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Agency

INVESTIGATION REPORT

Investigation of Simulated Sunlight in the Inactivation of *B. anthracis* and *B. subtilis* on Outdoor Materials

Office of Research and Development
National Homeland Security Research Center

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Investigation of Simulated Sunlight in the Inactivation of *B. anthracis* and *B. subtilis* on Outdoor Materials

U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

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Office of Research and Development
National Homeland Security Research Center

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

Joseph P. Wood
National Homeland Security Research Center
Office of Research and Development (NG16)
U.S. Environmental Protection Agency
Mail Code E343-06
Research Triangle Park, NC 27711
(919) 541-5029
wood.joe@epa.gov

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Contributors (Battelle)

Thomas Kelly

Young Choi

James Rogers

Karen Riggs

Zachary Willenberg

Peer reviewers

Worth Calfee (EPA NHSRC), Leroy Mickelson (EPA National Decontamination Team), and Jonathon Thornburg (RTI International)

Executive Summary

The U.S. Environmental Protection Agency's (EPA) National Homeland Security Research Center (NHSRC) helps protect human health and the environment by carrying out investigations on the persistence of biological agents in the environment. This report describes an investigation of the effect of UV-A/B light (representing sunlight) for inactivating spores of *Bacillus anthracis* (Ames) and *Bacillus subtilis* on test coupons of glass, bare pine wood, unpainted concrete, and topsoil.

Test Procedures. Test coupons were 1.9 cm by 7.5 cm, except for topsoil, which was prepared by filling a Parafilm®-lined 3.5 cm diameter by 1 cm deep Petri dish with uncompacted topsoil. For testing, coupons were "contaminated" by inoculation with approximately 108 colony forming units per coupon of the biological warfare agent *B. anthracis* (Ames), or the surrogate organism *B. subtilis* (American Type Culture Collection [ATCC] 19659). Test coupons were exposed to UV-A/B light, and the number of viable spores on each coupon was determined after a predefined duration of exposure. Positive control coupons were spiked in the same way and subjected to the same test environment except for being shielded from exposure to UV-A/B light. Five replicates of both test and positive control coupons were used for each coupon material in each test. Four different durations of UV-A/B exposure were used: 24, 168, 336, and 672 h. Those total exposures were accumulated by alternating 12-h periods of UV-A/B light and darkness, resulting in 12 h of UV-A/B exposure on each test day. For example, 168-h UV-A/B exposure was accumulated over 14 test days. Each UV-A/B exposure test with each organism was conducted separately and involved coupon inoculation, UV-A/B exposure, spore extraction, and enumeration, i.e., UV-A/B exposures were separate, non-simultaneous, and non-overlapping tests. All test procedures were conducted according to a peer-reviewed test/quality assurance (QA) plan.

UV-A/B/C intensities, temperature, and relative humidity (RH) were monitored at the locations of both the test and positive control coupons throughout all testing. Those measurements confirmed UV-A/B levels at the test coupons of approximately 100 $\mu W/cm^2$ UV-A and 70 $\mu W/cm^2$ UV-B, with no detectable UV-C (i.e., <1 $\mu W/cm^2$). There was also no detectable UV-A, UV-B, or UV-C at the positive control coupons. Temperature and RH at the positive control coupons were within about 3 °C and within about 10% RH, respectively, of the temperature and RH at the test coupons when the UV-A/B lights were on, and within 0.5 °C and 5% RH when the lights were off.

The effectiveness of UV-A/B exposure for inactivating spores was quantified as mean $\log_{10}$ reduction relative to the positive control coupons for each material with each spore type at each time point.

Results. Table ES-1 shows the mean log reduction values for *B. anthracis* (Ames) and *B. subtilis* on the four test materials at each of the four UV-A/B exposure time points. The 95% confidence interval (CI) is also shown for each mean log reduction value. UV-A/B inactivation was most effective for both *B. anthracis* and *B. subtilis* on glass, and least effective on topsoil. The maximum mean log reduction results on glass exceeded 5 logs for both *B. anthracis* and *B. subtilis*. Mean log reductions achieved on bare pine wood and unpainted concrete were primarily in the range of about 1 to 2 log reduction, and those mean log reductions were similar across both materials and both organisms. The topsoil mean log reductions varied around zero and never reached 1 log reduction for either organism. Table ES-1 indicates (by bold values) those cases in which statistically significant differences in mean log reduction were found between the two organisms. Of the 16 such comparisons, seven show significant differences in mean log reduction between *B. subtilis* and *B. anthracis*. No clear pattern is evident in the occurrence of these seven cases with coupon material or UV-A/B exposure duration.

Table ES-1. Summary of Mean Log Reduction Results with UV-A/B (Simulated Sunlight) Exposure on Four Materials

Material	Mean Log Reduction (± 95% CI) by UV-A/B Exposure Time[a]							
	24 h		168 h		336 h		672 h	
	B.a.	***B.s.***	***B.a.***	***B.s.***	***B.a.***	***B.s.***	***B.a.***	***B.s.***
Glass	3.77 (±0.29)	**2.59** (±0.24)	5.25 (±1.61)	3.84 (±0.15)	5.81 (±1.40)	**3.99** (±0.37)	4.72 (±0.88)	5.29 (±1.21)
Bare Pine Wood	0.69 (±0.15)	0.92 (±0.23)	1.16 (±0.34)	**0.11** (±0.23)	1.01 (±0.64)	0.94 (±0.64)	1.51 (±0.60)	1.27 (±0.20)
Unpainted Concrete	0.81 (±0.24)	0.44 (±0.37)	0.65 (±0.28)	**2.44** (±0.59)	2.21 (±0.83)	**0.48** (±0.15)	1.51 (±0.55)	2.15 (±0.10)
Topsoil	-1.10[b] (±0.09)	**0.31** (±0.50)	0.09 (±0.08)	**0.75** (±0.19)	0.17 (±0.10)	-0.08 (±0.38)	0.20 (±0.15)	0.15 (±0.09)

[a] *B.a. = B. anthracis* (Ames), *B.s. = B. subtilis*. Values in bold for *B. subtilis* are significantly different from corresponding values for *B. anthracis.*

[b] Unusual spore recoveries seen; see text.

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TABLES

FIGURES

ABBREVIATIONS/ACRONYMS

ATCC	American Type Culture Collection
B.a.	*Bacillus anthracis*
B.s.	*Bacillus subtilis*
BBRC	Battelle Biomedical Research Center
BSC	biosafety cabinet
C	Celsius
C_t	threshold cycle
CFU(s)	colony-forming unit(s)
CI	confidence interval
cm	centimeter
EPA	U.S. Environmental Protection Agency
h	hour
J	Joule
L	liter
LR	log reduction
min	minute
µL	microliter
µm	micrometer
µW	microwatts
mL	milliliter
mM	millimolar
NHSRC	National Homeland Security Research Center
nm	nanometer
ORD	EPA Office of Research and Development
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
QA	quality assurance
QC	quality control
QMP	quality management plan
RH	relative humidity
rpm	revolutions per minute
SD	standard deviation
SE	standard error
SFW	sterile filtered water
TOPO	Task Order Project Officer
TSA	technical systems audit
UV	ultraviolet light
UV-A	ultraviolet light (320 to 400 nm wavelength)
UV-A/B	combination of UV-A and UV-B light used in testing

ABBREVIATIONS/ACRONYMS (CONTINUED)

UV-B	ultraviolet light (290 to 320 nm wavelength)
UV-C	ultraviolet light (180 to 290 nm wavelength)
W	watts

1.0 INTRODUCTION

The U.S. Environmental Protection Agency's (EPA) National Homeland Security Research Center (NHSRC) evaluates the role that natural conditions play in counteracting chemical and biological homeland security threats, by working with stakeholders and subject matter experts to develop test plans, conduct evaluations, collect and analyze data, and prepare peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure the generation of high quality data and defensible results.

