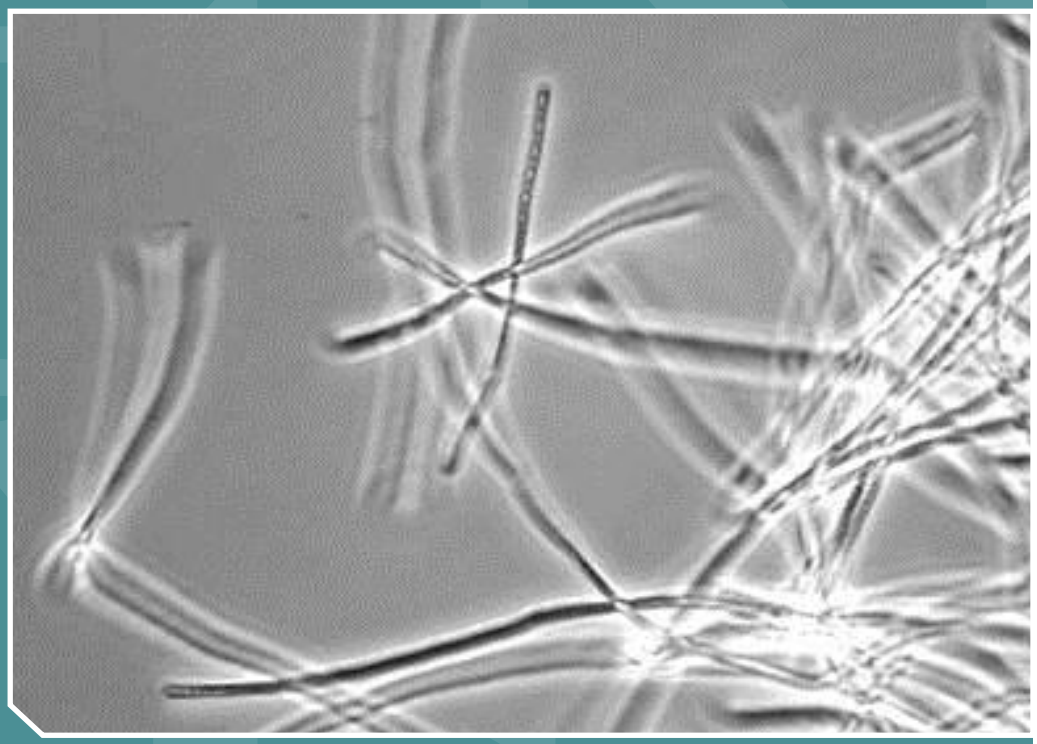
EPA/600/R-14/150 | July 2014 | www.epa.gov/ord

United States
Environmental Protection
Agency

Environmental Persistence of Vegetative *Bacillus anthracis* and *Yersinia pestis*



Office of Research and Development
National Homeland Security Research Center

EPA 600/R-14/150
July 2014

Environmental Persistence of Vegetative *Bacillus anthracis* and *Yersinia pestis*

United States Environmental Protection Agency
Research Triangle Park, North Carolina 27711

Disclaimer

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development's National Homeland Security Research Center (NHSRC), funded and directed this investigation through Task Order 4 of EPA Contract EP-C-11-038. This document has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names or commercial products in this document or in the methods referenced in this document does not constitute endorsement or recommendation for use.

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

Joseph P. Wood
National Homeland Security Research Center
Office of Research and Development
U.S. Environmental Protection Agency
Mail Code E343-06
Research Triangle Park, NC 27711
(919) 541-5029
wood.joe@epa.gov

Acknowledgments

Contributions of the following individuals and organization to the development of this document are gratefully acknowledged.

EPA Team Members
Worth Calfee, EPA National Homeland Security Research Center (NHSRC)
Shawn Ryan, EPA NHSRC
Leroy Mickelson EPA Office of Solid Waste and Emergency Response

Peer Reviewers
Erin Silvestri, EPA NHSRC
Martin Page, U.S. Army
Doris Betancourt, EPA National Risk Management Research Laboratory

Battelle Memorial Institute

Executive Summary

This report describes an investigation of the persistence of vegetative cells of *Bacillus anthracis* (Ames) and *Yersinia pestis (CO92)*, both with and without exposure to ultraviolet (UV)-A/B radiation (representing sunlight) on several surfaces and topsoil. The investigation of vegetative cells of *B. anthracis* is motivated by the possibility of using germinants to convert spores to vegetative cells, facilitating inactivation of the cells through natural attenuation or other decontamination processes.

ES.1 Test Procedures

The coupon materials used for *B. anthracis* testing were glass, bare pine wood, unpainted concrete, and topsoil. The coupon materials used for *Y. pestis* included glass, galvanized steel, painted wallboard paper, and topsoil. Testing was conducted at normal laboratory temperature, with relative humidity (RH) levels ranging from approximately 35 % to over 90 %. In all testing, the number of viable cells on each coupon was enumerated at predefined time points after inoculation of the coupons. In addition, qualitative growth tests were conducted to confirm the quantitative test results. In testing with *B. anthracis*, all coupon extracts (except as noted) were enumerated both with and without application of a heat shock procedure that distinguished heat-sensitive (presumed vegetative) *B. anthracis* cells from heat resistant cells (presumed spores).

ES.2 Results for Vegetative *Bacillus anthracis* (Ames)

ES.2.1 Vegetative Bacillus anthracis Persistence without UV Exposure

In a test in which the inoculum contained *B. anthracis* in both vegetative cell and spore form, both cells and spores were recovered from the test materials 56 days (the maximum duration tested) after inoculation.

Without the presence of spores, vegetative *B. anthracis* had minimal persistence on glass, wood and unpainted concrete. For these materials, *B. anthracis* cells persisted for less than 1 hour to a maximum of 12 hours, depending on the material and RH. Vegetative *B. anthracis* had the highest persistence in topsoil, which ranged between 4-5 days.

In every persistence test we confirmed that all *B. anthracis* cells remained vegetative throughout the entire test duration, with one exception. A different finding occurred in two tests in which the topsoil was wetted before inoculation, and the RH was maintained above 90 % to prevent drying of the soil. Results from those tests showed that the initially 100 % vegetative *B. anthracis* population grew by approximately a factor of 10 and showed extensive sporulation within one week after inoculation of the topsoil coupons. Over 10 % of the total *B. anthracis* population was in spore form at 48 hours after inoculation.

ES.2.2 Vegetative Bacillus anthracis Persistence with UV Exposure

The persistence of vegetative *B. anthracis* on topsoil with exposure to simulated sunlight was determined at six time points ranging from 1 hour to 120 hours after inoculation on to topsoil. The longest elapsed time tested in which *B. anthracis* cells were recovered from the soil was 96 hours. These results showed that exposure to simulated sunlight may have had only a minor impact on the persistence of vegetative *B. anthracis* in topsoil, presumably due to shading by soil particles.

ES.3 Results for *Yersinia pestis*

ES.3.1 Yersinia pestis Persistence without UV Exposure

The persistence of *Y. pestis* was tested at normal room temperature and RH levels from 54 to over 90 %. It was found that *Y. pestis* persisted at one hour after inoculation on all four test materials, but was completely inactivated within 24 hours after inoculation onto glass, painted wallboard paper, and galvanized metal. *Y. pestis* persisted to at least six days on topsoil, but when the soil was wetted and kept moist with elevated RH, viable *Y. pestis* was present at seven days after inoculation. No viable cells were observed at 14 days after inoculation.

ES.3.2 Yersinia pestis Persistence with UV Exposure

One test of the persistence of *Y. pestis* was conducted on all four test materials under UV-A/B exposure. In this test, *Y. pestis* did not persist on galvanized metal at the one-hour time point, but did persist at the one-hour time point on the other three materials.

Impact of Study

This work provides information on the persistence of vegetative *B. anthracis* on surfaces and soil, provided that the organism is completely germinated and sporulation is prevented. Such results may be useful in the development of wide area remediation plans that consider the possibility of germination and natural attenuation. To date, research has not shown such required effective germination of spores on surfaces.[1] For soil materials, natural attenuation may also be a viable decontamination option provided that longer attenuation times (e.g., approximately a week) are acceptable, and the soil can be kept relatively dry. Additional research is recommended to confirm that the persistence of *B. anthracis* cells produced through the germination of spores on materials is of similar duration to that of cells harvested in the laboratory during the exponential growth phase and inoculated onto materials.

Similar to *B. anthracis*, the results of the study on the persistence of *Y. pestis* show that natural attenuation may also be a viable option for the decontamination of non-soil materials. For soils, natural attenuation may also be a viable decontamination option provided that longer attenuation times (e.g., approximately a week) are acceptable, and that soils can be kept reasonably dry.

Contents

DISCLAIMER III
ACKNOWLEDGMENTS IV
EXECUTIVE SUMMARY V
ABBREVIATIONS/ACRONYMS X

1.0 INTRODUCTION 1

2.0 SUMMARY OF TEST PROCEDURES 3
2.1 Preparation of Test Coupons 3
2.2 Vegetative *B. anthracis* Production 4
2.3 UV-A/B Exposure Procedure 5
2.4 Non-UV Persistence Testing 8
2.5 Organism Recovery Procedures 8
2.6 Calculation of Quantitative Persistence 9
2.7 Qualitative Growth Testing 10

3.0 QUALITY ASSURANCE/QUALITY CONTROL 12
3.1 Equipment Calibration 12
3.2 QC Results 12
3.3 Audits 12
3.3.1 Performance Evaluation Audit 12
3.3.2 Technical Systems Audit 13
3.3.3 Data Quality Audit 13
3.4 QAPP Amendments and Deviations 13
3.5 QA/QC Reporting 14
3.6 Data Review 14

4.0 RESULTS FOR *BACILLUS ANTHRACIS* 15
4.1 QC Results 15
4.2 Vegetative *Bacillus anthracis* Persistence without UV Exposure 15
4.2.1 Uniformity of Test Conditions 16
4.2.2 Results of Persistence Tests with Vegetative *Bacillus anthracis* 17
4.3 Persistence of Vegetative *Bacillus anthracis* under Exposure to Simulated Sunlight 28
4.3.1 Uniformity of Test Conditions 29
4.3.2 Persistence Results in Simulated Sunlight Tests with Vegetative *Bacillus anthracis* 30

5.0 RESULTS FOR *YERSINIA PESTIS* 33
5.1 Recovery Testing 33
5.2 QC Results 33
5.3 *Yersinia pestis* Persistence without UV Exposure 34
5.3.1 Uniformity of Test Conditions 34
5.3.2 Results in Persistence Tests with *Yersinia pestis* 35

5.4 Persistence of *Yersinia pestis* under Exposure to Simulated Sunlight.....37
5.4.1 Uniformity of Test Conditions..... 37
5.4.2 Persistence Results in Simulated Sunlight Test with *Yersinia pestis* 38

6.0 SUMMARY..... 40
6.1 Results for Vegetative *Bacillus anthracis* (Ames).....40
6.1.1 Vegetative *Bacillus anthracis* Persistence without UV Exposure..... 40
6.1.2 Vegetative *Bacillus anthracis* Persistence with UV Exposure..... 41
6.2 Results for *Yersinia pestis*.....42
6.2.1 *Yersinia pestis* Persistence without UV Exposure..... 42
6.2.2 *Yersinia pestis* Persistence with UV Exposure..... 42

7.0 REFERENCES 44

Appendices

Appendix A: Description of Issues and Test Results Leading to Development of Procedure for Preparing Purely Vegetative *Bacillus anthracis*
Appendix B: Data Tables from Testing of Vegetative *Bacillus anthracis* Persistence under UV-A/B Exposure
Appendix C: Data Tables From Selected Persistence Tests with *Yersinia pestis*

Figures

Figure 2-1. Schematic Representation of Test Chamber 6
Figure 2-2. Schematic of Five Coupon Positions on the Support Trays..... 6
Figure 4-1. Vegetative *Bacillus anthracis* (Ames) Persists for at Least 96 Hours in Topsoil at Ambient Laboratory Temperature and Relative Humidity 22
Figure 4-2. Persistence of Vegetative *Bacillus anthracis* at One Hour on Glass Increases with RH 24
Figure 4-3. Enumeration of Spores and Total Cells of *Bacillus anthracis* Shows Growth and Sporulation in the 48-hour Test on Wet Soil at 93 % RH..... 28
Figure 4-4. Vegetative *Bacillus anthracis* Persistence with Exposure to Simulated Sunlight with a Low UV-A/UV-B Ratio 31
Figure 4-5. Vegetative *Bacillus anthracis* Persistence with Exposure to Simulated Sunlight with a High UV-A/UV-B Ratio..... 32

Tables

Table 3-1. Performance Evaluation Audit Results 13
Table 4-1. Temperature and Relative Humidity Conditions during Non-UV Exposure Persistence Tests with Vegetative *Bacillus anthracis* 17
Table 4-2. Persistence of Vegetative *Bacillus anthracis* (Four Materials, One Hour, 36 % RH; Test BA1) 18
Table 4-3. Persistence of Vegetative *Bacillus anthracis* on Glass (Eight Hours, 34 % RH;Test BA2) 19
Table 4-4. Persistence of Vegetative *Bacillus anthracis* (Four Materials, One Hour, 75 % RH; Test BA3) 20
Table 4-5. Persistence of Vegetative *Bacillus anthracis* on Topsoil (120 Hours, 46 % RH; Test BA4) 21
Table 4-6. Persistence of Vegetative *Bacillus anthracis* on Three Materials (24 Hours, 92 % RH; Test BA5) 23
Table 4-7. Persistence of Vegetative *Bacillus anthracis* on Wetted Topsoil (28 Days, 96 % RH; Test BA6) 25
Table 4-8. Persistence of Vegetative *Bacillus anthracis* on Wetted Topsoil (48 Hours, 93 % RH; Test BA7) 27
Table 4-7. Ultraviolet Radiation Intensity at the Topsoil Test Coupons in Testing with *Bacillus anthracis* 29
Table 4-8. Summary of Temperature and Relative Humidity Conditions in Simulated Sunlight Testing with Vegetative *Bacillus anthracis* 30
Table 5-1. Recovery Testing of *Yersinia pestis* 33
Table 5-2. Temperature and Relative Humidity Conditions during Non-UV Persistence Tests with *Yersinia pestis* 35
Table 5-3. Persistence of *Yersinia pestis* (Four Materials, 144 Hours, 65 % RH; Test YP3) 36
Table 5-4. Persistence of *Yersinia pestis* (Wetted Topsoil, 14 Days, 96 % RH; Test YP4) 37
Table 5-5. Ultraviolet Radiation Intensity at the Exposed Coupons in Test YPUV1 with *Yersinia pestis* 38
Table 5-6. Summary of Temperature and Relative Humidity Conditions at the Exposed Coupons in Test YPUV1 with *Yersinia pestis* 38
Table 5-7. UV-A/B Inactivation of *Yersinia pestis* on Four Coupon Materials (24-Hour Exposure at Low UV-A/UV-B Ratio; Test YPUV1) 39

Abbreviations/Acronyms

BSC	biosafety cabinet
C	Celsius
CFU	colony-forming unit(s)
CI	confidence interval
cm	centimeter(s)
DNA	deoxyribonucleic acid
EPA	U.S. Environmental Protection Agency
EAI	Etiologic Agent Inventory
ISO	International Standards Organization
J	Joule
L	liter
LR	log reduction
µL	microliter(s)
µm	micrometer(s)
µW	microwatt(s)
mL	milliliter(s)
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
nm	nanometer(s)
OD_{600}	optical density at 600 nanometers wavelength
PBS	phosphate-buffered saline
PE	performance evaluation
QA	quality assurance
QAPP	Quality Assurance Project Plan
QC	quality control
QMP	quality management plan
RH	relative humidity
rpm	revolutions per minute
SD	standard deviation
SE	standard error
SFW	sterile filtered water
T	temperature
TOPO	Task Order Project Officer
TSA	technical systems audit
TSB	tryptic soy broth
UV	ultraviolet
UV-A	ultraviolet light (320 to 400 nm wavelength)
UV-A/B	combination of UV-A and UV-B light used in testing
UV-B	ultraviolet light (290 to 320 nm wavelength)
UV-C	ultraviolet light (180 to 290 nm wavelength)
W	watt(s)

1.0 INTRODUCTION

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program (HSRP) is helping protect human health and the environment from adverse impacts resulting from the release of chemical, biological, or radiological agents. As part of fulfilling that mission, the HSRP evaluates the role that natural conditions play in counteracting chemical and biological homeland security threats. All evaluations are conducted in accordance with quality assurance (QA) protocols to ensure the generation of high quality data and defensible results.

This study investigated the persistence of *Bacillus anthracis* (Ames) vegetative cells and *Yersinia pestis* (CO92) on representative surface materials. The emphasis of this investigation on vegetative cells (rather than spores) of *B. anthracis* is driven by the potential to take advantage of vegetative cells in remediation activities after *B. anthracis* contamination. That is, a germinant might be used to convert *B. anthracis* spores to vegetative cells, which could then be inactivated without the need for sporicidal decontaminants. Vegetative cells might be inactivated largely or entirely by natural attenuation, including exposure to sunlight, thereby reducing or eliminating the need for chemical decontaminants. Thus to assess whether natural attenuation might be a viable option for remediation, the purpose of this study was to determine how long vegetative cells of *B. anthracis* would persist on different materials under various environmental conditions.

Separate persistence experiments were also conducted with *Y. pestis* in this study. This work builds on previous research[2] that investigated the inactivation of *Y. pestis* with chlorine dioxide and vapor-phase hydrogen peroxide and the persistence of *Y. pestis* on aluminum, carpet, computer keys, and wallboard paper. In particular, this study aimed to investigate the impact of materials not tested previously (such as topsoil) and the effect of simulated sunlight on *Y. pestis* persistence. As with *B. anthracis*, the purpose of the *Y. pestis* portion of the study was to assess whether natural attenuation might be a feasible remediation option by determining how long this agent might survive on different materials under various environmental conditions.

Persistence under normal laboratory conditions or simulated sunlight exposure was determined for each of the two test organisms (in separate experiments) on coupons of four test materials, which were chosen in part based on organism recovery tests conducted in this study prior to the persistence testing. Glass, bare pine wood, unpainted concrete, and topsoil coupons were used for testing with vegetative *B. anthracis*, to represent both porous and nonporous outdoor surfaces. For *Y. pestis*, the same four materials were initially chosen, but based on low recovery results with bare pine wood and unpainted concrete in the initial recovery tests, galvanized steel and painted wallboard paper were used instead of those two materials for testing with *Y. pestis*. The coupon preparation and recovery test results for *Y. pestis* are described in Chapters 2 and 5 of this report. Simulated sunlight testing involved exposure to a combination of ultraviolet (UV)-A (i.e., 320-400 nanometers [nm]) and UV-B (i.e., 290-320 nm) radiation (referred to as UV–A/B), and was conducted using alternating 12-hour periods of light and dark. Coupons of the test materials, inoculated with the test organisms, were placed in the same test environment as those exposed to UV-A/B radiation, but were shielded from any UV-A/B exposure. Persistence testing in the absence of simulated sunlight was conducted by exposing the test organisms only to normal laboratory lighting conditions.

The persistence of the two test organisms was determined by both quantitative and qualitative measures. Quantitative testing involved extracting and enumerating viable organisms from test coupons at each time point in each test. Qualitative testing involved placing an aliquot of the coupon extract in nutrient broth and assessing whether any growth occurred over periods of up to seven days of incubation. The qualitative testing provided a more sensitive measure of the presence of any remaining viable organisms at later time points in a test, when organism counts fell below the detection limit of the quantitative procedure.

The intent of the experiments with *B. anthracis* was to use inoculum containing purely vegetative cells. However, initial tests showed cell cultures containing predominantly vegetative cells, but with a few spores also present. Consequently, procedures were developed to ensure a purely vegetative *B. anthracis* cell population for subsequent tests. Additionally, a heat shock procedure was used in all subsequent tests with *B. anthracis* to confirm that only vegetative cells were inoculated onto test materials and to distinguish vegetative cells from spores in coupon extracts.

2.0 SUMMARY OF TEST PROCEDURES

Test procedures were performed in accordance with the Quality Assurance Project Plan (QAPP; which is available upon request) and are briefly summarized here. The activities conducted during this project consisted of persistence testing with either vegetative *B. anthracis* or with *Y. pestis* on four coupon materials, under different humidity conditions and under different levels of UV-A/B exposure. The following subsections describe the procedures that were used in carrying out these activities.

2.1 Preparation of Test Coupons

Test coupons of glass, galvanized metal, bare pine wood, and painted wallboard paper were each cut from larger pieces to 1.9 centimeters (cm) × 7.5 cm in size. Coupons of unpainted concrete were 1.0 cm × 3.5 cm in size, and were made by pouring into individual molds, rather than by cutting from a larger piece. The different size of the concrete coupons relative to the other coupons was solely due to the molds available and is not likely to affect the test results. Topsoil coupons consisted of 3.5 cm diameter Petri dishes having a height of 1 cm lined with Parafilm® and filled level with the top of the dish with uncompacted topsoil. The topsoil was obtained at a retail garden store and was shown by analysis to have approximately 30 % moisture content, 5 % organic carbon content, and a pH of approximately 7.3. Glass, concrete, and galvanized metal coupons were sterilized before use by autoclaving. Topsoil was autoclaved in bulk before being distributed into Petri dishes. Bare pine wood and painted wallboard paper coupons were sterilized before use by gamma irradiation. Test materials were sterilized to avoid confounding results from non-target organisms.

