while in the decontamination laboratory and during transport.

Sample Quantities

Each decontamination event was evaluated in triplicate. Table 4-2 lists the total number of each type of sample.

Table 4-2. Sample Quantities for Each Decontamination Test

Sample Type	Number of Replicates
Test Coupon	3 (Phases II, and III); 6 (Phase IV)
Test Coupon Wash Cycle Solution	3
Test Coupon Water Rinse	3
Procedural blank wash cycle liquid	1
Procedural blank rinse cycle liquid	1
Negative control coupon	1
Positive control coupon	3
Inoculation control	3-4
Swab sample	1 per item

Preventing Cross-Contamination During Sampling

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

Before each test, all materials and equipment needed for sampling were sterilized as discussed in Section 3-8, and handled using aseptic techniques. Non-powdered, surgical gloves were used during sampling. Individually wrapped, pre-moistened bleach wipes were placed in sterile sampling bags. Dispatch® bleach wipes were also used for sterilization of work surfaces, sample containers, and outside sampling bags. 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 each specific test. The following test protocol was implemented for each test.

Table 4-3 lists the cleaning methods and their frequency for common test materials and equipment.

Table 4-3. Cleaning Methods and Frequency for Common Test Materials and Equipment

Material or Equipment	Use	Cleaning Method	Frequency
Glassware, Nalgene bottles, and DI tanks	Testing and sample collection	Autoclaving	Before each test
All Work Surfaces	Throughout each test	Wiping with isopropyl alcohol	Before and after each use
ADAs, washer, and dryer	Inoculation and testing	VHP	Before each test

5.0 ANALYTICAL METHODS

The BioLab analyzed samples either qualitatively for spore presence (quality control, swab samples) or quantitatively for the number of viable spores recovered per sample. Results were reported in colony forming units (CFUs) per unit volume. Details of the extraction and analysis procedures are provided in the next sections.

Sample Extraction

Spores were extracted using the appropriate method for each sample type, as described below. After extraction, aliquots were removed for either dilution plating or filter plating, as appropriate for expected spore recoveries.

5.1.1 Coupon Extraction

Each material sample coupon was aseptically placed in Stomacher bags (P/N BA6141/CLR Seward Ltd., Worthing, West Sussex, United Kingdom) along with 100-ml of sterile PBST. The coupon was extracted in a Stomacher 400 Circulator machine (Seward Ltd., Worthing, West Sussex, United Kingdom) for 1 minute at 260 rpm. The excess liquid was recovered from the material coupon while in the bag, and then the coupon was aseptically retrieved and discarded. The eluent was transferred to a sterile specimen cup (P/N B12021O, Starplex Scientific Corp., Cleveland, TN, USA) and sonicated for 10 minutes in a Branson Ultrasonics™ cleaner (Model 8510R-MT, Emerson Electric Co., St Louis, MO, USA) and was then vortexed (Multi-Tube Vortexer, P/N 58816–115, VWR, Radnor, PA) continuously for 2 minutes prior to plating.

5.1.2 Wipe Sample

Wipe samples were extracted in 20 mL of sterile PBST as extraction buffer by agitating the tubes with a vortex mixer set to the maximum rotation for 2 min in 10-s intervals. After extraction, the sample buffer was subjected to serial dilution and analyzed using procedures described in Section 5.2. All extracts were stored at 4 ± 2 °C.

5.1.3 Swabs

Each swab was removed from the packaging in a Biological Safety Cabinet (BSC), then rotated across the tryptic soy agar (TSA) media surface in a zig-zag pattern until the entire circumference of the swab contacted the media surface. Plates were incubated at the appropriate temperature and time based on the *Bacillus* strain used in the test. Swab plates were manually observed to determine "growth" or "no growth" of the target organism.

Spiral- and Filter- Plating Procedures

Sample extracts requiring dilution were plated in triplicate using an Autoplate® 5000 spiral plater (Autoplate® 5000, Advanced Instruments Inc., Norwood, MA). The automated spiral plater deposits the sample in exponentially decreasing amounts across a rotating agar plate in concentric lines to achieve three 10-fold serial dilutions on each plate. Plates with *Bg* samples were incubated at 35 ± 2 °C for 18 to 20 hours. Colonies on each plate were enumerated using a QCount® colony counter (Advanced Instruments Inc., Norwood, MA).

Positive control samples were diluted 100-fold in PBST before spiral plating, while samples of unknown concentration were plated with no dilution and with a 100-fold dilution. Samples with known low concentrations were plated with no dilution. The QCount® colony counter automatically calculates the CFU/mL in a sample based on the dilution plated and the number of colonies that developed on the plate. The QCount® records the data in an MS Excel spreadsheet.

Only spiral plates meeting the threshold of at least 30 CFUs were used for spore recovery estimates. After quantitation with the QCount® colony counter, samples with plate results below the 30-CFU threshold were either re-spiral plated with a more concentrated sample aliquot or filter-plated to achieve a lower detection limit. The filter plate volume was based on the CFU data from the QCount® result. Filter plating was performed using 100-ml capacity micro funnel units with 0.45 µm Pall® filters (Item # 4852, Pall Corporation, Laboratory, Port Washington, NY) and a vacuum manifold (Pall Corporation, Laboratory, Port Washington, NY). The filters were placed onto TSA plates and incubated at 35 ± 2 °C for 20 to 24 hours before manual enumeration.

The test samples, procedural blanks, and sterility controls were filter plated with 1/10/100 ml aliquots, 1/10 ml/remainder aliquots or various aliquots until 2 plates were obtained within the countable range for filters (15-150 CFU/filter).

Other Analytical Procedures

5.1.4 Determination of FAC by Titration

Measurements of FAC were performed using an iodometric method that uses a HACH® digital titrator and a HACH® reagent titration kit (Model #16900, HACH Company, Loveland, CO). The titration procedure can be found in the HACH® digital titrator manual (https://pim-resources.coleparmer.com/instruction-manual/24908-00.pdf).

5.1.5 pH and Temperature Measurements

Measurements of pH and temperature of the bleach solutions were performed daily using a calibrated pH meter (Oakton® Acorn™ pH 5, OAKTON Instruments, USA). The temperature sensor included with the pH meter was factory-calibrated and checked monthly by comparison of the displayed value to a NIST-certified thermometer.

Decontamination Efficacy

Data reduction was performed on measurements of the total spores (CFU) recovered from each sampled surface coupon material, liquid samples and rinse samples. The following groups of samples were collected for each decontamination testing sequence and used to determine the effectiveness of the remediation technique.

- **MDI control coupons:** Stainless steel coupons inoculated at the same time as the fabric material coupons and sampled by sponge wipe. These coupons are inoculated at the beginning, middle, and end of each inoculation campaign to assess the stability of the MDI during the inoculation operation.
- **Positive control**: Sample from fabric material coupon contaminated with biological agent but not subjected to experimental conditions.
- **Procedural positive control:** Representative materials that were inoculated and subjected to a water wash cycle.
- **Test coupons**: Inoculated fabric coupons that went through a decontamination treatment.
- **Procedural blank:** Sterile non-inoculated material that was subjected to the same decontamination process as the inoculated test samples.
- **Negative control material**: Sterile non-inoculated material that was not subjected to a decontamination process.
- **Post-decontamination (Post-decon) liquid wash sample**: Aliquot sample of the decontamination solution following a decontamination event.
- **Post-decon liquid rinse sample**.: Aliquot sample of the water rinse following a decontamination event.
- **Procedural liquid wash sample**: Aliquot sample of the water solution following a water-only wash event.
- **Procedural liquid rinse sample**: Aliquot sample of the water rinse following a water-only wash event.

Decontamination/removal efficacy was expressed as a log reduction (LR) of the viable *Bg* colony forming units (CFU) recovered following treatment of coupon material from the average of the inoculated positive control samples recovered CFU. Typically, for laboratory assessments of decontamination efficacy, for a 1×10^6 colony forming units (CFU) challenge or greater, a LR ≥ 6 is considered effective (U.S. EPA, 2018), and when no viable spores are recovered after decontamination treatment, the method is considered highly effective.

Decontamination/Removal efficacy for each testing procedure is defined as log reduction (LR) of the average viable *Bg* spores recovered from the treated test material sample from the average of the inoculated positive control samples for that material. This value will be reported as a log reduction on the specific material cloth as defined in Equation 5-1.

$$\eta_i = \frac{\sum_{k=1}^{N_C} \log(CFU_{C,k})}{N_C} - \frac{\sum_{k=1}^{N_t} \log(CFU_{S,k})}{N_s} \tag{5-1}$$

where:

η_i	=	Surface decontamination effectiveness; the average log reduction of spores on a specific material coupon (designated by *i*).
$\frac{\sum_{k=1}^{N_C} \log(CFU_{C,k})}{N_C}$	=	The average of the logarithm (or geometric mean) of the number of viable spores (determined by CFUs) recovered on the positive control coupons (*C* indicates control and N_C is the number of control coupons).
$\frac{\sum_{k=1}^{N_t} \log(CFU_{S,k})}{N_s}$	=	The average of the logarithm (or geometric mean) of the number of viable spores (determined by CFUs) remaining on the coupon material (*S* indicates a decontaminated coupon and N_s is the number of coupons tested).

When no viable spores are detected on a decontaminated coupon, a value of 1 CFU will be assigned for extracted sample, k, and the efficacy will be reported as greater than or equal to the value calculated by Equation 5-1.

The standard deviation of the average log reduction of spores on a specific cloth material (η_i) is calculated using Equation 5-2:

$$SD_{\eta_i} = \sqrt{\frac{\sum_{k=1}^{N_s} \left(x_k - \eta_i\right)^2}{N_S - 1}} \tag{5-2}$$

where:

SD_{η_i}	=	Standard deviation of η_i the average log reduction of spores on a specific material cloth.
η_i	=	The average log reduction of spores on a specific material cloth (specific material designated by *i*).
x_k	=	The average of the log reduction from a decontaminated coupon (Equation 5-3).
N_S	=	Number of test coupons of a material coupon type.

$$x_k = \frac{\sum_{k=1}^{N_s}((\overline{\log(CFU_c)} - \log(CFU_{s,k}))}{N_s} \quad (5\text{-}3)$$

where:

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

$CFU_{s,k}$ = Number of CFUs recovered from the kth decontaminated coupon.

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

6.0 RESULTS AND DISCUSSION

This section discusses the impact of washing and drying as a potential method to reduce and/or inactivate biological spores from common clothing material. The overall process for data generation involved bench scale testing to simulate the washing and drying of contaminated clothes. Viable *Bg* spores, a surrogate for *B. anthracis* (*Ba*), were quantified throughout this process.

Section 6-1 describes the scoping phase (Phase I) during which a bench-scale washing machine simulation (without material coupons) was used to evaluate potential decontamination solutions and test parameters (e.g., temperature, decontamination solution dilution ratio, and wash cycle times) for use in subsequent test phases. Section 6-2 discusses Phase II in which bench-scale experiments were conducted with inoculated cloth coupons (3 in. x 3 in.) and the optimal decontamination parameters observed during Phase I. Section 6-3 discusses the Phase III pilot-scale experiments performed with off-the-shelf washers, decontamination solutions identified in Phase I, and larger inoculated cloth coupons (14 in. x 14 in.). Section 6-4 discusses the results of Phase IV using the combination of a washer/dryer during the decontamination process. Finally, Section 6-5 summarizes the results of the overall study.

Phase I: Scoping Phase

Experiments began with testing the extraction efficiency of the procedure to be used when processing the cloth material coupons and then evaluating the decontamination efficacy of proposed decontamination solutions at different temperatures. Subsequent tests determined neutralizer effectiveness to prevent residual decontamination past set washing time points. Finally, effective decontamination solutions were challenged by adjusting washing parameters,

including adjusting decontamination solution dilution ratios and adjusting wash cycle times. All tests in this phase, with the exception of the first extraction efficiency test, were performed using a liquid spore inoculation and without cloth coupons, which were used in the later phases of this project. The test progression results are described in the next sections.

6.1.1 Extraction Efficacy

The recovery of spores from five (5) cotton positive control coupons (3 in. × 3 in.) was tested. Coupons were placed in a Stomacher bag filled with 100-mL of sterile PBST, and extracted using the Stomacher 400 Circulator for 1 min at 260 rpm extraction method. Results (Section 5.1.1) were compared to the recovery of spores from three (3) 14 in. x 14 in. stainless-steel control coupons using the wipe sampling method and extracted in 20-mL of sterile PBST extraction buffer (Section 5.1.2). Table 6-1 displays average recoveries for the cloth extraction procedure compared to the stainless-steel controls.

Table 6-1. Material Coupon Sample Extraction Efficacy Evaluation

Coupon Type	Number of Samples	Average (CFU)	Stdev (CFU)
Stainless Steel	3	3.0×10^{7}	7.7×10^{6}
Cotton Material	5	2.7×10^{7}	2.4×10^{6}

CFU, colony forming units; Stdev, standard deviation

6.1.2 Challenges with Filter Plating Residual Decontamination

The filtration and subsequent plating of 1-mL and 10-mL aliquots through white Pall® filters (Pall Life Sciences, Ann Arbor, MI, P/N 4804 MicroFunnel™ with GN-6 Metricel® membrane with a 0.45 µm pore size made from a mixed cellulose ester) resulted in unexpected recoveries for Clorox bleach post-decon samples. The 1 ml filters had too many colonies to count while the 10 ml filters were either non-detect or had very few colonies. Filter plates from the same selected sample, plated at the same time, are shown in Figures 6-1 and 6-2, respectively.

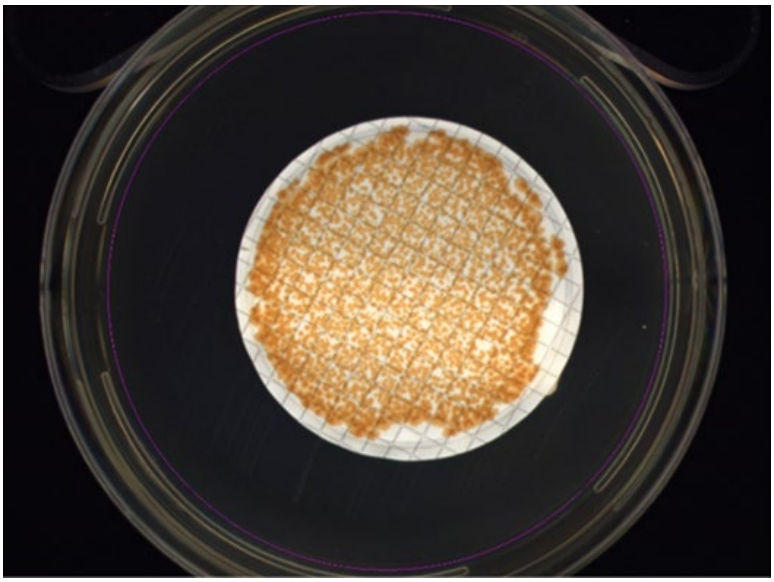

Figure 6-1. 1-mL aliquot filter plated from 10% bleach solution sample.