NHSRC investigated the effect of simulated sunlight (combination of UV-A (i.e., 320-400 nm) and UV-B (i.e., 290-320 nm) light, referred to as UV-A/B) in inactivating *Bacillus anthracis* (Ames) spores and *Bacillus subtilis* (American Type Culture Collection or ATCC 19659) spores on representative surface materials. The procedures of this investigation are specified in a peer-reviewed test/QA plan, that was developed according to the requirements of a previously established quality management plan (QMP).[1] In this investigation, the mean log reduction in viable spores due to UV-A/B exposure was determined at four successive non-zero time points for each of the two test organisms on each of four test materials: glass, bare pine wood, unpainted concrete, and topsoil. An initial 24-h UV-A/B exposure test was conducted; each successive time point was then chosen by EPA based on the results of the preceding test. The same four exposure times were used for both *B. subtilis and B. anthracis.*

In all tests, UV-A/B exposure was conducted in alternating 12-h periods of light and dark. Positive control coupons of the test materials, inoculated with the test organisms, were placed in the same test environment as those exposed to UV-A/B light, but were shielded from any UV-A/B exposure. The temperature and relative humidity (RH) of the test environment were monitored at both the position of the test coupons and the position of the positive control coupons throughout testing. UV-A and UV-B intensity was monitored at five locations in the array of test coupons and five corresponding locations in the array of positive control coupons. This monitoring was conducted to confirm both the UV-A and UV-B intensities reaching the test coupons and the absence of UV-A/B exposure on the positive control coupons.

2.0
Summary of Test Procedures

Test procedures were performed in accordance with the test/QA plan and are briefly summarized here.

2.1 Preparation of Test Coupons

B. anthracis Ames and *B. subtilis* spores were inoculated onto test coupons of bare wood, glass, unpainted concrete, and topsoil in an appropriate biosafety cabinet (BSC-II or -III) according to established Battelle Biomedical Research Center (BBRC) procedures. Inoculated coupons were prepared fresh for each day of experimental work. Test coupons were placed flat in the BSC and spiked at approximately 1 x 10^8 colony-forming units (CFUs) per coupon. This spiking was accomplished by dispensing a 100 µL aliquot of a spore stock suspension (approximately 1 x 10^9 CFUs/mL) using a micropipette as 10 droplets (each of 10 µL volume) across the surface of the test coupon. This approach provided more uniform distribution of spores across the coupon surface than would be obtained through a single drop of the suspension. It is possible that the application procedure produced multiple layers of spores on the test coupons. If this were the case, spores in the topmost layers could have diminished the UV-A/B exposure of spores in the underlying layers. However, this is a hypothesis that would have to be verified with additional research. After spiking, the test coupons remained undisturbed overnight in the BSC to dry. With the exception of topsoil (see Section 2.3), test coupons were sterilized before use by gamma irradiation (bare wood) or autoclaving (glass, unpainted concrete).

2.2 UV-A/B Exposure Procedure

On the day following spore inoculation, the test, blank, and positive control 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 light to which the coupons were exposed. Five test coupons and five positive control coupons of each material were used for each UV-A/B exposure time point. One blank coupon of each material was also included with the test coupons and with the positive control coupons for each time point. Test coupons and associated blank coupons were placed flat on top of the raised tray below the UV lamps, and positive control coupons and associated blank coupons were placed flat beneath that tray, shielded from direct UV-A/B light (Figure 2-1). The lower portions of the test chamber walls were covered with black paper to minimize reflected UV-A/B light.

The 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 test (or positive control) coupon of each of the four materials was placed at each of these five positions, 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 test and positive control trays.

All UV exposure testing started at normal room temperature and RH: approximately 22 °C and 50% RH. However, no attempt was made to control the chamber temperature or RH, and despite circulation of air through the chamber, the chamber temperature increased a few degrees Celsius and RH dropped by a few percent during the UV-A/B exposure periods. Temperature and RH were recorded at 5-minute intervals throughout all tests at the locations of both the test and control 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. Averages, standard deviations, maxima, and minima of the recorded temperature and RH data over the duration of each UV-A/B exposure are presented in Section 4 of this report to document the test conditions.

All testing consisted of alternating 12-h periods of UV-A/B exposure (lamps on) and darkness (lamps off). On the basis of mean log reduction results from an initial 24-h UV-A/B exposure, the next UV-A/B exposure period was selected by the EPA Task Order Project Officer (TOPO). This process was repeated upon completion of each UV-A/B exposure to select a range

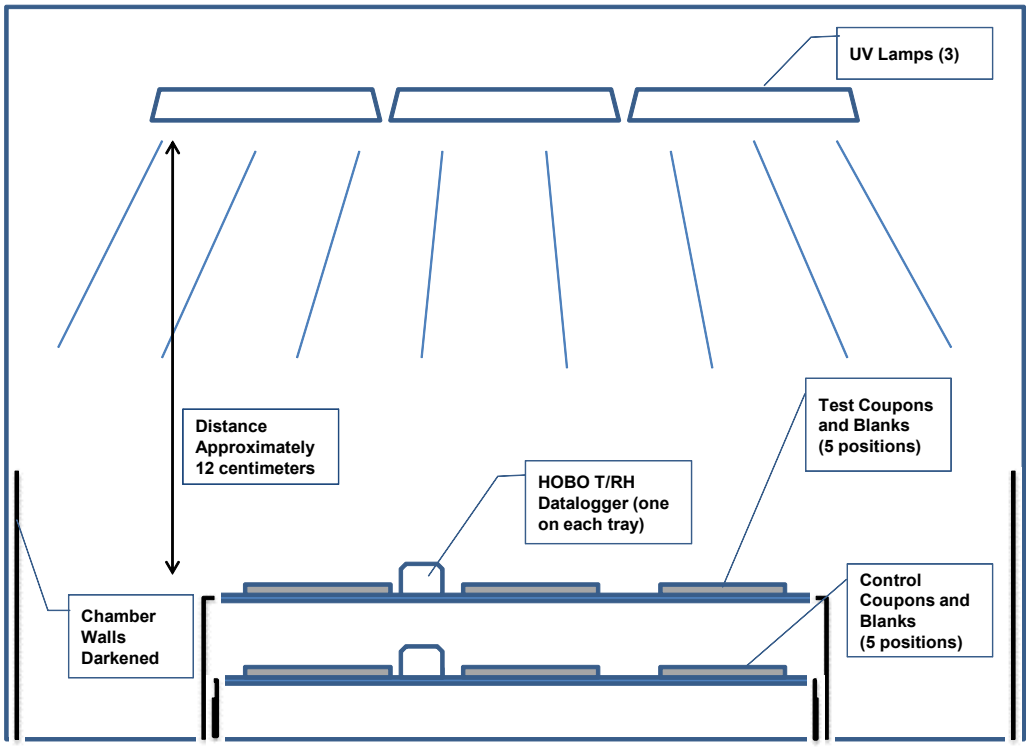

Figure 2-1. Schematic Representation of Test Chamber (not to scale).

