Article

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High-Dose Ultraviolet C Light Inactivates Spores of *Bacillus Atrophaeus* and *Bacillus Anthracis* Sterne on Nonreflective Surfaces

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Introduction

Ultraviolet light (UV) is frequently used in limited clinical settings to reduce the risk of nosocomial infections. The World Health Organization Global Solar UV Index (UVI) divides UV into A (315-400 nm), B (280-315 nm), and C (100-280 nm) bands. Light in the C band (UV-C) is listed in the UVI as the most harmful to living organisms because of its propensity to damage DNA and RNA. It is also the least relevant in the natural setting since it is completely filtered by the atmosphere and does not reach Earth's surface in levels measurable with commercially available equipment.

Often small surface areas or airflow in high-risk areas are treated with UV-C to decrease infectious microorganism populations. Other means of decontamination are sometimes employed when a large area has been contaminated. Gaseous disinfection with ethylene oxide, chlorine dioxide, or formaldehyde is costly, hazardous to workers and the environment, and requires prolonged evacuation of the treatment area (Rehork et al., 1990). Liquid disinfectants must be manually applied and removed and may damage exposed materials such as electrical devices. Ionizing radiation will kill in adequate doses but is hazardous to workers, difficult to contain, and not practical for general working space disinfection (Rehork et al., 1990).

The possibility of using UV-C (254 nanometer range) to decontaminate or sterilize work areas and to avoid the problems listed above led to the development by two of the authors (JLD, DRD) of the Ultraviolet Area Sterilizer (UVAS). The device is unique in that it generates intense levels of UV-C and then utilizes measured UV-C intensities reflected from the walls, ceilings, floors, or other treated areas to calculate the operation time to deliver the programmed lethal dose for infectious microorganisms. UV-C has been found to be highly effective against a wide spectrum of microorganisms (Banrud & Moan, 1999; Druce et al., 1995; Inamoto et al., 1979; Knudson, 1985). The development of a method to deliver a lethal and predictable UV-C dose can greatly increase the potential uses for UV-C in decontamination. Since the biological activity of UV-C is not limited to microorganisms, the UVAS has multiple safety features including remote controls, motion sensors, and audible voice warnings. These safety features were active during the course of this study.

The ability of the UVAS device to deliver lethal doses of UV-C to bacterial spores on nonreflective surfaces was evaluated by comparing the susceptibilities of *Bacillus atrophaeus* (formerly named *B. subtilis* var. *niger*, and *B. globigii*) and *Bacillus anthracis* Sterne spores to incremental UV-C doses. Additionally, the susceptibility of *Bacillus atrophaeus* spores in the presence of silica-containing powder to simulate a bioterrorism attack weapon was tested to see if this modification altered the efficacy of UV-C deactivation.

This investigation has shown that spore viability of both *Bacillus atrophaeus* as well as *B. anthracis* Sterne was significantly and reproducibly reduced by 3-5 logs under extreme contamination levels following dosimetric UV-C exposure. Complete kill can be achieved when the spore contamination level is lower. These findings are consistent with those of Nicholson and Galeano (2003) and Knudson (1986). Spores of *Bacillus atrophaeus* in 1%-2% silica were likewise susceptible to killing by UV-C. However, the presence of gross particulate matter such as visible powder containing extremely high concentrations of spores significantly inhibits spore susceptibility to UV-C inactivation.

Materials and Methods

Bacterial Spore Suspensions/Preparations

B. atrophaeus 93-PBA-1 spore mixture (provided by the Armed Forces Radiobiology Research Institute (AFRRI), Bethesda, MD) contained 1%-2% silica by weight and had an initial concentration of 2.5 x 10^{11} Colony Forming Units (CFU)/g. This spore mix was diluted in 50% ethanol to achieve concentrations of 10^9, 10^5, 10^4, and 10^3 CFU/ml. The dry, free-flowing silica spore mixture closely simulates a weapons-grade product used by bioterrorists. *B. anthracis* Sterne spores were produced at AFRRI using an inoculum from a live-spore veterinary vaccine (Colorado Serum Company, Denver, CO) as previously described (Elliott et al., 2002). The *B. anthracis* Sterne spores were washed twice in deionized sterile water and examined by phase-contrast microscopy to confirm that the refractile spore suspension was free of vegetative cells. The *B. atrophaeus* spores ATCC 9372 (Steris Corp., Mentor, OH) were at initial concentrations of 3.0 x 10^9 CFU/g and 2.5 x 10^{10} CFU/ml, respectively. Both of these spore products were suspended in 50% ethanol and used at a concentration of 10^9, 10^5, and 10^4 CFU/ml.