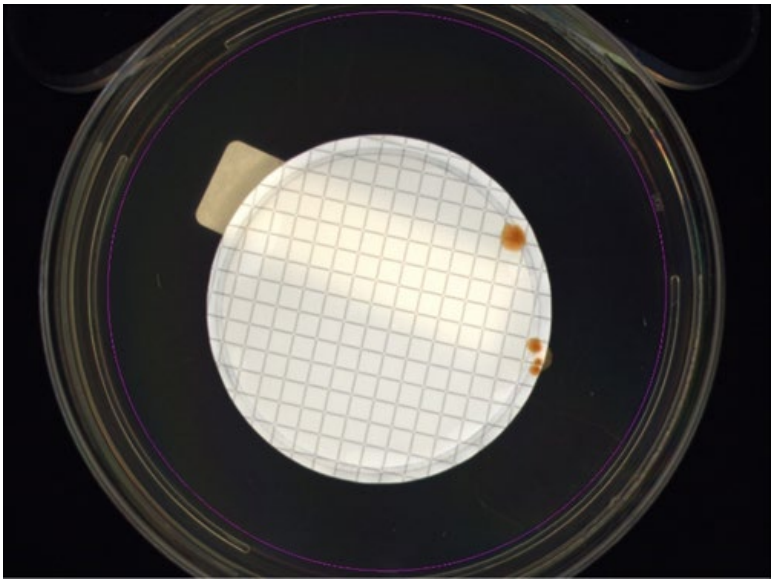

Figure 6-2. 10-mL aliquots filter plated from 10% bleach solution sample.

These unexpected results raised questions about all results obtained with the filter plating of the chlorinated bleach solutions. A scoping test (Appendix A) was performed to evaluate whether the different 0.45 µm Pall filter membrane materials (GN-6 white P/N 4804 mixed cellulose ester, black P/N 4805 and white P/N 4802 made of polyethersulfone [PES]) were influencing the recovery results. The black Pall filters are typically used to analyze lighter colored colonies (e.g., *Btk* colonies are white).

The results of the scoping test demonstrated that the GN-6 white Pall filters are not suitable for plating chlorine-containing samples. This is something that the EPA Biolab is looking into. All subsequent filter plating for this type of sample were performed with the PES type (black or white filters).

6.1.3 Neutralization Efficacy

This series of tests was performed to determine the neutralizer's ability to quench the sporicidal activity of a bleach-based decontamination solution before microbiological analysis. The neutralizer sodium thiosulfate (STS) was used in a 2× stoichiometry with the chlorinated bleach solutions. Both high (~ 7×10^7) and low (~5×10^2) *Bg* spore loading levels were tested to determine the effect of spore concentration on the neutralization effectiveness. The potential sporicidal activity of STS was also evaluated using the lower spore loading levels. The results of these tests are described in the next section.

6.1.3.1 High Spore Inoculated Solutions

Samples were prepared using chlorine-based Clorox® diluted bleach, hydrogen peroxide-based bleach solutions (Clorox 2®), or Tide® detergent diluted in a 1:10 ratio with DI water. Chlorine-based solution was neutralized using 2N-STS neutralizer that was added to each sample (using a 2× stoichiometric ratio) to evaluate its effectiveness in neutralizing the decontamination effects of the hypochlorite. The hydrogen peroxide-based bleach solutions and detergent (Tide®) were neutralized using 5% sulfuric acid (H_2S) and 1N-$KMnO_4$ (potassium permanganate). Samples containing the 10% dilute bleach-based solution and Tide® detergent with the neutralization agent were then inoculated with ~ 7×10^7 *Bg* spores. Spore recoveries from these samples were compared to recoveries from non-neutralized samples containing only phosphate buffered saline with 0.05% Tween 20 (PBST). All samples were analyzed for the presence of viable spores on the same day they were inoculated and again four 4 days later to evaluate the potential for residual decontamination.

The results illustrated in Figure 6-2, and detailed in Appendix B-1, show that same-day (Day 0) sample analysis yielded a mean spore recovery (6.8 x 10^7 spores) from the neutralized bleach samples that was surprisingly about 70% of the average recovery (9.8 x 10^7 spores) from the positive control PBST reference samples. These recoveries were expected to be more similar. However, the average recovery from these same chlorine-based bleach solution samples (3.9 x 10^7 spores), analyzed four days later, was only 48% of the average recovery from the PBST positive controls (8.1 x 10^7 spores), thereby suggesting some residual decontamination. Both neutralized hydrogen peroxide-based and the detergent (Tide®) solutions' recoveries were found to be stable over time.

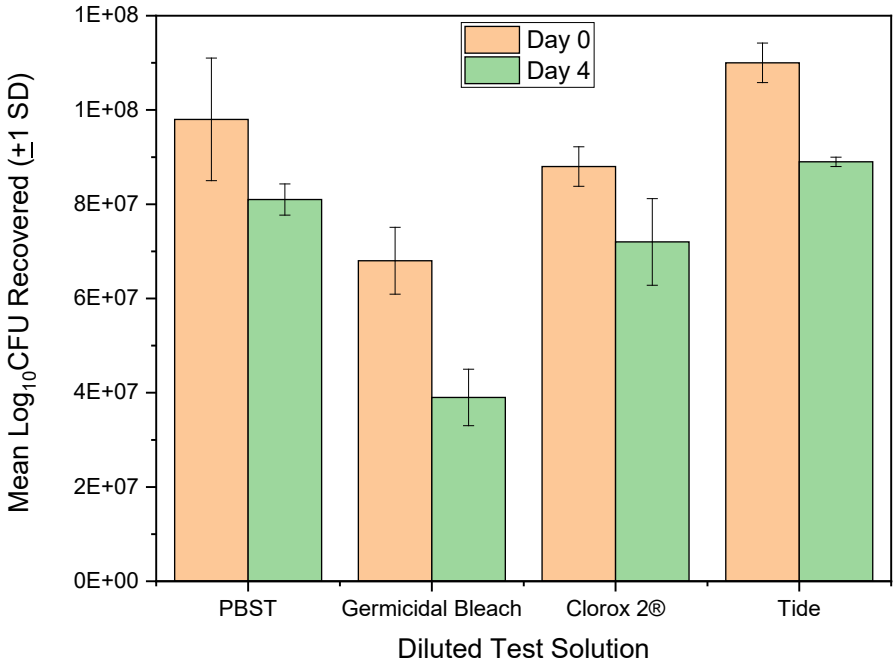

Figure 6-2. Temporal recoveries from neutralized test solutions inoculated at high-spore loading concentration.

6.1.3.2 Low Spore Inoculated Solutions

To test for the efficacy of neutralization of Clorox® chlorine-based diluted bleach solution at low spore concentration, 10% and 1% dilute bleach solutions were neutralized with appropriate amounts of 2N STS and then inoculated with *Bg* liquid inoculum at a tested concentration of 3.77×10^3. Each sample was inoculated with 133 µL for a target inoculation of 5×10^2 spores.

These samples were then plated immediately and again after 4 days to test for any residual decontamination effects. To test for the possible toxicity of the STS solution the amount of 2N STS used to neutralize the respective dilute bleach solutions was added to PBST. All samples were compared to positive controls consisting of inoculated PBST and inoculated DI water.

The results for this series of tests are illustrated in Figure 6-3 and detailed in Appendix B-2. The results confirm the high concentration results with a decrease in spore recovery over time. This decrease in recovery is also shown in DI water solution containing STS which suggests potential toxicity of the STS solution on viability of the spores. All the post-decontamination samples henceforth were analyzed the same day to minimize biasing the results.

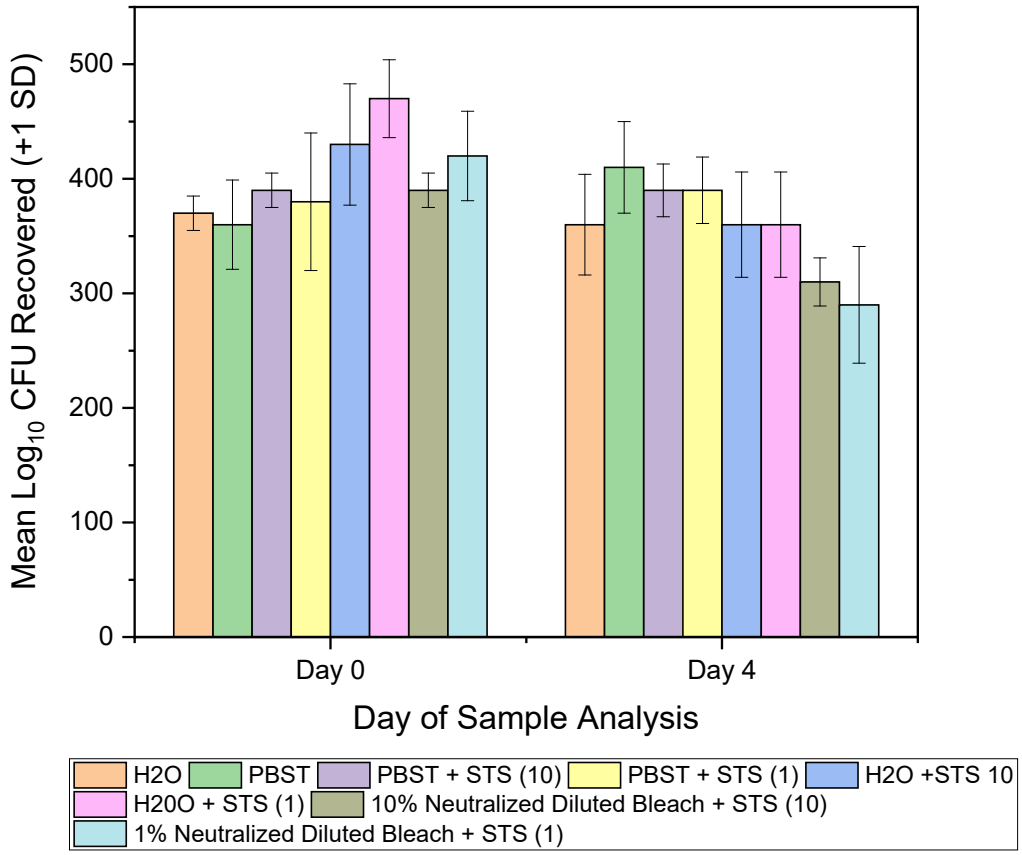

Figure 6-3. Temporal spore recoveries from neutralized solutions over time.

6.1.4 Preliminary Washing Solutions Evaluation

The target decontamination solutions were tested at two wash temperatures (72 ºF and 120 ºF) and at a dilution ratio of 1:10 in DI water to determine potential sporicidal efficacy. The test solutions were mixed in sterile 2-L Erlenmeyer flasks. Test solution aliquots (300 mL) were transferred to sterile 500-mL Nalgene bottles, heated to the desired test temperature in a water bath (the 120 °F samples only), and then inoculated with ~1×10^7 *Bg* spores from a liquid spore stock. Using an orbital shaker set at 250 rpm, each sample was agitated for a total duration of 9 min. After the 9-min cycle, samples were immediately processed and analyzed for potential viable spores. The tested solutions were as follows:

1. Tide® detergent
2. Clorox® diluted bleach
3. Clorox® 2

The spore recoveries for the two non-chlorinated solutions (Clorox® 2, and Tide® laundry detergent) were about the same levels as the spore recoveries for the DI water solution, as shown Figure 6-4 and detailed in Appendix B-3. Full decontamination with non-recoverable spores was observed for the chlorine-based Clorox® diluted bleach solution. A two-way ANOVA analysis showed that at the 0.05 level of confidence of the mean spore recoveries from the tested decontamination solutions were significantly different (P-Value =0.00) but the

interaction between the decontamination solutions and solution temperatures was not significant (P-Value = 0.98). The non-chlorinated bleach solutions, found not effective, were not tested further, and the wash solutions were maintained at ambient laboratory conditions henceforth.

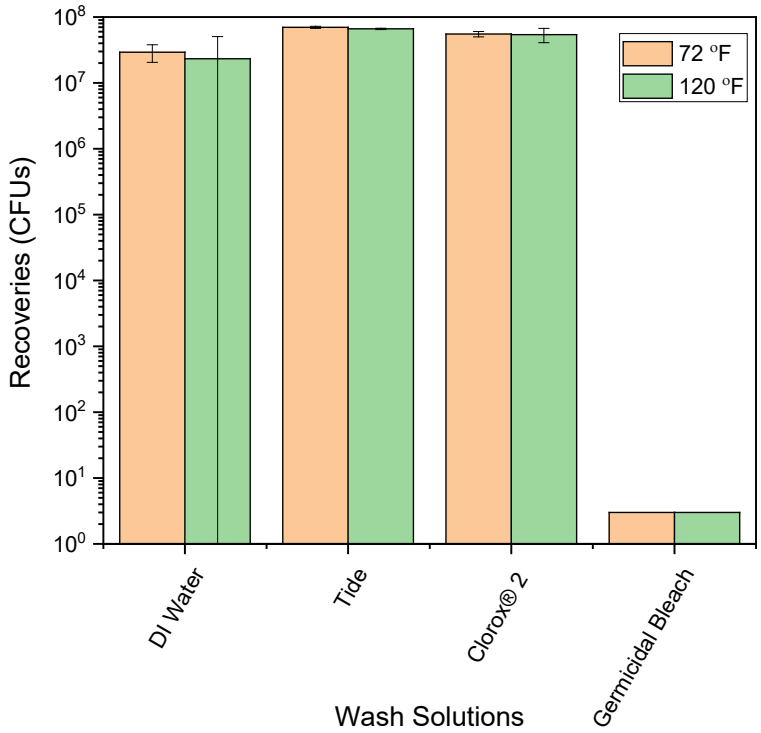

Figure 6-4. Wash liquid solutions recovery results.

6.1.5 Washing Parameters

This series of tests were performed to determine the optimum parameters for later tests. Clorox® concentrated germicidal bleach solution, designated as diluted bleach solution henceforth, was challenged with several dilution ratios and samples were subjected to various simulated wash cycle times. Later in this series, the use of Clorox® chlorine-based diluted bleach in combination with the addition of a detergent to decrease the hydrophobic reactivity and thus dispersion of the *Bg* spores in bleach solutions was evaluated. Surfactants are commonly used to reduce the surface tension and increase dispersion of bacterial spores in solution.

