

High-Dose Ultraviolet C Light Inactivates Spores of *Bacillus subtilis* var. *niger* and *Bacillus anthracis* Sterne on Non-reflective surfaces

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Introduction

Ultraviolet light (UV-C) is frequently used in limited clinical settings to reduce the risk of nosocomial infections. Often small surface areas or air-flow 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 (6). 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 is not practical for general working space disinfection (6).

The possibility of using UV-C (254 nanometer range) to decontaminate or sterilize work areas and avoid the problems listed above led to the development of TRU-D (Total Room Ultraviolet Disinfectant). The device is unique in that it utilizes measured UVC intensities reflected from the walls, ceilings, floors or other treated areas and calculates the operation time to deliver the programmed lethal dose for infectious microorganisms. UV-C has been found highly effective against a wide spectrum of micro-

organisms. Developing a method to deliver a lethal and predictable UV-C dose increased the potential use for UV-C in decontamination (1,3,4).

The ability of the TRU-D device to deliver lethal doses of UV-C to bacterial spores on non-reflective surfaces was evaluated by comparing the susceptibilities of *Bacillus subtilis* var. *niger* and *Bacillus anthracis* Sterne spores to incremental UV-C doses. Additionally, the susceptibility of *Bacillus subtilis* var. *niger* spores in the presence of 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 *B. subtilis* var *niger* as well as *B. anthracis* Sterne was significantly reduced, reproducibly by 3-5 logs, under extreme contamination levels following dosimetric UV-C exposure. Complete kill can be achieved when the contamination level is lower. These conclusions reflect the findings of Nicholson and Galeano (5) and Knudson (7). Spores of *B. subtilis* var *niger* 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 organisms inhibits spore susceptibility significantly.

Materials and Methods

Bacterial Spore Suspensions/Preparations.

B. subtilis var. *niger* (*B. globigii*) spores (93-PBA-1, Armed Forces Radiobiology Research Institute (AFRRI), Bethesda, MD) suspension containing 20% silica at a concentration of 9.2×10^{10} CFU/g was diluted in 50% ethanol to achieve concentrations of 10^9 , 10^5 , 10^4 , and 10^3 CFU/ml. Another stock suspension containing the same spore species at 2.5×10^{11} CFU/g was mixed with powder to produce a dry, free-flowing mixture that simulated a bioterrorist attack weapon. *B. anthracis* Sterne spores (AFRRI) and *B. subtilis* var. *niger* spores ATCC 9372 (Steris Corp., Mentor, OH) were at initial concentrations of 3.0×10^9 CFU/g and 2.5×10^{10} CFU/mL respectively. Both of these suspensions were diluted 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 cm², were painted with high heat-stable flat-black enamel (7778-822, Rust-Oleum, Vernon Hills, IL). Test surfaces were autoclaved prior to spore distribution. 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 spore powder, sterile 90 mm petri plates were used.

Spore Distribution.

Two milliliters of the liquid spore suspensions were distributed on the 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. *B. subtilis* var. *niger* (93-PBA-1) dry spore powder was spread in the base of sterile 90 mm petri plates by adding a uniform dry measure to sixteen 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 TRU-D device employs 14 medium pressure mercury bulbs (product #TUV 115W HHO, Philips Corporation, Sommerset, NJ). The device was used to expose test surfaces in a room measuring 25 X 35 ft. 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 was reported as the average between both devices.



TRU-D prototype in test room with sensors and contaminated test plates.

Spore recovery and culture media.

Two methods were used to recover spores from the 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 from 28.3 cm² sample areas of the test surfaces by direct contact of the plate. Swab Dilution Samplers (MT0010025, Millipore Corp., Billerica, MA) were used for recovery of 16-cm² sample areas of the test surfaces. For each inoculated test surface,

a minimum of three contact plates and one swab were used to obtain the 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- μ l 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 hr before colonies were counted. Data was reported as an average plate count. Spores in dry powder that were dispensed within the Petri plates were recovered by washing three times with sterile water (5 ml final volume) followed by suspending any material adhering to the bottom of the plate with a sterile rubber cell spreader. The suspensions were vortexed, diluted, inoculated as 150 μ l aliquots onto TSA plates in triplicate, and incubated at 35°C for 15 hours before colonies were counted. Average plate counts were reported.