Test Surfaces

Aluminum plates, which measured 1276 cm^2, were painted with high heat-stable, flat-black enamel (7778-822, Rust-Oleum, Vernon Hills, IL). These test surfaces were autoclaved prior to being evenly spread with suspensions of *B. atrophaeus* ATCC 9372 and *B. anthracis* Sterne spores in ethanol. The dark surface was intended to minimize reflectance and, therefore, measured primarily the effect of direct UV-C exposure. For the test using the dry, free-flowing *B. atrophaeus* (93-PBA-1) spore powder, sterile 90 mm Petri plates were used.

Spore Distribution

Two milliliters of the liquid spore suspensions in 50% ethanol were uniformly distributed on the metal test and control surfaces using sterile, plastic, cell spreaders. An additional 5 ml of 50% ethanol was used to facilitate the even distribution over the entire surface area. Test surfaces were air dried for a minimum of 2 hours before UV-C exposure.

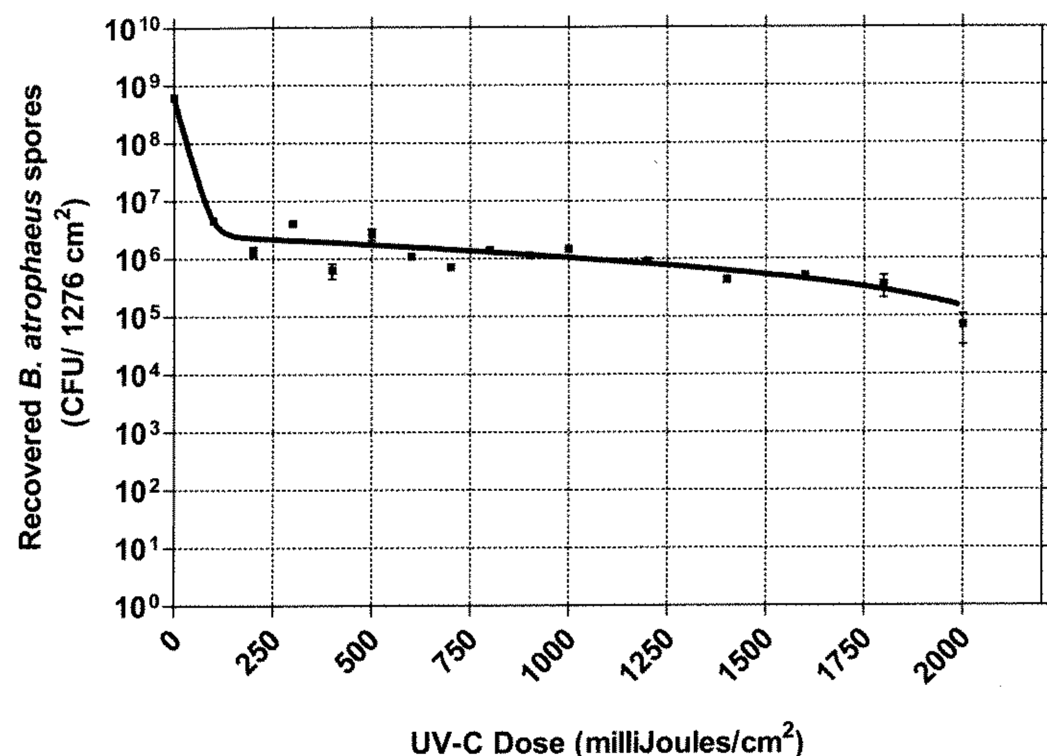
Dry *B. atrophaeus* (93-PBA-1) spore powder was spread in the base of sterile 90 mm Petri plates by adding a uniform dry measure to 16 dishes. Each dish was gently rotated and tilted by hand to achieve a relatively uniform distribution of the powder. Excess spore powder was removed into a beaker by inverting and tapping the plates. To reduce shadowing effects during UV-C exposure, the powder was wiped from the sides and corners of the dishes with sterile cotton swabs. The mass of spores remaining in each plate was determined by weighing each plate before and after addition of spores. Each plate received 20 to 50 mg of spore powder.