1. **Bleach Solution Dilution Ratio**
    1. 15:100 (15% diluted bleach)
    2. 10:100 (10% diluted bleach)
    3. 5:100 (5% diluted bleach)
    4. 3:100 (3% diluted bleach)
    5. 1:100 (1% diluted bleach)
2. **Wash Cycle Time**
    6. 9-min
    7. 18-min
    8. 27-min

The results, shown in Figure 6-5, and summarized in Appendix B-4, demonstrated that none of the solutions resulted in full decontamination of spores. The largest log reduction was obtained using a 10% bleach solution with a 27-minute simulated wash time. 1% bleach solutions had negligible decontamination efficacy at all wash times.

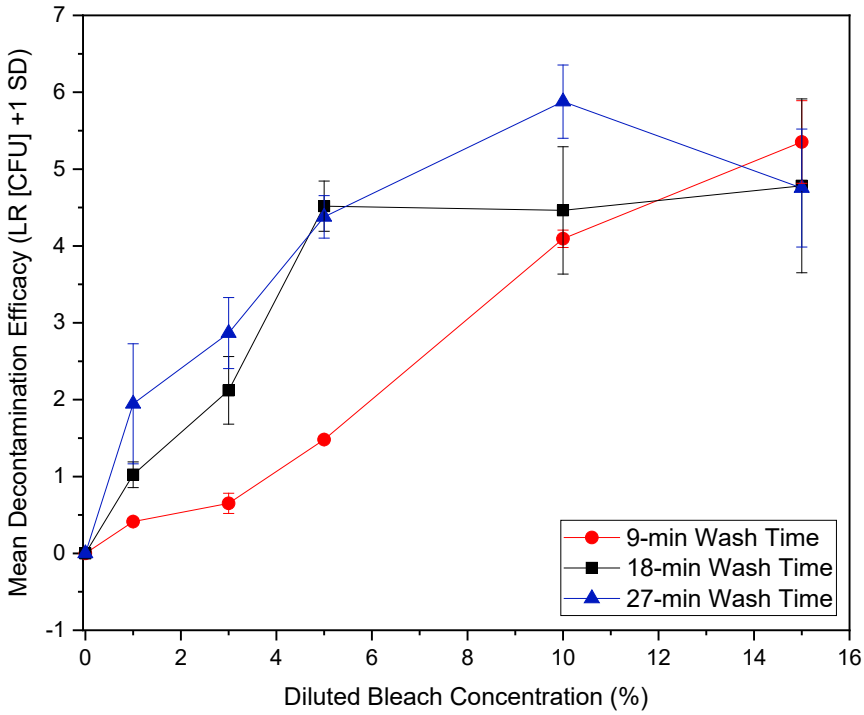

Figure 6-5. Chlorine-based bleach dilute solution decontamination efficacy.

6.1.6 Chlorine-Based Bleach with Surfactant

Following observations from the previous tests that none of the diluted bleach concentrations had full decontamination, it was postulated that the hydrophobic nature of the spores may have contributed to these results. The hydrophobic properties of the spores may have led to clumping and prevented full interactions between the spores and the decontamination solutions. A new series of tests was designed to include the addition of a commercial off-the-shelf surfactant (Tide®) detergent to the bleach decontamination solutions. Manufacturer's directions were used to add the appropriate amount of detergent to each of the bleach decontamination solutions, 300 µL of Tide® detergent per liter of decontamination solution. Samples were inoculated with ~2×10^7 spores and subjected to a series of tests evaluating simulated wash times and dilution ratios. The following wash times and dilution ratios were evaluated.

1. **Bleach Solution Dilution Ratio**
    1. 15:100 (15% diluted bleach)
    2. 10:100 (10% diluted bleach)
    3. 5:100 (5% diluted bleach)
    4. 3:100 (3% diluted bleach)

5. 1:100 (1% diluted Bleach)
6. 5:1,000 (0.5% diluted bleach)
7. 1:1,000 (0.1% diluted bleach)
8. 1:10,000 (0.01% diluted bleach)

2. Wash Cycle Time

1. 9-min
2. 18-min
3. 27-min

The results, shown in Figure 6-6 and summarized in Appendix B-5, indicate that full decontamination was obtained using a 1% diluted bleach solution with the addition of a detergent with simulated wash times of 18 and 27 minutes. Diluted bleach solutions containing less than 1% bleach had minimal decontamination efficacies with the exception of the 0.5% germicidal bleach solution with a 27-minute wash time which resulted in only a 3-log reduction of spores. Higher concentrated bleach solutions resulted in similar inactivation as with the 1% diluted bleach. The 18-minute wash times provided similar results to the 27-minute wash times and were used for all subsequent tests.

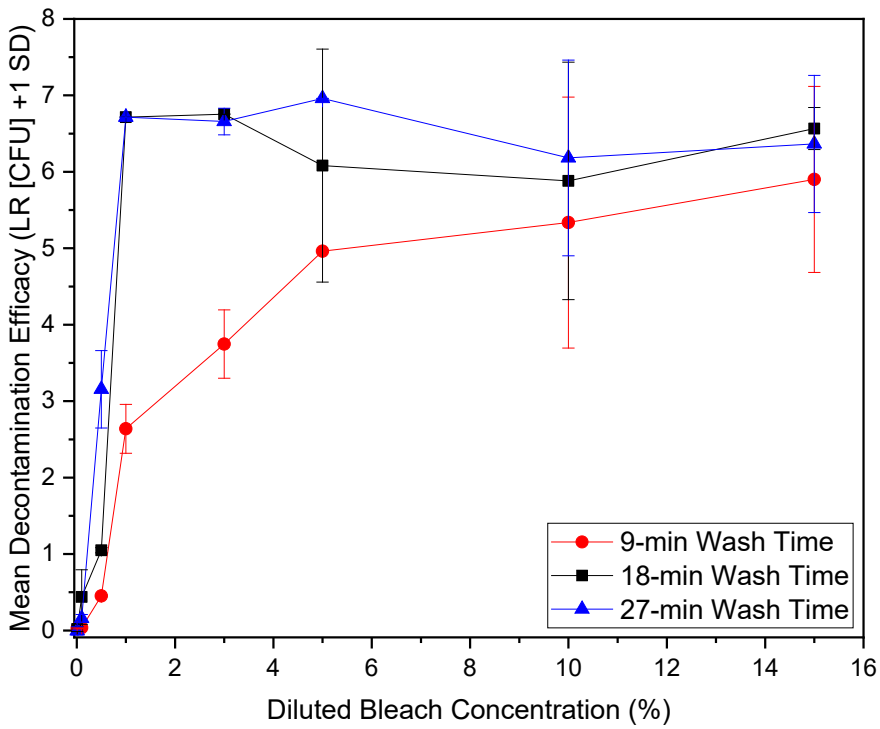

Figure 6-6. Diluted bleach solution with the addition of a detergent decontamination efficacy.

As shown in Figure 6-7, the addition of a detergent to the diluted bleach solutions, with an 18-minute wash time, increased the decontamination effectiveness by orders of magnitude. This effect is most pronounced with the 1% diluted bleach solutions in which there is ~7 log reduction difference (6.7 log reduction and 1.0 log reduction) between the solutions with and without detergent respectively. The hydrophobic nature of the spores may have led to spore

clumping in the liquid bleach solutions and offered some protection to the entrapped spores from the decontaminant agent. The addition of the detergent may have decreased the clumping of the spores and made them more susceptible to the bleach decontamination solution. Surfactants such as Tween 80 have been reported to improve spore extraction efficiency from various solutions (Da Silva et al., 2011). The surfactant-containing detergent may have reduced spore-spore interactions or spore-container interactions such that spores were more evenly dispersed in solution and thereby more readily in contact with the decontamination agent.

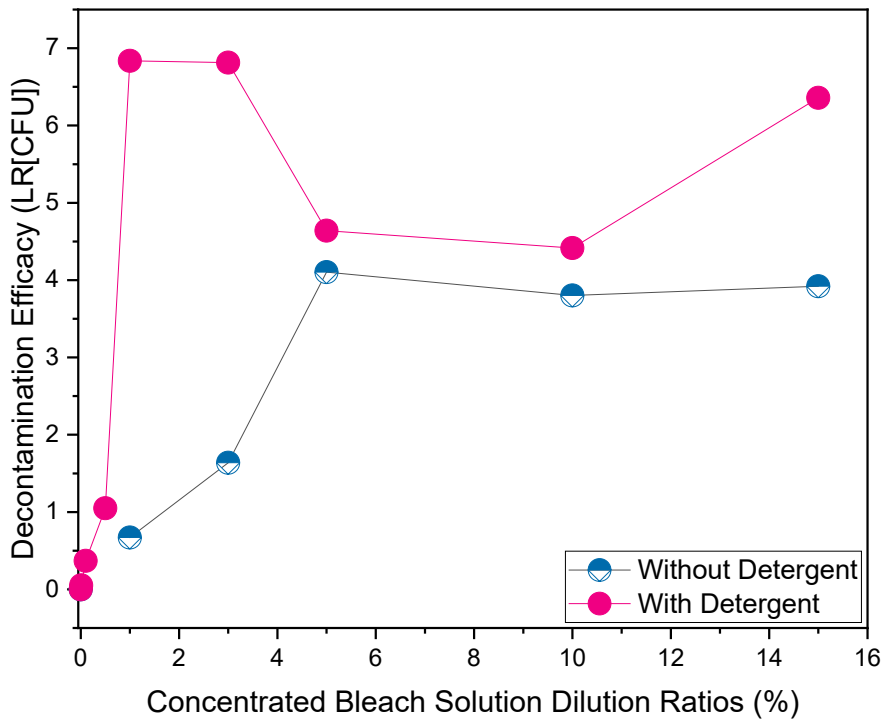

Figure 6-7. Decontamination efficacy of a diluted chlorine-based bleach solution with and without the addition of a detergent.

Phase II: (Bench-Scale Testing with Material Coupons)

A series of tests were conducted with the germicidal bleach solution with and without the addition of a surfactant (Tide® liquid detergent) to determine the decontamination efficacy of the different bleach formulations. The coupons used for this series of tests and the remainder of tests were all inoculated with ~ 10^7 *Bg* spores using the method described in Section 3.4.2.1. The following sections discuss the results of these tests.

6.1.7 Diluted Bleach Solution: Cotton Material

The method development tests showed that chlorinated germicidal bleach was the most effective off-the-shelf decontaminant of *Bacillus* spores. A series of tests was designed to determine the conditions that would provide optimal decontamination conditions while using the most diluted bleach. These tests were run using an 18-min wash cycle on inoculated cotton

materials with no detergent added. The tests were run at three bleach dilution ratios (1:10 (10% bleach), 1:100 (1% bleach), and 1:1000 (0.1% bleach).

The results for these tests are shown in Figure 6-8 and summarized in Appendix C-1. An increase in the concentration of the germicidal bleach solution resulted in a decrease of spores recovered on the cotton material coupons. The 10% bleach resulted in no-detectable spores on the cotton material as well as the post decontamination rinse samples. Close to a 6 LR (5.76 LR), and just over 4 LR (4.45 LR) were observed on the coupon material for the 1% and 0.1% bleach solutions with detectable spores found in both the post decontamination wash and rinse samples.

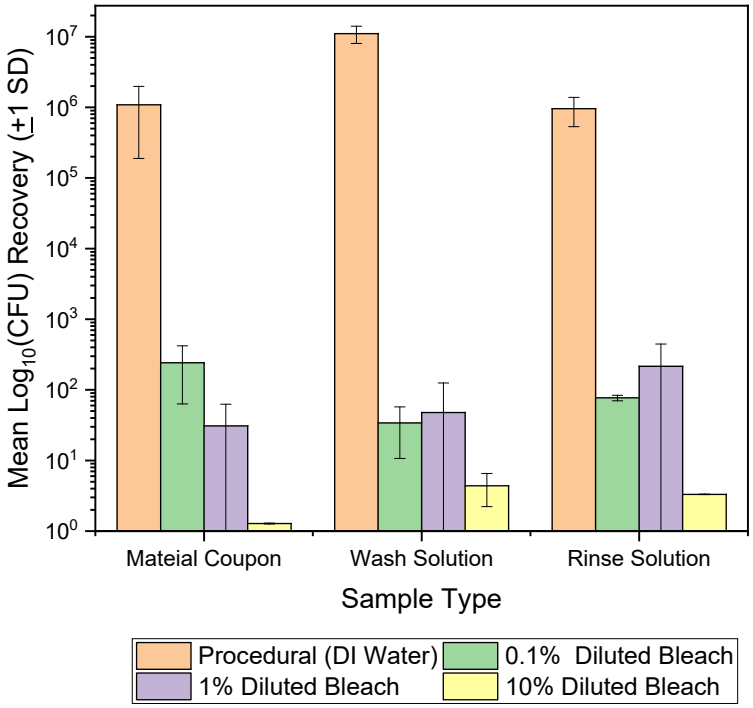

Figure 6-8. Spore recovery and redistribution for different diluted bleach solutions.

Procedural positive experiments were also conducted following the same parameters as the decontamination tests without any bleach added to the decontamination solutions. These tests were used to evaluate the redistribution of spores during the wash and rinse procedure. In both tests a < 2 LR was observed for the cloth surfaces and a large redistribution of spores to the wash and rinse samples were noted. These results suggest that the inactivation of the spores occurs both on the surface of the material coupons and in the wash solution.

6.1.8 Diluted Bleach Solution with the addition of Detergent: Cotton Material

After the previous tests showed a greater decontamination efficacy, using an aerosol deposition and cloth coupons, and the results from the Phase I tests demonstrated the increased decontamination efficacy by adding a surfactant to the wash solutions, an additional test was conducted to evaluate the effectiveness of lowering the concentration of the germicidal bleach decontamination solutions in conjunction with the addition of detergent. This test was conducted using the same parameters as the previous tests apart from the addition of a detergent to the 1% bleach solution.

Results shown in Figure 6-9 confirmed previous results that the addition of a detergent to the bleach solutions increased the decontamination efficacy. The 1% diluted bleach with the addition of a detergent achieved a similar log reduction compared to the 10% diluted bleach without the addition of a detergent (6.8 LR and 7.2 LR, respectively) with non-detectable spores on the material coupons. Both of these solutions were at least 1 log greater in their decontamination efficiencies compared to the 1% bleach without the addition of a detergent (<5.8 LR). Recoverable spores were found in both the 1% and 10% diluted bleach without detergent wash solutions.