The *B. anthracis* (Ames) used for this testing was prepared from a qualified stock of spores of the Ames strain at the Battelle Biomedical Research Center (BBRC, West Jefferson, OH). The spore lot was subject to stringent characterization and qualification processes required by the laboratory for spore production. Specifically, the spore lot was characterized prior to use by observation of colony morphology, direct microscopic observation of spore morphology and size, and determination of percent refractivity and percent encapsulation. Variations in the expected colony phenotypes were recorded. Endotoxin concentration of each spore preparation was determined by the Limulus Amebocyte Lysate assay to assess whether contamination from gram-negative bacteria occurred during the propagation and purification process of the spores. Genomic deoxyribonucleic acid (DNA) was extracted from the spores and DNA fingerprinting by the polymerase chain reaction was done by Dr. Paul Keim at Northern Arizona University to confirm the genotype. The virulence of the spore lot was measured by challenging guinea pigs intradermally with a dilution series of spore suspensions, and virulence was expressed as the intradermal median lethal dose. In addition, testing was conducted for robustness of the spores via hydrochloric acid resistance. The number of viable spores in the stock suspension was determined by colony count and expressed as colony forming units per milliliter (CFU/mL). Theoretically, once plated onto bacterial growth media, each viable spore germinates and yields 1 CFU.

The virulent *Y. pestis* strain CO92 was obtained from Etiological Agent Inventory (EAI) number YUC429, originally obtained from the University of Chicago and stored at -80 °C. Fresh

cultures were prepared in advance of each day that coupons were inoculated by transferring one or two colonies from a streak plate (freshly grown on tryptic soy agar (TSA) or stored less than two weeks at 2 to 8 °C) into 10 to 20 mL of tryptic soy broth (TSB). This culture was then incubated overnight at 26 ± 2 °C on an orbital shaker set to 200 revolutions per minute (rpm). The bacterial culture was then diluted with fresh media to an optical density at 600 nm (OD_{600}) of approximately 0.1 to 0.2 using a SPECTRAmax Plus^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA). A gram stain was performed on the cultured bacteria, and the colony morphologies were confirmed to be consistent with previous descriptions of *Y. pestis* (i.e., grayish-white colonies with a "fried egg" appearance) after approximately 72 hours at 26 ± 2 °C.

Y. pestis and vegetative *B. anthracis* were inoculated onto test coupons in an appropriate biosafety cabinet (BSC-II or -III) according to established procedures.[3] Inoculated coupons were prepared fresh for each day of experimental work. Test coupons were placed flat in the BSC and inoculated with vegetative *B. anthracis* at approximately 8×10^5 to 5×10^6 CFU per coupon, or with *Y. pestis* at approximately 3×10^7 to 2×10^8 CFU per coupon. The different inoculation levels of the two organisms were a result of the different growth characteristics of the two cultures. Inoculation was accomplished by dispensing a 100 microliter (µL) aliquot of a stock suspension as 10 droplets (each of 10 µL volume) across the surface of the test coupon using a micropipette. This approach provided more uniform distribution of cells across the coupon surface than would be obtained through a single drop of the suspension. A spreader was not used after application of the ten droplets to the coupon. In nearly all cases, the test coupons remained undisturbed in the BSC for one hour to dry after inoculation. In a single persistence test with vegetative *B. anthracis*, inoculated coupons were extracted before the usual one-hour drying period elapsed, to assess the persistence of that organism over time intervals shorter than one hour.

2.2 Vegetative *B. anthracis* Production

The intent of this project was to test with purely vegetative cells of *B. anthracis* (Ames). However, observations from initial tests (summarized in Appendix A) disclosed that *B. anthracis* cell cultures contained predominantly vegetative cells but also a few spores, indicating incipient endospore formation. In one initial persistence test in which the inoculum contained *B. anthracis* in both vegetative cell and spore form (distinguished by heat shock), cells were recovered from these materials at 56 days; refer to Table A-1.

Consequently, an improved procedure was developed that could be conducted reproducibly to produce a stock solution with a relatively high titer of purely vegetative *B. anthracis*. That procedure consisted of the following steps:

- Prepare a 1:100 dilution in sterile filtered water (SFW) of standard *B. anthracis* (Ames) spore stock, which is approximately 1×10^9 CFU/mL.
- Add 10 µL of the resulting 1:100 dilution (which is approximately 1×10^7 CFU/mL) to 200 mL of TSB.

- Allow the resulting culture (which is initially approximately 5×10^2 CFU/mL) to incubate at 37 °C while shaking on an orbital shaker at 200 rpm for 9 to 10 hours.
- During heating and shaking of the culture, remove an aliquot at 1-hour intervals for determination of OD_{600} as an indication of the titer of the culture. An OD_{600} of approximately 2 is expected at the end of the 9- to 10-hour incubation period.
- Remove the culture from heating and shaking and verify the purity of the vegetative cell culture by microscopic examination and heat shock treatment.

This procedure was used to prepare a fresh culture of vegetative *B. anthracis* for the start of each new test with that organism, and consistently provided a vegetative *B. anthracis* culture of approximately 1×10^7 to 5×10^7 CFU/mL, free of any detectable spores. Additional details on this procedure are presented in Appendix A.

2.3 UV-A/B Exposure Procedure

For the simulated sunlight tests with vegetative *B. anthracis*, blank coupons and inoculated coupons were transferred into a test chamber equipped with small UV lamps, shown schematically in Figure 2-1 (figure not to scale). The three UV lamps used were Reptisun® 10.0 UVB (15 Watts, 48 cm long), made by Zoo Med Laboratories, Inc. (San Luis Obispo, CA), which provided both the UV-A and UV-B radiation to which the coupons were exposed. These lamps emitted no UV-C (wavelength < 290 nm) radiation, consistent with the absence of UV-C in sunlight at ground level. In the UV exposure testing with vegetative *B. anthracis*, only coupons of topsoil were used. Five UV-exposed coupons and five non-exposed coupons of topsoil were used for each UV-A/B exposure time point. One blank topsoil coupon was also included with the exposed coupons and with the non-exposed coupons for each time point. The exposed coupons and the associated blank coupon were placed flat on top of the raised tray below the UV lamps, and non-exposed coupons and the associated blank coupon were placed flat beneath that tray, shielded from direct UV-A/B radiation (Figure 2-1). The lower portions of the test chamber walls were covered with black paper to minimize reflected UV-A/B radiation.

The topsoil coupons were arrayed in five separate positions on the support trays, as shown schematically in Figure 2-2, with Position 1 in the center of the tray and Positions 2 through 5 located toward the corners of the tray. One topsoil coupon was placed at each of these five positions, so that coupons were equally distributed across the support trays. A blank coupon was also placed at Position 1 of the appropriate tray.

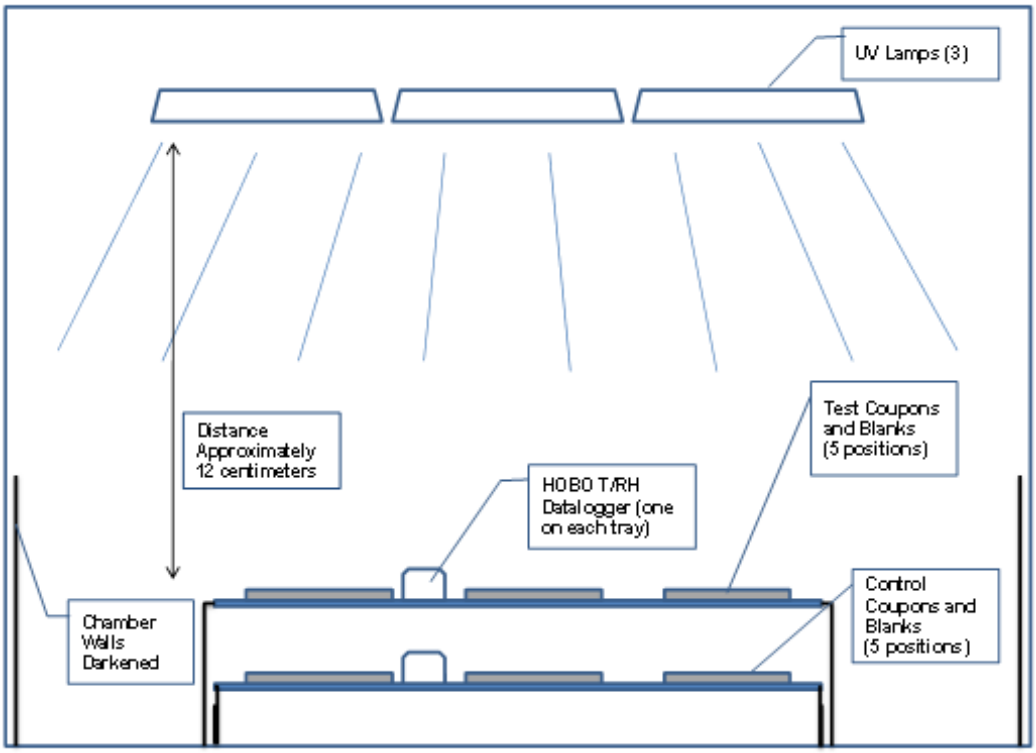


Figure 2-1. Schematic Representation of Test Chamber (not to scale)

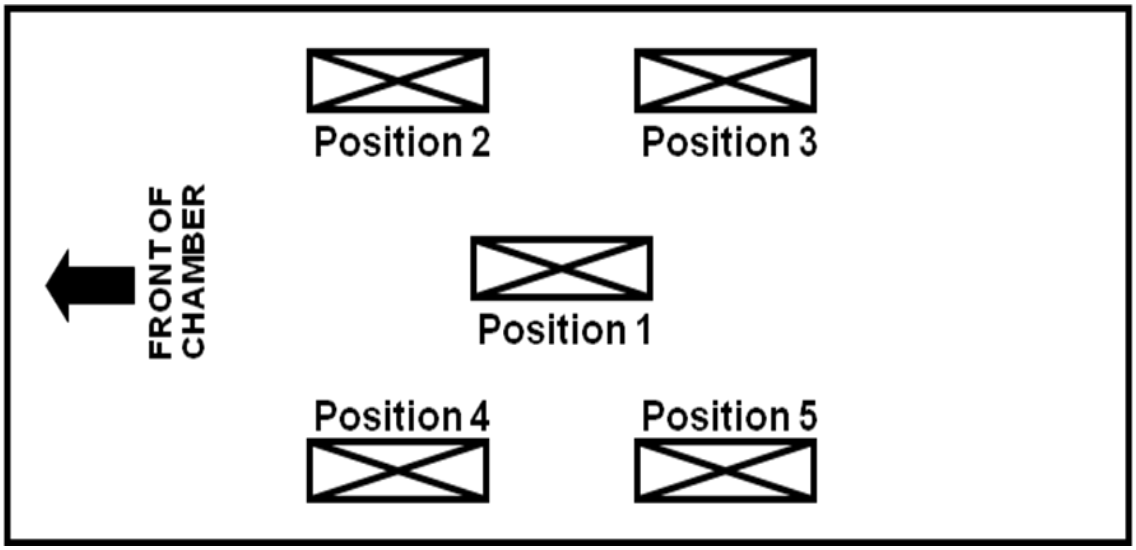


Figure 2-2. Schematic of Five Coupon Positions on the Support Trays

A single UV exposure test was conducted with *Y. pestis* in the same test chamber used for the UV exposure tests with *B. anthracis*. However, the *Y. pestis* UV exposure test used coupons of all four coupon materials placed in the UV test chamber at once, and only UV- exposed coupons were used (i.e., no non-exposed coupons were included in the test). Five inoculated UV-exposed coupons of each material were used, and one blank coupon of each material was also included with the exposed coupons. For that *Y. pestis* UV exposure test, the coupons were arrayed in the five separate positions on the UV-exposed support tray, as shown in Figure 2-2, with one exposed coupon of each of the four coupon materials placed at each of Positions 1 through 5, so that all coupon materials were equally distributed across the support trays. This approach ensured that all materials received equivalent UV-A/B exposures during testing. A blank coupon of one of the four materials was also placed at each of Positions 2 through 5, so that the four blanks were similarly distributed on both the exposed and non-exposed trays.

All UV exposure testing was conducted at normal room temperature (T) (i.e., approximately 22 °C) and over a range of RH from approximately 50 to 75 % RH. However, the chamber temperature and RH were not rigidly controlled, and despite circulation of air through the chamber, the chamber temperature typically increased slightly, and RH decreased slightly during the UV-A/B exposure periods. Temperature and RH were recorded at 5-minute intervals throughout the tests at the locations of both the exposed and non-exposed coupons by a HOBO® Model U12-011 temperature and RH sensor/data logger (Onset, Cape Cod, MA) placed near the center (Position 1, Figure 2-2) of each coupon support tray. The average and standard deviation (SD) of the recorded temperature and RH data at each tray over the duration of each UV-A/B exposure are presented in Sections 4 and 5 of this report to document the test conditions. The UV testing consisted of alternating 12-hour periods of UV-A/B exposure (lamps on) and darkness (lamps off), with the first 12-hour period always having lamps on. This alternating UV exposure schedule was designed to replicate natural diurnal conditions, allowing for potential repair of photo-induced damage in the test organisms between the periods of UV exposure. At the conclusion of each UV-A/B exposure period, exposed and non-exposed coupons of all materials were removed from the test chamber, and the test organisms were extracted and enumerated to determine persistence due to the UV-A/B exposure.

The UV lamps used for testing simulate natural sunlight by including both UV-A and UV-B components but without UV-C.[4] Wide variations in natural UV-A/B levels occur due to time of day, day of the year, location, cloud cover, air pollution levels, and altitude.[5-12] Peak (i.e., noontime) UV-B levels reported in a few studies range from approximately 20 to 150 microwatts (μW)/cm^2.[9-11] The target UV-B level chosen for testing was 44 μW/cm^2, which corresponds to a daily dose of approximately 1.9 Joules (J)/cm^2 with 12 hours of exposure per day. To put this in context, this UV-B dose is similar to the daily UV-B dose received during the summer months in Raleigh, North Carolina (see UV monitoring data at http://uvb.nrel.colostate.edu/UVB/index.jsf). The UV-A level was set at either approximately 100 μW/cm^2 or approximately 1,785 μW/cm^2, to assess the effect of UV-A relative to the more photobiologically active UV-B.[4] These UV-A levels correspond to a daily UV-A dose of about 4.3 J/cm^2 and 77 J/cm^2, respectively, with 12 hours of exposure per day. The former is a low UV-A level, and the latter is similar to the daily UV-A dose received during the summer months in Raleigh, North Carolina (see UV monitoring data at http://uvb.nrel.colostate.edu/UVB/index.jsf). A target of zero for UV-C radiation was

chosen because of the absence of this UV component in sunlight at ground level. The target UV intensities at the unexposed coupons were zero for UV-A, UV-B, and UV-C.

The actual UV intensities were measured at each of the five positions shown in Figure 2-2 on both the exposed and non-exposed coupon trays, at least near the start and end of every 12-hour UV-A/B exposure period. UV intensities were measured using Solarmeter® Digital Ultraviolet Radiometers, Model 5.7 (UV-A/B) (Serial No. 15957), Model 6.2 (UV-B) (Serial No. 01802), and Model 8.0 (UV-C) (Serial No. 00275) (Solartech, Inc., Harrison Twp., MI). The UV-A intensity was determined by subtracting the UV- B reading from the UV-A/B reading. The UV-A, UV-B, and UV-C intensities at each coupon position over each UV-A/B exposure test were determined, and the averages and SDs of those intensities are shown in Sections 4 and 5. The lamps used in testing are designed not to produce UV-C radiation, nevertheless UV-C measurements were made to confirm the absence of UV-C.

2.4 Non-UV Persistence Testing

The majority of the persistence tests were conducted without using UV-A/B radiation. The persistence testing was conducted in a laboratory chamber similar to the chamber used for the UV exposure testing, but with only minimal normal laboratory lighting and no UV lamps or UV sensors. The laboratory lighting was turned on and off manually by laboratory personnel on approximately a 12-hours on/12 hours off schedule. Initial tests involved all test materials and relatively long time points and were intended to provide a broad assessment of the persistence of the organisms. The conditions for subsequent tests were then chosen based on reviewing the results of previous tests. The resulting test matrix included targeted testing with individual coupon materials; determining persistence at multiple time points; varying the test RH; and varying the moisture content of topsoil coupons used in testing.

In the non-UV persistence testing, the temperature was not controlled but was always close to the temperature of the normal laboratory environment (i.e., approximately 22 ± 2 °C). The RH in different tests ranged from approximately 35 % to over 95 %. The RH of the normal laboratory environment provided sufficient control for tests at RH from approximately 35 % to 65 %; saturated salt solutions were used to control RH at approximately 75 % to over 95 % in other tests. Both T and RH were monitored in all testing.

2.5 Organism Recovery Procedures

Following the persistence test period or UV-A/B exposure period, each inoculated and associated blank coupon was transferred aseptically to a sterile 50-mL conical vial containing 10 mL of extraction solution. The extraction solution consisted of sterile phosphate-buffered saline (PBS) solution with Triton X-100 surfactant (i.e., 99.9 % PBS solution, 0.1 % Triton X-100 by volume). The coupons were then extracted by agitation on an orbital shaker for 15 minutes at approximately 200 rpm at room temperature. For all coupons, 1 mL of the coupon extract was removed following extraction, and a series of dilutions through 10^{-7} was prepared in SFW. For *B. anthracis*, an aliquot (0.1 mL) of the undiluted extract and of each serial dilution was spread plated in triplicate onto TSA plates and incubated overnight at 35 to 37 ºC. For *B. anthracis*, plates were enumerated within 18 to 24 hours of plating. For *Y. pestis*, an aliquot (0.1 mL) of the

undiluted extract and of each serial dilution was spread plated in triplicate onto TSA plates and incubated overnight at 26 ºC. For *Y. pestis*, plates were enumerated within 48 to 72 hours of plating. For both organisms, the number of CFU/mL was determined by multiplying the average number of colonies per plate by the reciprocal of the dilution and accounting for the 0.1 mL plated volume.

A heat shock procedure was applied to all coupon extracts in testing with *B. anthracis* to distinguish vegetative *B. anthracis* cells (which were killed by the heat shock) from *B. anthracis* spores (which survived the heat shock). Specifically, the 10 mL coupon extract was split into two equal portions, with one portion heat shocked and the other not, before plating of both aliquots for enumeration as described above. The heat shock procedure consisted of placing a small vial containing an aliquot of the extract into a water bath at 65 °C for one hour before the aliquot was serially diluted and plated.

Blank coupons controlled for viable organisms inadvertently introduced to test coupons, and were each inoculated with 100 µL of inoculum carrier liquid that did not contain *B. anthracis* or *Y. pestis* cells. The blank coupons underwent the same extraction process as the inoculated coupons, at the same time as those coupons. To be considered acceptable, extracts of blank coupons had to contain no CFU. The mean percent cell recovery from each coupon type was calculated by means of the following equation:

$$\text{Mean \% Recovery} = [\text{Mean } CFU_c/CFU_{pike}] \times 100 \qquad (1)$$

where Mean CFU_c is the mean number of CFU recovered from five replicate coupons of a single type, and CFU_{pike} is the number of CFU inoculated onto each of those coupons. The value of CFU_{pike} is known from enumeration of the stock suspension by the same dilution, plating, incubation, and enumeration procedures as were applied to the coupon extracts, as described above. Recovery was calculated for both *B. anthracis* and *Y. pestis* on each coupon type.

2.6 Calculation of Quantitative Persistence

The effect of coupon drying, different T/RH conditions, or UV-A/B exposure on the persistence of the test organisms was assessed by determining the number of viable organisms recovered from each test coupon after some period of time and/or UV-A/B exposure. In tests reported here, there were five test coupons at each time point for each material. When no viable CFU were found in a coupon extract, a CFU count of 1 was assigned, resulting in a base 10 logarithm (log) of zero for the CFU count for that coupon. When this occurred, the cell population on the coupon was considered to be completely inactivated within the detection limit of 33 CFU per extract sample. The possibility that viable cells still remained on the coupon despite the quantitative indication of zero CFU recovered was assessed by means of the qualitative testing described in Section 2.7.

Persistence of an organism on a coupon material was determined as the mean of the logs of the number of CFU recovered from each of the five replicate coupons of that coupon material at a test time point. First, the CFU count value from each coupon extract was determined, and then the mean of the log values was determined for each set of five replicate coupons, i.e.,

$$\text{Mean of Logs}_i = \overline{(\log_{10} CFUt_{ij})} \quad (2)$$

where $\log_{10} CFUt_{ij}$ refers to the j individual log values of the CFU extracted from the coupons of material i, with $j = 5$ and the overbar indicating a mean value.

Also, the number of CFU of an organism recovered in extracts of test coupons was compared to the number inoculated onto the coupons to calculate the mean log reduction (LR). Mean LR for a test organism on the i^{th} coupon material was calculated as the difference between the mean log value from Equation 2 and the log of the number of cells inoculated onto the coupons, which is known from the enumeration of the stock suspension as described in Section 2.5. Mean LR is calculated as:

$$LR = (\log_{10} CFUi) - \overline{(\log_{10} CFUt_{ij})} \quad (3)$$

where $\log_{10} CFUi$ refers to the log of the number of cells inoculated onto the material coupons and $\log_{10} CFUt_{ij}$ is as defined above. LR was determined for each coupon material at each time point in the testing.