Exposure

The UVAS device employs 14 medium-pressure mercury bulbs (product #TUV 115W HHO, Philips Corporation, Sommerset, NJ) with a combined power output of approximately 3,000 microwatts/cm^2 at 1 meter. The device was used to expose test surfaces in a room measuring 25 x 35 ft. For the flat-black metal surfaces, cumulative UV-C doses were measured using two National Institute of Standards and Technology-calibrated dosimeters (PMA2100, Solar Light Company, Philadelphia, PA) placed on either side of the test surfaces and were reported as the average between both devices. For the Petri plates containing dry spore powder, a single PMA2100 dosimeter was placed in the center of the plate array as shown in Figure 1.

Figure 1

Recovery of *B. atrophaeus* spores containing ~1-2% silica after exposure to UV-C



Incremental doses of UV-C were administered to a total surface inoculum of 6.2×10^8 CFU/1276 cm^2. Following each dose, spores were recovered using Millipore swabs and Rodac contact plates. The average number of colonies were reported and used in calculations to determine the total viable spore count over the entire test surface. The curve represents the best-fit of a single exponential decay equation to the data.

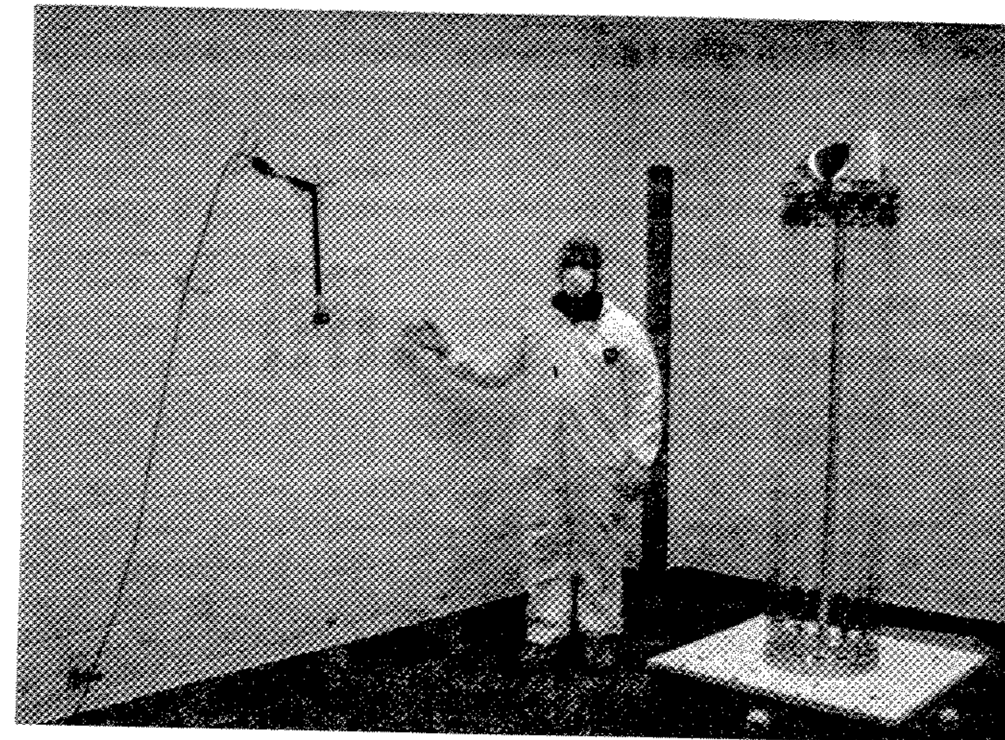
Spore Recovery and Culture Media

Two methods were used to recover spores from the metal test surfaces following each UV-C exposure. Rodac 45-mm contact culture plates containing trypticase soy agar with lecithin and polysorbate 80 (TSALP, PML Microbiologicals, Mississauga, ON, Canada) were used to recover viable spores from 28.3 cm^2 sample areas of the test surfaces by direct contact with the metal plates. Swab Dilution Samplers (MT0010025, Millipore Corp., Billerica, MA) were used for recovery of 16-cm^2 sample areas of the test surfaces. For each test surface, a minimum of three contact plates and one swab is used to obtain spore recovery at each targeted dose. The recovered spores adhering to the swabs were eluted in the Millipore buffer, subsequently diluted, and plated in triplicate as 150-µl aliquots onto 90 mm trypticase soy agar plates (TSA, BD Diagnostic Systems, Sparks, MD). Plates from both recovery methods were incubated at 35°C for 15 hours before colonies were counted. Average plate counts were used to calculate the number of viable spores remaining on the metal plates and were plotted as a function of UV-C dose to generate inactivation curves (Figures 1-3).