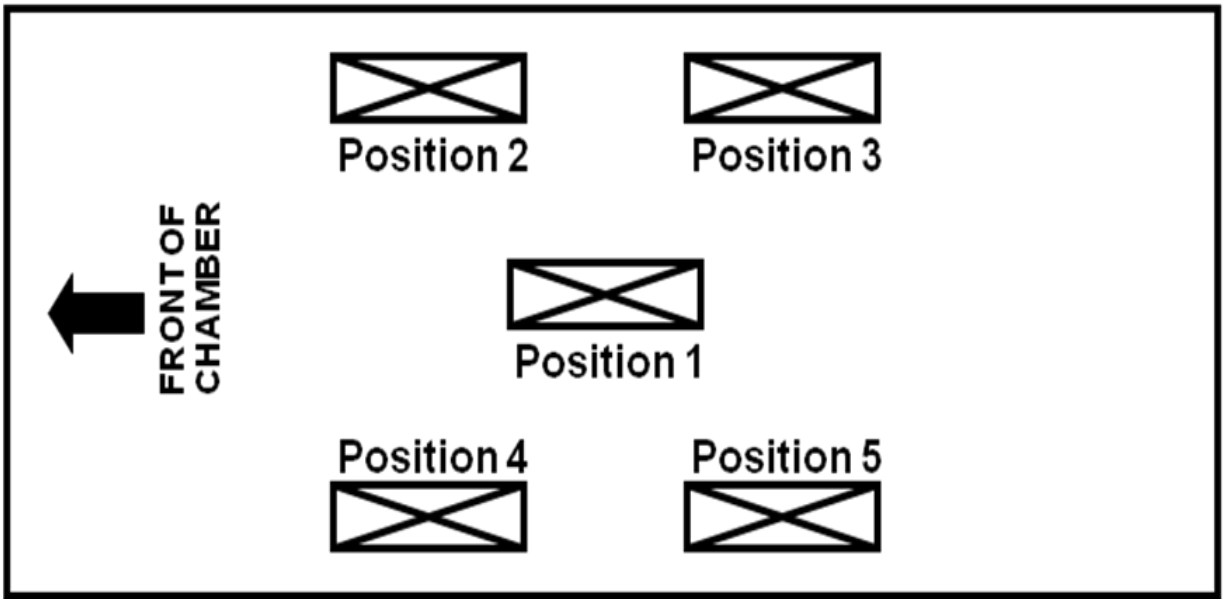

Figure 2-2. Schematic of Five Coupon Positions on the Support Trays.

of exposure times that clearly delineated the inactivation of spores on each material. The four UV-A/B exposure periods used were the same for all materials and for both organisms, and consisted of 24, 168, 336, and 672 h, accumulated through successive alternating 12-h periods of lights on/lights off. For example, the 168-h UV exposure required 14 calendar days to conduct. At the conclusion of each UV-A/B exposure period, test and positive control coupons of all materials were removed from the test chamber, and the spores were extracted and enumerated to determine mean log reduction 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, with emphasis on the more photobiologically active UV-B.[3] 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.[4-11] Peak (i.e., noontime) UV-B levels reported in a few studies range from about 20 to 150 µW/cm^2.[8-10] The target UV-B level chosen for testing was 70 µW/cm^2, which corresponds to a daily dose of about 3.0 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). Since UV-B is more photobiologically important, no specific UV-A level was targeted. However, the UV-A level was consistent throughout all experiments, at approximately 100 µW/cm^2. This UV-A level corresponds to a daily UV-A dose of about 4.3 J/cm^2 with 12 hours of exposure per day. The actual total UV-B doses during the 24, 168, 336, and 672 hour exposure periods were 5.8, 43.1, 85.6, and 169 J/cm^2, respectively, in testing with *B. anthracis,* and 5.8, 41.9, 83.8, and 169 J/cm^2, respectively, in testing with *B. subtilis.* The corresponding actual total UV-A doses during the 24, 168, 336, and 672 hour exposure periods were 8.8, 63.8, 126, and 243 J/cm^2, respectively, in testing with *B. anthracis,* and 8.7, 58.7, 124, and 243 J/cm^2, respectively, in testing with *B. subtilis.* (These doses are calculated based on the measured mean UV intensities in each test, which are tabulated in Section 4.) A target of zero for UV-C radiation (i.e., 180-290 nm) was chosen, because of the absence of this UV component in sunlight at ground level. The target UV intensities at the positive control 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 test coupon and positive control coupon trays, at least near the start and end of every 12-h 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 average, standard deviation (SD), maximum, and minimum of those position averages are shown in Section 4.

It must be stressed that each UV-A/B exposure test with each organism was a separate and unique test, involving inoculation of the four types of test coupons, accumulation of UV-A/B exposure in daily 12-hour doses up to the specified total duration, extraction of spores from the coupons, and enumeration of the recovered spores. The eight total tests (four exposure periods with each of two organisms) were conducted sequentially over a time period of approximately one year, and were not simultaneous, overlapping, or nested in any way. Each test included replicate coupons of all materials, but there were no replicate runs of the entire test procedure. Variability in the test results likely occurred due to variation in procedures, e.g., slightly different spore inoculations or coupon characteristics in each test, small variations in temperature, RH, and UV-A/B conditions from test to test, and variability in recovery and enumeration processes.

2.3 Spore Recovery Procedures

Following the UV-A/B exposure period, each test, positive control, 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). With the exception of bare concrete, the coupons were then extracted by agitation on an orbital shaker for 15 minutes at approximately 200 revolutions per minute (rpm) at room temperature. For bare concrete, recovery of spores required an alternate procedure in which 45 minutes of sonication was used instead of the period of agitation. For all coupons, following extraction 1 mL of the coupon extract was removed, and a series of dilutions through 10^{-7} was prepared in sterile filtered water (SFW). An aliquot (0.1 mL) of the undiluted extract and of each serial dilution was then spread plated onto tryptic soy agar plates and incubated overnight at 35 to 37 ºC. Plates were enumerated within 18 to 24 h of plating. The

number of CFUs/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.

The use of topsoil as a test coupon required techniques to ensure adequate recovery of spiked *B. anthracis* or *B. subtilis* spores, and the absence of interference from native topsoil microorganisms in counting of recovered spores. A heat shock procedure was found to minimize interference by native microorganisms. Specifically, spiked or blank topsoil was extracted in PBS/Triton X-100 solution as described above, and then the recovered supernatant was heat-shocked in a water bath at 65 °C for one hour before being serially diluted and plated. Topsoil samples spiked with *B. anthracis* or *B. subtilis* spores each showed the presence of a single homogeneous species, with all colonies of uniform size and morphologically characteristic of the respective *Bacillus* species. Some blank topsoil samples showed growth of colonies of other native species, which was not seen with the spiked topsoil samples. Consequently, although topsoil blanks showed some growth, that growth did not occur with extracts of spiked topsoil, so no interference existed in terms of counting recovered spores. The mechanism by which growth of native species is suppressed in the extracts of spiked topsoil was not investigated, but may involve monopolization of nutrients by the large numbers of spiked spores. By this procedure, spore recovery trials conducted before testing showed the recovery of spores spiked onto topsoil to be approximately 50% for *B. anthracis* and approximately 34% for *B. subtilis*.

Blank coupons controlled for viable spores inadvertently introduced to test coupons, and were spiked with an equivalent amount of 0.1 mL of "stock suspension" that did not contain *B. anthracis* or *B. subtilis* spores. The blank coupons underwent the same spore extraction process as the inoculated coupons, at the same time as those coupons (i.e., following completion of a UV-A/B exposure period). To be considered acceptable for quantitative determination of log reduction, extracts of blank coupons had to contain no CFUs showing the morphology characteristic of the respective *Bacillus* species. As noted above, the occurrence of native organisms on uninoculated topsoil coupons did not violate this blank acceptance criterion. The mean percent spore recovery from each coupon type was calculated using results from positive control coupons (inoculated, not exposed to UV-A/B light), by means of the following equation:

$$\text{Mean \% Recovery} = [\text{Mean } CFU_{pc}/CFU_{spike}] \times 100 \quad (1)$$

where Mean CFU_{pc} is the mean number of CFUs recovered from five replicate positive control coupons of a single type, and CFU_{spike} is the number of CFUs spiked onto each of those coupons. The value of CFU_{spike} is known from enumeration of the stock spore suspension. Spore recovery was calculated for both *B. anthracis* and *B. subtilis* on each coupon type. Spore recoveries from positive control coupons were within the target range specified in the test/QA plan in nearly all cases. Sections 2.4 and 3.2 describe the few exceptions.

2.4 Spore Growth and Confirmation

During the 24-h UV-A/B exposure testing, excessively high spore recoveries were found with *B. anthracis* on topsoil test coupons and with *B. subtilis* on topsoil positive control coupons. A spore recovery of 370% was found for *B. anthracis* on the topsoil test coupons (see Table 4-4a) and a spore recovery of 260% was found for *B. subtilis* on the topsoil positive control coupons (see Table 4-4b). Although experimental spore recoveries exceeding 100% can occur due to test variability, these two results greatly exceeded the expected variability. To assess whether these results could have been caused by growth of spore populations after inoculation onto topsoil coupons, or by misidentification of extracted spores, a series of tests was done (established by amendment to the test/QA plan; see Section 3.4). First, a growth test was conducted by retaining inoculated topsoil coupons at normal room conditions for one week before spore extraction. Then tests were done using qualitative polymerase chain reaction (PCR) to determine whether the spores found on the topsoil coupons from the initial testing were in fact *B. anthracis* (Ames) or *B. subtilis* spores, the organisms that were spiked onto the coupons. This PCR analysis was implemented to check on the identifications made by colony morphology.