Whenever the mean of the logs was calculated as in Equations 2 and 3, the SD of the five $\log_{10} CFUt_{ij}$ values was also calculated. The SD was then used to calculate the standard error (SE) of the result and in turn the 95% confidence interval (CI) for each result. The 95 % CI is:

$$95\%\ \text{CI} = \pm\ (1.96 \times \text{SE}) = \pm\ (1.96 \times [\text{SD}/\sqrt{5}]) \quad (4)$$

The significance of differences in persistence across different coupon materials, test conditions, or time points was assessed based on the 95 % CI of each result. Differences were judged to be significant if the 95 % CIs of two results did not overlap.

2.7 Qualitative Growth Testing

In addition to the extraction and quantitative enumeration described in Section 2.5, qualitative growth testing was conducted in all tests for vegetative *B. anthracis* and in some tests for *Y. pestis* by placing 1 mL of the coupon extract into an individual vial containing 10 mL of TSB. After one day of incubation, the solution in each vial was inspected visually for turbidity (cloudiness, indicating bacterial growth), and this inspection was then repeated after seven days of incubation. After the seven-day incubation a small amount of any solution showing turbidity was also streaked onto a tryptic soy agar plate, and the morphology of the resulting CFU was used to confirm the presence of the target organism. The qualitative results presented in Chapter 4 indicate whether bacterial growth was observed after 1 day and after 7 days. A "Y" indicates growth was visually observed with at least one of five coupon extracts, and an "N" indicates no growth was observed (complete inactivation of the organism). If at least one of the 7-day streak plates confirmed the presence of the target organism, this is indicated by using a bold letter "**Y**".

The qualitative growth test procedures provided a more sensitive indication of complete inactivation of the test organisms than did the extraction and enumeration alone, due to the inability of the extraction and enumeration to detect very small numbers of viable cells. In a few cases, the presence of viable cells was not indicated by the one-day qualitative test but was indicated by the seven-day test. Due to the greater sensitivity of the seven-day qualitative test, those results are emphasized in this report. The agreement or disagreement between quantitative enumeration and qualitative test results is noted where appropriate to document the persistence of the test organisms.

3.0 QUALITY ASSURANCE/QUALITY CONTROL

QA/QC procedures are summarized below.

3.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, BSCs) and monitoring devices (i.e., for T, RH, and UV-A/B, UV-B, and UV-C intensity) were verified as being certified, calibrated, or validated. Battelle's Instrumentation Services Laboratory, which is accredited by the American Association for Laboratory Accreditation to the International Standards Organization (ISO) 17025 standard, established NIST-traceable calibrations of the T and RH monitors used in this test. The three Solarmeter UV radiometers were obtained from the manufacturer certified with NIST-traceable calibrations, and that certification was in effect throughout all testing.

3.2 QC Results

QC efforts conducted during testing included inoculated non-exposed coupons in UV-A/B exposure testing, procedural blanks (not inoculated, UV-A/B-exposed), laboratory blanks (not inoculated, not UV-A/B-exposed), and spike control samples (analysis of the stock cell suspension). The results for these QC samples in each decontaminant evaluation are included in the results sections (see Sections 4 and 5).

3.3 Audits

3.3.1 Performance Evaluation Audit

Performance evaluation (PE) audits were conducted between January 2 and January 22, 2013, on equipment and measurements that factored directly into the test results, as specified in the QAPP. In the PE audit, the delivery of cell suspensions to coupons was audited by gravimetric checking of the micropipettes used for inoculation. The measurement of the concentration of cell suspensions by plating and enumeration was audited by recounting of the enumeration plates by a different laboratory staff member. The T and RH measurements were audited by comparison to a combination thermometer/hygrometer obtained from the laboratory and traceable to NIST. Table 3-1 lists the target criteria and results of the PE audits, and shows that all of the PE audit results met the target tolerances. Measurement of time was not subjected to a PE audit because the test durations were sufficiently long that high accuracy was not required.

Table 3-1. Performance Evaluation Audit Results

Measurement	Audit Procedure	Target Tolerance	Achieved Tolerance
Volume of cell suspensions or water	Gravimetric evaluation of micropipettes[(a)]	±10 %	-0.18 to -0.44 % 0.21 to 1.59 % 0.02 to 1.54 % -0.14 to -0.62 %
Enumeration of cell concentration	Recounting of enumeration plates by a different laboratory staff member	±10 %	0.8 % (*B.a.)* 1.7 % (*Y.p.)*
Temperature	Compared to independent calibrated thermometer[(b)]	±2 °C	0.1 °C[(c)] 0.2 °C
Relative humidity	Compare to independent calibrated hygrometer[(b)]	±10 %	3.2 %[c] 0.9 %

(a) Gravimetric check was performed by returning pipettes to the manufacturer (Rainin); results shown are from calibration certificates received for the four pipettes. Range of tolerances for each pipette results from calibration checks at delivered volumes ranging from 0.1 to 1.0 mL.

(b) Audit standard was a Vaisala (Boulder, CO, USA) Model HMT338 thermometer/hygrometer, serial number 111996775.

(c) Results shown are for the HOBO T/RH sensors positioned with the UV-exposed and shielded (non-exposed) coupons, respectively.

3.3.2 Technical Systems Audit

Laboratory QA staff conducted a technical systems audit (TSA) during testing on January 15, 2013, to ensure that the evaluation was being conducted in accordance with the QAPP and the QMP. As part of the TSA, test procedures were compared to those specified in the QAPP, and data acquisition and handling procedures were reviewed. Observations and findings from the TSA were documented and submitted to the laboratory lead for response. No adverse findings resulted from this TSA, but one QAPP amendment and one QAPP deviation were prepared as a result of this TSA to document changes made to improve the test procedures. The TSA report was submitted to EPA on January 31, 2013. TSA records were permanently stored with the laboratory QA Manager.

3.3.3 Data Quality Audit

All of the data acquired during the evaluation were audited. A QA auditor traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

3.4 QAPP Amendments and Deviations

Three amendments to the QAPP were prepared, reviewed, approved, and distributed to all parties involved in this evaluation. The subjects addressed by those three amendments were, respectively:

- The selection of different coupon materials (i.e., galvanized metal and painted wallboard paper) for testing with *Y. pestis* than were used in testing with *B. anthracis*, and the performance of UV-A/B exposure testing with only one of these organisms at a time, instead of with both organisms at once. The former change was made due to low recoveries observed with *Y. pestis* from concrete and bare pine wood (see Section 5.1). The latter change was made because the UV exposure system could not achieve a uniform UV-A/B intensity across the numerous test coupons when both organisms were tested at once.
- Dilution of stock cell suspensions to a target optical density was done for *Y. pestis* but not for *B. anthracis*. This change was made because the purely vegetative *B. anthracis* stock suspension was used without dilution to achieve a high enough inoculation of cells onto test coupons.
- Accommodation of a variety of exploratory test procedures other than UV-A/B exposure or persistence testing, including cell recovery testing, evaluation of heat shock procedures, experimentation to achieve purely vegetative *B. anthracis* cell cultures, and extraction of inoculated coupons after very short drying times (i.e., < one hour). This change was made to allow flexibility in investigating vegetative *B. anthracis* production procedures and the survivability of vegetative *B. anthracis* on coupon materials.

One QAPP deviation was prepared. That deviation stated that a saturated salt solution was not used as originally planned to control the RH in the UV-A/B exposure testing. The reason for this deviation was that the UV-A/B exposure tests were brief, and as a result the salt solution was ineffective in controlling the RH. The impact of this deviation on the testing was minimal, as the UV-A/B exposure tests were conducted at normal laboratory RH, and acceptable control was maintained by the laboratory climate control systems without the use of the salt solution.

3.5 QA/QC Reporting

Each audit was documented in accordance with the Quality Management Plan (QMP). The results of the audits were submitted to the EPA.

3.6 Data Review

Records and data generated in the evaluation received a QC/technical review before they were utilized in calculating or evaluating results and prior to incorporation in reports. All data were recorded by laboratory staff. The person performing the QC/technical review added his/her initials and the date to a hard copy of the record being reviewed. This hard copy was returned to the laboratory staff member who stored the record.

4.0 RESULTS FOR *Bacillus anthracis*

This section summarizes QC and persistence results for *B. anthracis*. Data are presented to document the uniformity of the UV exposure test conditions. Persistence results with and without UV-A/B exposure are reported for *B. anthracis* on the test materials.

4.1 QC Results

All procedural and laboratory blanks met the criterion of no observed CFU of the inoculated *B. anthracis* organism. Spike control samples were taken from the cell suspension on each day of testing, and serially diluted, nutrient plated, and counted to establish the cell suspension density used to inoculate the coupons. This process required approximately 24 hours, so the cell suspension density was known after completion of each day's testing. The target criterion was to maintain as high a cell suspension density as possible while producing a 100 % vegetative *B. anthracis* suspension. In actuality, *B. anthracis* suspensions of approximately 8×10^6 to 5×10^7 CFU/mL were produced, leading to actual spike values that ranged from approximately 8×10^5 CFU/coupon to 5×10^6 CFU/coupon. An aliquot of each *B. anthracis* cell suspension used to inoculate coupons and portions of all coupon extracts were subjected to the heat shock treatment noted in Section 2.5. No inoculation suspension subjected to the heat shock treatment showed any viable organisms, indicating the absence of *B. anthracis* spores and confirming that all coupons were inoculated with purely vegetative *B. anthracis* cells within the limit of detection.

4.2 Vegetative *Bacillus anthracis* Persistence without UV Exposure

Using the procedure for preparing a purely vegetative *B. anthracis* cell suspension (Section 2.2) seven persistence tests were conducted with vegetative *B. anthracis* without exposure to UV-A/B radiation. All those tests were conducted at normal laboratory temperatures. As noted in Section 2.4, the conditions for tests were chosen based on reviewing the results of previous tests. Those seven tests and the rationale for the test conditions in each test are listed below in chronological order.

- Test BA1 - Persistence on four coupon materials at a mean RH of 36 % (laboratory ambient RH) after a one-hour exposure time. Test BA1 was an initial scoping test to investigate relative persistence on the four test materials.
- Test BA2 - Persistence on glass coupons at a mean RH of 34 % (laboratory ambient RH) at time points from 15 minutes to 8 hours following inoculation. This test was conducted to obtain data on the persistence on glass coupons with greater time resolution than in Test BA1, and emphasized qualitative testing at all time points.
- Test BA3 - Persistence on four coupon materials at a mean RH of 75 % after a one-hour exposure time. This test was conducted for comparison to Test BA1, to investigate the effect of higher RH on vegetative *B. anthracis* persistence.
- Test BA4 - Persistence on topsoil coupons at a mean RH of 46 % at six time points from 1 hour to 120 hours following inoculation. Previous tests showed the highest persistence

of vegetative *B. anthracis* on topsoil, therefore this test focused on that test material with its normal moisture content of approximately 30 % by weight.

- Test BA5 - Persistence on wood, concrete, and glass coupons at a mean RH of 92 % at time points of 1, 8, 12, and 24 hours after inoculation. This test was conducted to assess the effect of high RH on persistence on these three materials before focusing exclusively on topsoil as the test material in the remaining tests.
- Test BA6 - Persistence on wetted topsoil coupons at a mean RH of 96 % at time points of 1 hour, and 7, 14, 21, and 28 days after inoculation. This test was designed to investigate whether sporulation would occur in wetted topsoil. Each topsoil coupon was wetted with 1 mL of SFW, resulting in wetted soil without producing a muddy consistency.
- Test BA7 - Persistence on wetted topsoil coupons at a mean RH of 93 % at time points of 1, 4, 8, 12, 18, 24, and 48 hours after inoculation. This test was conducted to investigate the onset of sporulation, which was observed to have reached completion by the seven-day time point in the previous test. Each topsoil coupon was wetted with 1 mL of SFW, resulting in wetted soil without producing a muddy consistency.

In all tests, the heat shock procedure was applied to an aliquot of the vegetative *B. anthracis* suspension used to inoculate the coupons and to all extracts of test coupons. Except in the last two tests conducted with wetted topsoil, no sporulation of the *B. anthracis* was observed at any point in the testing (i.e., only vegetative *B. anthracis* cells were present). Also, in each of these tests, the qualitative growth procedure described in Section 2.7 was used following the extraction of test coupons.

4.2.1 Uniformity of Test Conditions

Table 4-1 summarizes the temperature and RH conditions that were maintained during the persistence tests with vegetative *B. anthracis*. Not surprisingly, Table 4-1 shows that test temperature and RH showed a greater range of variation in the longer test periods.

Table 4-1. Temperature and Relative Humidity Conditions during Non-UV Exposure Persistence Tests with Vegetative *Bacillus anthracis*

Test No.	Persistence Test	Mean T (°C)	T Range (°C)	Mean RH (%)	RH Range (%)
BA1	Four Materials/1 hour/36 % RH	21.5	21.1 – 21.7	36.0	36.0 – 37.0
BA2	Glass/eight hours/34 % RH	22.1	20.6 – 23.9	33.9	27.5 – 45.3
BA3	Four Materials/one hour/75 % RH	21.8	21.4 – 22.2	75.4	73.7 – 76.4
BA4	Topsoil/120 hours/46 % RH	21.1	20.2 – 27.3	46.4	34.3 – 58.4
BA5	Three Materials/24 hours/92 % RH	21.1	20.9 – 22.3	92.4	92.0 – 92.6
BA6	Wet Topsoil/28 days/96 % RH[(a)]	21.0	20.2 – 22.1	95.7	89.4 - 96.8
BA7	Wet Topsoil/48 hours/93 % RH[(a)]	20.9	20.6 – 22.0	92.9	91.6 – 93.2

(a) Each topsoil coupon wetted with 1 mL of SFW at start of test.

4.2.2 Results of Persistence Tests with Vegetative Bacillus anthracis

Tables 4-2 through 4-8 show the test results from the non-UV persistence testing with vegetative *B. anthracis* in Tests BA1 through BA7, respectively. Each of these tables shows the vegetative *B. anthracis* inoculum onto each coupon, the mean log (± SD) of the recovered CFU, the mean (± SD) of the CFU percent recovery, the mean log reduction (± CI) relative to the inoculum, and the subsequent qualitative growth test results. Note that the format of these tables differs somewhat, in that Tables 4-2 and 4-4 show results with all four coupon materials at a single time point of one hour, Tables 4-3, 4-5, 4-7, and 4-8 show results from a single coupon material at multiple time points, and Table 4-6 shows results from three coupon materials at multiple time points. Also, Tables 4-7 and 4-8 include results from enumeration of aliquots of coupon extracts that were subjected to the heat shock treatment described in Section 2.5.

Table 4-2 shows results after one hour of exposure of the vegetative *B. anthracis* inoculum at a mean RH of 36 % RH and room temperature in Test BA1. No viable cells were recovered from glass, wood, or concrete in the quantitative testing, and the absence of viable cells on those materials was confirmed by the qualitative testing. Approximately 5 % of the vegetative *B. anthracis* in the inoculum survived the one-hour exposure time on topsoil in this test, and that result was confirmed by the qualitative results.

Table 4-2. Persistence of Vegetative *Bacillus anthracis*[(a)]

(Four Materials, One Hour, 36 % RH; Test BA1)

		Quantitative Testing			Qualitative Growth[(b)]	
Test Material	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
Topsoil						
Test Coupons	1.21×10^6	4.79 ± 0.10	5.2 ± 1.3	1.30 ± 0.09	Y	**Y**
Procedural Blank [(c)]	0	0	0	-		
Glass						
Test Coupons	1.21×10^6	0	0	6.08	N	N
Procedural Blank	0	0	0	-		
Bare Pine Wood						
Test Coupons	1.21×10^6	0	0	6.08	N	N
Procedural Blank	0	0	0	-		
Unpainted Concrete						
Test Coupons	1.21×10^6	0	0	6.08	N	N
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

Table 4-3 shows the results of Test BA2 with vegetative *B. anthracis* on glass coupons. In that test, coupons were removed after exposure for 15, 30, and 45 minutes and for 1, 2, 4, 6, and 8 hours after inoculation. Thus in this test, three sets of five coupons each were removed and extracted before one hour had elapsed (i.e., at 15, 30, and 45 minutes after inoculation). All coupon extracts were subjected to qualitative growth testing, but only extracts of coupons removed at one hour after inoculation were also quantitatively enumerated. Table 4-3 shows that after one hour of exposure, no viable cells were found in the quantitative testing. However, the seven-day qualitative growth testing showed the presence of viable *B. anthracis* at all drying times from 15 minutes through 6 hours after inoculation. Qualitative testing of coupon extracts from the eight-hour exposure showed no growth. These results indicate persistence of vegetative *B. anthracis* for at least six hours, but less than eight hours, on glass coupons at the conditions of Test BA2. While the quantitative persistence results for glass at 1 hour for Tests BA1 and BA2 are the same (no spores detected), the qualitative results for glass for the two tests are somewhat different (no persistence at 1 hour vs. persistence at 6 hours, respectively). This difference in qualitative results for the two tests may be attributed to slight differences in experimental procedures or conditions that may occur when performing separate experiments.

Table 4-3. Persistence of Vegetative *Bacillus anthracis*[(a)] on Glass

(Eight Hours, 34 % RH;Test BA2)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
15 Minutes						
Test Coupons	9.43×10^5	-	-	-	Y	**Y**
Procedural Blank[(c)]	0	-	-	-		
30 Minutes						
Test Coupons	9.43×10^5	-	-	-	Y	**Y**
Procedural Blank	0	-	-	-		
45 Minutes						
Test Coupons	9.43×10^5	-	-	-	N	**Y**
Procedural Blank	0	-	-	-		
1 Hour						
Test Coupons	9.43×10^5	0	0.00	5.97	N	**Y**
Procedural Blank	0	0	0	-		
2 Hours						
Test Coupons	9.43×10^5	-	-	-	N	**Y**
Procedural Blank	0	-	-	-		
4 Hours						
Test Coupons	9.43×10^5	-	-	-	N	**Y**
Procedural Blank	0	-	-	-		
6 Hours						
Test Coupons	9.43×10^5	-	-	-	N	**Y**
Procedural Blank	0	-	-	-		
8 Hours						
Test Coupons	9.43×10^5	-	-	-	N	N
Procedural Blank	0	-	-	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

Table 4-4 shows the results from one hour of exposure of the vegetative *B. anthracis* inoculum on all four materials at 75 % RH in Test BA3. The quantitative testing showed that approximately 10 % of the vegetative *B. anthracis* in the inoculum survived the one-hour exposure on topsoil, and approximately 2 % survived on glass. No viable cells were detected in the quantitative testing on wood and concrete coupons, but the qualitative testing showed the presence of viable cells on those materials. These results show that vegetative *B. anthracis* persisted on all four materials for at least one hour under 75 % RH conditions. Comparison of the quantitative results from Tests BA1 and BA3 (Tables 4-2 and 4-4) shows a significant increase in persistence at 75 % RH relative to 36 % RH on topsoil (based on comparison of 95 % CIs) and a significant increase on glass. The qualitative results suggest greater persistence on wood, glass, and concrete at the higher RH.

Table 4-4. Persistence of Vegetative *Bacillus anthracis*[(a)]

(Four Materials, One Hour, 75 % RH; Test BA3)

		Quantitative Testing			Qualitative Growth[(b)]	
Test Material	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
Topsoil						
Test Coupons	7.87×10^5	4.88 ± 0.20	10.4 ± 4.1	1.01 ± 0.17	Y	**Y**
Procedural Blank [(c)]	0	0	0	-		
Glass						
Test Coupons	7.87×10^5	4.15 ± 0.05	1.8 ± 0.24	1.74 ± 0.05	Y	**Y**
Procedural Blank	0	0	0	-		
Bare Pine Wood						
Test Coupons	7.87×10^5	0	0	5.90	N	**Y**
Procedural Blank	0	0	0	-		
Unpainted Concrete						
Test Coupons	7.87×10^5	0	0	5.90	N	**Y**
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

Because of the much greater persistence of vegetative *B. anthracis* on topsoil than on the other coupon materials, most subsequent testing focused on that material. Table 4-5 shows the results of Test BA4, a 120-hour test with vegetative *B. anthracis* on topsoil coupons, in which coupons were removed and extracted at 1, 24, 48, 72, 96, and 120 hours after inoculation. The quantitative test

results in Table 4-5 show that vegetative *B. anthracis* persisted on topsoil coupons at least 96 hours after inoculation, but no viable cells could be detected in the quantitative testing at 120 hours after inoculation. These quantitative test results were confirmed by the qualitative results. Also, Table 4-5 shows that approximately 9 % of vegetative *B. anthracis* inoculated on topsoil coupons were viable at one hour after inoculation, a result that is consistent with the results at one hour shown in Tables 4-2 and 4-4.