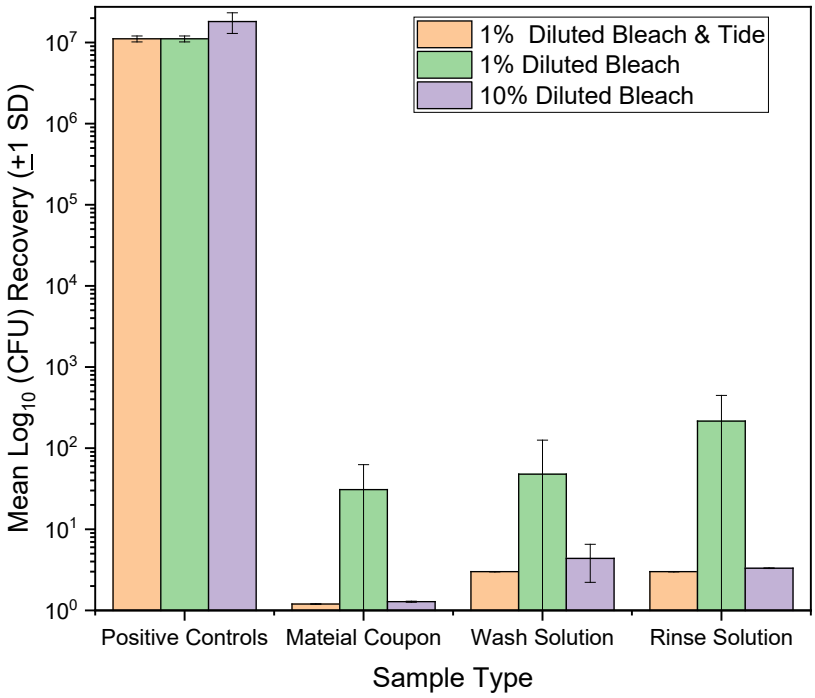

Figure 6-9. Spore recovery and redistribution of material coupons treated with 10% diluted bleach without detergent and 1% diluted bleach with and without detergent.

6.1.9 1% Diluted Bleach with the Addition of a Detergent: All Materials

Following the results from the previous tests showing that 1% bleach with detergent is as efficacious as 10% bleach without detergent and provided almost a 7 LR, the remaining materials were tested following the same parameters. The results, shown in Figure 6-10 and Figure 6-11, and summarized in Appendix C-2, demonstrated full decontamination efficacy on cotton, blend, spandex, and polyester materials. The denim and military materials only had a 4.7 LR. The lower decontamination efficiencies of the denim and military material may have been due to the thicker and more complex weaves compared to the other materials. There were detectable spores on both the post decontamination wash and rinse samples for all materials, with the denim and military materials having the highest number of spores detected in both the post decontamination wash and rinse samples.

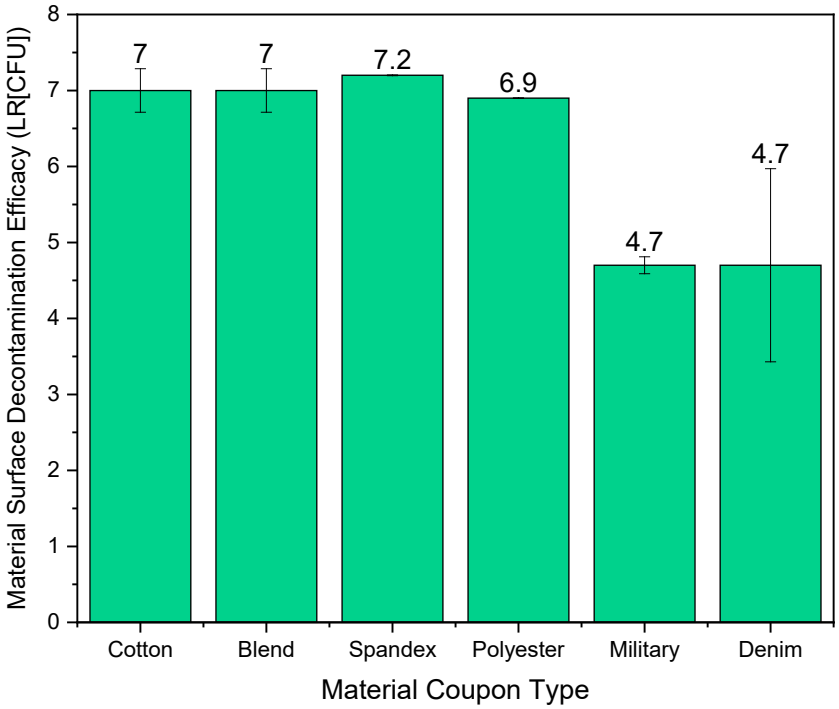

Figure 6-10. Bench-scale decontamination efficacy results for all material coupons using 1% diluted bleach.

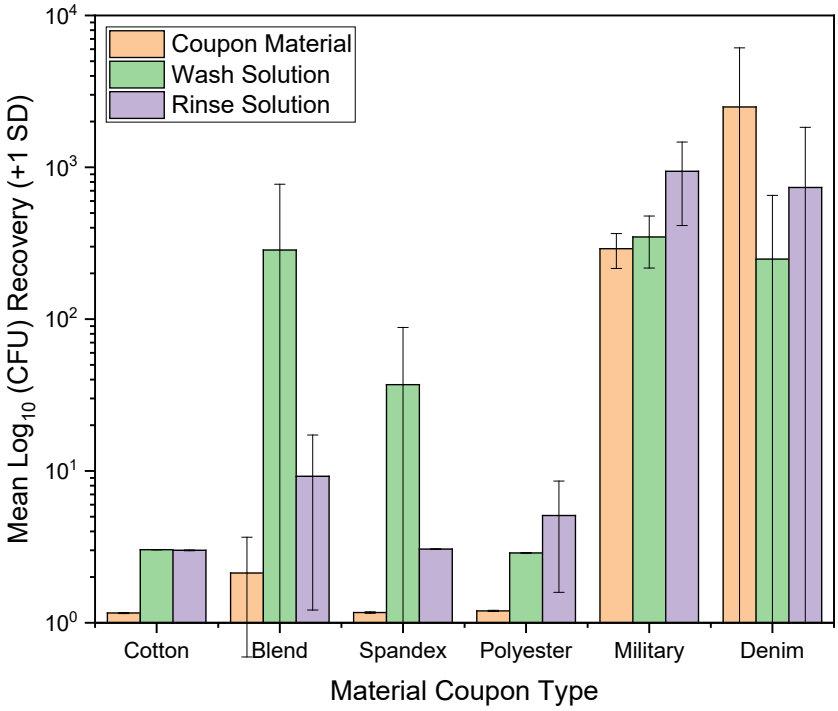

Figure 6-11. Spores recovered from material coupons using 1% diluted bleach solution with detergent.

Each material also underwent a procedural positive wash during which they were subjected to the same wash and rinse times without the addition of bleach. The procedural positive test results are shown in Figure 6-12, < 2 LR was obtained on the procedural positive material samples regardless of the type of material used. These results suggest that the spores were completely inactivated by the bleach solution rather than just redistributed during the procedure.

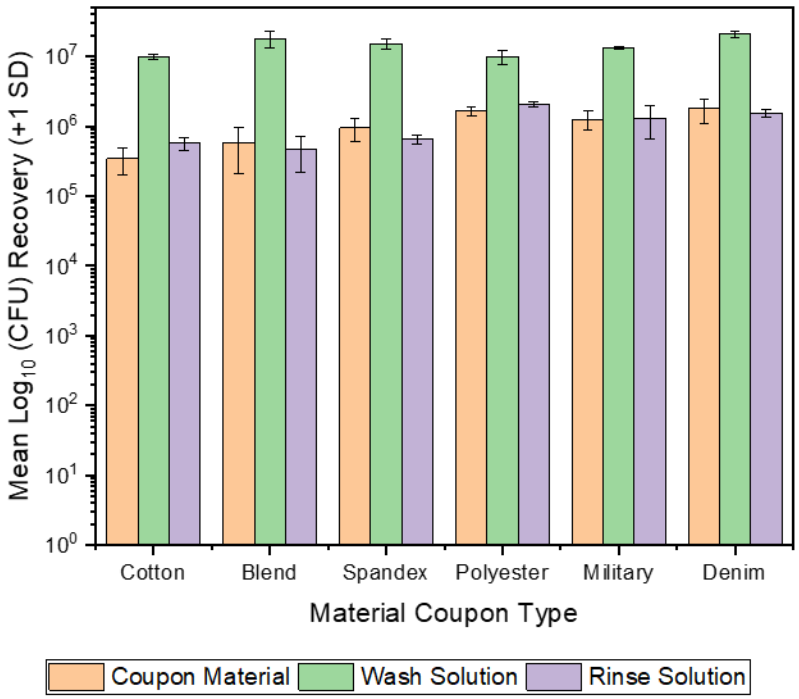

Figure 6-12. Spores recovered from material coupons subjected to DI water procedural testing process.

Phase III: (Decontamination Testing using a Washing Machine)

Results from Phase II tests showed that a 1:100 (1% Clorox® diluted bleach) dilution ratio with the addition of a detergent provided acceptable decontamination results (> 6 LR) for all materials with the exception of the military and denim cloth. The series of tests in Phase III used this optimized decontamination solution on larger material coupons, 14 in. × 14 in., using off-the-shelf washing machines (see Section 3.7.2).

6.1.10 1% Diluted Bleach with Detergent: All Materials

The decontamination results for all six materials tested with the washers are shown in Figure 6-13 and summarized in Appendix D-1. The diluted bleach solution (dilution ratio: 1:100) with the addition of a detergent resulted in non-detectable spores on the surface of the material and in both the liquid washing and rinse solutions. These results confirm the results obtained in Phase II. Interestingly, both the military and denim materials had no-detectable spores on their surfaces. This effect may have been due to the increased agitation from the washing machines, which may have liberated spores embedded within the materials, making them more susceptible to decontamination.

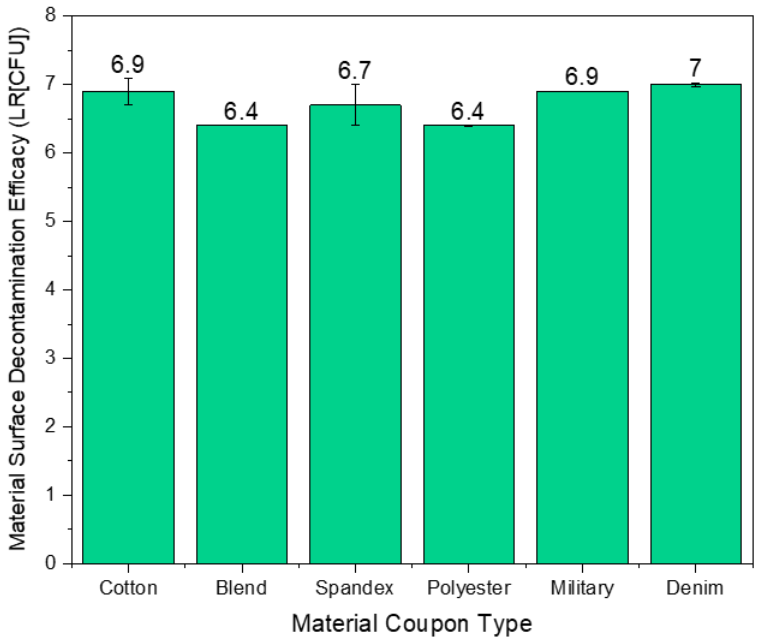

Figure 6-13. Pilot-scale washer decontamination efficacy results for all material coupons using 1% diluted bleach solution with detergent.

Confirming the observation that the increased agitation of the washing machines liberated more spores from the clothing materials, procedural positive coupon samples all had a greater log reduction during this phase of testing compared to the bench scale tests in Phase II. These results are shown in Figure 6-14.

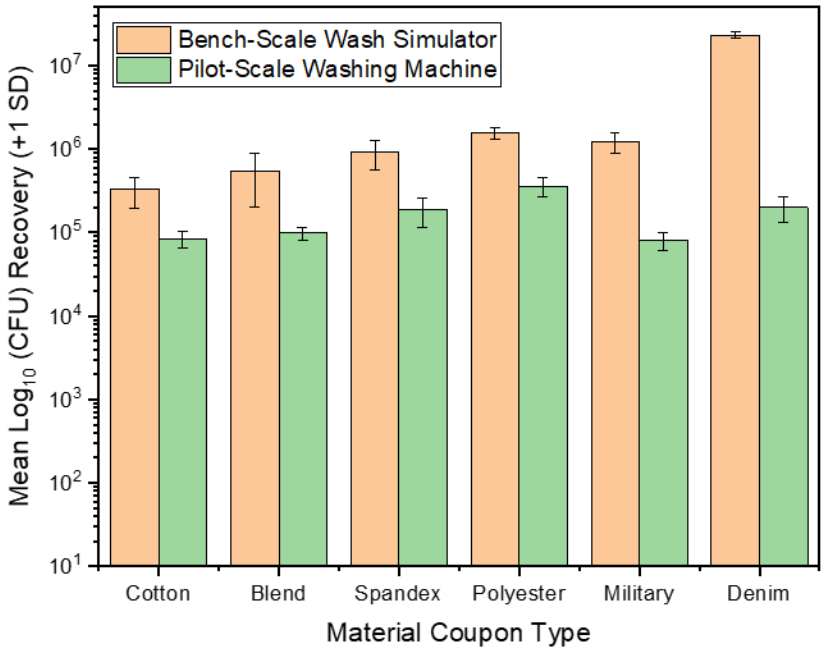

Figure 6-14. Bench-scale versus pilot-scale spores recovered from material coupons subjected to DI water procedural testing process.

6.1.11 1% Diluted Bleach Without the Addition of a Detergent: Cotton and Military Materials

A limited number of tests were performed to determine whether the use of detergent increased the decontamination efficacy of the bleach solutions given the increased agitation from the washing machines. Cotton and military cloth coupons were tested in the washing machines using a 1:100 dilution ratio (1% diluted bleach solution) with and without the addition of a detergent. Cotton was chosen because it was the material with the most data having been used for all scoping tests. The military material was chosen for this series of tests because it had the lowest surface log reduction during the Phase II tests and was used to challenge these test conditions.

The results for these tests are shown in Figure 6-15, and detailed in Appendix D-2. The germicidal bleach solution (dilution ratio: 1:100), with and without the addition of detergent, resulted in similar decontamination efficacies. These results confirm that the agitation dynamic of the washers increased physical removal of the spores from the surface of the coupons to the wash solution, thus resulting in a more efficient decontamination process.