For the PCR confirmation, the topsoil extracts from the 24-h UV-A/B exposure tests with *B. anthracis* (Ames) and with *B. subtilis* were replated onto tryptic soy agar and incubated for 18 to 24 h at 37 °C and 35 °C, respectively. Then 50 individual colonies from the suspected *B. anthracis* (Ames) extract and 50 individual colonies from the suspected *B. subtilis* extract were picked with a 1 µL disposable sterile loop. Each picked colony was suspended in 200 µL of 10 mM Tris-HCl (pH 8) in a 1.5 mL microcentrifuge tube containing a 0.22 µm filter unit (Millipore, Bedford, MA). Each

microcentrifuge tube was heated at 95 °C for 20 minutes and then centrifuged at 6,000 × gravity for 2 minutes. The near-boiling temperature lysed the vegetative cells, and resulted in release of their DNA. The filter unit was used to collect cellular debris, and was discarded after use. The bacterial lysate was stored at -20 °C until PCR testing.

A commercial off-the-shelf PCR assay (Invitrogen, Carlsbad, CA) was used to confirm that the DNA isolated from the suspected *B. anthracis* (Ames) colonies was in fact that of *B. anthracis* origin. This assay utilized primers specific for the *B. anthracis* Cap B Domain. A custom PCR assay (Applied Biosystems, Forster City, CA) was created by special order to confirm *B. subtilis*. Primers were designed that target a conserved region of *B. subtilis* chromosomal DNA, since multiple strains of this bacterium exist. The results of the PCR analysis are summarized in Section 4.1.

2.5 Calculation of Mean Log Reduction

The effect of UV-A/B on inactivation of spores was assessed by determining the number of viable organisms remaining on each test coupon after UV-A/B exposure. The numbers of colony-forming units (CFUs) of *B. anthracis* (or *B. subtilis*) in extracts of test and positive control coupons were compared to calculate the mean log reduction attributable to the UV-A/B exposure.

First, the base 10 logarithm of the CFU count value from each coupon extract was determined, and then the mean of those logarithm values was determined for each set of test and associated control coupons, respectively. Mean log reduction (LR) due to UV-A/B exposure for a test organism on the ith coupon material was calculated as the difference between those mean log values, i.e.:

$$LR = \overline{(\log_{10} CFUc_{ij})} - \overline{(\log_{10} CFUt_{ij})} \quad (2)$$

where $\log_{10} CFUc_{ij}$ refers to the j^{th} individual logarithm values obtained from the positive control coupons and $\log_{10} CFUt_{ij}$ refers to the j^{th} individual logarithm values obtained from the corresponding test coupons, and the overbar designates a mean value.

In tests conducted under this plan, there were five controls and five corresponding test coupons (i.e., j = 5) at each time point for each material. When no viable CFUs were found in a coupon extract, a CFU count of 1 was assigned, resulting in a $\log_{10}$ CFU of zero for that coupon. When no viable CFUs were found in any of the five extracts of decontaminated coupons, the final mean log reduction was reported as greater than or equal to (≥) the LR value from Equation 2.

The variances (i.e., the square of the standard deviation) of the $\log_{10} CFUc_{ij}$ and $\log_{10} CFUt_{ij}$ values were also calculated for both the control and test coupons (i.e., S^2c_{ij} and S^2t_{ij}), and were used to calculate the pooled standard error (SE) for the mean log reduction value calculated in Equation 2, as follows:

$$SE = \sqrt{\frac{S^2 c_{ij}}{5} + \frac{S^2 t_{ij}}{5}} \quad (3)$$

where the number 5 again represents the number j of coupons in both the control and test data sets. Thus each mean log reduction result is reported with an associated SE value.

The significance of differences in mean log reduction across different coupon materials and spore types was assessed based on the 95% confidence interval of each mean log reduction result. The 95% confidence interval (CI) is:

$$95\%\ CI = \pm (1.96 \times SE) \quad (4)$$

Differences in mean log reduction were judged as significant if the 95% CIs of the two mean log reduction results did not overlap.

3.0 QUALITY ASSURANCE/QUALITY CONTROL

Quality assurance/quality control (QC) procedures were performed in accordance with the program QMP[1] and the test/QA plan for this evaluation, except as noted below. QA/QC procedures are summarized below.

3.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, biological safety cabinets) and monitoring devices (i.e., for temperature, relative humidity, 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 (A2LA) to the ISO 17025 standard, established National Institute of Standards and Technology (NIST)-traceable calibrations of the temperature 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

Quality control efforts conducted during testing included positive control coupons (inoculated with spores, not UV-A/B-exposed), 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 spore suspension). The results for these QC samples in each decontaminant evaluation are included in the results section (see Section 4).

As noted in Section 2.4, excessively high spore recoveries were observed in the 24-h UV-A/B exposure test with *B. anthracis* on topsoil test coupons, and in that same test with *B. subtilis* on topsoil positive control coupons. Those two occurrences were investigated as described in Sections 2.4 and 4.1. The cause of those high recovery values was not identified, and the data were retained in the test results.

3.3 Audits

3.3.1 Performance Evaluation Audit

The test/QA plan called for a Performance Evaluation (PE) audit of the UV meters used in testing, by comparison with responses from independent UV sensors for the same wavelength ranges (UV-A/B, UV-B, UV-C). However, that PE audit procedure is unrealistic, as it has been demonstrated that nominally similar UV meters from different manufacturers may give very different readings on the same light source, due to differences in spectral sensitivity, cosine response, and type of calibration.[12, 13] In fact, efforts were largely unsuccessful to identify UV meters from other manufacturers applicable to the same wavelength intervals, with nearly the same spectral response curves, as the Solarmeters used in testing. Nevertheless, a comparison was made between the Solarmeter Model 5.7 UV-A/B meter (Serial No. 15957) and a Lutron® Model UV-340A UV-A/B Light Meter (Serial No. AC89597) using the Reptisun lamps as the light source. The Model 5.7 reading (174 µW/cm^2) was in only qualitative agreement with the Model UV-340A reading (304 µW/cm^2), as expected. A comparison was also made between the Solarmeter Model 8.0 UV-C meter (Serial No. 00275) and a UVP Model UVX Digital Radiometer (Serial No. E28265) with Model UVX-25 UV-C sensor (Serial No. 31898). That comparison confirmed that the Reptisun lamps used in testing produced no detectable UV-C (i.e., less than the 1 µW/cm^2 detection limit on both meters). No appropriate meter could be found with which to make a corresponding comparison of UV-B measurements. However, the average UV-B intensity of 70 µW/cm^2 measured at the test coupons during testing is in reasonable agreement with expected UV-B intensities at comparable distances from the Reptisun lamps

(http://www.uvguide.co.uk/fluorescenttuberesults.htm).

To augment the PE audit, the Solarmeter UV-A/B and UV-B radiometers purchased for this study were compared to a similar set purchased from the same manufacturer for another program. Both sets have NIST-traceable calibrations established by the manufacturer, but were obtained separately and have different histories of use. Table 3-1 shows the results for this comparison,

Table 3-1. Qualitative Performance Evaluation Audit of Solarmeter UV Radiometers

Solarmeter Radiometer Set 1[a]	Solarmeter Radiometer Set 2[b]	Set 1 Reading ($\mu W/cm^2$)	Set 2 Reading ($\mu W/cm$)	% Difference[c]
Model 5.7 (UVA+B) (S. No. 15957)	Model 5.7 (UVA+B) (S. No. 17493)	174	176	-1.1
Model 6.2 (UV-B) (S. No. 01802)	Model 6.2 (UV-B) (S. No. 02988)	76	77	-1.3

a Used in this study.

b Obtained from a separate study.

c ((Set 1 Reading/Set 2 Reading)-1) x 100.

which was conducted in the test configuration described in Section 2.2. Table 3-1 indicates agreement within about 1% between the two sets of meters.