Table 4-5. Persistence of Vegetative *Bacillus anthracis*[(a)] on Topsoil (120 Hours, 46 % RH; Test BA4)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Test Coupons	8.23×10^5	4.84 ± 0.15	8.9 ± 3.4	1.08 ± 0.15	Y	**Y**
Procedural Blank[(c)]	0	0	0	-		
24 Hours						
Test Coupons	8.23×10^5	2.01 ± 0.42	0.018 ± 0.015	3.91 ± 0.37	Y	**Y**
Procedural Blank	0	0	0	-		
48 Hours						
Test Coupons	8.23×10^5	0.90 ± 1.3	0.011 ± 0.017	5.01 ± 1.10	Y	**Y**
Procedural Blank	0	0	0	-		
72 Hours						
Test Coupons	8.23×10^5	0.67 ± 0.92	0.0025 ± 0.0036	5.25 ± 0.81	Y	**Y**
Laboratory Blank	0	0	0	-		
96 Hours						
Test Coupons	8.23×10^5	0.30 ± 0.68	0.00091 ± 0.0018	5.61 ± 0.60	Y	**Y**
Procedural Blank	0	0	0	-		
120 Hours						
Test Coupons	8.23×10^5	0	0	5.92	N	N
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).
(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Figure 4-1 shows the persistence curve of vegetative *B. anthracis* on topsoil coupons in the 120-hour Test BA4 summarized in Table 4-5. This figure shows the log of the number of CFU recovered at each time point in that test, along with error bars showing the 95 % CI of the recovery results. Figure 4-1 illustrates that much of the loss of vegetative cells in topsoil occurs in the first 24 hours after inoculation (i.e., less than 0.02 % of the inoculated cells are viable at the 24-hour time point). As stated in Section 2.6, a heat shock procedure was applied to aliquots

of all coupon extracts to distinguish vegetative *B. anthracis* cells from spores. In Test BA4, the heat shock procedure confirmed at every time point that the organisms present were vegetative cells and not spores. Thus, Figure 4-1 and the heat shock results show that the vegetative *B. anthracis* either persisted or died off, but did not sporulate in topsoil under the conditions of this test.

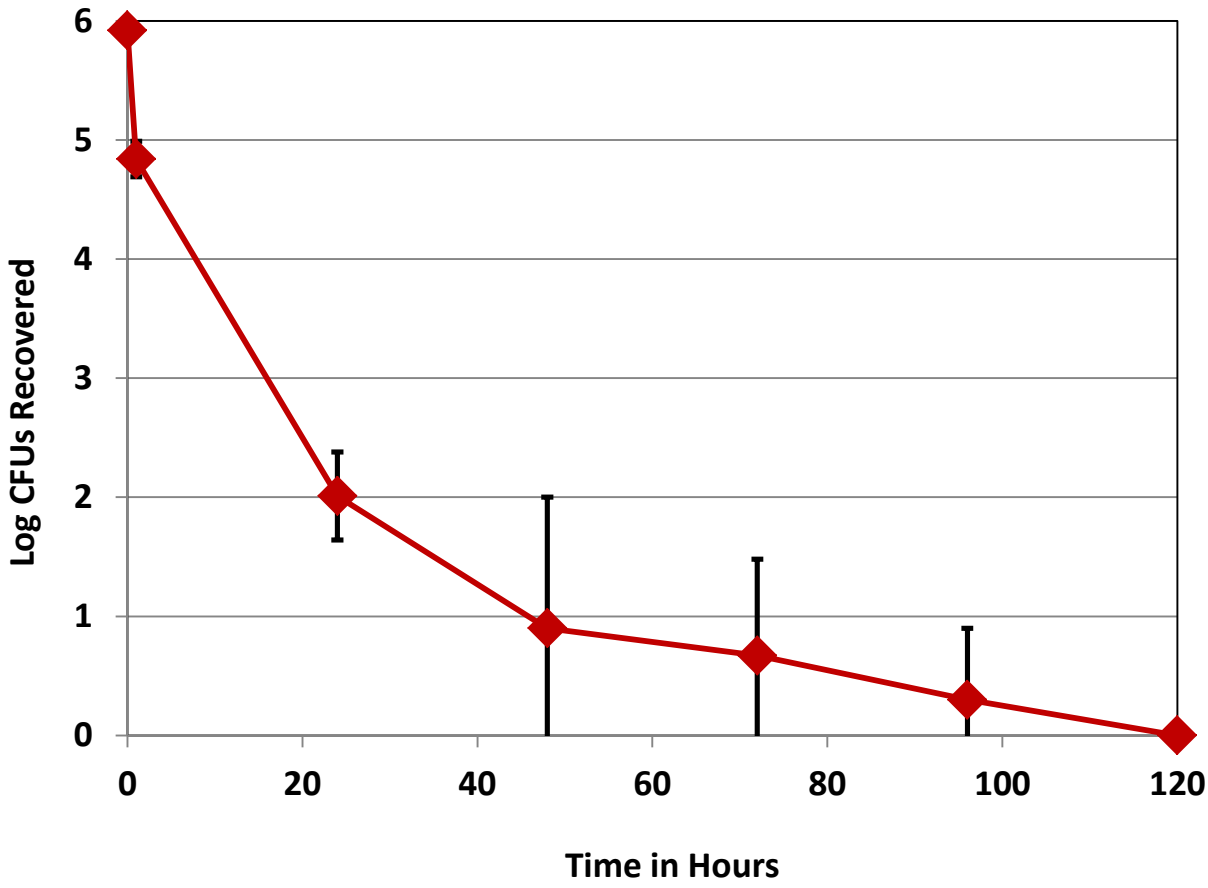


Figure 4-1. Vegetative *Bacillus anthracis* (Ames) Persists without Sporulation for at Least 96 Hours in Topsoil at Ambient Laboratory Temperature and Relative Humidity

Table 4-6 shows the results of persistence testing of vegetative *B. anthracis* on glass, bare pine wood, and unpainted concrete coupons over a 24-hour period at 92 % RH in Test BA5. Coupons were extracted for enumeration at 1, 8, 12, and 24 hours after inoculation, and also subjected to qualitative growth testing. Both the quantitative and qualitative testing showed that at this elevated RH, the vegetative *B. anthracis* cells persisted on glass through the 12-hour time point, but were completely inactivated by the 24-hour time point. The quantitative and qualitative test results also agreed in showing persistence on unpainted concrete at one hour (with less than 0.003 % of the inoculated vegetative *B. anthracis* cells viable at that time point), but with no persistence at eight hours. Both the quantitative and qualitative testing showed no persistence on bare pine wood, even at the one hour time point.

Table 4-6. Persistence of Vegetative *Bacillus anthracis*[(a)] on Three Materials (24 Hours, 92 % RH; Test BA5)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Test Coupons						
Glass	1.43×10^6	5.95 ± 0.09	63.0 ± 12.5	0.21 ± 0.08	Y	**Y**
Wood	1.43×10^6	0	0	6.16	N	N
Concrete	1.43×10^6	0.79 ± 1.09	0.0028 ± 0.0041	5.37 ± 0.95	Y	**Y**
Laboratory Blank[(c)]	0	0	0	--		
Procedural Blank[(d)]	0	0	0	--		
8 Hours						
Test Coupons						
Glass	1.43×10^6	1.28 ± 2.11	1.05 ± 2.34	4.88 ± 1.85	N	**Y**
Wood	1.43×10^6	0	0	6.16	N	N
Concrete	1.43×10^6	0	0	6.16	N	N
Laboratory Blank	0	0	0	--		
Procedural Blank	0	0	0	--		
12 Hours						
Test Coupons						
Glass	1.43×10^6	2.64 ± 0.43	0.045 ± 0.047	3.52 ± 0.38	N	**Y**
Wood	1.43×10^6	0	0	6.16	N	N
Concrete	1.43×10^6	0	0	6.16	N	N
Laboratory Blank	0	0	0	--		
Procedural Blank	0	0	0	--		
24 Hours						
Test Coupons						
Glass	1.43×10^6	0	0	6.16	N	N
Wood	1.43×10^6	0	0	6.16	N	N
Concrete	1.43×10^6	0	0	6.16	N	N
Laboratory Blank	0	0	0	--		
Procedural Blank	0	0	0	--		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Laboratory Blank = Not inoculated.

(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

The data in Table 4-6 are consistent with a positive effect of higher RH on the persistence of vegetative *B. anthracis* on glass, as suggested by the data in Tables 4-2 to 4-4. The results supporting this RH effect are shown in Figure 4-2, which shows the log of the CFU of vegetative *B. anthracis* recovered after one hour of exposure on glass at RH from 36 % to 92 %. At 36 % RH, no viable cells were recovered after one hour, but at the higher RH levels, recovery increased with higher RH. The 95 % CI values are shown as error bars in Figure 4-2, and clearly illustrate that the persistence on glass at 92 % was significantly greater than at 75 % RH, and that persistence at both 75 % and 92 % RH was significantly greater than persistence at 36 % RH.

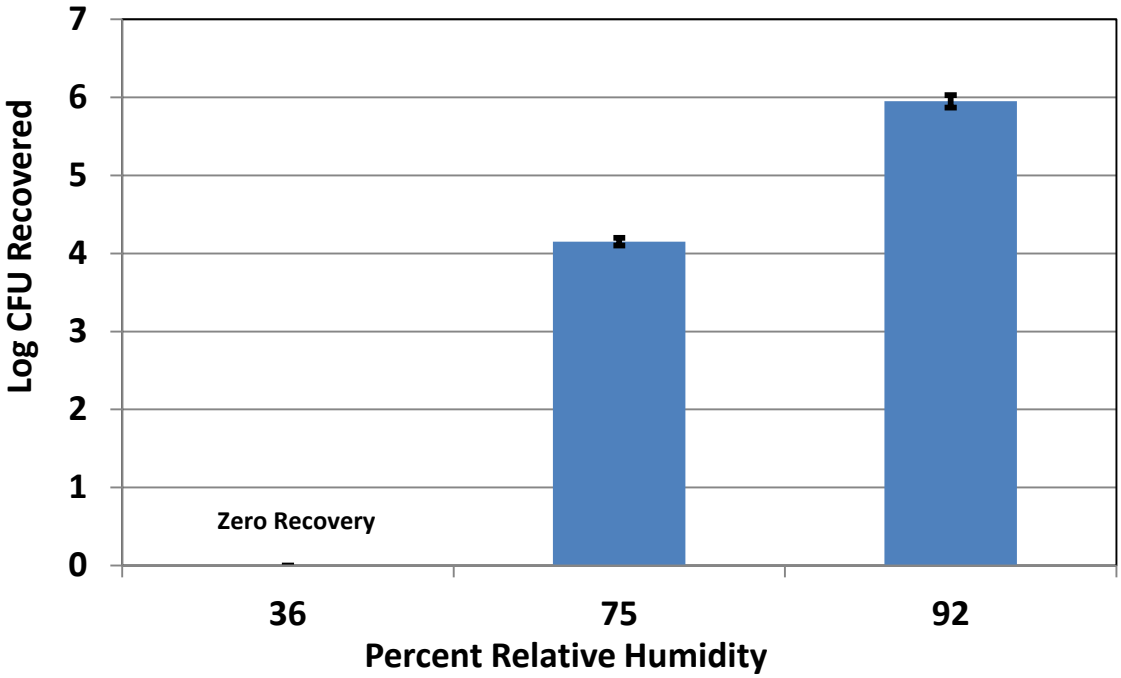


Figure 4-2. Recovery of Vegetative *Bacillus anthracis* at One Hour on Glass Increases with RH

Table 4-7 shows the results of persistence testing of vegetative *B. anthracis* on topsoil coupons over a 28-day period in Test BA6. In this test, the topsoil coupons were each wetted with 1 mL of SFW, and the RH in the test chamber was maintained above 95 % throughout the 28-day test duration. This test was conducted to assess the effect of elevated RH as well as elevated soil moisture on persistence of vegetative *B. anthracis*. Coupons were extracted for enumeration at one hour, and then at 7, 14, 21, and 28 days after inoculation, and also subjected to qualitative growth assessment at one and seven days after extraction. Table 4-7 shows that in contrast to all other tests described above, growth and sporulation of *B. anthracis* occurred in the wetted topsoil coupons, with the result that the total organism counts in the topsoil coupons increased by more than an order of magnitude within the first week of the test. Consequently, the recovery values at all time points longer than one hour in Table 4-7 greatly exceeded 100 % recovery.

Table 4-7. Persistence of Vegetative *Bacillus anthracis*[(a)] on Wetted Topsoil (28 Days, 96 % RH; Test BA6)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Test Coupons	2.90×10^6	6.32 ± 0.05	72.7 ± 8.5	0.14 ± 0.04	Y	**Y**
Test Coupons HS[(c)]	2.90×10^6	0	0	6.46		
Procedural Blank[(d)]	0	0	0	--		
7 Days						
Test Coupons	2.90×10^6	7.37 ± 0.01	811 ± 21	-0.91 ± 0.01	Y	**Y**
Test Coupons HS	2.90×10^6	7.36 ± 0.01	794 ± 19	-0.90 ± 0.01		
Procedural Blank	0	0	0	--		
14 Days						
Test Coupons	2.90×10^6	7.55 ± 0.37	1586 ± 1103	-1.09 ± 0.32	Y	**Y**
Test Coupons HS	2.90×10^6	7.61 ± 0.45	1957 ± 1406	-1.14 ± 0.39		
Procedural Blank	0	0	0	--		
21 Days						
Test Coupons	2.90×10^6	7.48 ± 0.49	1597 ± 1400	-1.02 ± 0.43	Y	**Y**
Test Coupons HS	2.90×10^6	7.72 ± 0.39	2290 ± 1386	-1.25 ± 0.34		
Procedural Blank	0	0	0	--		
28 Days						
Test Coupons	2.90×10^6	7.21 ± 0.69	1110 ± 1015	-0.75 ± 61	Y	**Y**
Test Coupons HS	2.90×10^6	7.20 ± 0.72	1126 ± 1093	-0.73 ± 63		
Procedural Blank	0	0	0	--		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).
(c) HS = heat shocked. Each extract of a test coupon is split, and one half is heat shocked prior to enumeration to inactivate any vegetative *B. anthracis* cells and leave only spores.
(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-7 also includes the results of enumerating coupon extracts that were subjected to the heat shock treatment. The heat shock procedure conducted on the coupon extracts at one hour after inoculation showed no viable organisms, confirming that the cell population was still 100 % vegetative *B. anthracis* at that time point. However, the heat shock procedures conducted at all later time points showed that the organisms present were predominantly to entirely *B. anthracis* spores. These results indicate growth and subsequent sporulation of the *B. anthracis* cell population within approximately the first seven days of the test. The results of this and previous tests suggest the impact of moisture in determining whether vegetative *B. anthracis* cells persist

as cells or die out (i.e., under relatively dry conditions), or grow and sporulate (i.e., under wet conditions as in this test).

Table 4-8 shows the results of persistence testing of vegetative *B. anthracis* on topsoil coupons over a 48-hour period in Test BA7. In this test the topsoil coupons were each wetted with 1 mL of SFW, and the RH in the test chamber was maintained at approximately 93 % throughout the 48-hour test duration. The purpose of this test was to further investigate the sporulation observed in the previous test, by attempting to determine more precisely when the onset of sporulation occurred in the first days after coupon inoculation. Consequently in this test, coupons were extracted for enumeration at 1, 4, 8, 12, 18, 24, and 48 hours after inoculation, and also subjected to qualitative growth assessment at one and seven days after extraction. The heat shock procedure conducted on the coupon extracts showed no viable organisms through the 18-hour time point, confirming that the initial inoculation and subsequent *B. anthracis* populations were 100 %vegetative until at least the 18-hour time point. However, Table 4-8 shows that growth of *B. anthracis* occurred in the wetted topsoil coupons within approximately eight hours after inoculation, and the onset of sporulation was observed within 24 hours after inoculation (as indicated by the heat shocked results). The total *B. anthracis* population in the topsoil coupons increased by about a factor of two relative to the inoculated amount within the 48-hour duration of the test, and more than 10 % of the *B. anthracis* organisms were in spore form at the end of the 48-hour time point.

The results of the 48-hour test with *B. anthracis* on wetted topsoil are illustrated in Figure 4-3, which shows the total number of recovered *B. anthracis* CFU (vegetative cells plus spores) and the number of only spores on the soil coupons at each time point of this test. Until the 24-hour time point, no spores were present, consistent with the purely vegetative nature of the inoculation. However, at the 24- and 48-hour time points, the sporulation of the *B. anthracis* is clearly evident. The results of this test are consistent with those of the previous 28-day test with *B. anthracis* and may show the impact of moisture in determining whether vegetative *B. anthracis* cells die out or grow and sporulate.

Table 4-8. Persistence of Vegetative *Bacillus anthracis*[(a)] on Wetted Topsoil (48 Hours, 93 % RH; Test BA7)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
1 Hour						
Test Coupons	2.27×10^6	6.10 ± 0.07	56.5 ± 9.3	0.25 ± 0.06	Y	**Y**
Test Coupons HS[(c)]	2.27×10^6	0	0	6.36		
Procedural Blank[(d)]	0	0	0	--		
4 Hours						
Test Coupons	2.27×10^6	6.36 ± 0.03	101 ± 6.5	0.00 ± 0.03	Y	**Y**
Test Coupons HS	2.27×10^6	0	0	6.36		
Procedural Blank	0	0	0	--		
8 Hours						
Test Coupons	2.27×10^6	6.59 ± 0.10	177 ± 41.9	-0.24 ± 0.09	Y	**Y**
Test Coupons HS	2.27×10^6	0	0	6.36		
Procedural Blank	0	0	0	--		
12 Hours						
Test Coupons	2.27×10^6	6.65 ± 0.05	198 ± 23.7	-0.29 ± 0.05	Y	**Y**
Test Coupons HS	2.27×10^6	0	0	6.36		
Procedural Blank	0	0	0	--		
18 Hours						
Test Coupons	2.27×10^6	6.61 ± 0.05	182 ± 19.3	-0.26 ± 0.04	Y	**Y**
Test Coupons HS	2.27×10^6	0	0	6.36		
Procedural Blank	0	0	0	--		
24 Hours						
Test Coupons	2.27×10^6	6.71 ± 0.07	230 ± 41.9	-0.36 ± 0.06	Y	**Y**
Test Coupons HS	2.27×10^6	3.07 ± 1.81	0.48 ± 0.69	3.28 ± 1.58		
Procedural Blank	0	0	0	--		
48 Hours						
Test Coupons	2.27×10^6	6.66 ± 0.05	202 ± 21.9	-0.30 ± 0.04	Y	**Y**
Test Coupons HS	2.27×10^6	5.72 ± 0.22	25.9 ± 14.1	0.63 ± 0.19		
Procedural Blank	0	0	0	--		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) HS = heat shocked. Each extract of a test coupon is split, and one half is heat shocked prior to enumeration to inactivate any vegetative *B. anthracis* cells and leave only spores.

(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

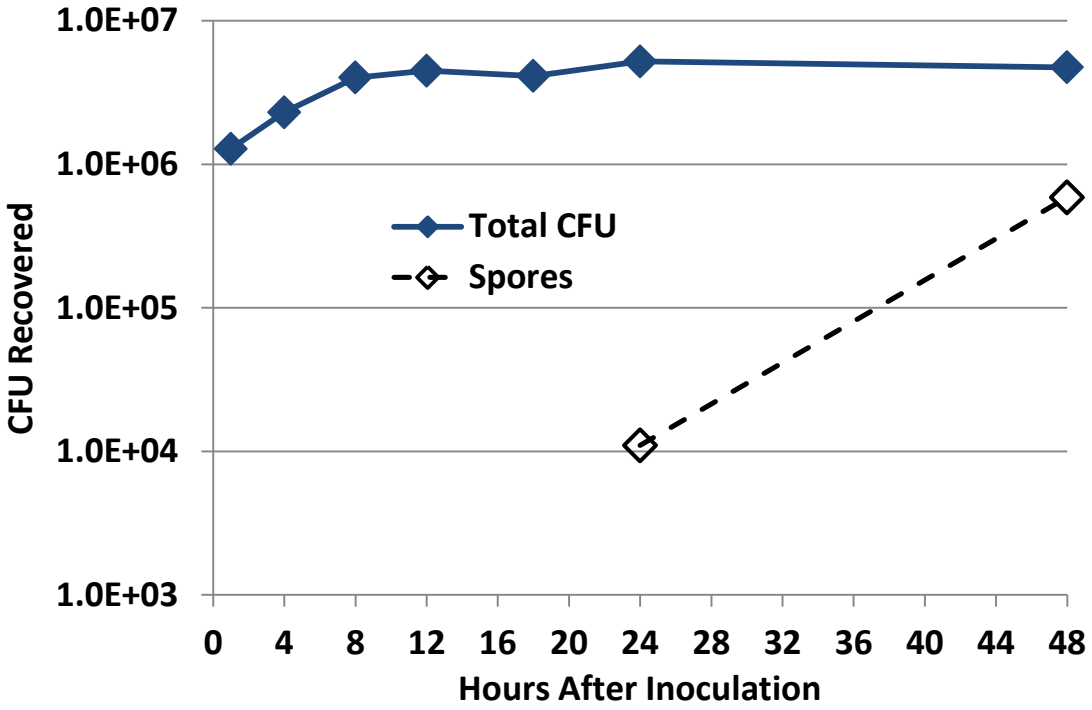


Figure 4-3. Enumeration of Spores and Total CFU of *Bacillus anthracis* Shows Growth and Sporulation in the 48-hour Test on Wet Soil at 93 % RH

4.3 Persistence of Vegetative *Bacillus anthracis* under Exposure to Simulated Sunlight

Using the procedure for preparing a purely vegetative *B. anthracis* cell suspension (Section 2.2), tests were conducted to assess the effect of simulated sunlight on the persistence of vegetative *B. anthracis* in topsoil. Those tests consisted of:

- Test BAUV1 - Persistence on topsoil over 120 hours with a low UV-A/UV-B ratio
- Test BAUV2 - Persistence on topsoil over 120 hours with a high UV-A/UV-B ratio.