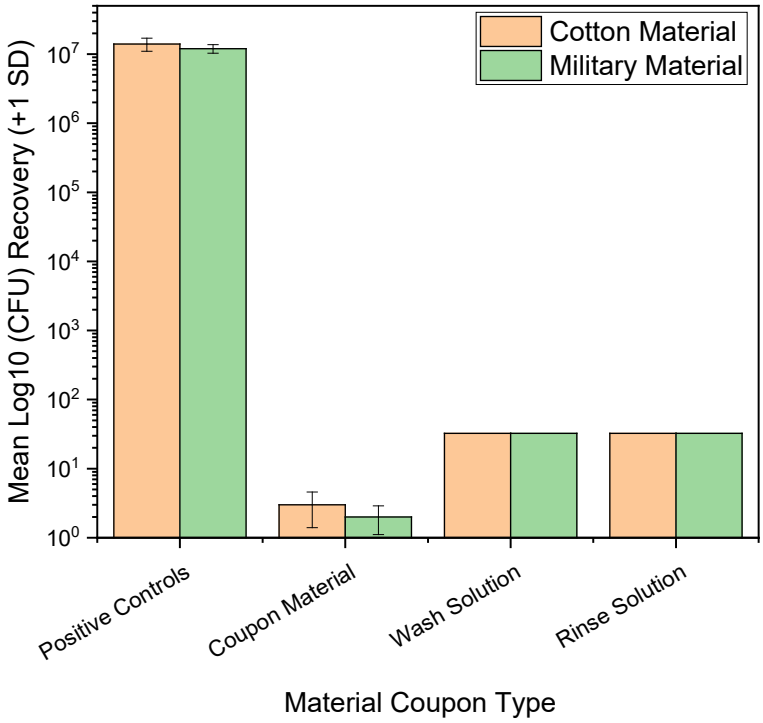

Figure 6-15. Spores recovered from cotton and military material coupons using 1% diluted bleach solution without the addition of detergent using washing machine.

Phase IV: Decontamination Testing using a Washing Machine/Dryer

A series of tests was designed in this phase to evaluate the effect of adding a drying step to the decontamination process. Re-aerosolization of spores which were not decontaminated during the wash cycle were also of interest. HEPA filters were placed on the air inlet and outlet of the dryer to eliminate any cross contamination from outside sources and to capture any spores

potentially released during the drying process. 14 in. × 14 in. cotton coupons were inoculated with 10^7 spores and were first subjected to an 18-minute wash cycle followed by a 5-minute spin cycle, 9-minute rinse, additional 5-minute spin and finally a drying cycle using "High Heat" for 30 minutes.

Diluted bleach solution with a high dilution ratio (1:1000) with the addition of a liquid detergent that was used previously in Phase II did not result in full decontamination of the inoculated fabric material; hence, this condition was tested here to determine if the addition of heating improved the inactivation of spores, when compared to a washing step alone.

The results for this test are illustrated in Figure 6-16 and summarized in Appendix E-1. The addition of a drying sequence resulted in no apparent increase in the decontamination efficacy of the diluted bleach solution. A similar number of spores were detected on the cotton coupons following both procedures. The difference between the number of spores recovered between the two procedures is made up almost entirely of spores recovered from the HEPA filters placed on the air outlet of the dryers. Due to the potential for spore re-aerosolization and lack of an increase in decontamination efficacy, drying of washed contaminated clothing may not be the best approach for a potential self-help practice to reduce cross-contamination among an affected population.

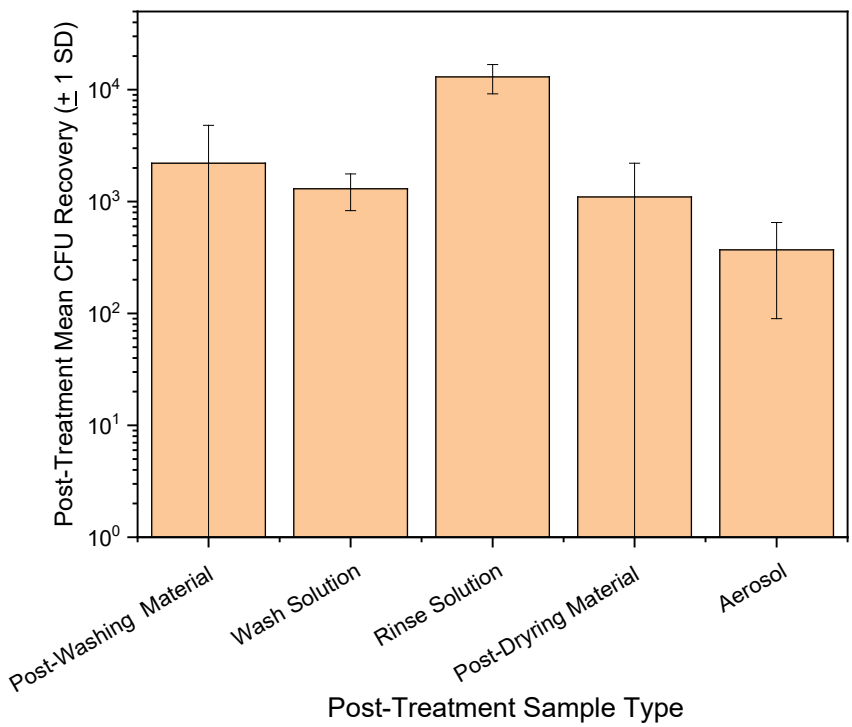

Figure 6-16. Spore distribution following a post-decontamination washing/drying cycle using 0.1% diluted bleach solution with the addition of a detergent.

7.0 QUALITY ASSURANCE AND QUALITY CONTROL

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

The following sections discuss the measurement equipment calibration, the criteria for the critical measurements and parameters, data quality indicators, and the quality assurance (QA) and quality control (QC) checks for the project.

Supplies and Consumables

Test materials must be uniform to ensure reliable test results. Uniformity was maintained by obtaining a large enough quantity of material so that multiple coupons could be constructed with presumably uniform characteristics.

To determine the sampling efficiency, there must be assurances that the enumeration of viable spores is representative of the actual number of spores present and not the result of contamination or faulty culture. The QC checks are discussed in Section 7.4 to provide this assurance.

Measurement Equipment Calibration

The DTRL and Biolab have standard operating procedures for the maintenance and calibration of all laboratory equipment. All equipment is verified as being certified calibrated or by having the calibration validated by the Air Methods and Characterization Division's Metrology Laboratory at the time of use. Table 7-1 summarizes the instrument calibration frequency.

Table 7-1. Instrument Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
Thermometer	Compare to independent NIST thermometer (a thermometer recertified annually by either NIST or an ISO-17025 facility) value once per quarter	±1 ºC
Stopwatch	Compare to official U.S. time at time.gov every 30 days	±1 minute every 30 days
Micropipettes	Certified as calibrated at time of use; recalibrated by gravimetric evaluation of performance to manufacturer's specifications every year	±5%
Scale	Compare reading to Class S weights	±1%
Graduated cylinder	Collect effluent at specified time.	± 1 mL

Criteria for Critical Measurements and Parameters

The data quality objects define the critical measurements needed to address the stated objectives and specify tolerable levels of potential error associated with the decontamination event. The following measurements have been deemed critical to accomplishing the project objectives:

- pH and temperature of the diluted bleach solution and any other liquid solution being used
- Sodium hypochlorite concentration as free available chlorine (FAC) of the diluted bleach decontamination solution (quantitative)
- Washing solution volume
- Water rinse volume
- Washing time
- Drying time
- Washing solution temperature

Data quality indicator goals for the critical measurements are used to determine if the collected data meet the objectives established for this project. All the acceptance criteria for the critical parameters listed in Table 7-2 were met for this project.

Table 7-2. Data Quality Acceptance Criteria for Critical Measurements

Critical Measurement	Analysis Method	Accuracy/Precision	Acceptance Criteria
CFU per plate	Spiral plater/QCount	50% RSD among the triplicate plating	50% RSD
Temperature of incubation chamber	NIST-traceable thermometer (daily)	± 2 °C	38 ± 2 °C
Decontamination time	NIST-calibrated stopwatch	± 1 minute/hour	± 2 minutes (2 × ± 1 min)
FAC	$Na_2S_2O_3$/KI titration	± 0.06 gram per liter	± 10% of target value
pH	pH meter /NIST-traceable buffer solutions	± 0.01 pH unit	10% RSD
Titration/extraction/sample/neutralizer volumes volume and extraction volumes	Burette	± 1 mL	± 10% of target value
Water bath Temperature	Thermometer	± 1 °C	± 10% of target value

RSD = Relative standard deviation

Control Checks

Quantitative standards do not exist for biological agents. Viable spores were counted using a QCount® colony counter. Counts generated that were either greater than 300 or less than 30 were considered outside of the targeted range. If the count of colony-forming units for bacterial growth did not fall within the target range, the sample was either filtered or replated. Replates and filter plates were enumerated manually.

Before each batch of plates were enumerated on the QCount®, a QC plate was analyzed, and the result was verified to be within the range indicated on the back of the QC plate. As the plates were being counted, a visual inspection of colony counts made by the QCount® software was performed. Obvious count errors made by the software were corrected by adjusting the settings (e.g., colony size, light, and field of view) and recounting or by manually removing or adding colonies as needed.

The acceptance criteria for the critical CFU measurements were set at the most stringent level that could be achieved routinely. Positive controls were included along with the test samples in the experiments so that spore recovery from the different surface types could be assessed. Background checks were also included as part of the standard protocol to check for unanticipated contamination. Replicate coupons were included for each set of test conditions to characterize the variability of the test procedures.

Additional QC samples were collected and analyzed to check the ability of the BioLab to culture the test organism, as well as to demonstrate that materials used in this effort did not contain spores. The checks included the following:

- Procedural blank coupons: Material coupons sampled in the same fashion as test coupons but not contaminated with surrogate organism prior to sampling.
- Stainless-steel positive control coupons: Coupons inoculated in tandem with the test coupons and meant to demonstrate the highest level of contamination recoverable from an inoculation event.

Additional QC checks for BioLab procedures are listed in Table 7-3. These provide assurances against cross-contamination and other biases in microbiological samples.

Table 7.3. QA/QC Sample Acceptance Criteria

QC Sample	Information Provided	Frequency	Acceptance Criteria
Positive control (sample from material coupon contaminated with biological agent but not subjected to decontamination	Initial contamination level on the coupons; allows determination of log reduction *(LR)	3 or more replicates per test	Target loading of 1×10^7 CFU per sample, with standard deviation of < 0.5 log (5×10^6 to 5×10^7 CFU per sample)
Procedural blank (test material coupon without biological agent)	Controls for sterility of materials and methods used in the procedure	1 per test	No observed CFU

QC Sample	Information Provided	Frequency	Acceptance Criteria
Laboratory blank (TSA sterility control; plate incubated but not inoculated)	Controls for sterility of plates	Each plate incubated for at least 18 but less than 24 hours	No observed growth after incubation
Inoculation control (replicate of microbiological dilution plates)	Controls for repeatability of results	3 per dilution	Counts greater than 30, and less than 300 are reportable; standard deviation must be < 100%; Grubbs outlier test or equivalent
Blank plating of microbiological supplies	Controls for sterility of supplies used in dilution plating	3 of each supply per plating event	No observed growth after incubation
Procedural blank	Contamination level present during sampling	1 per sampling event	Non-detect
Field blank coupon	Verify the sterility of materials used to analyze viable spore count	3 per material per test	No detectable spores
MDI control (wipe sample from stainless-steel coupon contaminated with biological agent)	Initial contamination level on coupons; shows plate's ability to support growth	3 replicates per MDI use	Target loading CFU per sample, with a standard deviation of < 0.5 log

The QA/QC control test results for the whole sampling campaign are listed for each test result tabulated in Appendices B, C, D, and E for Phases I, II, III, and IV respectively. Sterility checks were conducted for all the equipment and materials and listed for each test in the result section. Most of the control blanks (negative controls), sampling procedure blanks (procedural blanks), and the inoculum control blanks (stainless-steel control blanks) were non-detectable. Some control blanks were found to be contaminated, but they had little or no effect on the final results. The detection limit for each sample was set at 1 CFU per sample volume extracted.

8.0 Summary

The main purpose of this study was to evaluate potential decontamination solutions that can be efficacious in removal and/or inactivation of bacterial spores from contaminated clothing. The overall process of data generation included bench scale testing to simulate the washing and drying of contaminated clothes with viable spores of *Bg*. The washing/drying decontamination approach was evaluated as a function of different parameters such as wash temperature, wash cycle, material clothing type, wash solution type (water versus bleach-type solution), water rinse, and drying. Six materials (cotton, stretch denim, polyester fleece, spandex, cotton/polyester blend, and US Navy camouflage)) were evaluated to determine if the clothing material type had any effect on the overall decontamination approach.

Results from the first phase indicated that oxygen-based diluted (10%) bleach solutions (Clorox® 2, and Tide® Free & Gentle laundry detergent) were not sporicidal. Full decontamination with non-recoverable spores was observed for the Clorox® chlorine-based diluted bleach solution. The effect of temperature on the decontamination efficacy of the wash solutions was found to be negligible at the two temperatures that were evaluated.

The non-chlorinated bleach solutions were not tested further, and the wash solutions were maintained at ambient laboratory conditions henceforth. Additionally, Phase I experiments demonstrated that the addition of a detergent to the chlorine-based diluted solution allowed a significant decrease in concentration (down to 1%) while still maintaining adequate decontamination efficacy (> 6 LR). The addition of Tide® detergent may have caused the reduction of spore clumping in liquid-contaminated solution; hence increasing the effectiveness of the bleach solution.

Phase II testing confirmed results from the Phase I tests that the 1% Clorox® chlorine-based bleach solution was a highly effective decontamination approach. This solution provided a > 6 LR for all cloth materials (cotton, blend, spandex, and polyester) except for denim and military fabrics (5.7 and 4.7 LR, respectively). The lower decontamination efficiencies of these two fabrics may have been the result of a denser more complex weave and a greater number of spores being entrapped within these cloths compared to the other 4 materials. It should also be noted that greater decontamination efficacies were observed during this phase of testing compared to the Phase I tests. This effect may have been caused by the aerosol inoculation of material coupons during Phase II tests compared to the liquid inoculation used during Phase I tests.

Results from Phase III confirmed results obtained in Phase II, that 1% diluted Clorox® chlorine-based bleach solution with the addition of a detergent using an 18-minute wash time was effective in decontaminating fabric inoculated with ~10^7 spores per square foot. All materials had surface log reductions > 6. The increased surface decontamination efficacies seen during this phase may have been due to the increased agitation dynamic from the washing machines.