The test/QA plan also called for a PE audit of the timepiece used to monitor the UV-A/B exposure periods. The long exposure times in this testing made high accuracy in the timing of those exposures unnecessary. A deviation from the test/QA plan, documenting the decision not to conduct that PE audit, was prepared, approved, and placed in the study file.

3.3.2 Technical Systems Audit

Battelle QA staff conducted a technical systems audit (TSA) at the BBRC during testing on July 15, 2009 to ensure that the evaluation was being conducted in accordance with the test/QA plan and the QMP.[1] As part of the TSA, test procedures were compared to those specified in the test/QA plan, and data acquisition and handling procedures were reviewed. Observations and findings from the TSA were documented and submitted to the Battelle Task Order Leader for response. No adverse findings resulted from this TSA. TSA records were permanently stored with the Battelle QA Manager.

3.3.3 Data Quality Audit

All of the data acquired during the evaluation were audited. A Battelle 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 Test/QA Plan Amendments and Deviations

One amendment to the test/QA plan was prepared, reviewed, approved, and distributed to all parties involved in this evaluation. That amendment called for three efforts: performance of a one-week spore growth test on topsoil coupons; confirmation by PCR analysis of the identity of spores recovered from topsoil coupons in the 24-h UV-A/B exposure test; and monitoring of UV intensity at the positive control coupons to ensure UV-A, UV-B, and UV-C levels were zero. Two deviations were prepared, one documenting the acceptance of the three positive control spore recoveries noted in Section 3.2, which were below the minimum target recovery of 1% but still suitable for determining the mean log reduction due to UV exposure, and one documenting the absence of a PE audit for timing of the UV-A/B exposure periods.

3.5 QA/QC Reporting

Each audit was documented in accordance with the QMP.[1] The results of the audits were submitted to the EPA (i.e., to the NHSRC Quality Assurance Manager and the TOPO).

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 Battelle 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 Battelle staff member who stored the record.

4.0 RESULTS

In this chapter spore confirmation efforts, QC results, and spore inactivation (log reduction) results are summarized. Data are presented documenting the uniformity of test conditions, and the mean log reductions due to UV-A/B inactivation are reported for each of the organisms on each of the test materials.

4.1 Spore Growth and Confirmation

The extraction and enumeration of spores at the end of the one-week spore growth period did not indicate any growth of inoculated spores on the topsoil coupons. Consequently, the high spore recoveries in the 24-h test could not be attributed to growth of the inoculated organisms.

All 50 colonies picked from the replated *B. anthracis* (Ames) topsoil extracts were positively confirmed as *B. anthracis* by PCR (i.e., threshold cycle ($C_{t)}$ values of 18 to 20). A liquid culture of *B. anthracis* (Ames) was used as a positive control, and a liquid culture of *B. subtilis* was used as a negative control. The former culture gave similar positive PCR results (i.e., $C_t < 20$), and the latter gave negative PCR results for *B. anthracis* (i.e., no positive response through 45 cycles). In addition, blank culture solutions (sterile filtered water) gave negative PCR results for *B. anthracis*.

All 50 colonies picked from the replated *B. subtilis* topsoil extracts were also positively confirmed as *B. subtilis* by PCR (again, C_t values of 18 to 20). A liquid culture of *B. subtilis* was used as a positive control, and a liquid culture of *B. anthracis* (Ames) was used as a negative control. The former culture gave positive PCR results ($C_t < 20$), and the latter gave negative PCR results for *B. subtilis* (i.e., no positive response through 45 cycles). In addition, blank culture solutions (sterile filtered water) gave negative PCR results for *B. subtilis*.

In summary, the PCR results confirmed that the organisms found on the topsoil coupons were the inoculated species, *B. anthracis* (Ames) or *B. subtilis,* and the week-long growth study suggested that growth of spores on the topsoil coupons was not the cause of the high spore recoveries observed in the initial testing. As a result, those initial unusual results may be attributed to some undisclosed error in inoculation of the topsoil coupons. No other unusual results were observed in any other testing throughout this project.

4.2 QC Results

The positive control spore recovery results were within the target range of 1 to 150% of the spiked spores, with a few exceptions. As noted in Sections 2.4 and 3.2, an excessively high spore recovery was observed with topsoil positive control coupons in the 24-h UV-A/B exposure test with *B. subtilis* and also with *B. anthracis*. Those two occurrences were investigated as described in Sections 2.4 and 4.1. The cause of those high recovery values was not identified, and the data were retained in the test results. Also, positive control spore recoveries below 1% were observed with *B. subtilis* on unpainted concrete in the 24 h UV-A/B exposure (0.25% recovery), with *B. anthracis* on bare pine wood in the 672 h UV-A/B exposure (0.93% recovery), and with *B. subtilis* on unpainted concrete in the 672 h UV-A/B exposure (0.40% recovery). While those spore recoveries were lower than the acceptance criterion in the test/QA plan, they were more than sufficient for determining the mean log reduction due to UV-A/B exposure, and thus were retained in the test results. The low recoveries in the 672 h UV-A/B exposure tests may be due in part to some loss in viability over that extended time period. A test/QA plan deviation concerning these spore recovery values was prepared, approved, and retained in the test files.

All procedural and laboratory blanks met the criterion of no observed CFUs of the inoculated organism. Growth of native organisms, with colonies morphologically distinct from those of *B. anthracis* or *B. subtilis,* was observed from some blank topsoil coupons.

Spike control samples were taken from the spore suspension on each day of testing, and serially diluted, nutrient plated, and counted to establish the spore density used to spike the coupons. This process takes approximately 24 hours, so the spore density is known after completion of each day's testing. The target criterion is to maintain a spore suspension density of 1×10^{9}/mL (± 25%), leading to a spike of 1×10^{8} spores (± 25%) on each test coupon. The actual spike values for the four UV-A/B exposure periods with *B. anthracis* were 9.43×10^{7}/coupon, 8.37×10^{7}/coupon, 1.11×10^{8}/coupon, and 1.04×10^{8}/coupon, respectively. The actual spike values for the four UV-A/B exposure periods with *B. subtilis* were 9.93×10^{7}/coupon, 1.01×10^{8}/coupon, 1.04×10^{8}/coupon, and 7.83×10^{7}/coupon, respectively. Thus all the spike values met the target criterion.

4.3 Uniformity of Test Conditions

Tables 4-1 through 4-3 summarize the test conditions of UV intensity, temperature, and RH monitored during the testing.

Table 4-1 shows the average (± standard deviation) and range of intensities (in $\mu W/cm^2$) of UV-B, UV-A, and UVA/B measured at five positions in the test coupon arrays in each of the eight UV-A/B exposure tests. The intensity of UV-C at both the test and positive control 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 positive control coupons were also below detection in all tests, so these parameters are not shown in Table 4-1. The data in Table 4-1 show that close consistency of the average UV-B, UV-A, and UV-A/B intensities was maintained across all four exposure time periods with both *B. anthracis* and *B. subtilis*. The UV intensities found at the five different positions in the test coupon arrays typically ranged from about 5% less than to 10% greater than the average UV intensity over all five positions.

Table 4-2 shows the average (± standard deviation) and range of the temperature and RH monitored near the center of the test coupon array and the positive control coupon array in all tests with *B. anthracis*, both with the UV-A/B lights on and the lights off. Table 4-3 shows the corresponding information for all tests with *B. subtilis*. These tables show close consistency in the test conditions across all four UV-A/B exposure time periods. The only substantial departure from test conditions occurred over a three-day period in the middle of the 672-h exposure with *B. subtilis* (Table 4-3, right-hand columns), when RH fell to between 10 and 20%. The cause of this occurrence is not known, and the RH readings had returned to normal before the occurrence was discovered. The temporarily low RH values in the 672-h *B. subtilis* test are not likely to have any significant effect on the mean log reduction results. As expected, when the UV-A/B lights were off, the test and control coupons experienced closely similar temperature and RH conditions (i.e., within about 0.5 °C and 5% RH). When the lights were on, the test coupons experienced slightly higher temperatures (i.e., by approximately 3 °C) than did the positive control coupons, and somewhat lower RH (i.e., by about 10% RH). These small differences are not expected to have any impact on the mean log reduction due to UV-A/B exposure.