Persistence under UV exposure was assessed only with topsoil as the test material, because the testing described in Section 4.2 showed that vegetative *B. anthracis* persisted for only a few hours on the other materials under ambient RH conditions. In each of tests BAUV1 and BAUV2, the heat shock procedure was applied to the vegetative *B. anthracis* suspension used to inoculate the coupons, and to all extracts of UV-exposed and non-exposed coupons. No organisms survived the heat shock treatment in these tests, indicating that the inoculum and the coupon extracts were free of *B. anthracis* spores. Also, in each of these tests, aliquots of the coupon extracts were placed in a nutrient broth and assessed for growth at one and seven days after coupon extraction. There were a few test results in which CFU were not recovered quantitatively from soil coupons, but qualitatively showed growth after seven days of incubation. These results are further described below and in Appendix B.

4.3.1 Uniformity of Test Conditions

Tables 4-9 and 4-10 summarize the test conditions of UV intensity, temperature, and RH monitored during the UV exposure testing with *B. anthracis*. Table 4-9 shows the average (± SD) intensities (in $\mu W/cm^2$) of UV-A, UV-B, and UVA/B measured at five positions in the UV-exposed coupon arrays in each of the two 120-hour UV-A/B exposure tests with *B. anthracis*. The intensity of UV-C at both the UV-exposed and non-exposed coupons was below detection (i.e., < 1 $\mu W/cm^2$) in all tests, and the intensities of UV-B and UV-A/B at the non-exposed coupons were also below detection in all tests.

Table 4-10 shows the average (± SD) of the temperature and RH monitored near the center of the UV-exposed coupon array and the non-exposed coupon array in the two 120-hour UV exposure tests with vegetative *B. anthracis*. Table 4-10 shows data both with the UV lights on and the lights off, and shows consistency in the test conditions. When the UV lights were off, the UV-exposed and non-exposed coupons experienced closely similar temperature conditions (i.e., within about 0.7 °C). When the lights were on, the UV-exposed coupons experienced slightly higher temperatures than did the non-exposed coupons, by 1.6 °C with a low UV-A/UV-B ratio, and by 4.4 °C with a high UV-A/UV-B ratio (and overall much higher UV intensity). In both UV tests, the RH at the non-exposed coupons was higher than that at the UV-exposed coupons, both with the lights on and lights off. The reason is not certain but is probably related to the temperature of the coupon arrays. All the mean RH values were in the range of approximately 55 to 72 % RH, and the RH difference between UV-exposed and non-exposed coupons is not expected to affect persistence significantly.

Table 4-9. Ultraviolet Radiation Intensity at the Topsoil Test Coupons in Testing with *Bacillus anthracis*[(a)]

	UV-A/B Exposure Test	
UV Condition[(b)]	**Test BAUV1: 120 Hour Low UV-A to UV-B Ratio**	**Test BAUV2: 120 Hour High UV-A to UV-B Ratio**
UV-A Average ± SD	99.3 ± 4.3	1,824 ± 53
UV-B Average ± SD	46.9 ± 3.4	44.9 ± 1.7
UV-A/B Average ± SD	146.2 ± 6.6	1,869 ± 54

(a) All entries are in $\mu W/cm^2$ at the test coupons; all UV intensities were zero at the non-exposed coupons.
(b) Average ± SD refers to average over all five measurement positions.
SD = standard deviation.
UV-A determined by subtracting UV-B reading from UV-A/B reading.

Table 4-10. Summary of Temperature and Relative Humidity Conditions in Simulated Sunlight Testing with Vegetative *Bacillus anthracis*

	UV-A/B Exposure Test			
	Test BAUV1: 120 Hour Low UV-A/UV-B Ratio		Test BAUV2: 120 Hour High UV-A/UV-B Ratio	
Test Condition[(a)]	Test[(b)]	Non-Exposed[(c)]	Test	Non-Exposed
UV ON				
T Average ± SD (°C)	22.1 ± 0.8	20.5 ± 0.2	25.2 ± 2.0	20.8 ± 0.5
RH Average ± SD (%)	55.6 ± 3.7	71.5 ± 4.0	55.2 ± 10.6	63.1 ± 3.2
UV OFF				
T Average ± SD (°C)	19.8 ± 0.7	20.5 ± 0.2	20.5 ± 1.7	20.7 ± 0.6
RH Average ± SD (%)	57.1 ± 2.8	71.9 ± 3.6	57.8 ± 5.7	64.6 ± 5.0

(a) UV ON and UV OFF refer to 12-h periods of alternating illumination and darkness in test chamber; averages shown are over all UV ON or UV OFF periods in the indicated UV exposure period. T = temperature, RH = relative humidity,

(b) Test coupons (UV-A/B-exposed); readings taken at a central position in the coupon array.

(c) Non-exposed coupons (not exposed to UV-A/B); readings taken at a central location in the coupon array.

SD = standard deviation.

4.3.2 Persistence Results in Simulated Sunlight Tests with Vegetative Bacillus anthracis

In both Tests BAUV1 and BAUV2, topsoil coupons were extracted at time points of 1, 24, 48, 72, 96, and 120 hours, and coupon extracts were subjected to both quantitative enumeration and qualitative growth testing. The heat shock procedure was applied to all coupon extracts and showed that only vegetative *B. anthracis* cells were present at all times during these tests. Tables showing the detailed results from Tests BAUV1 and BAUV2 are included in Appendix B of this report.

Refer to Figure 4-4 for a comparison of trends in the persistence of vegetative *B. anthracis* on the UV-exposed (Test BAUV1; low UV-A) and non-UV exposed topsoil coupons. Viable cells were recovered from the non-UV exposed topsoil samples at 120 hours but not from the UV exposed topsoils. In general, the quantitative persistence results in Figure 4-4 show that vegetative *B. anthracis* recovery was lower at each time point for the UV-exposed topsoil coupons than for the non-exposed coupons. However, the 95 % CIs of the UV-exposed and non-exposed persistence results overlap at all time points except at the one-hour and 24 hour time points. Thus, within the first 24 hours of exposure the data indicate a significant difference in persistence due to the simulated sunlight exposure.

We note that per the qualitative results of Test BAUV1, *B. anthracis* vegetative cells not exposed to UV persisted to 120 hours in soil, whereas from Test BA4, *B. anthracis* cells in soil (no UV exposure) persisted up to 96 hours. This difference in results exemplifies the inherent variability working with microorganisms, and may also be due to minor uncontrolled differences in experimental procedures or conditions that may occur when performing separate experiments. Thus, from these data, the persistence in soil may be best characterized as ranging from 4-5 days.

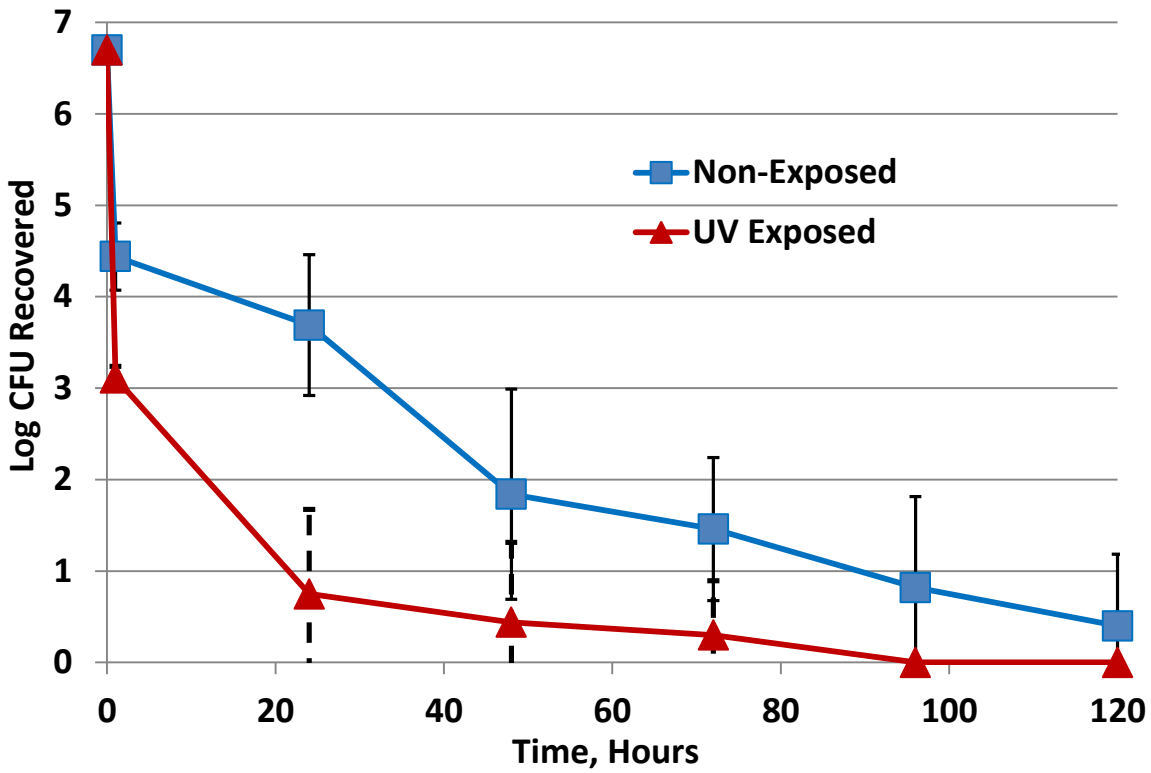


Figure 4-4. Vegetative *Bacillus anthracis* Persistence with Exposure to Simulated Sunlight with a Low UV-A/UV-B Ratio

Figure 4-5 shows the corresponding results from Test BAUV2 conducted with a high UV-A/UV-B ratio. Although the persistence seen on the UV-exposed topsoil coupons at each time point with this high UV-A/UV-B ratio is less than the persistence on the non-exposed coupons, the differences are statistically significant only at the one-hour time point. Based on the qualitative test results (Table B-2, in Appendix B), vegetative *B. anthracis* survived for 96 hours, but no growth was seen at the 120 hour time point, for both the UV and non-UV exposure conditions.

The results for the CFU recovery from soil when exposed to the high UV-A level are significantly different from the recovery from soil when exposed to the low UV-A level only at the one-hour time point (refer to Figure B-1 in Appendix B), and at that time point the recovery with the high UV-A level was greater than the recovery with the low UV-A level. This result provides some confirmation of the limited impact that UV-A may have in the inactivation of *B. anthracis* cells.

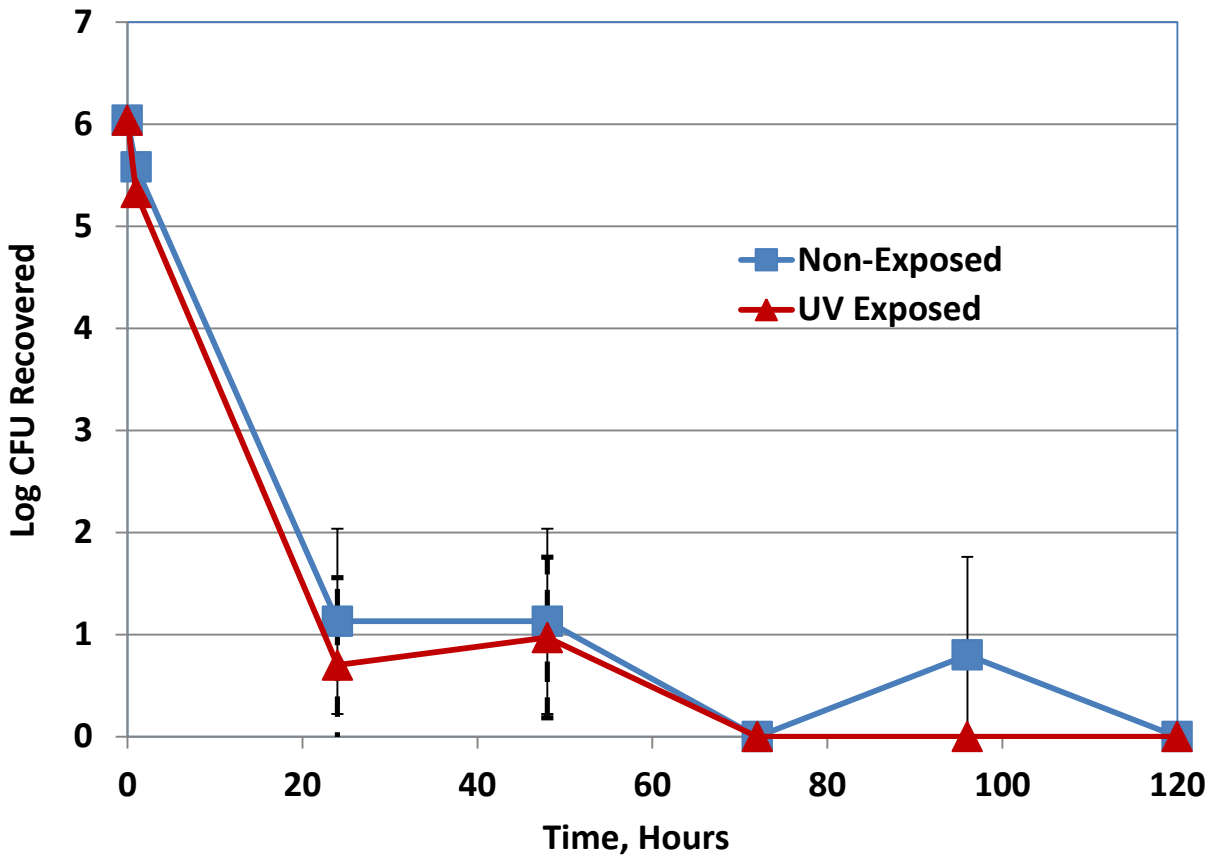


Figure 4-5. Vegetative *Bacillus anthracis* Persistence with Exposure to Simulated Sunlight with a High UV-A/UV-B Ratio

Overall, the results of Tests BAUV1 and BAUV2 indicate that exposure to simulated sunlight appears to have minor impact (more so during the first 24 hours) on the persistence of vegetative *B. anthracis* on topsoil, presumably due to the shading effect of soil particles.

Comparison of Figures 4-4 and 4-5 also shows that the two persistence curves for the non-exposed topsoil coupons in Tests BAUV1 and BAUV2 differed significantly at the one hour time point (with the Test BAUV2 coupons showing greater persistence) and at the 24 hour and 72 hour time points (with the Test BAUV1 coupons showing greater persistence). (The two persistence curves are compared directly in Figure B-2 in Appendix B.) The reason for these differences in persistence on non-UV-exposed topsoil coupons is not clear. The slightly higher RH at the non-exposed coupons in Test BAUV1 relative to Test BAUV2 (see Table 4-10) is not likely to have been sufficient to cause the observed difference in persistence.

5.0 RESULTS FOR *Yersinia pestis*

This section summarizes QC and persistence results for *Y. pestis*. Data are presented to document the uniformity of the UV exposure test conditions, and persistence results with and without UV-A/B exposure are reported for *Y. pestis* on the test materials.

5.1 Recovery Testing

In previous studies, recovery of *Y. pestis* from some coupon materials (including bare wood and unpainted concrete) immediately after inoculation was found to be very low.[2,13] Consequently, prior to any persistence testing in this project, the recovery of *Y. pestis* from the coupon materials was tested. In these tests, the *Y. pestis* inoculation was allowed to dry for one hour after inoculation before extraction of the coupons and enumeration of the organisms as CFU. Although relatively high recovery of *Y. pestis* from topsoil and glass coupons was observed in initial tests, the recovery of *Y. pestis* from wood and concrete coupons was found to be approximately 0.005 % and 0.001 %, respectively. These recovery values were judged to be too low for adequate persistence testing, so initial recovery testing was repeated with galvanized metal ductwork and painted wallboard paper in place of the wood and concrete. The results of both the initial and further recovery tests with *Y. pestis* are shown in Table 5-1.

Table 5-1. Recovery Testing of *Yersinia pestis*[(a)]

Test Material	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery
Glass	3.97×10^7	7.56 ± 0.02	91.0 ± 3.8
Topsoil	3.97×10^7	7.33 ± 0.03	54.5 ± 3.2
Bare Pine Wood	3.97×10^7	3.28 0.21	0.0052 ± 0.0028
Unpainted Concrete	3.97×10^7	2.29 0.77	0.0013 ± 0.0019
Galvanized Metal	4.13×10^7	7.29 ± 0.10	47.7 ± 10.9
Painted Wallboard Paper	4.13×10^7	4.03 ± 0.54	0.042 ± 0.047

(a) Data are expressed as mean of the logs of total number of cells (CFU) inoculated onto and recovered from individual coupons and mean percent recovery.

Table 5-1 shows that recoveries of *Y. pestis* from glass, topsoil, and galvanized metal were all near or above 50 %, whereas the recovery from painted wallboard paper was much lower, at approximately 0.04 %. Nevertheless, the recovery of *Y. pestis* from painted wallboard paper was much higher than had been found with wood or concrete, and as a result, that coupon material was used along with glass, topsoil, and galvanized metal in persistence testing of *Y. pestis*.

5.2 QC Results

All procedural and laboratory blanks met the criterion of no observed CFU of the inoculated *Y. pestis* organism. Spike control samples were taken from the cell suspension on each day of testing and serially diluted, nutrient plated, and counted to establish the cell suspension density used to spike the coupons. This process required approximately 48 to 72 hours, so the cell suspension density was known after completion of each day's testing. *Y. pestis* suspensions of

approximately 3×10^8 to 2×10^9 CFU/mL were produced, leading to actual spike values that ranged from approximately 3×10^7 CFU/coupon to 2×10^8 CFU/coupon.

5.3 *Yersinia pestis* Persistence without UV Exposure

Four persistence tests were conducted with *Y. pestis* without exposure to UV-A/B radiation. All those tests were conducted at normal laboratory temperatures. As noted in Section 2.4, the conditions for tests were chosen based on reviewing the results of previous tests. Those four tests and the rationale for the test conditions in each test are listed below in chronological order.

- Test YP1 - Persistence on four coupon materials at a mean RH of 57 % RH, at time points of one hour and 14 days. This test was an initial scoping test to investigate relative persistence on the four test materials.
- Test YP2 - Persistence on four coupon materials at a mean RH of 54 % RH, at time points of one hour and seven days. The lack of recovery of *Y. pestis* at 14 days in Test YP1 was the motivation for this shorter test on all four materials.
- Test YP3 - Persistence on four coupon materials at a mean RH of 65 %, at time points of 1, 24, 48, 96, and 144 hours. The absence of persistence of *Y. pestis* at seven days in Test YP2 was the motivation for this shorter test on all four materials with multiple time points after inoculation.
- Test YP4 - Persistence on wetted topsoil coupons at a mean RH of 96 %, at time points of one hour, seven days, and 14 days. This test was planned for 28 days but was truncated after 14 days due to inactivation of the *Y. pestis*. This test focused on topsoil as the coupon material because of the much greater persistence observed in Test YP3 on that material relative to the other materials. The test conditions were chosen to assess persistence of *Y. pestis* under conditions where drying of the organism after inoculation was not a concern. Each topsoil coupon was wetted with 1 mL of sterile filtered water, resulting in wetted soil without producing a muddy consistency.

The qualitative growth procedure described in Section 2.7 was used following the extraction of test coupons in Tests YP3 and YP4 but not in Tests YP1 or YP2.

5.3.1 Uniformity of Test Conditions

Table 5-2 summarizes the temperature and RH conditions that were maintained during the non-UV persistence tests with *Y. pestis*.

Table 5-2. Temperature and Relative Humidity Conditions during Non-UV Persistence Tests with *Yersinia pestis*

Test No.	Persistence Test	Mean T (°C)	T Range (°C)	Mean RH (%)	RH Range (%)
YP1	Four Materials/50% RH/14 Days	20.7	20.0 – 21.4	57.0	44.7 – 65.1
YP2	Four Materials/50 % RH/7 Days	20.7	20.3 – 21.2	53.6	45.4 – 56.5
YP3	Four Materials/65 % RH/144 hours	20.8	20.3 – 23.5	64.6	52.5 – 72.6
YP4	Wet Topsoil/90 % RH/28 Days[(a)]	21.0	20.5 – 21.5	96.0	91.6 – 96.5

(a) Each topsoil coupon wetted with 1 mL of SFW at start of test.

5.3.2 Results in Persistence Tests with Yersinia pestis

Tests YP1 and YP2 both showed recovery of *Y. pestis* at one hour after inoculation on all four test materials, with the greatest recovery on topsoil. However, *Y. pestis* was not recovered at the 14-day time point in Test YP1 or at the seven-day time point in Test YP2. Data tables for those two tests are shown in Appendix C.