Spores in dry powder that were dispensed within the Petri plates were recovered by washing the Petri plates three times with sterile water (5 ml final volume) followed by recovering any material adhering to the bottom of the plate with a sterile rubber cell spreader. The suspensions were vortexed, diluted, inoculated as 150 µl aliquots onto TSA plates in triplicate, and incubated at 35°C for 15 hours before colonies were counted. Average plate counts were

Photo 1

UVAS prototype in test room with sensor and test plates containing a thin layer of dry *B. atrophaeus* spore powder



used to calculate the number of viable spores remaining in each exposure plate and plotted as a function of UV-C dose (Figure 4).

Calculations

Total Population per Aluminum Plate Surface Area:

Swab Dilution Samplers:

= Mean Plate Count x Dilution Factor ÷ Vol. Plated (0.150 ml) x Swab Dilution Sampler Vol. (18 ml) ÷ Aluminum Plate Surface Area Swabbed (16 cm^2) x Aluminum Plate Total Surface Area (1276 cm^2)

Rodac Contact Plates:

= Mean Plate Count ÷ Aluminum Plate Surface Area Contacted (28.3 cm^2) x Aluminum Plate Total Surface Area (1276 cm^2)

Results

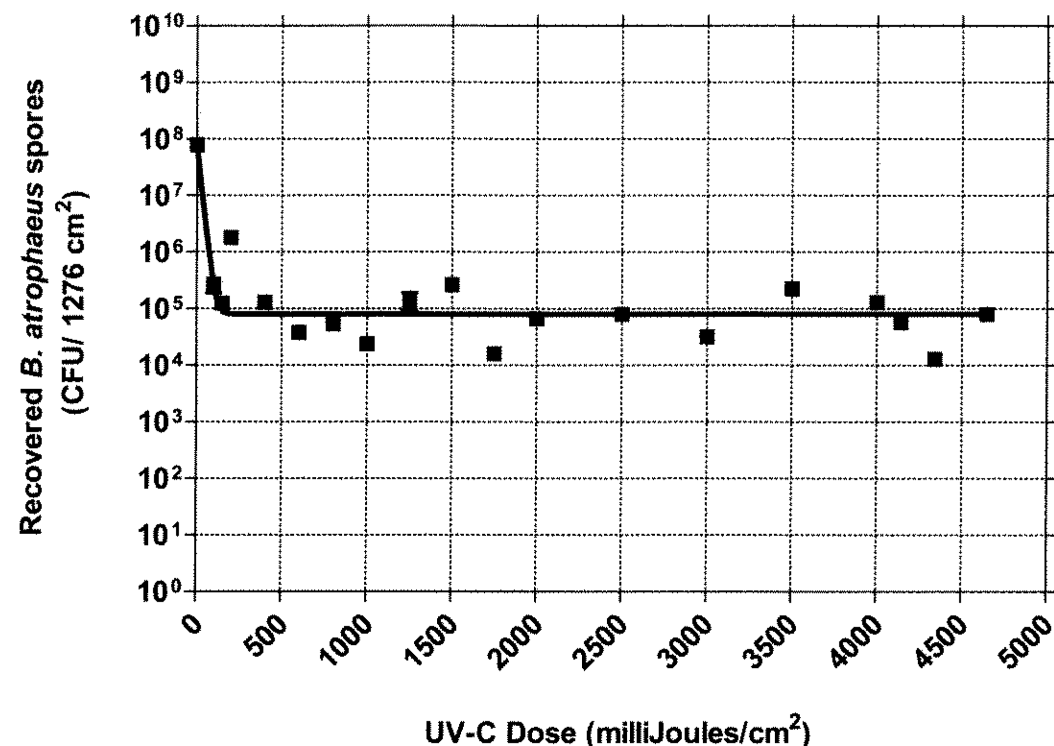
Inactivation of *B. atrophaeus* (93-PBA-1) Spores with Silica