Table 4-1. Summary of Ultraviolet (UV-A/B) Light Intensity at the Test Coupons[a]

	UV-A/B Exposure Time Points							
	24 h		168 h		336 h		672 h	
Test Condition[a]	Test[b]	Control[c]	Test	Control	Test	Control	Test	Control
UV-B Average ± SD	67.0 ± 4.7	66.9 ± 4.5	71.4 ± 3.3	69.3 ± 3.7	70.9 ± 3.8	69.4 ± 3.7	69.6 ± 4.4	70.0 ± 3.9
UV-B Range	63.3-75.0	64.3-75.0	68.5-76.8	66.8-75.8	67.2-76.5	66.0-75.2	65.3-75.6	67.0-76.5
UV-A Average ± SD	102 ± 4.3	101 ± 3.5	106 ± 7.7	97.5 ± 4.0	105 ± 7.1	103 ± 6.6	101 ± 8.1	101 ± 8.5
UV-A Range	98.5-108	99.3-108	99.2-119	92.9-101	97.6-114	94.1-108	90.7-109	91.3-108
UV-A/B Average ± SD	169 ± 8.4	168 ± 8.0	177 ± 10.9	167 ± 6.3	176 ± 10.8	172 ± 10.1	171 ± 12.3	171 ± 11.7
UV-A/B Range	163-83	164-183	168-196	160-174	165-190	160-183	156-184	158-185

[a]All entries are in μW/cm^2 at the test coupons; all UV intensities were zero at the positive control coupons.
[b]Average ± SD refers to average over all five measurement positions; range refers to range of the means from the five measurement positions. SD = standard deviation. UV-A determined by subtraction of UV-B reading from UV-A/B reading.
[c]B.a. = results from testing with *Bacillus anthracis* (Ames).
[d]B.s. = results from testing with *Bacillus subtilis*.

Table 4-2. Summary of Temperature and Relative Humidity Conditions in UV-A/B Exposure Testing with *Bacillus anthracis* (Ames) Spores

	UV-A/B Exposure Time Points							
	24 h		168 h		336 h		672 h	
Test Condition[a]	**Test[b]**	**Control[c]**	**Test**	**Control**	**Test**	**Control**	**Test**	**Control**
UV ON								
T Average ± SD (°C)	28.3 ± 1.4	25.0 ± 0.7	26.3 ± 2.8	23.6 ± 1.6	26.6 ± 1.0	22.6 ± 0.5	28.4 ± 1.1	24.4 ± 0.6
T Range (°C)	23.4-28.9	22.8-25.5	20.0-29.0	19.8-25.5	20.9-28.2	20.8-23.8	21.7-31.0	21.7-27.0
RH Average ± SD (%)	31.3 ± 3.6	41.1 ± 4.6	46.2 ± 5.4	57.7 ± 5.1	35.4 ± 2.7	47.1 ± 1.8	32.0 ± 3.0	41.0 ± 2.9
RH Range (%)	25.5-44.9	33.6-57.9	29.7-60.0	38.1-68.3	30.7-49.6	41.4-59.0	23.6-52.7	30.1-57.4
UV OFF								
T Average ± SD (°C)	23.8 ± 1.0	23.4 ± 0.5	21.6 ± 1.1	21.4 ± 0.9	21.8 ± 0.9	21.7 ± 0.5	22.8 ± 0.9	22.6 ± 0.6
T Range (°C)	23.3-28.6	23.0-25.3	20.4-28.4	20.3-25.0	20.8-27.6	20.7-23.5	21.4-27.6	21.4-25.0
RH Average ± SD (%)	32.5 ± 0.6	36.5 ± 0.8	50.9 ± 3.5	56.3 ± 4.2	45.9 ± 2.8	49.9 ± 1.6	41.4 ± 3.3	44.6 ± 3.0
RH Range (%)	29.7-33.2	35.8-39.1	33.7-60.0	38.2-68.3	33.1-62.4	44.8-65.4	28.8-54.5	33.1-58.2

[a]UV ON and UV OFF refer to 12-h periods of alternating illumination and darkness in test chamber; averages and ranges shown are over all UV ON or UV OFF periods in the indicated UV exposure period. T = temperature, RH = relative humidity. SD = standard deviation.
[b]Test coupons (UV-A/B-exposed); readings taken at a central position in the coupon array.
[c]Positive control coupons (not exposed to UV-A/B); readings taken at a central location in the coupon array.

Table 4-3. Summary of Temperature and Relative Humidity Conditions in UV-A/B Exposure Testing with *Bacillus subtilis* Spores

	UV-A/B Exposure Time Points							
	24 h		168 h		336 h		672 h	
Test Condition[a]	Test[b]	Control[c]	Test	Control	Test	Control	Test	Control
UV ON								
T Average ± SD (°C)	28.3±1.3	25.0±0.7	25.0±0.8	22.4±0.4	26.8±1.0	23.2±0.7	29.0 ± 1.1	24.8 ± 0.8
T Range (°C)	23.6-28.9	22.8-25.4	21.1-26.6	20.8-23.5	21.3-28.7	20.8-25.1	21.9-30.8	22.0-28.9
RH Average ± SD (%)	33.0±2.9	42.1±2.5	37.6±2.3	45.1±2.5	38.4±2.9	46.5±2.6	26.5 ± 5.3	33.2 ± 7.0
RH Range (%)	25.7-44.7	33.7-53.6	33.4-46.1	39.8-51.4	32.1-53.0	41.4-59.9	8.8-44.2	8.4-45.9
UV OFF								
T Average ± SD (°C)	23.8±1.0	23.3±0.5	21.3±0.5	21.3±0.4	21.6±1.1	21.5±0.8	23.1 ± 0.9	23.0 ± 0.6
T Range (°C)	23.2-28.4	22.9-25.1	20.4-24.0	20.3-24.0	20.1-27.3	20.5-24.7	21.8-28.5	21.6-25.5
RH Average ± SD (%)	36.2±1.0	40.4±2.0	44.2±2.2	47.6±2.3	46.7±3.1	50.9±2.3	34.1 ± 6.4	36.2 ± 7.3
RH Range (%)	34.4-38.3	37.4-44.9	38.3-59.7	41.6-66.9	33.6-61.3	42.1-64.2	9.8-45.0	9.3-47.6

[a]UV ON and UV OFF refer to 12-h periods of alternating illumination and darkness in test chamber; averages and ranges shown are over all UV ON or UV OFF periods in the indicated UV exposure period. T = temperature, RH = relative humidity. SD = standard deviation.
[b]Test coupons (UV-A/B-exposed); readings taken at a central position in the coupon array.
[c]Positive control coupons (not exposed to UV-A/B); readings taken at a central position in the coupon array.

4.4 Mean Log Reduction Results

Tables 4-4 through 4-7 show the inactivation (as mean log reduction) of *B. anthracis* (Ames) and *B. subtilis* spores by UV-A/B exposures lasting 24, 168, 336, and 672 h, respectively. Each of these tables consists of a part "a" showing the *B. anthracis* results, and a part "b" showing the corresponding *B. subtilis* results. For each organism on each of the four test materials, the tables show the spore inoculum; the mean log of the observed spores on the test, positive control, and blank coupons; the spore recovery (to two significant figures); and the resulting mean log reduction (± 95% CI) due to the UV-A/B exposure. As appropriate, footnotes to Tables 4-4 through 4-7 denote unusual spore recoveries observed in the initial test (see Section 2.4) or the presence of endogenous organisms in uninoculated topsoil blanks (see Section 2.3). The significance of differences in mean log reduction results among the four coupon materials and between the two organisms was assessed by means of the 95% CI values shown in Tables 4-4 through 4-7. Inspection of Tables 4-4 through 4-7 shows that the mean log reductions found on glass at any UV-A/B exposure time were always significantly greater than those found on any other material; mean log reductions on bare pine wood and unpainted concrete were almost always significantly greater than those on topsoil. Tables 4-4 through 4-7 show seven cases in which the mean log reduction found for *B. subtilis* was significantly different from the corresponding mean log reduction found for *B. anthracis*. However, there is no clear dependence of those cases on the type of coupon material or the duration of UV-A/B exposure.