Table 5-3 shows persistence results (Test YP3) for *Y. pestis* on the four coupon materials at 65 % RH at several time points up to 144 hours (six days) after inoculation. The quantitative test results indicated that *Y. pestis* was recovered on all four materials at the one-hour time point. Recovery at that time point was greatest on topsoil, with approximately 36 % recovered, while 15 % of the cells were recovered on galvanized metal. However, at the 24-hour time point, no *Y. pestis* was recovered on glass, galvanized metal, or painted wallboard paper. In contrast, *Y. pestis* persisted on topsoil at all time points including the 144 hour time point. The qualitative results from Test YP3 were consistent with the quantitative results, with one exception: the qualitative growth testing did not confirm the presence of *Y. pestis* on the painted wallboard paper at the one-hour time point. One possible explanation is the inhibition of growth by some compound extracted from the wallboard paper. However, this hypothesis was not explored in this study.

In Table 5-4, the persistence results (Test YP4) are presented for *Y. pestis* on wetted topsoil at one hour, seven days and 14 days after inoculation. The quantitative test results in Table 5-4 show the agent persists at seven days after inoculation. However, at 14 days after inoculation, the *Y. pestis* was completely inactivated. These quantitative results were confirmed by the corresponding qualitative growth tests. The results of Tests YP1 through YP4 indicate that *Y. pestis* can persist for at least one hour, but less than 24 hours, on glass, galvanized metal, and painted wallboard paper under normal temperature and RH conditions but can persist for several days in topsoil. *Y. pestis* persists for up to six days in topsoil of normal moisture content but persists for between one and two weeks on wetted topsoil that remains wet due to high RH.

Table 5-3. Persistence of *Yersinia pestis*[(a)] (Four Materials, 144 Hours, 65 % RH; Test YP3)

		Quantitative Testing			Qualitative Growth[(b)]	
Test Material	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
Topsoil						
Test Coupons 1 Hour	2.70×10^7	6.98 ± 0.07	35.7 ± 6.4	0.45 ± 0.07	N	**Y**
Test Coupons 24 Hour	2.70×10^7	3.00 ± 0.75	0.0084 ± 0.01	4.43 ± 0.66	N	**Y**
Test Coupons 48 Hour	2.70×10^7	1.72 ± 1.04	0.00059 ± 0.00063	5.71 ± 0.91	N	**Y**
Test Coupons 96 Hour	2.70×10^7	1.40 ± 0.87	0.00027 ± 0.00040	6.03 ± 0.77	N	**Y**
Test Coupons 144 Hour	2.70×10^7	1.07 ± 1.02	0.00020 ± 0.00031	6.36 ± 0.90	N	**Y**
Laboratory Blank[(c)]	0	0	0	-		
Procedural Blank[(d)]	0	0	0	-		
Glass						
Test Coupons 1 Hour	2.70×10^7	4.55 ± 0.09	0.13 ± 0.029	2.88 ± 0.08	N	**Y**
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
Galvanized Metal						
Test Coupons 1 Hour	2.70×10^7	6.34 ± 0.69	15.0 ± 14.1	1.09 ± 0.61	N	**Y**
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
Painted Wallboard Paper						
Test Coupons 1 Hour	2.70×10^7	3.46 ± 0.61	0.028 ± 0.047	3.97 ± 0.69	N	N
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *Y. pestis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Laboratory Blank = Not inoculated.

(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable

.

Table 5-4. Persistence of *Yersinia pestis*[(a)] (Wetted Topsoil, 14 Days, 96 % RH; Test YP4)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Test Coupons	5.90×10^7	7.78 ± 0.25	115 ± 51	-0.01 ± 0.22	Y	**Y**
Laboratory Blank[(c)]	0	0	0	--		
Procedural Blank[(d)]	0	0	0	--		
7 Days						
Test Coupons	5.90×10^7	4.44 ± 0.65	0.12 ± 0.20	3.33 ± 0.64	N	**Y**
Laboratory Blank	0	0	0	--		
Procedural Blank	0	0	0	--		
14 Days						
Test Coupons	5.90×10^7	0	0	7.78	N	N
Laboratory Blank	0	0	0	--		
Procedural Blank	0	0	0	--		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth visually confirmed to be *Y. pestis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).
(c) Laboratory Blank = Not inoculated.
(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

5.4 Persistence of *Yersinia pestis* under Exposure to Simulated Sunlight

A single test was conducted to assess the effect of exposure of *Y. pestis* to UV-A/B radiation. That test (TestYPUV1) evaluated the recovery of *Y. pestis* on four coupon materials over 24 hours with a low UV-A/UV-B ratio. In this test, the persistence of *Y. pestis* on the four coupon materials was determined after the one hour drying time and after 24 hours of UV exposure of the coupons. The UV radiation intensity consisted of nominally 100 $\mu W/cm^2$ UV-A and 44 $\mu W/cm^2$ UV-B. This test was performed at ambient laboratory temperature and RH (approximately 40 % RH). As noted in Section 2.3, no non-exposed coupons were used in this UV test.

5.4.1 Uniformity of Test Conditions

Tables 5-5 and 5-6 summarize the test conditions of UV intensity, temperature, and RH monitored during the UV exposure test with *Y. pestis*. Table 5-5 shows the average (± SD) of intensities (in $\mu W/cm^2$) of UV-B, UV-A, and UVA/B measured at five positions in the test

coupon array in the UV-A/B exposure test with *Y. pestis*. The intensity of UV-C was below detection (i.e., < 1 μW/cm^2). The data in Table 5-5 show that the consistency of the average UV-B, UV-A, and UV-A/B intensities was maintained in this test with *Y. pestis*, though the radiation intensity was not as uniform as in the UV testing with *B. anthracis* (see Section 4.3).

Table 5-6 shows the average (± SD) of the temperature and RH monitored near the center of the exposed coupon array in the UV exposure test with *Y. pestis*. Table 5-6 shows data both with the UV lights on and the lights off, and shows consistency in the test conditions, i.e., temperature and RH values when the UV lamps were on were closely similar to the values when the UV lamps were off.

Table 5-5. Ultraviolet Radiation Intensity at the Exposed Coupons in Test YPUV1 with *Yersinia pestis*[(a)]

UV Condition[(b)]	UV Intensity
UV-A Average ± SD	88.2 ± 10.7
UV-B Average ± SD	39.7 ± 3.5
UV-A/B Average ± SD	127.9 ± 13.2

(a) All entries are in μW/cm^2 at the test coupons; all UV intensities were zero at the non-exposed coupons.
(b) Average ± SD refers to average over all five measurement positions.
SD = standard deviation. UV-A determined by subtraction of UV-B reading from UV-A/B reading

Table 5-6. Summary of Temperature and Relative Humidity Conditions at the Exposed Coupons in Test YPUV1 with *Yersinia pestis*

Test Condition[(a)]	Result
UV ON	
T Average ± SD (°C)	22.3 ± 0.5
RH Average ± SD (%)	41.4 ± 1.1
UV OFF	
T Average ± SD (°C)	20.1 ± 0.6
RH Average ± SD (%)	42.4 ± 1.4

UV ON and UV OFF refer to 12-hour periods of alternating illumination and darkness in test chamber; averages shown are over all UV ON or UV OFF periods in the indicated UV exposure period.
T = temperature, RH = relative humidity,
SD = standard deviation.

5.4.2 Persistence Results in Simulated Sunlight Test with Yersinia pestis

Table 5-7 shows results from Test YPUV1 in terms of the cell inoculum; the mean log of the observed cells on the exposed and blank coupons; the cell recovery; and the resulting mean log reduction (±CI) on the exposed coupons relative to the initial inoculum due to the UV-A/B

exposure. In this test, coupons were extracted at time points of one hour after the initial drying period and after 24 hours (12 on, 12 off) of UV exposure. Extracted coupons were not subjected to qualitative growth testing after extraction.

Table 5-7 shows that *Y. pestis* did not persist on galvanized metal at the one-hour time point. *Y. pestis* did persist to that point on the other three materials, with the greatest recovery on glass and the lowest quantity recovered on painted wallboard paper. Under simulated sunlight exposure, *Y. pestis* did not persist at the 24-hour time point on any of the four materials. This result differs from the observed persistence of *Y. pestis* on topsoil out to at least six days in the absence of UV exposure (Table 5-3).

Table 5-7. UV-A/B Inactivation of *Yersinia pestis* on Four Coupon Materials[(a)] (24-Hour Exposure at Low UV-A/UV-B Ratio; Test YPUV1)

		Quantitative Testing		
Test Material	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**
Topsoil				
Exposed Coupons 1 Hour	2.05×10^8	6.93 ± 0.26	4.7 ± 1.9	1.38 ± 0.23
Exposed Coupons 24 Hour	2.05×10^8	0	0	8.31
Laboratory Blank[(b)]	0	0	0	-
Procedural Blank[(c)]	0	0	0	-
Glass				
Exposed Coupons 1 Hour	2.05×10^8	8.15 ± 0.02	68.9 ± 3.7	0.16 ± 0.02
Exposed Coupons 24 Hour	2.05×10^8	0	0	8.31
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Galvanized Metal				
Exposed Coupons 1 Hour	2.05×10^8	0	0	8.31
Exposed Coupons 24 Hour	2.05×10^8	0	0	8.31
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Painted Wallboard Paper				
Exposed Coupons 1 Hour	2.05×10^8	4.43 ± 0.38	0.018 ± 0.016	3.88 ± 0.33
Exposed Coupons 24 Hour	2.05×10^8	0	0	8.31
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Laboratory Blank = Not inoculated.
(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

6.0 SUMMARY

This project evaluated the persistence of vegetative *B. anthracis* (Ames) on several materials under a range of temperature and RH conditions. Persistence testing was conducted both under normal laboratory lighting conditions and under exposure to controlled levels of UV-A/B radiation simulating sunlight. Similar persistence tests were also conducted separately for *Y. pestis*.

The intent of this project was to test with purely vegetative cells of *B. anthracis* due to the possibility of using germinants to convert spores to vegetative cells, thereby facilitating inactivation of the cells through natural attenuation or decontamination. However, initial tests showed that *B. anthracis* cell cultures contained predominantly vegetative cells but also a few spores. In one initial test in which the inoculum contained *B. anthracis* in both vegetative cell and spore form, both cells and spores were recovered from the test materials at 56 days.

Consequently, an improved procedure was developed to reliably produce a stock cell culture consisting of purely vegetative *B. anthracis* at a relatively high titer for inoculation onto coupons (see Appendix A). To ensure purely vegetative cells, heat shock was applied to aliquots of all stock cell cultures and all coupon extracts in testing with *B. anthracis*, to clearly distinguish *B. anthracis* vegetative cells from spores (heat shock inactivates all cells, but does not affect spores). The use of these procedures confirmed that in all subsequent tests, only vegetative *B. anthracis* cells were inoculated onto test coupons, and allowed clear observation of the occurrence of *B. anthracis* sporulation under specific test conditions.

Coupon extracts were subjected to quantitative enumeration of viable cells and to qualitative growth testing, which provided a more sensitive indication of complete inactivation than did the quantitative testing. There were several instances when no CFU were detected via quantitative tests, but bacterial growth did occur in the qualitative tests, thus demonstrating the utility of the latter test.

The persistence results for *B. anthracis* (purely vegetative cells, no spores) and *Y. pestis*, both with and without UV exposure, are summarized separately below. The persistence of the vegetative *B. anthracis* cells produced and used in this study may differ from the persistence of cells newly germinated from *B. anthracis* spores by nutrient or non-nutrient germinants in the environment. Investigation of such potential differences may be a valuable topic for further research.

6.1 Results for Vegetative *Bacillus anthracis* (Ames)

6.1.1 Vegetative Bacillus anthracis Persistence without UV Exposure

The persistence of vegetative *B. anthracis* was tested in the absence of UV radiation on glass, bare pine wood, unpainted concrete, and topsoil at normal room temperature (approximately 22 °C) and over a range of RH from 34 to over 90 %. The quantitative test results were confirmed by the qualitative growth testing in all cases. The results of testing were as follows.

- Vegetative *B. anthracis* persisted for less than one hour after inoculation on bare pine wood under all test conditions.
- Vegetative *B. anthracis* also persisted for less than one hour after inoculation on glass and unpainted concrete when the RH was approximately 50 % or lower.
- Vegetative *B. anthracis* persistence on glass and unpainted concrete increased when the RH exceeded approximately 50 %. At 92 % RH, on unpainted concrete, vegetative *B. anthracis* persisted for one hour after inoculation; on glass, vegetative *B. anthracis* persisted for at least 12 hours after inoculation.
- Vegetative *B. anthracis* persisted in topsoil for at least 96 hours at 46 % RH. However, during the simulated sunlight test with low UV-A exposure, CFU were recovered from the control soil samples (samples not exposed to the UV light) at the 120 hour time point.

In every persistence test summarized above, the heat shock treatment of coupon extracts confirmed that all *B. anthracis* cells were vegetative throughout the entire test duration. That is, no CFU were found in any coupon extract subjected to the heat shock procedure. However, a different result was found in two tests in which the topsoil was wetted before inoculation, and the RH was maintained above 90 % to prevent drying of the soil. Results from those tests are summarized below.

- In wet topsoil, the initially 100 % vegetative *B. anthracis* population grew by approximately a factor of 10 and showed extensive sporulation within one week after inoculation of the topsoil coupons.
- In the second test, we found that the onset of sporulation occurred within 24 hours after inoculation of the wetted topsoil, and over 10 % of the total *B. anthracis* population was in spore form at 48 hours after inoculation.

6.1.2 Vegetative Bacillus anthracis *Persistence with UV Exposure*

The persistence of vegetative *B. anthracis* on topsoil under UV-A/B exposure simulating sunlight was determined at six time points ranging from one hour to 120 hours after inoculation onto coupons. Two such tests were conducted. One test used levels of UV-A and UV-B representative of a mid-summer day in Raleigh, North Carolina. The other test used the same UV-B level but only a minimal level of UV-A. In these tests, all *B. anthracis* cells were 100 % vegetative throughout the test duration. The quantitative test results were confirmed by the qualitative growth testing in all cases; there were a few test results in which CFU were not recovered quantitatively from soil coupons, but qualitatively showed growth after seven days of incubation. The results of those tests were as follows.

- From the qualitative results for the UV exposure tests, the longest elapsed time tested in which *B. anthracis* cells were recovered from soil was 96 hours, for both the high and low UV-A test conditions.

- The recovery of vegetative *B. anthracis* was lower on topsoil coupons exposed to simulated sunlight than on coupons shielded from that exposure, but the differences in recovery were generally not significant.
- The results for the CFU recovery from soil when exposed to the high UV-A level are not significantly different from the CFU recovery from soil when exposed to the low UV-A level. This finding provides some confirmation of the limited role that UV-A may have in the inactivation of *B. anthracis* cells when exposed to sunlight.

6.2 Results for *Yersinia pestis*

Recovery of *Y. pestis* cells from bare pine wood and unpainted concrete was found to be unacceptably low in the initial recovery testing conducted before the start of persistence testing. Further recovery testing showed acceptable recoveries from galvanized metal and painted wallboard paper, so those materials were used in testing with *Y. pestis*.

6.2.1 Yersinia pestis *Persistence without UV Exposure*

The persistence of *Y. pestis* was tested in the absence of UV-A/B radiation on glass, painted wallboard paper, galvanized metal, and topsoil, at normal room temperature (approximately 22 °C) and over a range of RH from 54 to over 90 %. The results of that testing were as follows.

- *Y. pestis* persisted beyond one hour after inoculation on all four test materials.
- *Y. pestis* was not recovered within 24 hours after inoculation onto glass, painted wallboard paper, and galvanized metal.
- *Y. pestis* persisted to at least six days after inoculation on topsoil.
- Persistence of *Y. pestis* was higher on wetted topsoil and RH maintained above 90 % to prevent drying of the soil. In that test, viable *Y. pestis* was present at seven days after inoculation. Complete inactivation (no viable cells) was observed at 14 days after inoculation.

6.2.2 Yersinia pestis *Persistence with UV Exposure*

One test was conducted to assess persistence of *Y. pestis* on all four test materials under UV-A/B exposure. This test used a level of UV-B representative of a mid-summer day in Raleigh, North Carolina, and a minimal level of UV-A. The results of this test were as follows.

- Under simulated sunlight exposure *Y. pestis* did not persist on galvanized metal at the one-hour time point, but did persist at the one-hour time point on the other three materials.
- Under simulated sunlight exposure, *Y. pestis* did not persist at the 24-hour time point on any of the four materials.

Impact of Study

This work provides information on the persistence of vegetative *B. anthracis* on surfaces and soil, provided that the organism is completely germinated and sporulation is prevented. Such results may be useful in the development of wide area remediation plans that consider the possibility of germination and natural attenuation. To date, research has not shown such required effective germination of spores on surfaces.[1] For soil materials, natural attenuation may also be a viable decontamination option provided that longer attenuation times (e.g., approximately a week) are acceptable, and the soil can be kept relatively dry. Additional research is recommended to confirm that the persistence of *B. anthracis* cells produced through the germination of spores on materials is of similar duration to that of cells harvested in the laboratory during the exponential growth phase and inoculated onto materials.

Similar to *B. anthracis*, the results of the study on the persistence of *Y. pestis* show that natural attenuation may also be a viable option for the decontamination of non-soil materials. For soils, natural attenuation may be a viable decontamination option provided that longer attenuation times (e.g., approximately a week) are acceptable, and that soils can be kept relatively dry.

7.0 REFERENCES

1. Kane, S., Wollard, J., Bourguet, F., Murphy, G., Alfaro, T., Scher, H. Final Report: Germination-Lysis Approach for Outdoor Surface Decontamination of *Bacillus anthracis* Spores. Lawrence Livermore National Laboratory Report LLNL-TR-625913. March 29, 2103.

2. Persistence Testing and Evaluation of Fumigation Technologies for Decontamination of Building Materials Contaminated with Biological Agents, EPA/600/R-10/086, U.S. EPA Office of Research and Development, National Homeland Security Research Center, August 2010.

3. Rogers, J.V., Sabourin, C.L.K., Choi, Y.W., Richter, W.R., Rudnicki, D.C., Riggs, K.B., Taylor, M.L., and Chang, J., Decontamination assessment of *Bacillus anthracis, Bacillus subtilis,* and *Geobacillus stearothermophilus* spores on indoor surfaces using a hydrogen peroxide gas generator, *J. Applied Microbiol.* 99(4), 739-748, 2005.

4. Sinha, R.P. and Häder, D.-P., UV-Induced DNA damage and repair: A review, *Photochem. Photobiol. Sci.*, 1, 225-236, 2002.

5. Mecherikunnel, A.T., Gatlin, J.A., and Richmond, J.C., Data on total and spectral solar irradiance, *Applied Optics*, 22, 1354-1359, 1983.

6. Acra, A., Jurdi, M., Muallem, H., Karahagopian, Y., and Raffoul, Z. *Water Disinfection by Solar Radiation: Assessment and Application*, published by International Development Research Center Canada, Ottawa, Canada, 1990.

7. Diffey, B.L., Sources and measurement of ultraviolet radiation, *Methods*, 28, 4-13, 2002.

8. Tavakoli, M.B., and Shahi, Z., Solar ultraviolet radiation on the ground level of Isfahan, *Iran. J. Radiat. Res*., 5, 101-104, 2007.

9. Jeanmougin, M. and Civatte, J., Dosimetry of solar ultraviolet radiation: Daily and monthly changes in Paris (in French), *Ann. Dermatol. Venereol.*, 114, 671-676, 1987.

10. Kolari, P.J., Lauharanta, J., and Hoikkala, M., Midsummer solar UV radiation in Finland compared with the UV radiation from phototherapeutic devices measured by different techniques, *Photodermatol.*, 3, 340-345, 1986.

11. Balasaraswathy, P., Kumar, U., Srinivas, C.R., and Nair, S., UVA and UVB in Sunlight, optimal utilization of UV rays in sunlight for phototherapy, *Indian J. Dermatol. Venereol. Leprol*., 68, 198-201, 2002.

12. Frederick, J.E., Slusser, J.R., and Bigelow, D.S., Annual and interannual behavior of solar ultraviolet irradiance revealed by broadband measurements, *Photochem. and Photobiol.*, 72, 488-496, 2000.

13. Calfee, M.W. and Wendling, M., Inactivation of vegetative bacterial threat agents on environmental surfaces, *Science of the Total Environment*, 443, 387-396, 2013.

APPENDIX A:

Description of Issues and Test Results Leading to Development of Procedure for Preparing Purely Vegetative *Bacillus anthracis*

Introduction

In cases where the intent is to test with vegetative cells (as in this project), there is the potential for vegetative cells to sporulate on any surface, including soil, if nutrients are not available. Thus germination and sporulation have the potential to confound testing with *B. anthracis* if not prevented or at least accounted for. These concerns were obviously of great importance for this project, in which the intent was to test with 100 % vegetative *B. anthracis* cells.