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 cm²) x
Aluminum Plate Total Surface Area (1276
cm²)

Rodac Contact Plates:

= Mean Plate Count /Aluminum Plate
Surface Area Contacted (28.3 cm²) x
Aluminum Plate Total Surface Area (1276
cm²)

Results.

Inactivation of *B. subtilis* var. *niger* (93-PBA-1) Spores from Silica Preparation.

At extremely high contamination levels contact plates could not provide population data from the control, unexposed plates, due to the high number of microorganisms, but the swabbing method indicated 6.2 x10⁸ CFU existed over the entire plate surface. The swab method was also the only procedure that could be used for recovering at lower doses of 100 to 600 MilliJoules/cm² for the

same reason and indicated a three log reduction could be obtained at these exposure levels. At higher doses between 600 to 1,800 MilliJoules/cm², a three to four log reduction was demonstrated with the swab recovery method and a five log reduction was obtained using the contact plates. A five log reduction was obtained from both methods at 2,000 milliJoules/cm² (Figure 1). For the less concentrated test plates (theoretical 10⁵, 10⁴, and 10³ CFU per test surface) recoveries were conducted using the contact plates alone after exposures of 1,000 to 2,000 milliJoules/cm². All plates indicated no survivors. Recoveries from unexposed control plates were one log lower than anticipated; therefore, at least a four 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. subtilis* var. *niger* (ATCC 9372) Spores.

Swab recoveries used for determining viable spore counts from the extremely concentrated test plate indicated a spore survival count of 10⁸ CFU per test surface. This method demonstrated a two-log reduction at 50 MilliJoules/cm² and a three to four log reduction at 100 to 4,647 MilliJoules/cm². The variability was attributed to the use of data from plates in which usable counts were less than 30 CFU per plate, and quite often less than 10 CFU per plate. Contact plates that were used only at the doses from 2,000 to 4,000 MilliJoules/cm² indicated that a five log reduction could be obtained in comparison to the unexposed population determined by swabbing (Figure 2). The control, contact plate population counts from the less concentrated test plates, theoretically 10⁴ and 10⁵ CFU, indicated a two log lower population than expected. The number of survivors was quite often zero for all the doses evaluated from 50 to 2,000 MilliJoules/cm² except in some instances low level growth was indicated, which may have been environmental contamination.

***B. anthracis* Sterne Spores**

Millipore swab recoveries from unexposed test surfaces indicated 10^8 CFU. Swab recoveries from doses of 150 and 1,000 MilliJoules/cm² resulted in a three and four log reduction respectively. Contact plates used for recoveries on the test surface exposed to doses of 2,000 to 4,000 MilliJoules/cm² demonstrated a 4 log reduction (Figure 3). Test surfaces contaminated with the diluted inoculum indicate 10^4 CFU per test surface and indicated total kill when exposed to 50 to 1,000 MilliJoules/cm².

Inactivation of Dry Spore Powder.

The last evaluation in which the spores were laden with powder to represent an extremely high challenge for UV-C efficacy indicated 10^8 to 10^9 CFU from control plates. A one log reduction was obtained from duplicated plates after exposures of 10,000 or 16,000 milliJoules/cm² (Figure 4).

Discussion

This investigation has demonstrated that UV-C generated by the TRU-D in the absence of visible particulate matter can be delivered at lethal doses on non-reflective, 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 three to five log reduction can be assured following UV-C exposure to contamination levels simulating those used in bioterrorist weapons, 10^8 to 10^9 CFU/1276 cm² or 10^5 to 10^6 CFU/cm², using doses of one hundred to several thousand milliJoules/cm² (Figures 1,2 and 3). In situations where the bioburden levels are more 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 total 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 UVC. 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 one 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 the 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 significant reduction of bacterial loads: however, the addition of a chemical disinfectant followed by UV-C treatment was “particularly successful, reducing bacterial loads to extremely low levels.”(2) 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 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 biosimetry model for the UV inactivation of *B. anthracis* spores.”(5) The investigations conducted have confirmed this finding. Previous experience with the TRU-D device suggested smooth materials that reflected UV-C may be more readily decontaminated than rough, non-reflective 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 conclusions one may reasonably assume the TRU-D 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.

Figures and legends

Figure 1. Recovery of *B. subtilis* var. *niger* spores containing ~1- 2% silica after exposure to UV-C.

Incremental doses of UVC were administered to a total surface inoculum of 6.2×10^8 CFU/1276 cm². 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.