The main conclusion from the results in Tables 4-4 through 4-7 is that UV-A/B inactivation of *B. anthracis* and *B. subtilis* is partly effective on bare wood and unpainted concrete, ineffective on topsoil, but relatively effective on glass surfaces.

Tables 4-4 through 4-7 also show that the mean log reduction results did not always increase uniformly with increasing UV-A/B exposure time. In some tests with a particular microorganism/material combination the LR remained statistically the same (i.e., the 95% CIs overlapped) despite increased UV-A/B exposure. For example, with *B. anthracis* on glass the LR values were not significantly different for the 168-, 336-, and 672-h time points. This may be attributed to the relatively large CIs for these LR values, which are due to the variability of the coupon replicates. Additionally, the relatively low effectiveness of UV-A/B exposure on bare wood, unpainted concrete, and topsoil (i.e., mean log reductions rarely exceeding 1.5 logs) coupled with the 95% CIs may contribute to the lack of consistent increase in LR. An example case is the 336 h test with *B. subtilis* on unpainted concrete, for which the LR is low compared to the 168 h result, and without any overlap of the 95% CIs. This observation may be due to variability introduced by the separate and unique nature of each UV-A/B exposure test (as discussed in Section 2.2).

Table 4-4a. UV-A/B Inactivation of *Bacillus anthracis* (Ames) Spores[a] – 24 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	9.43×10^7	7.87 ± 0.13	82 ± 23	-
Test Coupons[c]	9.43×10^7	4.10 ± 0.30	0.016 ± 0.011	3.77 ± 0.29
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	9.43×10^7	6.86 ± 0.09	7.9 ± 1.5	-
Test Coupons	9.43×10^7	6.17 ± 0.15	1.6 ± 0.52	0.69 ± 0.15
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	9.43×10^7	7.60 ± 0.27	47 ± 21	-
Test Coupons	9.43×10^7	6.79 ± 0.05	6.6 ± 0.72	0.81 ± 0.24
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	9.43×10^7	7.43 ± 0.20	31 ± 11	-
Test Coupons	9.43×10^7	8.53 ± 0.10[f]	370 ± 86[f]	-1.10 ± 0.19
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B
[e]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.
[f]Unusual result; investigation of this result discussed in Section 4.1.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-4b. UV-A/B Inactivation of *Bacillus subtilis* Spores[a] – 24 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	9.93×10^7	7.49 ± 0.02	31 ± 1.7	-
Test Coupons[c]	9.93×10^7	4.90 ± 0.27	0.092 ± 0.046	**2.59 ± 0.24**
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	9.93×10^7	6.50 ± 0.06	3.2 ± 0.45	-
Test Coupons	9.93×10^7	5.58 ± 0.25	0.43 ± 0.23	0.92 ± 0.23
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	9.93×10^7	5.31 ± 0.31	0.25 ± 0.18	-
Test Coupons	9.93×10^7	4.87 ± 0.29	0.087 ± 0.050	0.44 ± 0.37
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	9.93×10^7	8.18 ± 0.56[f]	260 ± 220[f]	-
Test Coupons	9.93×10^7	7.87 ± 0.08	77 ± 15	**0.31 ± 0.50**
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction. Mean log reductions shown in bold are significantly different from mean log reductions for *B. anthracis* with the same UV-A/B exposure and coupon material.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d] Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e]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.
[f]Unusual result; investigation of this result discussed in Section 4.1.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-5a. UV-A/B Inactivation of *Bacillus anthracis* (Ames) Spores[a] – 168 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	8.37×10^7	7.20 ± 0.17	20 ± 8.6	-
Test Coupons[c]	8.37×10^7	1.95 ± 1.83	0.0019 ± 0.0025	5.25 ± 1.61
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	8.37×10^7	6.41 ± 0.26	3.6 ± 2.0	-
Test Coupons	8.37×10^7	5.26 ± 0.29	0.27 ± 0.24	1.16 ± 0.34
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	8.37×10^7	5.93 ± 0.14	1.1 ± 0.30	-
Test Coupons	8.37×10^7	5.28 ± 0.29	0.27 ± 0.17	0.65 ± 0.28
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	8.37×10^7	7.11 ± 0.07	16 ± 2.6	-
Test Coupons	8.37×10^7	7.02 ± 0.05	13 ± 1.6	0.09 ± 0.08
Laboratory Blank	0[f]	0	0	-
Procedural Blank	0[f]	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e] 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.
[f]Endogenous organisms were found in uninoculated topsoil blanks; no organisms other than *B. anthracis* Ames were found on inoculated coupons.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-5b. UV-A/B Inactivation of *Bacillus subtilis* Spores[a] – 168 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	1.01×10^8	7.85 ± 0.14	73 ± 23	-
Test Coupons[c]	1.01×10^8	4.01 ± 0.09	0.010 ± 0.0023	3.84 ± 0.15
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	1.01×10^8	6.00 ± 0.09	1.0 ± 0.21	-
Test Coupons	1.01×10^8	5.89 ± 0.28	0.87 ± 0.43	**0.11 ± 0.23**
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	1.01×10^8	7.03 ± 0.02	11 ± 0.59	-
Test Coupons	1.01×10^8	4.59 ± 0.67	0.094 ± 0.12	**2.44 ± 0.59**
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	1.01×10^8	7.76 ± 0.08	58 ± 10	-
Test Coupons	1.01×10^8	7.02 ± 0.20	11 ± 5.1	**0.75 ± 0.19**
Laboratory Blank	0[f]	0	0	-
Procedural Blank	0[f]	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction. Mean log reductions shown in bold are significantly different from mean log reductions for *B. anthracis* with the same UV-A/B exposure and coupon material.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e]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.
[f]Endogenous organisms were found in uninoculated topsoil blanks; no organisms other than *B. subtilis* were found on inoculated coupons.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-6a. UV-A/B Inactivation of *Bacillus anthracis* (Ames) Spores[a] – 336 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	1.11×10^8	6.94 ± 0.33	9.8 ± 6.5	-
Test Coupons[c]	1.11×10^8	1.14 ± 1.56	0.00025 ± 0.00035	5.81 ± 1.40
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	1.11×10^8	6.18 ± 0.33	1.7 ± 1.4	-
Test Coupons	1.11×10^8	5.17 ± 0.66	0.35 ± 0.56	1.01 ± 0.64
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	1.11×10^8	6.37 ± 0.28	2.5 ± 1.7	-
Test Coupons	1.11×10^8	4.16 ± 0.90	0.082 ± 0.17	2.21 ± 0.83
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	1.11×10^8	7.35 ± 0.11	21 ± 5.0	-
Test Coupons	1.11×10^8	7.18 ± 0.02	14 ± 0.54	0.17 ± 0.10
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e]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 4-6b. UV-A/B Inactivation of *Bacillus subtilis* Spores[a] – 336 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	1.04×10^8	7.49 ± 0.17	31 ± 9.1	-
Test Coupons[c]	1.04×10^8	3.50 ± 0.39	0.0039 ± 0.0025	**3.99 ± 0.37**
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	1.04×10^8	6.16 ± 0.31	1.7 ± 1.3	-
Test Coupons	1.04×10^8	5.22 ± 0.66	0.35 ± 0.43	0.94 ± 0.64
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	1.04×10^8	6.99 ± 0.14	9.8 ± 2.7	-
Test Coupons	1.04×10^8	6.51 ± 0.09	3.2 ± 0.80	**0.48 ± 0.15**
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	1.04×10^8	6.95 ± 0.40	11 ± 6.4	-
Test Coupons	1.04×10^8	7.02 ± 0.17	11 ± 3.8	-0.08 ± 0.38
Laboratory Blank	0[f]	0	0	-
Procedural Blank	0[f]	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction. Mean log reductions shown in bold are significantly different from mean log reductions for *B. anthracis* with the same UV-A/B exposure and coupon material.