At extremely high spore-inoculum levels, contact plates could not provide population data from the control, unexposed plates due to the high number of colonies, but the swabbing method gave a viable spore count of 6.2×10^8 CFU existed over the entire plate surface. The swab method was also the only procedure that could be used for recovery studies at lower UV-C doses of 100-600 milliJoules/cm^2 for the same reason. Results from the swab method indicated a 3-log reduction could be obtained at these UV-C exposure levels. At higher UV-C doses between 600-1,800 milliJoules/cm^2, a 3-4-log reduction was demonstrated with the swab recovery method and a 5-log reduction was obtained using the contact plates (Figure 1).

For the test plates spread with less concentrated spore suspensions (theoretically 10^5, 10^4, and 10^3

Figure 2

Recovery of *B. atrophaeus* (ATCC9372) spores from black-plate surfaces after exposure to UV-C.



Recovery of *B. atrophaeus* spores using the Millipore swab sampling recovery method. Curves represent best-fit of a single exponential decay equation to data.

CFU per test surface) recoveries were conducted using the contact plates alone after UV-C exposures of 1,000 to 2,000 milliJoules/cm^2. All plates indicated no survivors. Recoveries from unexposed control plates were 1 log lower than anticipated; therefore, at least a 4-log reduction can be ensured.

Control surfaces, which were not exposed to UV-C, did not show any reduction in colony counts during the course of the experiment, eliminating the possibility that recoveries varied with time.

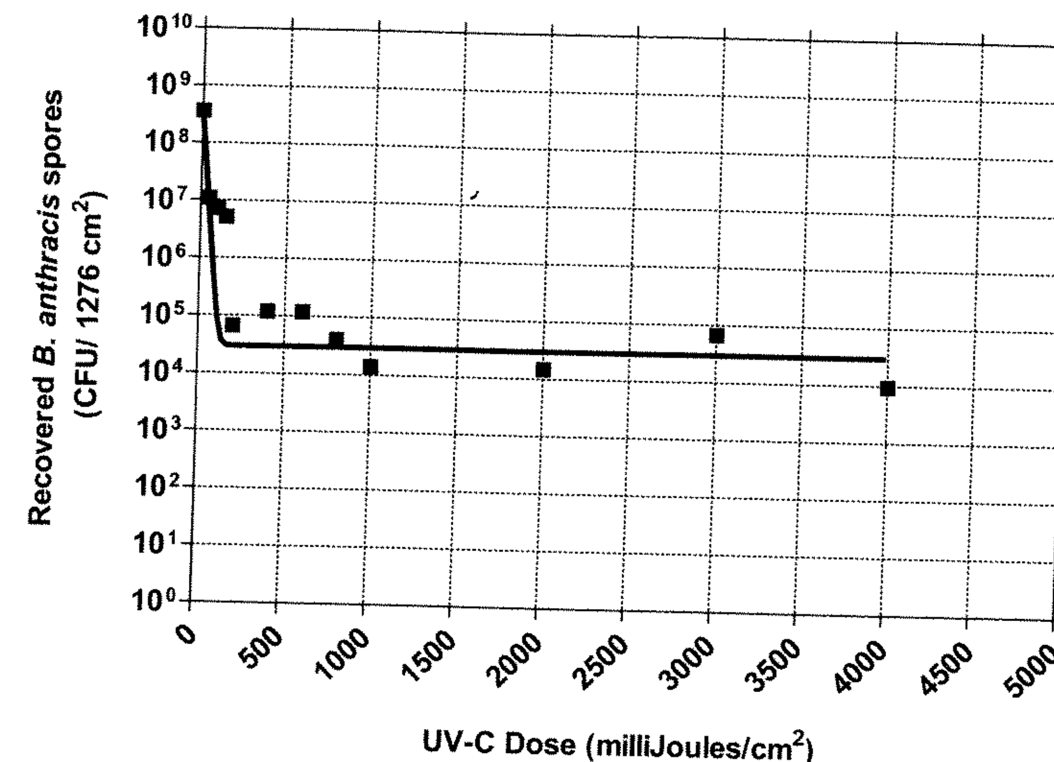
Inactivation of *B. atrophaeus* (ATCC 9372) Spores