In Phase IV a drying sequence was added to the wash sequence. The addition of a drying sequence resulted in no apparent increase in the decontamination efficacy of the diluted bleach solution. A similar number of spores were detected on cotton material coupons following the wash cycles with and without a drying step. The difference between the number of spores recovered from the coupons between washing and drying is made up almost entirely of spores

recovered from HEPA filters placed on the air outlet of the dryers. Due to the potential for spore re-aerosolization and lack of an increase in decontamination efficacy, drying of washed contaminated clothing may not be the best approach for a potential self-help practice to reduce cross-contamination among an affected population.

This approach can be used to lessen potential cross-contamination among people such as first responders, security personnel, or normal citizens. As with any process there are many variables which can have an impact on the efficacy of this approach. Some of these variables include how the material is agitated in the washing machine (e.g., how the material folds or crumples in the wash/dryer cycle), volume of the washing machine and impact of the agitation mechanism, clothing load, and organic load (e.g., soiling of clothing).

One of the primary goals of this project was to demonstrate the effectiveness of washing and drying cycles for inactivating *Bacillus* spores for clothing. The process parameters identified in this project could be used as a risk reduction option in the event of a wide area release of *Bacillus anthracis*.

Safety Considerations

• Spores can be released to the air and can therefore be a significant source of cross-contamination during a remediation and can pose health risks to persons performing decontamination tasks.

• When using bleach or other disinfectants and cleaners follow the instructions on the labels for precautions, use, and personal protective equipment (PPE) recommendations.

• Never mix bleach products together with vinegar or ammonia.

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Appendix A. Challenges with Filter Plating Residual Decontamination

While processing the Clorox® bleach post-decontamination samples, the filtration and subsequent plating of 1-mL and 10-mL aliquots through white Pall® filters (P/N 4804 MicroFunnel™ with GN-6 Metricel® membrane with a 0.45 µm pore size made from a mixed cellulose ester) resulted in unexpected recoveries. The 1 ml filters had too many colonies to count (too numerous to count, TNTC) while the 10 ml filters were either non-detect or had very few colonies. Photos of filter plates from the same selected sample, plated at the same time, are shown in Figures A-1 and A-2, respectively.

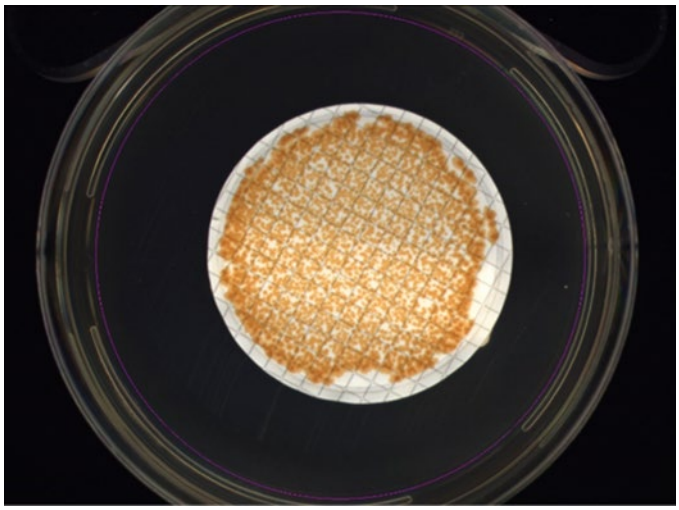


Figure A-1. 1-mL aliquot filter plated from 10% bleach solution sample.

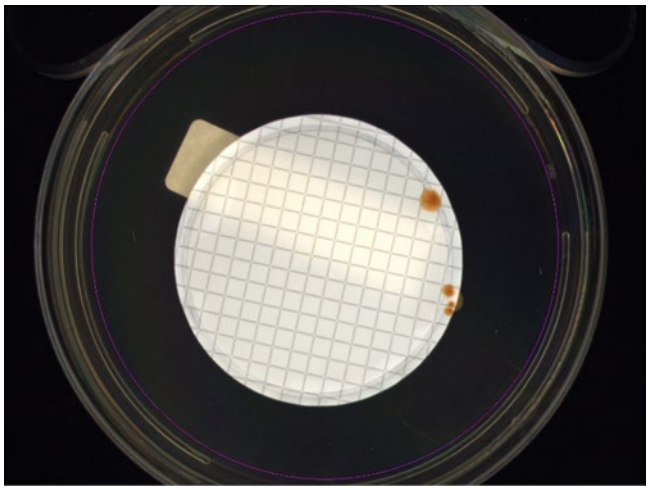


Figure A-2. 10-mL aliquots filter plated from 10% bleach solution sample.

These unexpected results raised questions about all the results obtained via filter plating of chlorinated bleach solutions. A scoping test was performed to evaluate whether the different 0.45 µm Pall filter membrane materials (GN-6 white P/N 4804 mixed cellulose ester, black P/N 4805 and white P/N 4802 made of polyethersulfone [PES]) were influencing the recovery results.

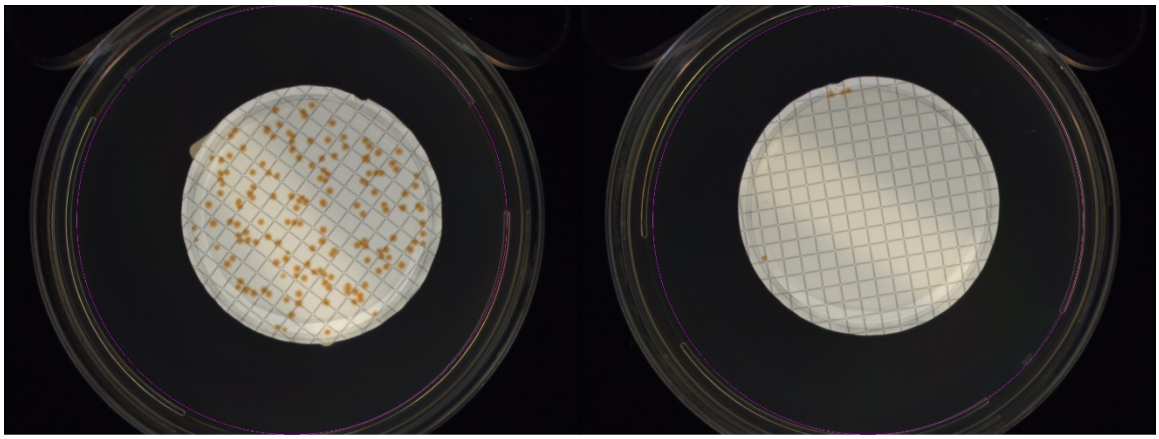
The same samples with 10% bleach and 1× and 2× STS were filter plated and plated on all three types of filters. Aliquots of 1, 10, and 100 ml were filter plated with no extra rinse. All the PES (black and white filters) filters had colony growth at the expected levels, while the GN-6

white filters were found to have no growth at the 10-ml and 100-ml aliquots. The data results for the scoping tests are presented in Table A-1 and pictures of the 10% bleach filtered samples are shown in Figures A-3 through A-5 for all three filters for the 1-ml and 10-mL aliquots.

These results demonstrate that the GN-6 white Pall filters are not suitable for plating chlorine-containing samples. All subsequent filter plating for this type of sample was performed with the PES type (black or white filters).

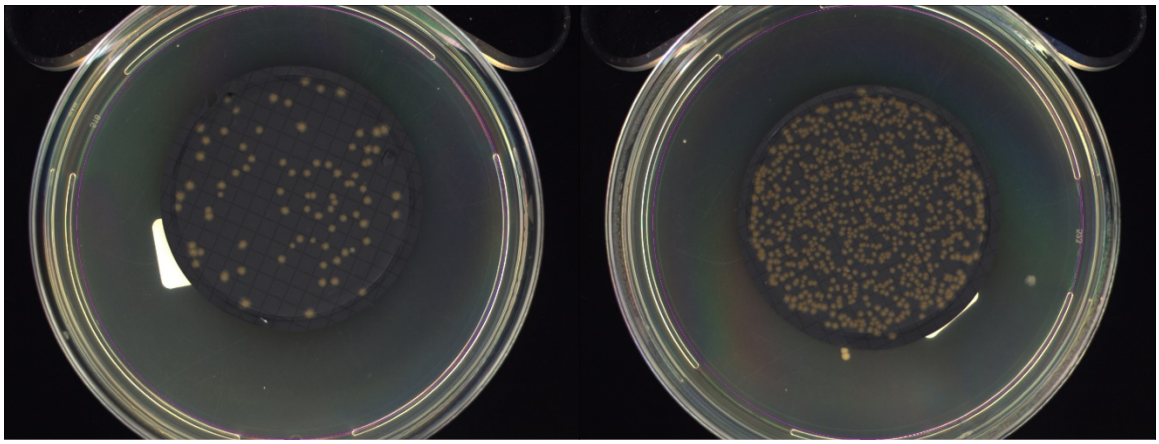
Table A-1. Filter Plating Results for Different Types of Filters

Sample ID	CFU GN-6 White Filters	CFU PES White Filters	CFU PES Black Filters	Volume Plated (mL)
152-10%-2×STS-01	150	91	79	1.0
	4	TNTC	TNTC	10.0
	6	TNTC	TNTC	100.0
152-10%-2×STS-02	141	88	49	1.0
	1	TNTC	TNTC	10.0
	2	TNTC	TNTC	100.0
152-10%-2×STS-03	87	123	64	1.0
	2	TNTC	TNTC	10.0
	3	TNTC	TNTC	100.0
152-Water-2×STS-01	117	109	104	1.0
	TNTC	TNTC	TNTC	10.0
	TNTC	TNTC	TNTC	100.0



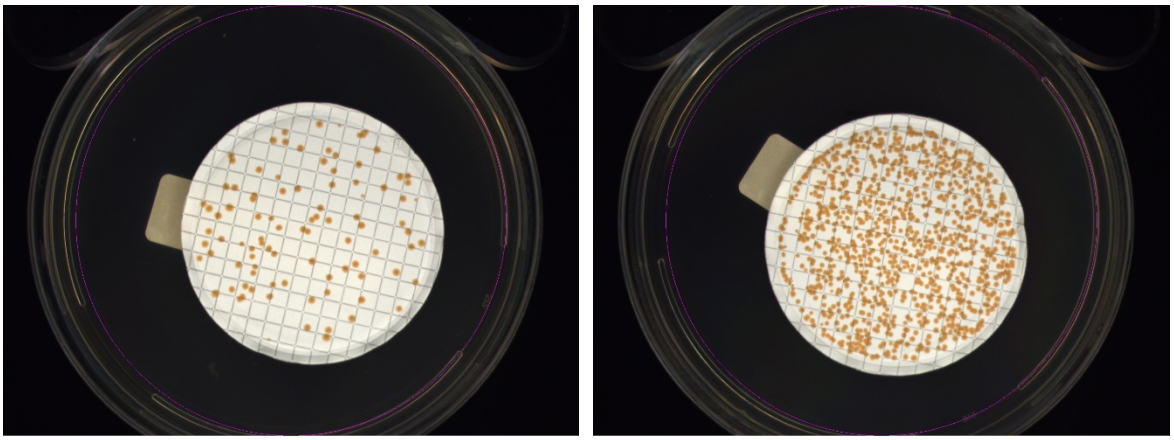
A B

Figure A-3. Aliquots filter plated on GN-6 white filter from 10% bleach solution (A: 1-, B: 10-mL)



A B

Figure A-4. Aliquots filter plated on PES black filter from 10% bleach solution (A: 1-, B: 10-mL)



A B

Figure A-5. Aliquots filter plated on PES white filter from 10% bleach solution (A: 1-, B: 10-mL)

Appendix B-1: Temporal Recoveries from Neutralized Test Solutions at High-Spore Loading Concentrations

Wash Solution		PBST	Diluted Bleach		Clorox 2® Free and Clear, H_2O_2 Bleach		Tide®	
Analysis (Day)	Test #	CFU	CFU	%	CFU	%	CFU	%
Day 0	1	1.0×10^8	6.1×10^7	62	9.2×10^7	95	1.0×10^8	106
	2	1.1×10^8	7.0×10^7	71	8.4×10^7	86	1.1×10^8	110
	3	8.3×10^7	7.5×10^7	77	8.8×10^7	90	1.1×10^8	115
	Average	**9.8×10^7**	**6.8×10^7**	**70**	**8.8×10^7**	**90**	**1.1×10^8**	**110**
	Stdev	**1.3×10^7**	**7.1×10^6**	**7.2**	**4.2×10^6**	**4.3**	**4.2×10^6**	**4.3**
Day 4	1	8.3×10^7	3.5×10^7	43	7.2×10^7	89	9.0×10^7	111
	2	8.2×10^7	3.7×10^7	46	7.1×10^7	87	9.0×10^7	112
	3	7.7×10^7	4.6×10^7	57	7.2×10^7	89	8.8×10^7	109
	Average	**8.1×10^7**	**3.9×10^7**	**48**	**7.2×10^7**	**89**	**8.9×10^7**	**111**
	Stdev	**3.3×10^6**	**6.0×10^6**	**7.5**	**9.2×10^5**	**1**	**1.0×10^6**	**1.2**

Appendix B-2: Temporal Recoveries from Neutralized Test Solutions at Low-Spore Loading Concentrations

Processing Day	Day 0			Day 4		
Recoveries (CFU/sample)	Average	Stdev	%	Average	Stdev	%
H_2O	3.7×10^2	1.5×10^1	NA	3.6×10^2	4.4×10^1	NA
PBST	3.6×10^2	3.9×10^1	99	4.1×10^2	4.0×10^1	116
PBST + STS10	3.9×10^2	1.5×10^1	106	3.9×10^2	2.3×10^1	109
PBST + STS1	3.8×10^2	6.0×10^1	104	3.9×10^2	2.9×10^1	109
H2O + STS10	4.3×10^2	5.3×10^1	118	3.6×10^2	4.6×10^1	100
H2O + STS1	4.7×10^2	3.4×10^1	128	3.6×10^2	4.6×10^1	100
10% Neutralized Diluted Bleach	3.9×10^2	1.5×10^1	105	3.1×10^2	2.1×10^1	87
1% Neutralized Diluted Bleach	4.2×10^2	3.9×10^1	115	2.9×10^2	5.1×10^1	82

Note: STS 1 or 10 corresponds to the same amount of STS added to 1 or 10% neutralized diluted bleach solution

Appendix B-3: Phase I (Scoping). Decontamination Results with Wash Liquid Solutions

Wash Solution	Wash Solution Temperature	Spores Recovered (CFU)			Decontamination Efficacy (LR [CFU])		
	°F	Per test	Average	Stdev	Per test	Average	Stdev
Procedural Positive Control (DI Water)	72	3.54×10^7	2.9×10^7	8.7×10^6	Not Applicable		
		2.30×10^7					
	120	4.26×10^7	2.3×10^7	2.7×10^7			
		3.87×10^6					
Tide® Detergent	72	6.81×10^7	7.0×10^7	2.5×10^6	-0.4	-0.4	0.02
		7.17×10^7			-0.4		
	120	6.72×10^7	6.6×10^7	1.5×10^6	-0.5	-0.5	0.01
		6.51×10^7			-0.4		
Clorox® 2 Hydrogen Peroxide-based Bleach	72	5.88×10^7	5.5×10^7	5.1×10^6	-0.2	-0.2	0.04
		5.16×10^7			-0.2		
	120	4.47×10^7	5.4×10^7	1.3×10^7	-0.3	-0.4	0.11
		6.33×10^7			-0.4		
Clorox® Concentrated Germicidal Bleach	72	3*	3*	0.0*	7.0	7.0	0.00
		3*			7.0		
	120	3*	3*	0.0*	6.9	6.9	0.00
		3*			6.9		

Green shading denotes the sample detection limit.