[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).

[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.

[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.

[e]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.

[f]Endogenous organisms were found in uninoculated topsoil blanks; no organisms other than *B. subtilis* were found on inoculated coupons.

CI = Confidence interval (± 1.96 × SE).

"-" Not Applicable.

Table 4-7a. UV-A/B Inactivation of *Bacillus anthracis* (Ames) Spores[a] – 672 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	1.04×10^8	6.15 ± 0.29	1.6 ± 0.71	-
Test Coupons[c]	1.04×10^8	1.43 ± 0.96	0.00028 ± 0.00061	4.72 ± 0.88
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	1.04×10^8	5.97 ± 0.12	0.93 ± 0.24	-
Test Coupons	1.04×10^8	4.46 ± 0.67	0.050 ± 0.041	1.51 ± 0.60
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	1.04×10^8	6.59 ± 0.10	3.9 ± 0.95	-
Test Coupons	1.04×10^8	5.08 ± 0.61	0.30 ± 0.49	1.51 ± 0.55
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	1.04×10^8	7.93 ± 0.14	85 ± 32	-
Test Coupons	1.04×10^8	7.73 ± 0.09	52 ± 9.4	0.20 ± 0.15
Laboratory Blank	0[f]	0	0	-
Procedural Blank	0[f]	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e]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.
[f]Endogenous organisms were found in uninoculated topsoil blanks; no organisms other than *B. anthracis* Ames were found on inoculated coupons.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

Table 4-7b. UV-A/B Inactivation of *Bacillus subtilis* Spores[a] – 672 Hour Exposure

Test Material	Inoculum (CFUs)	Mean of Logs of Observed CFUs	Mean % Recovery	Mean Log Reduction ± CI
Glass				
Positive Controls[b]	7.83×10^7	6.76 ± 0.17	7.8 ± 2.3	-
Test Coupons[c]	7.83×10^7	1.47 ± 1.37	0.00027 ± 0.00034	5.29 ± 1.21
Laboratory Blank[d]	0	0	0	-
Procedural Blank[e]	0	0	0	-
Bare Pine Wood				
Positive Controls	7.83×10^7	6.02 ± 0.09	1.4 ± 0.27	-
Test Coupons	7.83×10^7	4.76 ± 0.21	0.081 ± 0.046	1.27 ± 0.20
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Unpainted Concrete				
Positive Controls	7.83×10^7	5.49 ± 0.06	0.40 ± 0.066	-
Test Coupons	7.83×10^7	3.34 ± 0.09	0.0029 ± 0.00059	2.15 ± 0.10
Laboratory Blank	0	0	0	-
Procedural Blank	0	0	0	-
Topsoil				
Positive Controls	7.83×10^7	7.74 ± 0.05	70 ± 7.6	-
Test Coupons	7.83×10^7	7.59 ± 0.09	50 ± 10	0.15 ± 0.09
Laboratory Blank	0[f]	0	0	-
Procedural Blank	0[f]	0	0	-

[a]Data are expressed as mean of the logs of total number of spores (CFUs) observed on individual coupons, percent recovery, and mean log reduction.
[b]Inoculated, not exposed to UV-A/B (spore recovery conducted after conclusion of UV-A/B exposure).
[c]Inoculated, exposed to UV-A/B. UV-A/B exposure accumulated in alternating 12-h periods of light and darkness.
[d]Laboratory Blank = Not inoculated, placed with positive control coupons and not exposed to UV-A/B.
[e]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.
[f]Endogenous organisms were found in uninoculated topsoil blanks; no organisms other than *B. subtilis* were found on inoculated coupons.
CI = Confidence interval (± 1.96 × SE).
"-" Not Applicable.

5.0 SUMMARY

Spores of *B. anthracis* (Ames) and *B. subtilis* were exposed to UV-A/B radiation simulating normal sunlight on test surfaces of glass, bare pine wood, unpainted concrete, and topsoil, for periods of 24, 168, 336, and 672 h. Those UV-A/B exposures were accumulated in alternating 12-h periods of light and darkness, e.g., the 168-h UV-A/B exposure was accumulated over 14 days. The numbers of viable spores remaining on each test coupon after UV-A/B exposure were compared to the numbers remaining on positive control coupons kept in the same test environment for the same time period but not exposed to UV-A/B light. The inactivation of spores by each UV-A/B exposure was calculated as the mean log reduction in the number of spores on coupons of each material.

Table 5-1 shows the mean log reduction results for both *B. anthracis* and *B. subtilis* on each of the four materials at each of the four successive UV-A/B exposure time points. The 95% confidence interval (CI) is also shown for each mean log reduction value. Table 5-1 shows that UV-A/B inactivation was far more effective for both *B. anthracis* and *B. subtilis* on glass than on any of the other coupon materials. Maximum mean log reduction results for both *B. anthracis* and *B. subtilis* on glass exceeded 5 logs. Mean log reductions on bare pine wood and unpainted concrete were similar across both materials and both organisms, primarily falling in the range of about 1 to 2 logs at all UV-A/B exposure time points. Topsoil exhibited the lowest UV-A/B inactivation results, with mean log reductions that varied around zero and never reached 1 log reduction for either organism.

Mean log reduction results did not always increase significantly with increasing UV-A/B exposure time, probably because of test coupon replicate variability (resulting in a large CI relative to the mean LR observed), and test-to-test variability in spore inoculation, temperature and RH conditions, and other test procedures. Furthermore, it is possible that the spore application procedure did not produce a single layer of spores on the test coupons, leading to protection of underlying spores from the UV-A/B exposure. This may also explain some of the observed variability in results. Additional research would be needed to investigate this hypothesis.

Table 5-1 indicates those cases in which statistically significant differences in mean log reduction were found between the two organisms. Of the 16 such comparisons, seven show significant differences in mean log reduction between *B. subtilis* and *B. anthracis*. No clear pattern is evident in the occurrence of these seven cases with coupon material or UV-A/B exposure duration.

Table 5-1. Summary of Mean Log Reduction Results with UV-AB (Simulated Sunlight Exposure on Four Materials

Material	Mean Log Reduction (± 95% CI) by UV-A/B Exposure Time[a]							
	24 h		168 h		336 h		672 h	
	B.a.	***B.s.***	***B.a.***	***B.s.***	***B.a.***	***B.s.***	***B.a.***	***B.s.***
Glass	3.77 (±0.29)	**2.59** (±0.24)	5.25 (±1.61)	3.84 (±0.15)	5.81 (±1.40)	**3.99** (±0.37)	4.72 (±0.88)	5.29 (±1.21)
Bare Pine Wood	0.69 (±0.15)	0.92 (±0.23)	1.16 (±0.34)	**0.11** (±0.23)	1.01 (±0.64)	0.94 (±0.64)	1.51 (±0.60)	1.27 (±0.20)
Unpainted Concrete	0.81 (±0.24)	0.44 (±0.37)	0.65 (±0.28)	**2.44** (±0.59)	2.21 (±0.83)	**0.48** (±0.15)	1.51 (±0.55)	2.15 (±0.10)
Topsoil	-1.10[b] (±0.09)	**0.31** (±0.50)	0.09 (±0.08)	**0.75** (±0.19)	0.17 (±0.10)	-0.08 (±0.38)	0.20 (±0.15)	0.15 (±0.09)

[a]*B.a.* = *B. anthracis* (Ames), B.s. = *B. subtilis*. Values in bold for *B. subtilis* are significantly different from corresponding values for B. anthracis.

[b]Unusual spore recoveries seen; see text.

6.0 REFERENCES

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