Swab recoveries used for determining viable spore counts from the test plates spread with highly concentrated spore suspensions indicated a spore survival count of 10^8 CFU per test surface. This recovery method demonstrated a 2-log reduction at 50 milliJoules/cm^2 and a 3–4-log reduction at 100 to 4,647 milliJoules/cm^2 UV-C. The variability was attributed to the use of data from plates in which usable counts were less than 30 CFU per plate. Contact plates, which were used only at the doses from 2,000 to 4,000 milliJoules/cm^2 UV-C, indicated that a 5-log reduction could be obtained in comparison to the unexposed spore population determined by swabbing (Figure 2).

The control, contact plate viability counts from the less-concentrated test plates, theoretically 10^4 and 10^5 CFU, indicated a 2-log lower population than expected. The number of survivors was quite often zero for all the UV-C doses evaluated from 50 to 2,000 milliJoules/cm^2 except in some instances low colony counts were obtained, which may have been environmental contamination.

Figure 3

Recovery of *B. anthracis* Sterne spores from black plate surfaces after exposure to UV-C.



Recovery of *B. anthracis* Sterne spores using the Millipore swab sampling recovery method. Curves represent best-fit of a single exponential decay equation to data.

B. anthracis Sterne Spores

Millipore swab recoveries from nonirradiated test surfaces indicated 3.6 x 10^8 CFU. Swab recoveries from UV-C doses of 150 and 1,000 milliJoules/cm^2 resulted in a three and four log reduction respectively. Contact plates used for recoveries on the test surface exposed to UV-C doses of 2,000 to 4,000 milliJoules/cm^2 demonstrated a 4 log reduction (Figure 3).

Test surfaces spread with the diluted spore inoculum had 10^4 CFU per test surface and showed total kill when exposed to 50 to 1,000 milliJoules/cm^2.

Inactivation of Dry Spore Powder

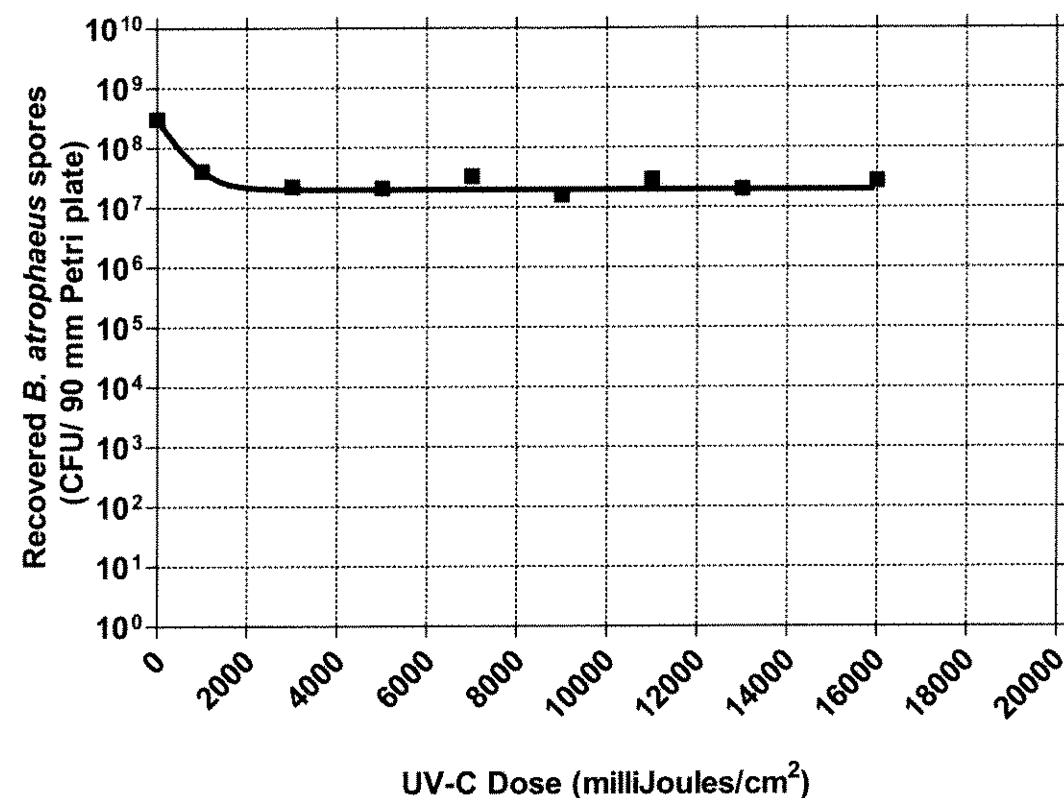
The last evaluation used dry, free-flowing *B. atrophaeus* spore powder in open Petri plates, with spore counts of 10^8 to 10^9 CFU per plates. A 1-log reduction was obtained from duplicated plates after exposures of 10,000 or 16,000 milliJoules/cm^2 (Figure 4).