Initially in this project, only microscopy was used to check for the presence of spores in the inoculum. To ensure that the inoculum consisted of purely vegetative cells, revised procedures were implemented. The primary revision made to the test procedures was to apply a heat shock procedure to aliquots of the inoculum and to aliquots of the extracts of all test coupons to distinguish *B. anthracis* spores (which survive the heat shock) from vegetative cells (which do not survive). That heat shock procedure was well established before the current project (see, for example, J.V. Rogers et al., *Journal of Applied Microbiology*, **99**, 739-748, 2005) and consists of subjecting the sample extract to a temperature of 65 °C in a water bath for 60 minutes. The heat shock procedure was first applied in this project in a test of *B. anthracis* persistence on four test materials, and the results clearly indicated that the intent of testing with purely vegetative *B. anthracis* cells was not being achieved. That finding motivated efforts to improve the production method for vegetative *B. anthracis*, and those efforts in turn led to successful development of a method for producing a 100 % vegetative cell culture. That method was then used in all subsequent testing in this project, as described in the body of this report.

The following sections of this Appendix provide more detail on this subject, by presenting 1) the data from the persistence test that disclosed the lack of a purely vegetative *B. anthracis* population, and 2) the development of the successful production method for 100 % vegetative *B. anthracis* that was used in all subsequent testing. All test results for *B. anthracis* in the main body of this report were obtained using a 100 % vegetative *B. anthracis* culture to inoculate test coupons, verified by heat shock, and that every coupon extract in those tests was subjected to the heat shock treatment.

Test Results Indicating Lack of Purely Vegetative *B. anthracis* Cells

In early 2013, a test was conducted to assess the persistence (without any UV exposure) of vegetative *B. anthracis* on topsoil, glass, bare pine wood, and unpainted concrete at normal room conditions (i.e., temperature approximately 20 °C and relative humidity approximately 50 %). The test was of 56 days duration and was the first in which the heat shock procedure was applied. Coupons of each type were extracted at one hour after inoculation (i.e., immediately after the inoculation culture had dried) and at 56 days after inoculation. The extract of each control and test coupon was split in half, and one half was subjected to the heat shock procedure before both aliquots were diluted, plated, and enumerated as described in Section 2.5 of this report.

The results from the 56-day test are summarized in Table A-1, which shows for each coupon material the total number of *B. anthracis* CFU recovered at the 1-hour and 56-day time points, the number of *B. anthracis* spores (determined by the heat shock treatment), and the percentage of *B. anthracis* present in spore form.

Table A-1. Heat Shock Data from 56-Day *B. anthracis* Persistence Test

Time Point	Parameter	Topsoil	Glass	Wood	Concrete
1 Hour	Total Number of CFU	5.62×10^6	2.40×10^4	3.89×10^4	3.16×10^4
	Total Number of Spores	1.74×10^4	1.35×10^4	1.41×10^4	1.35×10^4
	Percent Spores	0.3	56.2	36.3	42.7
56 Days	Total Number of CFU	7.59×10^4	2.75×10^4	1.15×10^4	5.89×10^3
	Total Number of Spores	3.31×10^4	9.55×10^3	7.41×10^3	3.63×10^3
	Percent Spores	43.7	34.7	64.6	61.7

Table A-1 shows that at the one-hour time point, the *B. anthracis* population in the topsoil coupons was only 0.3 % in spore form, or almost entirely (99.7 %) vegetative. However, on the other three materials, the percentage of spores ranged from about 36 to 56 % at that time point, indicating that overall roughly half the *B. anthracis* on those materials was in spore form. Similarly at the 56-day time point, a large portion of the total *B. anthracis* on all four materials was in spore form, including on the topsoil coupons (approximately 44 % spores). These data clearly showed the need for improved procedures to assure that testing was conducted with vegetative *B. anthracis* cells, leading to the development efforts described in the next section.

Development of Production Procedure for 100 % Vegetative *B. anthracis* (Ames)

The development effort to assure production of vegetative *B. anthracis* (Ames) with no spores or endospores present focused on achieving a sufficiently high cell titer for use in testing and a purely vegetative cell population. The approach taken was to monitor the growth and nature of *B. anthracis* cells closely throughout the 16-hour cell culturing process used previously, to identify a stopping point that met these goals. Specifically, a growth curve investigation was conducted as follows:

- A 1:100 dilution was prepared in SFW of standard *B. anthracis* (Ames) spore stock, which is approximately 1×10^9 CFU/mL.
- Ten µL of the resulting 1:100 dilution (which is approximately 1×10^7 CFU/mL) was added to 200 mL of TSB.
- The resulting culture (which is initially approximately 5×10^2 CFU/mL) was allowed to incubate at 37 °C while shaking on an orbital shaker at 200 rpm for up to 16 hours.
- During heating and shaking of the culture, an aliquot was removed at one-hour intervals for performance of three separate measurements:
    - determination of optical density at 600 nm wavelength (OD_{600}) as an indication of the density of the culture.
    - microscopic examination to assess the nature of cells in the culture. The focus of this investigation was to determine at what point in the culturing process the onset of endospore formation was first observed.
    - determination of the concentration of *B. anthracis* cells in the culture by dilution, plating, and enumeration.

The results of the growth curve investigation are shown in Figure A-1, which displays the cell enumeration results (left vertical axis) and OD_{600} results (right axis) over 15 hours of the cell culturing process. Figure A-1 shows that at approximately 10 hours into the culturing process, the log linear (exponential) growth in the log of *B. anthracis* was near its end, having reached a concentration of approximately 2×10^7 cells/mL, and the OD_{600} value was approximately 2. The microscopic inspection of the cell culture also showed that at this 10 hour time point, there was no indication of spore or endospore formation. This observation is illustrated in Figures A-2 through A-6, which show photomicrographs (at 100× magnification) of cells in the culture at 8, 10, 12, 14, and 15 hours into the culturing process. These figures show that at ten hours, only long chains of vegetative cells are observed (Figure A-3), at 12 hours those chains are starting to break up (Figure A-4), at 14 hours endospore formation is beginning (Figure A-5), and at 15 hours (Figure A-6) endospores are becoming refractile (and consequently resistant to heat shock). Based on these observations, for all testing, the incubation of vegetative *B. anthracis* was stopped when the OD_{600} reading reached a value of 2, which occurred reliably after 9 or 10 hours of incubation. As noted in the body of this report, heat shock testing of the cell cultures was conducted in all tests and confirmed that the identified procedure produced 100 % vegetative *B. anthracis* cells.

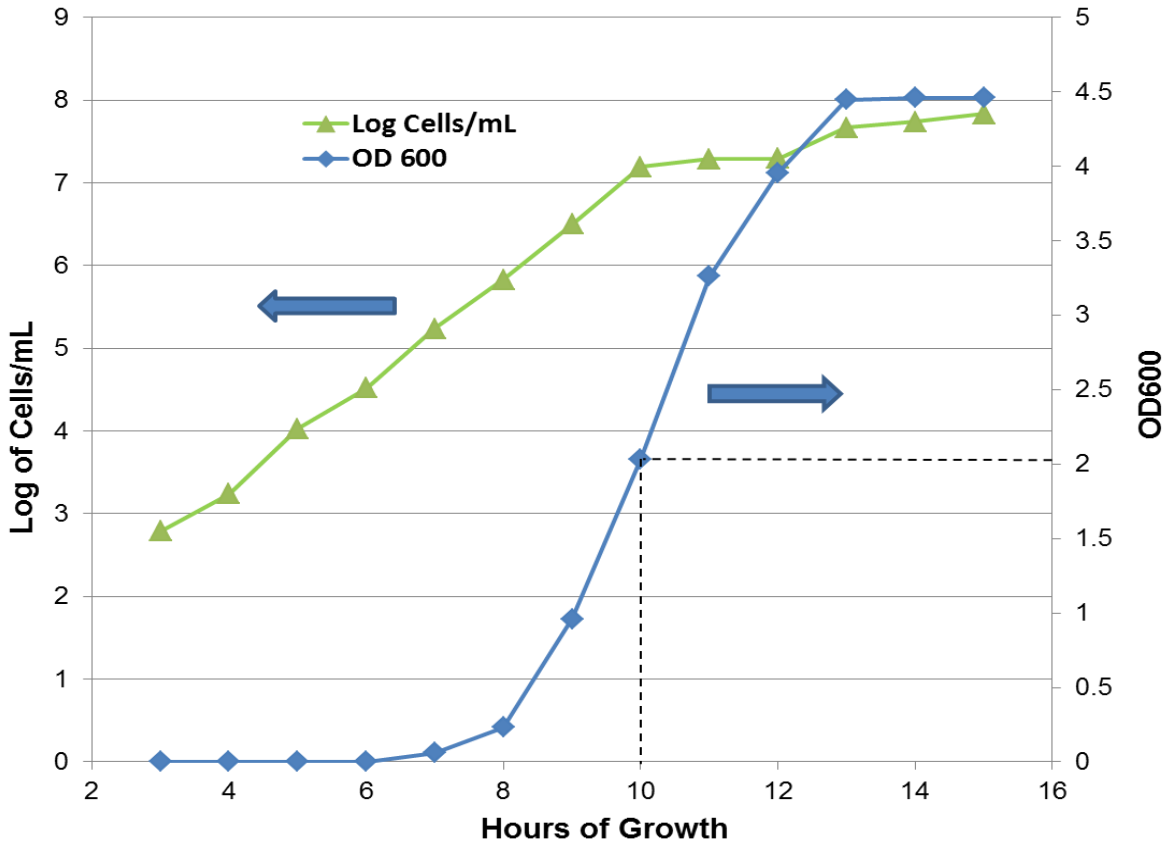


Figure A-1. Results of Growth Curve Investigation of Culturing of Vegetative *B. anthracis*

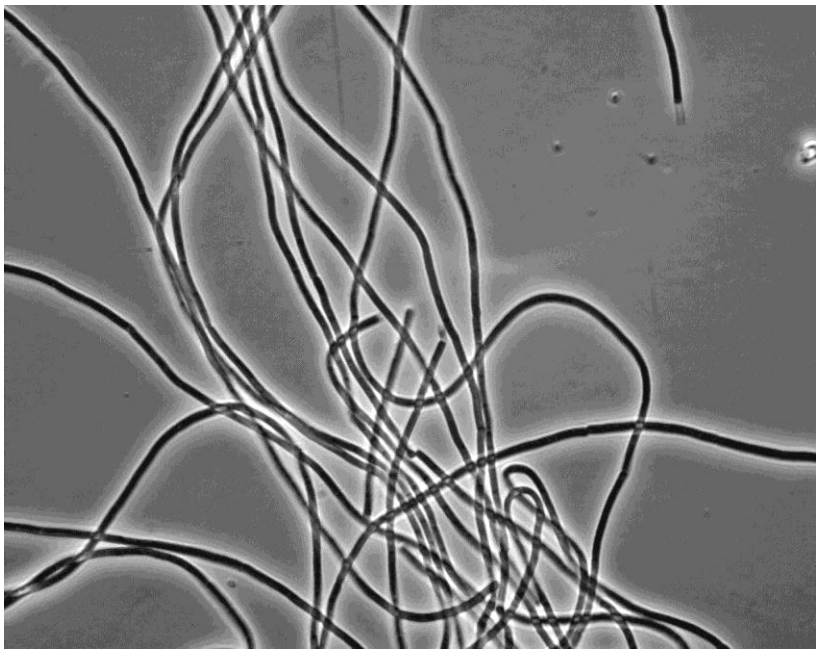


Figure A-2. Photomicrograph of *B. anthracis* Cell Culture at Eight Hours of Incubation, Showing Tangling of Long Vegetative Cell Chains (magnification 100×)

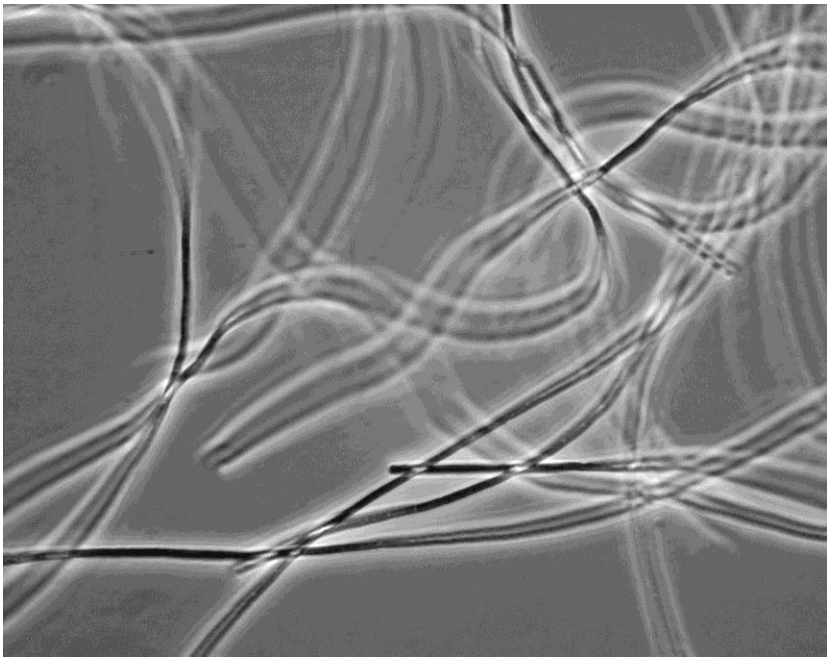


Figure A-3. Photomicrograph of *B. anthracis* Cell Culture at Ten Hours of Incubation; Cells Shortening within Long Vegetative Chains, No Sign of Endospores (magnification 100×)

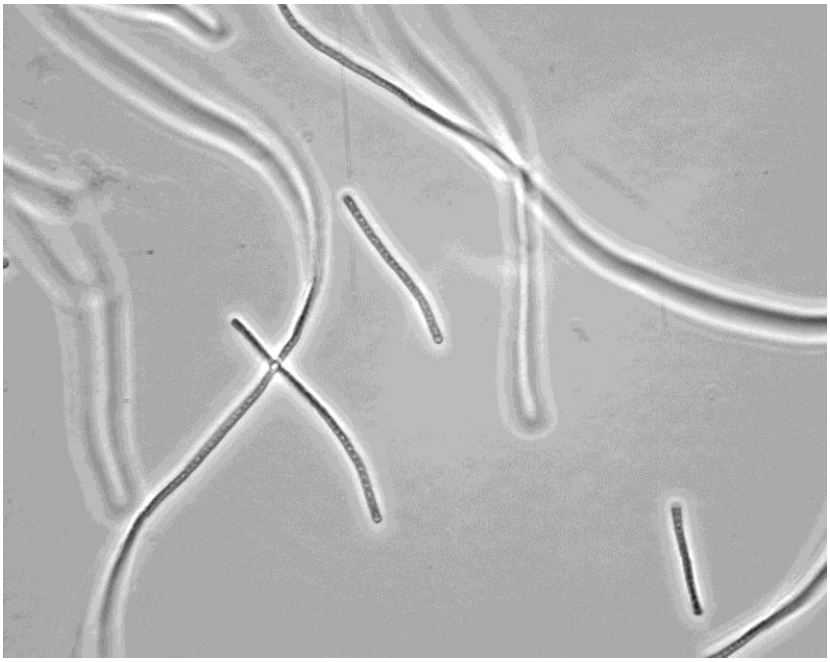


Figure A-4. Photomicrograph of *B. anthracis* Cell Culture at 12 Hours of Incubation; Long Vegetative Chains Starting to Break Apart, No Sign of Endospores (magnification 100×)

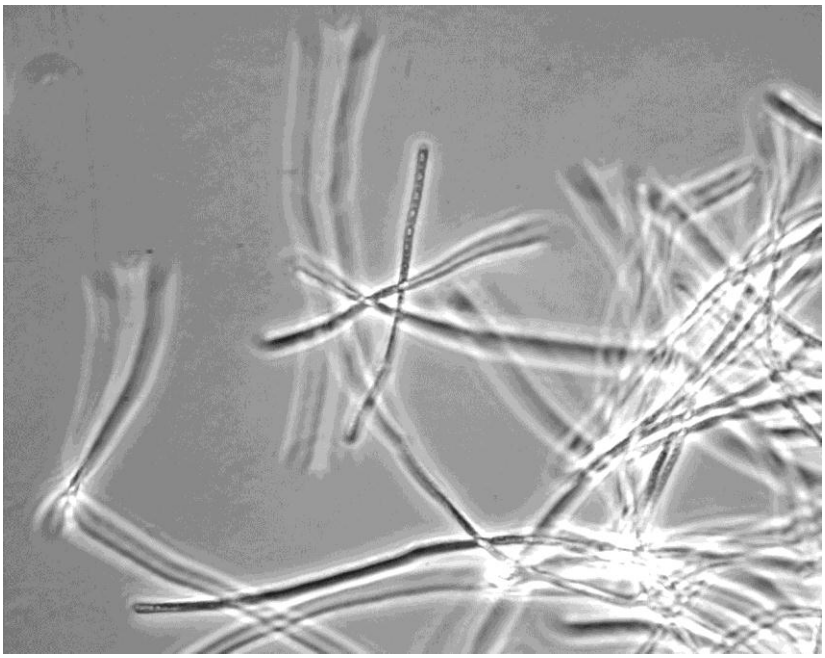


Figure A-5. Photomicrograph of *B. anthracis* Cell Culture at 14 Hours of Incubation; Long Vegetative Chains Continuing to Break Apart, Endospores Forming (magnification 100×)

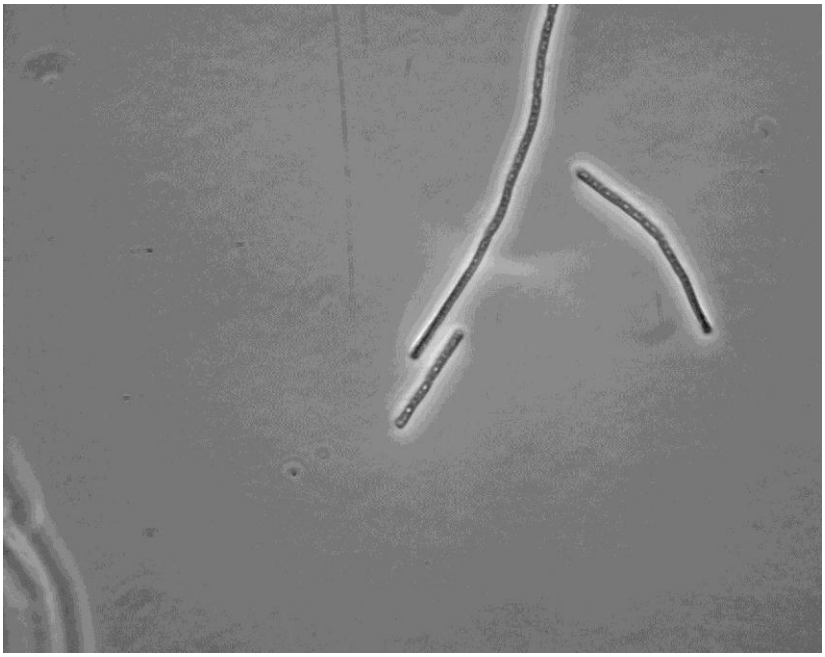


Figure A-6. Photomicrograph of *B. anthracis* Cell Culture at 15 Hours of Incubation; Endospores Forming and Becoming Refractile (magnification 100×)

APPENDIX B:

Data Tables from Testing of Vegetative *Bacillus anthracis* Persistence under UV-A/B Exposure

Tables B-1 and B-2 show the results from testing the persistence of vegetative *B. anthracis* (Ames) on topsoil over 120 hours in Test BAUV1 and BAUV2, under UV-A/B exposures involving low and high UV-A/UV-B ratios, respectively. In both tests, topsoil coupons were extracted at time points of 1, 24, 48, 72, 96, and 120 hours, and extracted coupons were subjected to qualitative growth testing and assessed for growth at one and seven days after extraction. These tables show the cell inoculum; the mean log of the observed cells on the non-exposed, exposed, and blank coupons; the cell recovery; the resulting mean log reduction (±CI) on the non-exposed and test coupons relative to the initial inoculum; and the results of the qualitative growth testing at each time point. The test results shown in Tables B-1 and B-2 are the basis for Figures 4-4 and 4-5, respectively, in the body of this report.

Comparison of Tables B-1 and B-2 shows that the persistence under exposure to the high UV-A/UV-B ratio (Table B-2) was similar to that at the same time point under exposure to the low UV-A/UV-B ratio (Table B-1). This finding is illustrated in Figure B-1, which compares the persistence on UV-exposed topsoil coupons in the two 120-hour UV tests. At the one hour time point, recovery of cells at the high UV-A/UV-B condition was significantly greater than with the low UV-A/UV-B ratio; at 24 hours and at all later time points, the results from the two tests were not significantly different.