Appendix B-4: Phase I (Scoping): Decontamination Results with Diluted Bleach Alone

Wash Time	9 min				18 min				27 min			
Recovery	Recovered (CFU)		LR (CFU)		Recovered (CFU)		LR (CFU)		Recovered (CFU)		LR (CFU)	
Dilution Ratio	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
1%	5.3×10^6	4.4×10^5	**0.41**	**0.04**	1.4×10^6	5.6×10^5	**1.02**	**0.17**	3.0×10^5	2.7×10^5	**1.95**	**0.78**
3%	3.1×10^6	9.1×10^5	**0.65**	**0.13**	1.5×10^5	1.5×10^5	**2.12**	**0.44**	2.5×10^4	2.1×10^4	**2.87**	**0.46**
5%	4.5×10^5	2.9×10^4	**1.48**	**0.03**	5.0×10^2	3.8×10^2	**4.52**	**0.33**	6.4×10^2	3.2×10^2	**4.38**	**0.28**
10%	1.1×10^3	3.1×10^2	**4.09**	**0.11**	1.0×10^3	9.8×10^2	**4.46**	**0.83**	2.6×10^1	2.5×10^1	**5.88**	**0.48**
15%	9.6×10^1	1.0×10^2	**5.35**	**0.54**	7.6×10^2	8.9×10^2	**4.78**	**1.13**	5.1×10^2	6.0×10^2	**4.75**	**0.77**
PCs[1]	6.4×10^6	7.3×10^6	NA[1]									

PCs[1]; Positive Controls; NA[2]: Not Applicable

Appendix B-5: Phase I (Scoping): Decontamination Results with Diluted Bleach Plus Detergent

Wash Time	9 min				18 min				27 min			
Recovery	Recovered (CFU)		LR (CFU)		Recovered (CFU)		LR (CFU)		Recovered (CFU)		LR (CFU)	
Dilution Ratio	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
0.01%	1.8×10^7	3.1×10^5	**0.03**	**0.01**	1.9×10^7	1.4×10^6	**0.02**	**0.03**	1.8×10^7	2.2×10^5	**0.00**	**0.05**
0.10%	1.8×10^7	4.4×10^5	**0.04**	**0.01**	8.9×10^6	7.7×10^6	**0.44**	**0.36**	1.3×10^7	2.2 ×106	**0.16**	**0.05**
0.50%	7.4×10^6	1.7×10^5	**0.45**	**0.01**	1.9×10^6	1.5×10^5	**1.05**	**0.03**	2.0×10^4	1.5×10^4	**3.16**	**0.51**
1%	4.3×10^4	3.0×10^4	**2.64**	**0.32**	3	0.01	**6.72**	**0.00**	3	0.01	**6.72**	**0.00**
3%	4.4×10^3	3.6×10^3	**3.75**	**0.45**	3	0.004	**6.75**	**0.00**	4	1.9	**6.66**	**0.17**
5%	3.3×10^2	9.5×10^{-1}	**4.96**	**0.00**	4.8×10^2	8.2×10^2	**6.08**	**1.52**	3	0.001	**6.96**	**0.00**
10%	2.3×10^3	3.9×10^3	**5.34**	**1.64**	8.1×10^2	1.4×10^3	**5.88**	**1.55**	2.0×10^2	3.4×10^2	**6.18**	**1.28**
15%	3.0×10^2	5.0×10^2	**5.90**	**1.22**	9	4.5	**6.57**	**0.28**	5.0×10^1	7.9×10^1	**6.36**	**0.90**
0%									2.1×10^7	6.1×10^6		

Green shading denotes the sample detection limit.

Appendix C-1: Phase II Spore Recovery and Redistribution for Diluted Bleach Solution on Cotton Material

Table C1-1. Phase II (Benchscale): Decontamination Results for Cotton Material with a Bleach Dilution Ratio of 1:1000

Sample Media	Type of Samples	Recoveries	Average (CFUs)	Stdev
Coupon Samples	Inoculation Controls	CFU	1.1×10^7	1.9×10^6
		Log (CFU)	**7.04**	**0.07**
	Positive Controls	CFU	5.9×10^6	1.5×10^6
		Log (CFU)	**6.76**	**0.11**
	Procedural Positive Controls	CFU	4.6×10^5	3.3×10^5
		Log (CFU)	**5.57**	**0.35**
		Log Reduction	**1.47**	**0.35**
	Post Decon Fabric Sample	CFU	242	179
		Log (CFU)	**2.31**	**0.30**
		Log Reduction	**4.45**	**0.30**
Liquid wash water Samples	Procedural Wash Liquid Sample	CFU	8.2×10^6	1.1×10^6
		Log (CFU)	**6.91**	**0.06**
	Post Decon Wash Liquid Sample	CFU	34	23
		Log (CFU)	**1.46**	**0.96**
	Procedural Rinse Liquid Sample	CFU	5.4×10^5	9.4×10^4
		Log (CFU)	**5.73**	**0.08**
	Post Decon Rinse Sample	CFU	77	7
		Log (CFU)	**1.89**	**0.04**
Procedural Blanks	Test Coupon	CFU/coupon	**2**	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C1-2. Phase II (Benchscale): Decontamination Results for Cotton Material with a Bleach Dilution Ratio of 1:100

Sample Media	Type of Samples	Recoveries	Average (CFUs)	Stdev
Coupon Samples	Inoculation Controls	CFU	1.1×10^7	9.2×10^5
		Log (CFU)	**7.04**	**0.04**
	Positive Controls	CFU	7.3×10^6	1.0×10^6
		Log (CFU)	**6.86**	**0.07**
	Procedural Positive Controls	CFU	3.4×10^5	1.4×10^5
		Log (CFU)	**5.50**	**0.20**
		Log Reduction	**1.54**	**0.20**
	Post Decon Fabric Sample	CFU	31	32
		Log (CFU)	**1.10**	**0.92**
		Log Reduction	**5.76**	**0.92**
Liquid wash water Samples	Procedural Wash Liquid Sample	CFU	9.9×10^6	1.0×10^6
		Log (CFU)	**6.99**	**0.04**
	Post Decon Wash Liquid Sample	CFU	47.8	77.53
		Log (CFU)	**1.03**	**0.96**
	Procedural Rinse Liquid Sample	CFU	5.7×10^5	1.2×10^5
		Log (CFU)	**5.75**	**0.09**
	Post Decon Rinse Sample	CFU	215	231
		Log (CFU)	**2.12**	**0.56**
Procedural Blanks	Test Coupon	CFU/coupon	**1***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**7**	
	Inoculation Control Blank	CFU/coupon	**5**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C1-3. Phase II (Benchscale): Decontamination Results for Cotton Material with a Bleach Dilution Ratio of 1:10

Sample Media	Type of Samples	Recoveries	Average (CFUs)	Stdev
Coupon Samples	Inoculation Controls	CFU	1.8×10^7	5.2×10^6
		Log (CFU)	**7.25**	**0.13**
	Positive Controls	CFU	2.2×10^7	6.8×10^6
		Log (CFU)	**7.32**	**0.15**
	Procedural Positive Controls	CFU	2.5×10^6	2.7×10^5
		Log (CFU)	**6.39**	**0.05**
		Log Reduction	**0.86**	**0.05**
	Post Decon Fabric Sample	CFU	1.3*	0.02*
		Log (CFU)	**0.1**	**0.01**
		Log Reduction	**7.2**	**0.01**
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.5×10^7	3.28×10^6
		Log (CFU)	**7.18**	**0.10**
	Post Decon Wash Liquid Sample	CFU	4.4*	2.16*
		Log (CFU)	**0.61**	**0.20**
	Procedural Rinse Liquid Sample	CFU	1.8×10^6	6.2×10^5
		Log (CFU)	**6.23**	**0.16**
	Post Decon Rinse Sample	CFU	3.3*	0.02*
		Log (CFU)	**0.52**	**0.00**
Procedural Blanks	Test Coupon	CFU/coupon	**1***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**1***	
	Inoculation Control Blank	CFU/coupon	**3**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Appendix C-2: Phase II (Benchscale): 1% Diluted Bleach Solution, with the Addition of a Detergent, Decontamination Results: All Materials

Table C2-1: Phase II (Benchscale): Decontamination Results for Cotton Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.1×10^7	9.2×10^5
		Log (CFU)	**7.04**	**0.04**
	Positive Controls	CFU	7.3×10^6	1.1×10^6
		Log (CFU)	**6.86**	**0.07**
	Procedural Positive Controls	CFU	3.5×10^5	1.4×10^5
		Log (CFU)	**5.51**	**0.20**
		Log Reduction	**1.35**	**0.20**
	Post Decon Fabric Sample	CFU	1.2*	0.01*
		Log (CFU)	**0.08***	**0.003***
		Log Reduction	**6.78***	**0.28***
Liquid Samples	Procedural Wash Liquid Sample	CFU	9.9×10^6	9.9×10^5
		Log (CFU)	**6.99**	**0.04***
	Post Decon Wash Liquid Sample	CFU	3.0*	0.01*
		Log (CFU)	**0.48***	**0.001***
	Procedural Rinse Liquid Sample	CFU	5.8×10^5	1.2×10^5
		Log (CFU)	**5.75**	**0.09**
	Post Decon Rinse Sample	CFU	3.0*	0.02
		Log (CFU)	**0.48***	**0.002***
Procedural Blanks	Test Coupon	CFU/coupon	**1***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.10**	
	Negative Control Coupon	CFU/coupon	**7**	
	Inoculation Control Blank	CFU/coupon	**1**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C2-2. Phase II (Benchscale): Decontamination Results for Blend Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.2×10^7	6.7×10^6
		Log (CFU)	**7.33**	**0.14**
	Positive Controls	CFU	3.0×10^7	2.8×10^6
		Log (CFU)	**7.30**	**0.06**
	Procedural Positive Controls	CFU	5.8×10^5	3.7×10^5
		Log (CFU)	**5.69**	**0.32**
		Log Reduction	**1.64**	**0.32**
	Post Decon Fabric Sample	CFU	2.1	1.53
		Log (CFU)	**0.26**	**0.29**
		Log Reduction	**7.04**	**0.29**
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.8×10^7	4.8×10^6
		Log (CFU)	**7.25**	**0.12**
	Post Decon Wash Liquid Sample	CFU	285	489
		Log (CFU)	**1.30**	**1.41**
	Procedural Rinse Liquid Sample	CFU	4.7×10^5	2.5×10^5
		Log (CFU)	**5.63**	**0.22**
	Post Decon Rinse Sample	CFU	9.2	8.01
		Log (CFU)	**0.85**	**0.39**
Procedural Blanks	Test Coupon	CFU/coupon	**3**	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**0.03**	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C2-3. Phase II (Benchscale): Decontamination Results for Military Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.4×10^7	2.3×10^6
		Log (CFU)	**7.14**	**0.07**
	Positive Controls	CFU	1.3×10^7	1.9×10^6
		Log (CFU)	**7.10**	**0.07**
	Procedural Positive Controls	CFU	1.3×10^6	3.7×10^5
		Log (CFU)	**6.09**	**0.13**
		Log Reduction	**1.05**	**0.13**
	Post Decon Fabric Sample	CFU	291	75
		Log (CFU)	**2.44**	**0.11**
		Log Reduction	**4.65**	**0.11**
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.4×10^7	5.8×10^5
		Log (CFU)	**7.13**	**0.02**
	Post Decon Wash Liquid Sample	CFU	348	130
		Log (CFU)	**2.52**	**0.19**
	Procedural Rinse Liquid Sample	CFU	1.3×10^6	6.5×10^5
		Log (CFU)	**6.08**	**0.22**
	Post Decon Rinse Sample	CFU	940	527
		Log (CFU)	**2.93**	**0.25**
Procedural Blanks	Test Coupon	CFU/coupon	**1**	
	Wash Liquid Solution	CFU/mL	**0.01**	
	Rinse Liquid Solution	CFU/mL	**0.01**	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01**	
	Sterility Check for DI-H20	CFU/mL	**0.01**	
	Negative Control Coupon	CFU/coupon	**1**	
	Inoculation Control Blank	CFU/coupon	**1**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C2-4. Phase II (Benchscale): Decontamination Results for Polyester Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.0×10^7	3.0×10^6
		Log (CFU)	**6.99**	**0.13**
	Positive Controls	CFU	1.2×10^7	8.5×10^5
		Log (CFU)	**7.09**	**0.03**
	Procedural Positive Controls	CFU	1.7×10^6	2.5×10^5
		Log (CFU)	**6.21**	**0.06**
		Log Reduction	**0.77**	**0.06**
	Post Decon Fabric Sample	CFU	1*	0.01*
		Log (CFU)	**0.10***	**0.00***
		Log Reduction	**6.99***	**0.00***
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.0×10^7	2.2×10^6
		Log (CFU)	**6.99**	**0.10**
	Post Decon Wash Liquid Sample	CFU	3*	0.01*
		Log (CFU)	**0.46***	**0.00***
	Procedural Rinse Liquid Sample	CFU	2.1×10^6	1.6×10^5
		Log (CFU)	**6.32**	**0.03**
	Post Decon Rinse Sample	CFU	5	3.5
		Log (CFU)	**0.64**	**0.27**
Procedural Blanks	Test Coupon	CFU/coupon	**1.0***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**1***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C2-5. Phase II (Benchscale): Decontamination Results for Spandex Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.5×10^7	1.3×10^6
		Log (CFU)	**7.18**	**0.04**
	Positive Controls	CFU	1.9×10^7	2.8×10^6
		Log (CFU)	**7.27**	**0.06**
	Procedural Positive Controls	CFU	9.5×10^5	3.5×10^5
		Log (CFU)	**5.96**	**0.15**
		Log Reduction	**1.22**	**0.15**
	Post Decon Fabric Sample	CFU	1.2*	0.01*
		Log (CFU)	**0.08***	**0.00***
		Log Reduction	**7.19***	**0.00***
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.5×10^7	2.6×10^6
		Log (CFU)	**7.18**	**0.07**
	Post Decon Wash Liquid Sample	CFU	37	51
		Log (CFU)	**1.24**	**0.65**
	Procedural Rinse Liquid Sample	CFU	6.6×10^5	9.8×10^4
		Log (CFU)	**5.82**	**0.06**
	Post Decon Rinse Sample	CFU	3	0.01*
		Log (CFU)	**0.49***	**0.00***
Procedural Blanks	Test Coupon	CFU/coupon	**1**	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.02**	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**1***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table C2-6. Phase II (Benchscale): Decontamination Results for Denim Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.5×10^7	8.6×10^6
		Log (CFU)	**7.14**	**0.22**
	Positive Controls	CFU	2.3×10^7	1.4×10^6
		Log (CFU)	**7.36**	**0.03**
	Procedural Positive Controls	CFU	2.3×10^7	2.1×10^6
		Log (CFU)	**6.71**	**0.18**
		Log Reduction	**0.44**	**0.18**
	Post Decon Fabric Sample	CFU	113	122
		Log (CFU)	**1.64**	**0.95**
		Log Reduction	**5.73**	**0.95**
Liquid Samples	Procedural Wash Liquid Sample	CFU	2.1×10^7	2.1×10^6
		Log (CFU)	**7.32**	**0.04**
	Post Decon Wash Liquid Sample	CFU	100	76
		Log (CFU)	**1.90**	**0.39**
	Procedural Rinse Liquid Sample	CFU	1.5×10^6	2.1×10^5
		Log (CFU)	**6.18**	**0.06**
	Post Decon Rinse Sample	CFU	5.0	1.73
		Log (CFU)	**0.68**	**0.17**
Procedural Blanks	Test Coupon	CFU/coupon	**1***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Appendix D-1: Phase III (Washer): 1% Diluted Bleach Solution, with the Addition of a Detergent, Decontamination Results: All Materials.