Discussion

This investigation has demonstrated that UV-C generated by the UVAS in the absence of visible particulate matter can be delivered at lethal doses on nonreflective, non-porous surfaces for partial spore reduction even when the contamination levels are extremely high and for total spore reduction in the presence of less-concentrated spore populations. A 3-5-log reduction can be assured following UV-C exposure to contamination levels simulating those used in bioterrorist weapons—10^8 to10^9 CFU/1276 cm^2 or 10^5 to 10^6 CFU/cm^2—using doses of one hundred to several thousand milliJoules/cm^2 (Figures 1, 2, and 3). In situations where the bioburden levels are more

Figure 4

Recovery of a visible layer of dry *B. atrophaeus* spores containing ~1% silica on Petri plates after exposure to UV-C.



The curve represents the best-fit of a single exponential decay equation to the data.

representative of the contamination in areas such as operating/emergency rooms ($\leq 10^2$ CFU/cm²), UV-C is capable of completely deactivating the entire population at lower doses, most likely less than 100 milliJoules/cm². Test surfaces contaminated with high numbers of spores that are subsequently spread across the surface area as may occur during precleaning were readily decontaminated or sterilized with adequate doses of UV-C.

Spores at contamination levels of 10^5 to 10^6 CFU/cm² and applied in powder dense enough to be visible to the naked eye indicated a 1-log reduction after UV-C doses of 1,000 to 16,000 milliJoules/cm² (Figure 4). These findings emphasize the need for precleaning contaminated surfaces soiled with gross material. Use of a precleaning step, such as HEPA-vacuuming or damp wiping, for heavily contaminated surfaces in the presence of visible soil, followed by UV-C exposure, should effectively decontaminate the area or surface. This is substantiated by the data in which total kill was demonstrated from surfaces contaminated with less-concentrated spore suspensions in the absence of visible powder.

The presence of 1%-2% silica does not impede the germicidal effect of UV-C since the lethality was similar to that observed in the absence of silica. This finding demonstrates that the efficacy of UV-C is not altered in the presence of low concentrations of particulate matter or soil that could be present after precleaning.

Prior studies in animal laboratory settings using UV light showed a significant reduction of bacterial loads, and the addition of a chemical disinfectant followed by UV-C treatment was "particularly successful, reducing bacterial loads to extremely low levels" (Dix et al., 1992). In this study where the organisms were spread on the test surface without deactivation or removal by any cleaning agent, a significant spore reduction was observed after UV-C irradiation.

A recent study by Nicholson and Galeano (2003) found "the data indicate that standard UV treatments that are effective against *B. subtilis* spores are likely also sufficient to inactivate *B. anthracis* spores, and spores of standard *B. subtilis* strains could reliably be used as a biodosimetry model for the UV inactivation of *B. anthracis* spores" Our investigations have confirmed this finding.

Previous experience with the UVAS device suggested smooth materials that reflected UV-C may be more readily decontaminated than rough, nonreflective materials. Data obtained through this investigation will be useful in planning surface decontamination for many environmental applications, since determining the required decontamination doses should be based upon information obtained using the least reflective test surfaces, thus avoiding underexposure.

From these experimental findings one may reasonably conclude that the UVAS device, or other UV-C generating devices, could decontaminate areas in which surface contamination was 10^2 CFU/cm² and could be used to decontaminate extremely concentrated surfaces as long as a precleaning step was instituted.

Author's Note

The *B. anthracis* Sterne spores were washed twice in deionized sterile water and examined by phase-contrast microscopy to confirm that the refractile spore suspension was free of vegetative cells.

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