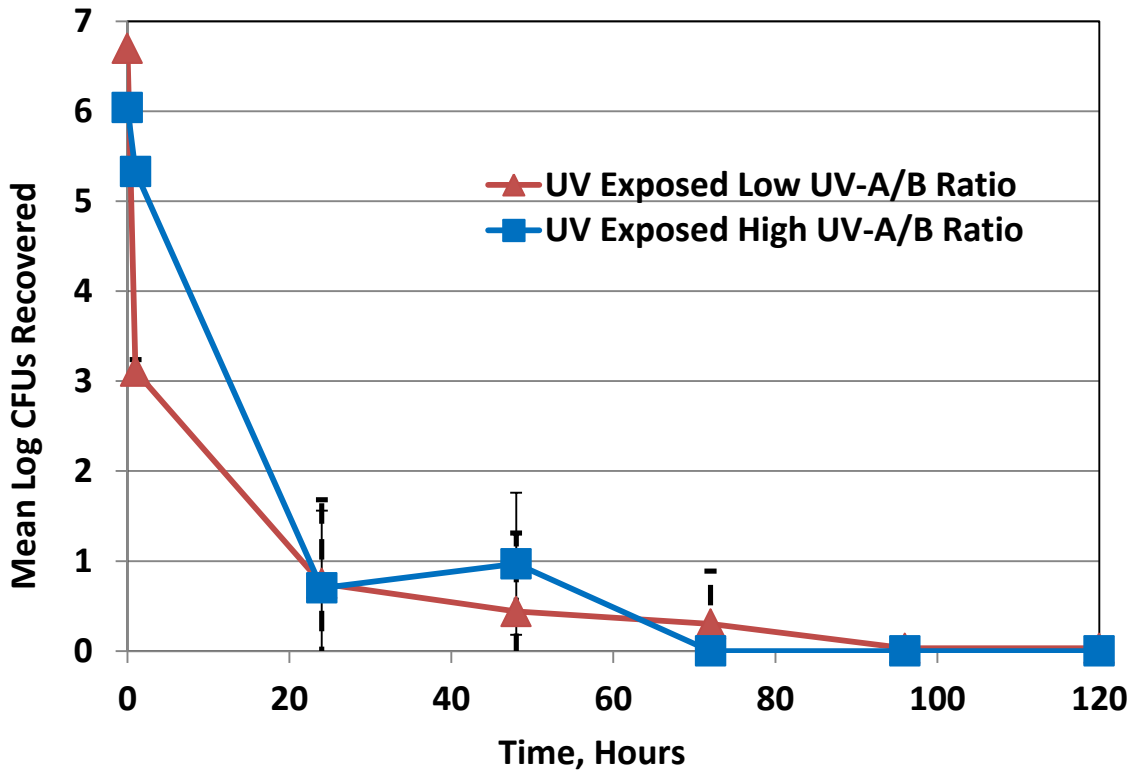


Figure B-1. A High UV-A/UV-B Ratio does not Reduce Persistence of Vegetative *B. anthracis* Relative to a Low UV-A/UV-B Ratio with the same UV-B level

Table B-1. UV-A/B Inactivation of Vegetative *Bacillus anthracis* on Topsoil[a] (120-Hour Exposure at Low UV-A/UV-B Ratio; Test BAUV1)

		Quantitative Testing			Qualitative Growth[b]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Nonexposed Coupons[c]	5.03×10^6	4.44 ± 0.42	0.70 ± 0.35	2.26 ± 0.37	Y	**Y**
Test Coupons[d]	5.03×10^6	3.11 ± 0.15	0.027 ± 0.009	3.59 ± 0.13	Y	**Y**
Laboratory Blank[e]	0	0	0	-		
Procedural Blank[f]	0	0	0	-		
24 Hours						
Nonexposed Coupons	5.03×10^6	3.69 ± 0.88	0.36 ± 0.48	3.01 ± 0.77	Y	**Y**
Test Coupons	5.03×10^6	0.75 ± 1.06	0.0008 ± 0.0014	5.95 ± 0.92	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
48 hours						
Nonexposed Coupons	5.03×10^6	1.84 ± 1.31	0.021 ± 0.045	4.87 ± 1.15	Y	**Y**
Test Coupons	5.03×10^6	0.44 ± 0.99	0.0007 ± 0.0015	6.26 ± 0.87	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
72 hours						
Nonexposed Coupons	5.03×10^6	1.46 ± 0.89	0.0016 ± 0.0021	5.24 ± 0.78	Y	**Y**
Test Coupons	5.03×10^6	0.30 ± 0.68	0.0002 ± 0.0003	6.40 ± 0.60	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
96 hours						
Nonexposed Coupons	5.03×10^6	0.82 ± 1.13	0.0009 ± 0.0013	5.88 ± 0.99	Y	**Y**
Test Coupons	5.03×10^6	0	0	6.70	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
120 Hours						
Nonexposed Coupons	5.03×10^6	0.40 ± 0.89	0.0004 ± 0.0009	6.30 ± 0.78	Y	**Y**
Test Coupons	5.03×10^6	0	0	6.70	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Inoculated, not exposed to UV-A/B (recovery conducted after conclusion of UV-A/B exposure).

(d) Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.

(e) Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.

(f) Procedural Blank = Not inoculated, placed with test coupons and exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

Table B-2. UV-A/B Inactivation of Vegetative *Bacillus anthracis* on Topsoil[(a)] (120-Hour Exposure at High UV-A/UV-B Ratio; Test BAUV2)

		Quantitative Testing			Qualitative Growth[(b)]	
Time Point	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Mean Log Reduction ± CI	One day	Seven days
1 Hour						
Nonexposed Coupons[(c)]	1.09×10^6	5.58 ± 0.14	36.3 ± 12.3	0.46 ± 0.12	Y	**Y**
Test Coupons[(d)]	1.09×10^6	5.33 ± 0.006	19.8 ± 0.3	0.70 ± 0.01	Y	**Y**
Laboratory Blank[(e)]	0	0	0	-		
Procedural Blank[(f)]	0	0	0	-		
24 Hours						
Nonexposed Coupons	1.09×10^6	1.13 ± 1.03	0.0043 ± 0.0041	4.91 ± 0.91	N	**Y**
Test Coupons	1.09×10^6	0.70 ± 0.98	0.0025 ± 0.0039	5.33 ± 0.86	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
48 hours						
Nonexposed Coupons	1.09×10^6	1.13 ± 1.03	0.0043 ± 0.0041	4.91 ± 0.91	Y	**Y**
Test Coupons	1.09×10^6	0.97 ± 0.90	0.0025 ± 0.0025	5.06 ± 0.79	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
72 hours						
Nonexposed Coupons	1.09×10^6	0	0	6.04	N	**Y**
Test Coupons	1.09×10^6	0	0	6.04	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
96 hours						
Nonexposed Coupons	1.09×10^6	0.80 ± 1.10	0.0037 ± 0.0050	5.24 ± 0.96	N	**Y**
Test Coupons	1.09×10^6	0	0	6.04	N	**Y**
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
120 Hours						
Nonexposed Coupons	1.09×10^6	0	0	6.04	N	N
Test Coupons	1.09×10^6	0	0	6.04	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, ***Y*** indicates growth confirmed to be *B. anthracis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Inoculated, not exposed to UV-A/B (recovery conducted after conclusion of UV-A/B exposure).

(d) Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.

(e) Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.

(f) Procedural Blank = Not inoculated, placed with test coupons and exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

The primary conclusion to be drawn from the UV test results shown above is that UV-A/B has a small effect, at most, on the persistence of vegetative *B. anthracis* on topsoil. Consideration of these data raised the question of how reproducible persistence results are with vegetative *B. anthracis*. To address this question, a comparison was made of the persistence of *B. anthracis* on topsoil (no UV exposure) in three nominally identical sets of topsoil coupons.

- Test 1 - the test coupons used in the 120-hour persistence test with no UV exposure (Table 4-5 and Figure 4-1);
- Test 2 - the non-exposed coupons (controls) used in the 120-hour UV exposure test with the low UV-A/UV-B ratio (Table B-1 and Figure 4-4); and
- Test 3 - the non-exposed coupons (controls) used in the 120-hour UV exposure test with the high UV-A/UV-B ratio (Table B-2 and Figure 4-5).

Figure B-2 shows the persistence curves of vegetative *B. anthracis* on these three sets of topsoil coupons. Overall, the three persistence curves are quite similar, all reaching or nearing complete inactivation of the vegetative *B. anthracis* within 120 hours. The 95 % CI intervals are plotted at each time point for each of the three tests. The CI intervals of at least two data points overlap at every time point, and in most cases all three overlap. Thus the three persistence curves are not consistently significantly different from one another.

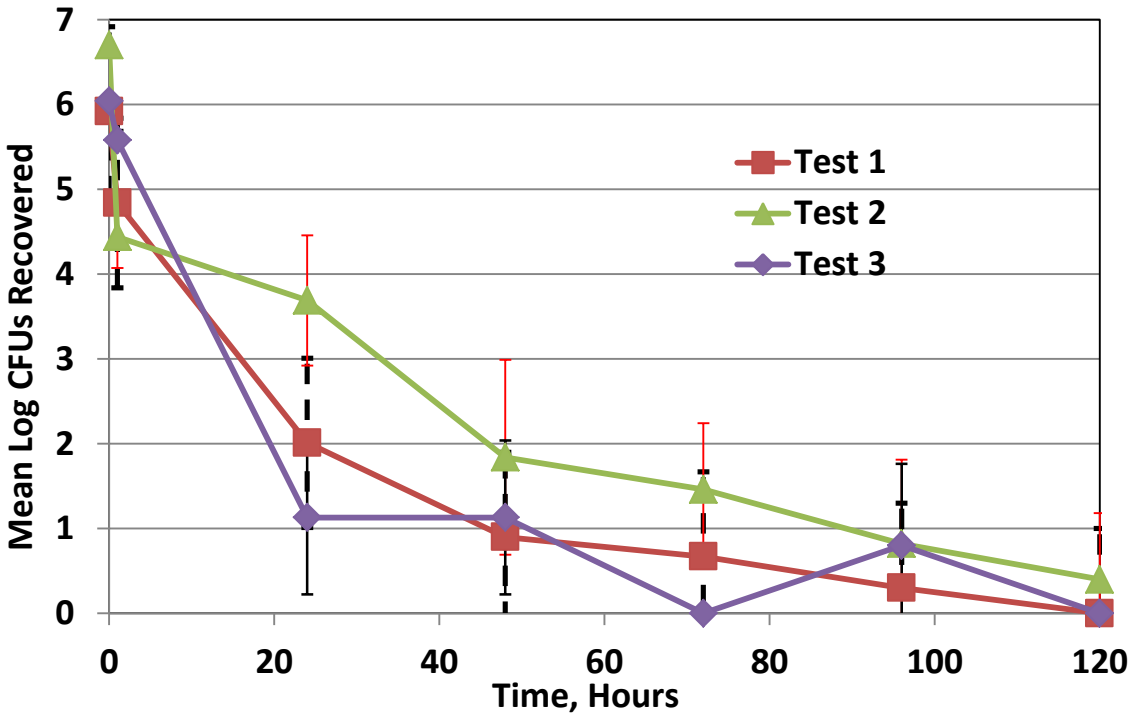


Figure B-2. Recovery of Vegetative *B. anthracis* in Soil, No UV exposure

Figure B-3 shows a more quantitative comparison of the persistence results from these three nominally identical sets of topsoil coupons. In this figure, the mean log CFU recovered results

from Tests 2 and 3 are plotted against the corresponding results from Test 1 at the same time points. Figure B-3 includes a one-to-one line indicating perfect agreement, and parallel lines indicating differences of +1 and -1 log. Figure B-3 shows that the persistence results in the three replicate tests agree within approximately 1 log at all time points. A similar degree of agreement has been seen in previous decontamination tests conducted by Battelle with *B. anthracis* (Ames) spores. The implication of Figure B-3 is that persistence can be replicated within about ±1 log in testing with vegetative *B. anthracis*.

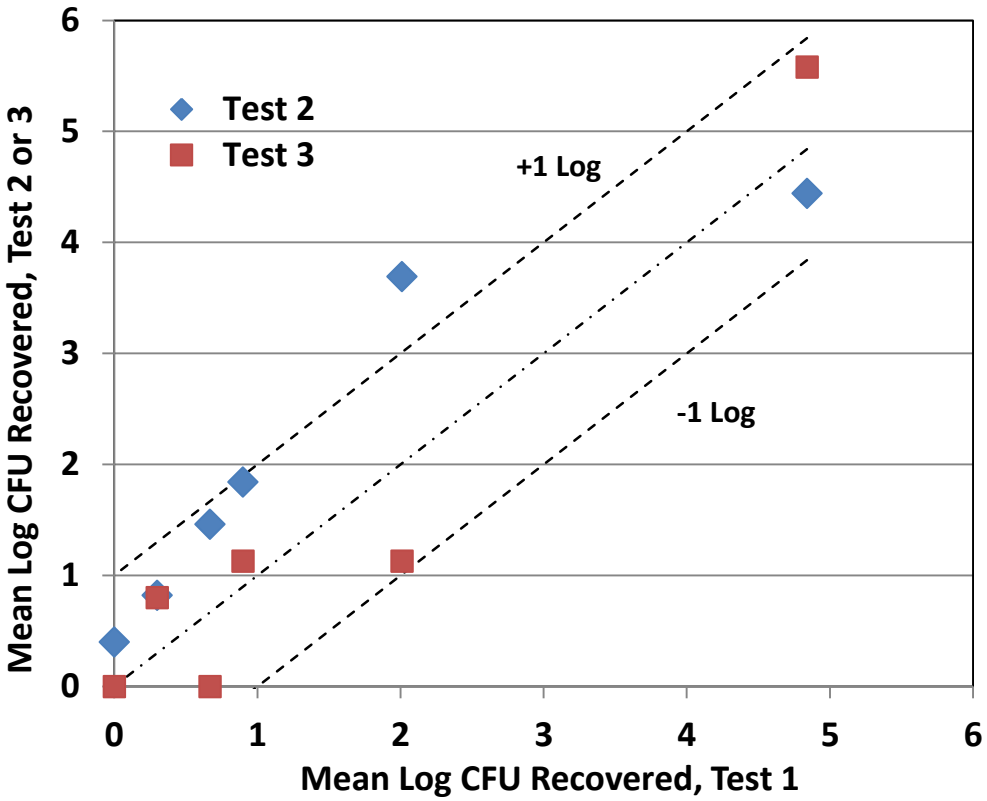


Figure B-3. Persistence Results in Three Comparable 120-hour Tests are within ± 1 Log

APPENDIX C:

Data Tables from Selected Persistence Tests with *Yersinia pestis*

Tables C-1 through C-3 show detailed results from testing of *Y. pestis* persistence without exposure to simulated sunlight. These tables augment the data shown in Section 5 of this report. Each of these tables shows the *Y. pestis* inoculum onto each coupon, the mean log (± SD) of the recovered CFU, the mean (± SD) of the CFU percent recovery, the mean log reduction (± CI), and the qualitative results of subsequent growth testing, when performed. All three tables show results for all four coupon materials used with *Y. pestis*, for each time point used in the respective test.

Table C-1 shows the results from Test YP1, in which *Y. pestis* persistence was assessed at one hour and at 14 days after inoculation onto the four coupon materials, at ambient laboratory temperature and 57 % RH. *Y. pestis* was recovered on all four materials at 1 hour after inoculation, but no viable cells were present at 14 days after inoculation. Qualitative testing was not conducted in Test YP1, but the results of which would not be expected to differ from the quantitative results.

Table C-2 shows the results from Test YP2, in which *Y. pestis* persistence was assessed at one hour and at seven days after inoculation onto the four coupon materials, at ambient laboratory temperature and 54 % RH. *Y. pestis* persisted on all four materials at one hour after inoculation, but no viable cells were present at seven days after inoculation. Qualitative testing was not conducted in Test YP2, but the results of which would not be expected to differ from the quantitative results.
.

Table C-3 shows the complete set of results from Test YP3, in which *Y. pestis* persistence was assessed at 1, 24, 48, 96, and 144 hours after inoculation onto the four test materials, at ambient laboratory temperature and 65 % RH. Some of the data in Table C-3 are shown in Table 5-3 in the body of this report. Table C-3 shows data for glass, galvanized metal, and painted wallboard paper at 48, 96, and 144 hours after inoculation; those data are not shown in Table 5-3 because *Y. pestis* did not persist on those coupons at those time points. Despite the inactivation observed at 24 hours and the later time points, testing was continued through those time points for consistency with the results on topsoil. Table C-3 shows that the qualitative test results confirmed the quantitative test results in all cases in Test YP3.

Table C-1. Persistence of *Yersinia pestis*[a] (Four Materials, 14 Days, 57 % RH; Test YP1)

Test Material	Inoculum (CFU)	Quantitative Testing: Mean of Logs of Observed CFU	Quantitative Testing: Mean % Recovery	Quantitative Testing: Mean Log Reduction ± CI
Topsoil				
Test Coupons 1 Hour	3.67×10^7	7.37 ± 0.11	65.2 ± 17.9	0.20 ± 0.10
Test Coupons 14 Days	3.67×10^7	0	0	7.56
Laboratory Blank[b]	0	0	0	-
Procedural Blank[c]	0	0	0	-
Glass				
Test Coupons 1 Hour	3.67×10^7	7.23 ± 0.03	46.1 ± 3.1	0.34 ± 0.02
Test Coupons 14 Days	3.67×10^7	0	0	7.56
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Galvanized Metal				
Test Coupons 1 Hour	3.67×10^7	7.43 ± 0.16	77.1 ± 27.4	0.14 ± 0.14
Test Coupons 14 Days	3.67×10^7	0	0	7.56
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Painted Wallboard Paper				
Test Coupons 1 Hour	3.67×10^7	6.21 ± 0.30	5.4 ± 3.7	1.35 ± 0.26
Test Coupons 14 Days	3.67×10^7	0	0	7.56
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Laboratory Blank = Not inoculated.
(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table C-2. Persistence of *Yersinia pestis*[a] (Four Materials, Seven Days, 54 % RH; Test YP2)

Test Material	Inoculum (CFU)	Quantitative Testing: Mean of Logs of Observed CFU	Quantitative Testing: Mean % Recovery	Quantitative Testing: Mean Log Reduction ± CI
Topsoil				
Test Coupons 1 Hour	3.13×10^7	7.08 ± 0.03	38.7 ± 2.4	0.41 ± 0.03
Test Coupons 7 Days	3.13×10^7	0	0	7.50
Laboratory Blank[b]	0	0	0	-
Procedural Blank[c]	0	0	0	-
Glass				
Test Coupons 1 Hour	3.13×10^7	5.06 ± 0.11	0.38 ± 0.11	2.44 ± 0.10
Test Coupons 7 Days	3.13×10^7	0	0	7.50
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Galvanized Metal				
Test Coupons 1 Hour	3.13×10^7	2.58 ± 0.23	0.0013 ± 0.0006	4.92 ± 0.21
Test Coupons 7 Days	3.13×10^7	0	0	7.50
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Painted Wallboard Paper				
Test Coupons 1 Hour	3.13×10^7	4.27 ± 0.45	0.085 ± 0.067	3.23 ± 0.39
Test Coupons 7 Days	3.13×10^7	0	0	7.50
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).
(b) Laboratory Blank = Not inoculated.
(c) Procedural Blank = Not inoculated, placed with test coupons during persistence test.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table C-3. Persistence of *Yersinia pestis*[(a)] (Four Materials, 144 Hours, 65 % RH; Test YP3)

		Quantitative Testing			Qualitative Growth[(b)]	
Test Material	**Inoculum (CFU)**	**Mean of Logs of Observed CFU**	**Mean % Recovery**	**Mean Log Reduction ± CI**	**One day**	**Seven days**
Topsoil						
Test Coupons 1 Hour	2.70×10^7	6.98 ± 0.07	35.7 ± 6.4	0.45 ± 0.07	N	**Y**
Test Coupons 24 Hour	2.70×10^7	3.00 ± 0.75	0.0084 ± 0.01	4.43 ± 0.66	N	**Y**
Test Coupons 48 Hour	2.70×10^7	1.72 ± 1.04	0.00059 ± 0.00063	5.71 ± 0.91	N	**Y**
Test Coupons 96 Hour	2.70×10^7	1.40 ± 0.87	0.00027 ± 0.00040	6.03 ± 0.77	N	**Y**
Test Coupons 144 Hour	2.70×10^7	1.07 ± 1.02	0.00020 ± 0.00031	6.36 ± 0.90	N	**Y**
Laboratory Blank[(c)]	0	0	0	-		
Procedural Blank[(d)]	0	0	0	-		
Glass						
Test Coupons 1 Hour	2.70×10^7	4.55 ± 0.09	0.13 ± 0.029	2.88 ± 0.08	N	**Y**
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 48 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 96 Hour	2.70×10^7	0	0	7.73	N	N
Test Coupons 144 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
Galvanized Metal						
Test Coupons 1 Hour	2.70×10^7	6.34 ± 0.69	15.0 ± 14.1	1.09 ± 0.61	N	**Y**
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 48 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 96 Hour	2.70×10^7	0	0	7.73	N	N
Test Coupons 144 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		
Painted Wallboard Paper						
Test Coupons 1 Hour	2.70×10^7	3.46 ± 0.61	0.028 ± 0.047	3.97 ± 0.69	N	N
Test Coupons 24 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 48 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 96 Hour	2.70×10^7	0	0	7.43	N	N
Test Coupons 144 Hour	2.70×10^7	0	0	7.43	N	N
Laboratory Blank	0	0	0	-		
Procedural Blank	0	0	0	-		

(a) Data are expressed as mean (± SD) of the logs of total number of cells (CFU) observed on individual coupons, percent recovery (± SD), and mean log reduction (± CI).

(b) Growth in nutrient medium at one day and seven days after coupon extraction. Y indicates growth visually observed with at least one of five coupon extracts, **Y** indicates growth confirmed to be *Y. pestis* with at least one of five coupon extracts, N indicates no growth (complete inactivation).

(c) Laboratory Blank = Not inoculated.

(d) Procedural Blank = Not inoculated, placed with test coupons during persistence test.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

EPA
United States
Environmental Protection
Agency

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

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

Official Business
Penalty for Private Use
$300