Table D1-1. Phase III (Washer): Decontamination Results for Cotton Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.6×10^7	6.2×10^6
		Log (CFU)	**7.40**	**0.10**
	Positive Controls	CFU	1.4×10^7	3.0×10^6
		Log (CFU)	**7.12**	**0.10**
	Procedural Positive Controls	CFU	8.4×10^4	1.8×10^4
		Log (CFU)	**4.92**	**0.09**
		Log Reduction	**2.49**	**0.09**
	Post Decon Fabric Sample	CFU	2	0.9
		Log (CFU)	**0.26***	**0.19***
		Log Reduction	**6.87***	**0.19***
Liquid Samples	Procedural Wash Liquid Sample	CFU	3.0×10^7	5.7×10^6
		Log (CFU)	**7.48**	**0.09**
	Post Decon Wash Liquid Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
	Procedural Rinse Liquid Sample	CFU	3.2×10^6	6.2×10^5
		Log (CFU)	**6.50**	**0.09**
	Post Decon Rinse Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
Procedural Blanks	Test Coupon	CFU/coupon	**1***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D1-2. Phase III (Washer): Decontamination Results for Blend Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	3.2×10^7	6.9×10^6
		Log (CFU)	**7.49**	**0.10**
	Positive Controls	CFU	2.4×10^6	3.4×10^5
		Log (CFU)	**6.37**	**0.06**
	Procedural Positive Controls	CFU	9.8×10^4	1.7×10^4
		Log (CFU)	**4.99**	**0.07**
		Log Reduction	**2.50**	**0.07**
	Post Decon Fabric Sample	CFU	1*	0.12*
		Log (CFU)	**0.23***	**0.03***
		Log Reduction	**5.91***	**0.03***
Liquid Samples	Procedural Wash Liquid Sample	CFU	2.9×10^7	4.4×10^6
		Log (CFU)	**7.46**	**0.07**
	Post Decon Wash Liquid Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**1.41***
	Procedural Rinse Liquid Sample	CFU	3.5×10^6	7.9×10^6
		Log (CFU)	**6.53**	**0.10**
	Post Decon Rinse Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
Procedural Blanks	Test Coupon	CFU/coupon	**2***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D1-3. Phase III (Washer): Decontamination Results for Spandex Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	verage	Stdev
Coupon Samples	Inoculation Controls	CFU	2.4×10^7	2.3×10^6
		Log (CFU)	**7.37**	**0.04**
	Positive Controls	CFU	2.2×10^7	2.1×10^6
		Log (CFU)	**7.34**	**0.04**
	Procedural Positive Controls	CFU	1.9×10^5	7.3×10^4
		Log (CFU)	**5.25**	**0.16**
		Log Reduction	**2.08**	**0.16**
	Post Decon Fabric Sample	CFU	5	3.2
		Log (CFU)	**0.64**	**0.30**
		Log Reduction	**6.69**	**0.30**
Liquid Samples	Procedural Wash Liquid Sample	CFU	2.6×10^7	3.3×10^6
		Log (CFU)	**7.41**	**0.06**
	Post Decon Wash Liquid Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
	Procedural Rinse Liquid Sample	CFU	3.6×10^6	9.1×10^5
		Log (CFU)	**6.55**	**0.11**
	Post Decon Rinse Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
Procedural Blanks	Test Coupon	CFU/coupon	**2**	
	Wash Liquid Solution	CFU/mL	**0.01**	
	Rinse Liquid Solution	CFU/mL	**0.01**	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01**	
	Sterility Check for DI-H20	CFU/mL	**0.01**	
	Negative Control Coupon	CFU/coupon	**2**	
	Inoculation Control Blank	CFU/coupon	**1**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D1-4. Phase III (Washer): Decontamination Results for Polyester Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	1.7×10^7	6.9×10^6
		Log (CFU)	**7.18**	**0.23**
	Positive Controls	CFU	1.0×10^7	2.1×10^6
		Log (CFU)	**6.89**	**0.40**
	Procedural Positive Controls	CFU	3.6×10^5	9.0×10^4
		Log (CFU)	**5.55**	**0.11**
		Log Reduction	**1.53**	**0.11**
	Post Decon Fabric Sample	CFU	3.2	0.06
		Log (CFU)	**0.50**	**0.01**
		Log Reduction	**6.39**	**0.01**
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.7×10^7	3.5×10^6
		Log (CFU)	**7.21**	**0.10**
	Post Decon Wash Liquid Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
	Procedural Rinse Liquid Sample	CFU	2.8×10^6	2.9×10^5
		Log (CFU)	**6.45**	**0.04**
	Post Decon Rinse Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
Procedural Blanks	Test Coupon	CFU/coupon	**3**	
	Wash Liquid Solution	CFU/mL	**0.01**	
	Rinse Liquid Solution	CFU/mL	**0.01**	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.03**	
	Sterility Check for DI-H20	CFU/mL	**0.01**	
	Negative Control Coupon	CFU/coupon	**3.7×10^4**	
	Inoculation Control Blank	CFU/coupon	**3**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D1-5. Phase III (Washer): Decontamination Results for Denim Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.6×10^7	1.9×10^7
		Log (CFU)	**7.26**	**0.48**
	Positive Controls	CFU	2.4×10^7	6.5×10^5
		Log (CFU)	**7.39**	**0.01**
	Procedural Positive Controls	CFU	2.0×10^5	6.8×10^4
		Log (CFU)	**5.28**	**0.14**
		Log Reduction	**2.10**	**0.14**
	Post Decon Fabric Sample	CFU	3	0.16
		Log (CFU)	**0.44**	**0.03**
		Log Reduction	**6.95**	**0.03**
Liquid Samples	Procedural Wash Liquid Sample	CFU	4.8×10^7	6.3×10^6
		Log (CFU)	**7.67**	**0.06**
	Post Decon Wash Liquid Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
	Procedural Rinse Liquid Sample	CFU	5.4×10^6	4.2×10^5
		Log (CFU)	**6.73**	**0.03**
	Post Decon Rinse Sample	CFU	43	19
		Log (CFU)	**1.61**	**0.17**
Procedural Blanks	Test Coupon	CFU/coupon	**2**	
	Wash Liquid Solution	CFU/mL	**0.0**	
	Rinse Liquid Solution	CFU/mL	**0.0**	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.0**	
	Sterility Check for DI-H20	CFU/mL	**0.0**	
	Negative Control Coupon	CFU/coupon	**2**	
	Inoculation Control Blank	CFU/coupon	**5**	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D1-6. Phase III (Washer): Decontamination Results for Military Material with 1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.1×10^7	6.2×10^6
		Log (CFU)	**7.30**	**0.14**
	Positive Controls	CFU	1.2×10^7	1.7×10^6
		Log (CFU)	**7.09**	**0.06**
	Procedural Positive Controls	CFU	8.1×10^4	2.0×10^4
		Log (CFU)	**4.90**	**0.10**
		Log Reduction	**2.19**	**0.10**
	Post Decon Fabric Sample	CFU	2*	0.03*
		Log (CFU)	**0.20***	**0.01***
		Log Reduction	**6.89***	**0.01***
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.7×10^7	3.6×10^6
		Log (CFU)	**7.22**	**0.09**
	Post Decon Wash Liquid Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
	Procedural Rinse Liquid Sample	CFU	2.7×10^6	4.3×10^5
		Log (CFU)	**6.43**	**0.07**
	Post Decon Rinse Sample	CFU	32.5	0.00
		Log (CFU)	**1.51**	**0.00**
Procedural Blanks	Test Coupon	CFU/coupon	**2***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Appendix D-2: Phase III: 1% Diluted Bleach Solution without the Addition of a Detergent Decontamination Results: Cotton and Military Materials.

Table D2-1. Phase III (Washer): Decontamination Results for Cotton Material with 1% Diluted Bleach and without Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.6×10^7	6.2×10^6
		Log (CFU)	**7.40**	**0.10**
	Positive Controls	CFU	1.4×10^7	3.0×10^6
		Log (CFU)	**7.12**	**0.10**
	Procedural Positive Controls	CFU	8.4×10^4	1.8×10^4
		Log (CFU)	**4.92**	**0.09**
		Log Reduction	**2.49**	**0.09**
	Post Decon Fabric Sample	CFU	3	1.6
		Log (CFU)	**0.47**	**0.27**
		Log Reduction	**6.66**	**0.27**
Liquid Samples	Procedural Wash Liquid Sample	CFU	3.0×10^7	5.7×10^6
		Log (CFU)	**7.48**	**0.09**
	Post Decon Wash Liquid Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.000***
	Procedural Rinse Liquid Sample	CFU	3.2×10^6	6.2×10^5
		Log (CFU)	**6.50**	**0.09**
	Post Decon Rinse Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.000***
Procedural Blanks	Test Coupon	CFU/coupon	**37**	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Table D2-2. Phase III (Washer): Decontamination Results for Military Material with 1% Diluted Bleach and without Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	3.7×10^7	1.6×10^7
		Log (CFU)	**7.54**	**0.18**
	Positive Controls	CFU	1.2×10^7	1.7×10^6
		Log (CFU)	**7.09**	**0.06**
	Procedural Positive Controls	CFU	8.1×10^4	2.0×10^4
		Log (CFU)	**4.90**	**0.10**
		Log Reduction	**2.19**	**0.10**
	Post Decon Fabric Sample	CFU	2	0.89
		Log (CFU)	**0.30**	**0.17**
		Log Reduction	**6.79**	**0.17**
Liquid Samples	Procedural Wash Liquid Sample	CFU	1.7×10^7	3.6×10^6
		Log (CFU)	**7.22**	**0.09**
	Post Decon Wash Liquid Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
	Procedural Rinse Liquid Sample	CFU	2.7×10^6	4.3×10^5
		Log (CFU)	**6.43**	**0.07**
	Post Decon Rinse Sample	CFU	32.5*	0.00*
		Log (CFU)	**1.51***	**0.00***
Procedural Blanks	Test Coupon	CFU/coupon	**2***	
	Wash Liquid Solution	CFU/mL	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

Appendix E-1: Phase IV (Washer and Dryer): 0.1% Diluted Bleach Solution, with the Addition of a Detergent, Decontamination Results Using a Washer/Dryer: Cotton Material.

Table E-1. Phase IV (Washer and Dryer): Decontamination Results for Cotton Material with 0.1% Diluted Bleach Plus Detergent

Sample Media	Type of Samples	Recoveries	Average	Stdev
Coupon Samples	Inoculation Controls	CFU	2.4×10^7	7.1×10^6
		Log (CFU)	**7.37**	**0.11**
	Positive Controls	CFU	1.4×10^7	3.0×10^6
		Log (CFU)	**7.12**	**0.10**
	Procedural Positive Controls	CFU	8.4×10^4	1.8×10^4
		Log (CFU)	**4.92**	**0.09**
		Log Reduction	**2.49**	**0.09**
	Post Wash Fabric Sample	CFU	2.2×10^3	2.6×10^3
		Log (CFU)	**3.14**	**0.52**
		Log Reduction	**3.98**	**0.52**
	Post Drying Fabric Sample	CFU	1.1×10^3	1.1×10^3
		Log (CFU)	**2.87**	**0.44**
		Log Reduction	**4.25**	**0.44**
	Aerosol Samples	CFU	3.7×10^2	2.8×10^2
		Log (CFU)	**2.48**	**0.34**
Liquid Samples	Procedural Wash Liquid Sample	CFU	3.0×10^7	5.7×10^6
		Log (CFU)	**7.48**	**0.09**
	Post Decon Wash Liquid Sample	CFU	1.3×10^3	4.7×10^2
		Log (CFU)	**3.08**	**0.15**
	Procedural Rinse Liquid Sample	CFU	3.2×10^6	6.2×10^5
		Log (CFU)	**6.50**	**0.09**
	Post Decon Rinse Sample	CFU	1.3×10^4	3.8×10^3
		Log (CFU)	**4.11**	**0.12**
Procedural Blanks	Washer Test Coupon	CFU/coupon	**1**	
	Dryer Test Coupon	CFU/coupon	**1**	
	Dryer Filter	CFU/coupon	**1**	

Sample Media	Type of Samples	Recoveries	Average	Stdev
	Wash Liquid Solution	CFU/ml	**0.01***	
	Rinse Liquid Solution	CFU/mL	**0.01***	
Sterility Checks	Sterility Check for Decon Solution	CFU/ml	**0.01***	
	Sterility Check for DI-H_20	CFU/mL	**0.01***	
	Negative Control Coupon	CFU/coupon	**2***	
	Inoculation Control Blank	CFU/coupon	**1***	

Green shading denotes the sample detection limit and blue shading denotes log CFU.

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