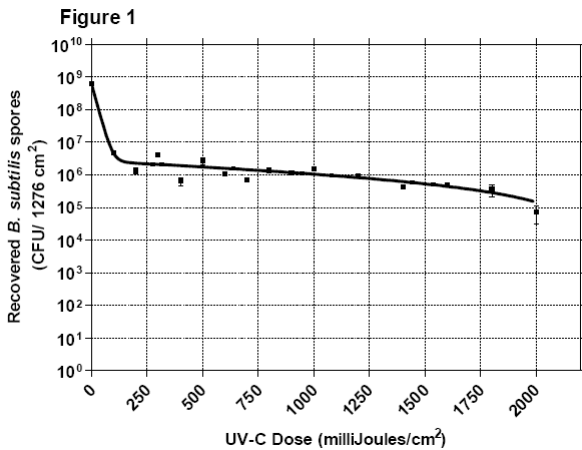


Figure 2. Recovery of *B. subtilis* var. *niger* (ATCC9372) spores from black plate surfaces after exposure to UV-C.

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

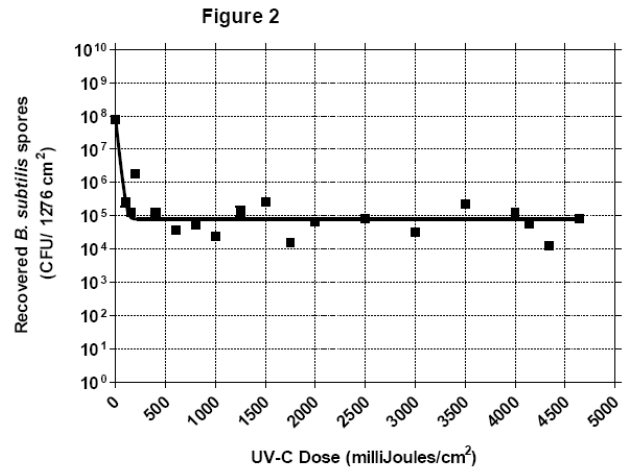


Figure 3. Recovery of *B. anthracis* Sterne spores from black plate surfaces after exposure to UV-C

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.

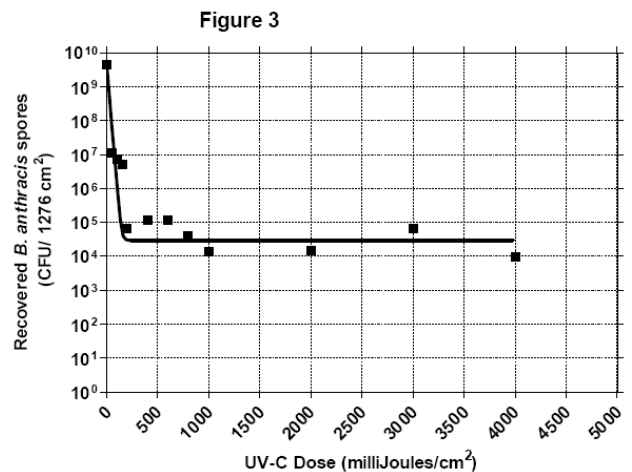
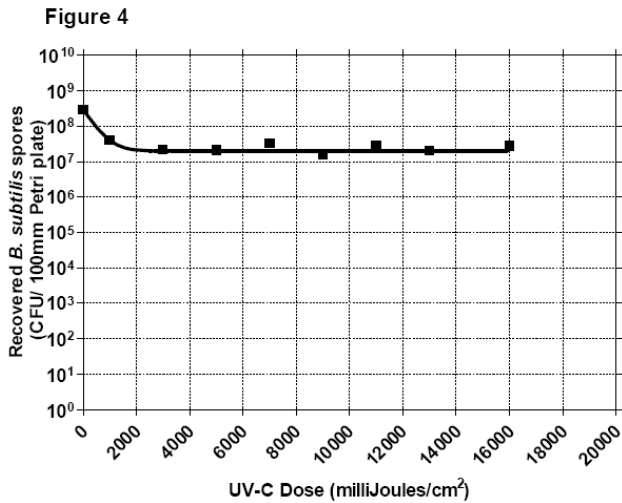


Figure 4 Recovery of a visible layer of dry *B. subtilis* var. *niger* spores containing ~1% silica on Petri plates after exposure to UV-C.

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



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