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BIOLOGICAL EFFICACY OF THE JOINT BIOLOGICAL AGENT DECONTAMINATION SYSTEM, INTERIOR-DECONTAMINATION CAPABILITY (JBADS LITE)

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14. ABSTRACT
This study evaluated microorganisms with varying levels of resistance to disinfection and provides data to expand the utility of the JBADS process. Protocols were established for preparing biological indicators (BIs) containing various microorganisms to compliment JBADS field testing efforts. The BIs were qualified in terms of concentration, stability, and resistance to a range of Biothermal Decontamination (BTD) treatments under controlled laboratory conditions. Estimated the kill rates of the BIs at various BTD conditions ranging in dew points from 50 to 139 °F were determined. Laboratory efficacy results provided a basis for estimating minimum target dew points or equivalent temperature and humidity conditions needed to achieve a 4-log reduction (LR) within 8 hours for each type of pathogen. Thereafter, BIs were incorporated in two JBADS Lite field demonstrations to assess the effectiveness of the BTD treatment. The kill rates generated for the diverse types of BIs in this study can be used as an aid in selecting the decontamination cycle (time, temperature, and relative humidity) to achieve a desired LR for a given type of pathogen using the JBADS Lite.

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JBADS, log reduction, biological indicators, SARS-CoV-2, pathogens

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 EXECUTIVE SUMMARY

The Joint Biological Agent Decontamination System (JBADS) Interior Decontamination Capability (JBADS Lite) was created for the decontamination of public transport vehicles using BioThermal Decontamination (BTD). During the development, field demonstrations were performed to assess the decontamination performance of the systems by measuring the viability of Biological Indicators (BIs) before, during, and after a decontamination cycle. Historically, the BIs used were small coupons inoculated with bacterial spores, indicative of the hardiest biological threats; specifically, *Bacillus thuringiensis kurstaki*, a surrogate to the traditional biological warfare agent *Bacillus anthracis*. However, there has been growing interest in combatting public health threats beyond the traditional biological warfare targets, especially during and after the Coronavirus (COVID) pandemic. Therefore, this study was performed to demonstrate the utility of JBADS Lite to address a broad range of organisms that could more closely resemble the next major threat to human health. To do so, BIs were prepared for microorganisms with varying levels of resistance to disinfection and evaluated against a variety of BTD conditions in the lab, using an environmental chamber, and in the field, using JBADS Lite.

Methods were established for preparing BIs with surrogates for various pathogens, including enveloped and non-enveloped virus (Phi6 and MS2 bacteriophages), Gram positive cells (*Staphylococcus epidermidis*), Gram negative vegetative cells (*Escherichia coli*), fungal spores (*Aureobasidium sp., Cladosporium sp.*, and *Penicillium sp.)*, and bacterial spores (*Bacillus thuringiensis kurstaki* (Btk) and *Bacillus atrophaeus* (Bg)). The BIs were qualified in terms of concentration, stability, and resistance to a range of BTD treatments under controlled laboratory conditions. Thereafter, BIs were incorporated in JBADS field demonstrations to assess the effectiveness of BTD treatment.

Laboratory tests were conducted to determine the Log_{10} Reduction (LR) versus time for the BIs against six BTD conditions representing a range of target dew points from 50 degrees Fahrenheit (°F) to 139 °F. The results were used to approximate the BTD condition(s) that achieved a 1 LR within 2 hours (or 3-4 LR in 6-8 hours) per organism. A field study was performed at Guardian Center (Perry, GA) to assess the kill rates of BIs prepared with Phi6 bacteriophage, a surrogate to Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-CoV-2) virus, when placed in a public transport vehicle (railcar) and treated with JBADS Lite. A second field demonstration was performed at the manufacturer's facility in Orlando, FL to assess kill rates of a broader range of BIs in a railcar treated with JBADS Lite. Field data were compared to corresponding lab-scale BTD tests.

In the Guardian Center Demonstration, all three demonstration trials achieved >4 log reduction (LR) of Phi6 within 2 hours, even though JBADS Lite did not reach the desired dew point of 139 °F. The maximum dew points achieved were 129, 128, and 125 °F for demonstrations 1-3, respectively. The time required to transition from one railcar to another was demonstrated to be 45 minutes, allowing multiple railcars to be decontaminated in a single workday with only one JBADS Lite unit.

The Orlando Demonstration demonstrated the utility of JBADS Lite to decontaminate a subway railcar that was contaminated with diverse types of organisms including virus, vegetative bacteria, and fungal spores. Three BTD conditions were evaluated [143 °F @ 50% relative

humidity (RH); 120 °F @ 90% RH; 120 °F @ 50% RH] and all achieved 4 LR for the virus and bacterial cell BIs in <6 hours. Two conditions also achieved 4 LR in <6 hours for the fungal BIs. As expected, these conditions did not significantly impact the viability of the bacterial spore BIs. Overall, the field efficacy data were comparable to the laboratory efficacy data, particularly at the higher temp/RH conditions. While it is difficult to make conclusions based on a limited data set (one experiment per condition), reasonable correlation and reasonable kill times (<6 hours) for a variety of organisms were observed.

The kill rates generated for the diverse types of BIs in this study can be used as an aid in selecting the decontamination cycle (time, temperature, and RH) to achieve a desired LR for a given type of pathogen using the JBADS Lite. Table 1 shows the D-values (time to reduce the population by 1 log or 90%) as determined in laboratory testing. These values can be multiplied by the desired LR to determine the total decontamination time required. Field kill rates were generally comparable to these laboratory results; however, this comparison is based on a limited number of data points. Replicate tests are recommended to refine this correlation and the calculated D-values, particularly those less than 0.5 hours.

Table 1. Summary of D-values from Laboratory Testing

Decontamination Conditions						
Temperature	143 °F	143 °F	143 °F	120 °F	120 °F	120 °F
Relative Humidity	90%	50%	10%	90%	50%	10%
Dew Point	138 °F	118 °F	67 °F	117 °F	97 °F	50 °F
Organism	**D-values in Hours**					
Phi6	0.07	0.12	1.08	0.10	0.27	6.7
MS2	nd	0.09	nd	0.20	nd	nd
S. epidermidis	0.08	0.14	8.1	1.4	2.6	4.9
E. coli	0.04	0.07	4.1	0.2	0.8	6.3
Aureobasidium	0.06	0.12	3.8	0.2	0.9	4.0
Cladosporium	0.16	0.25	3.5	0.3	1.2	10.4
Penicillium	0.16	0.22	6.7	0.3	1.6	83
Bt	3,068	nd	nd	nd	nd	nd
Bg	22,430	nd	nd	nd	nd	nd

nd = not determined

2.0 MISSION DESCRIPTION

JBADS was originally designed as a biological decontamination system for simultaneous interior and exterior decontamination of a C-130J or J-stretch aircraft. The original JBADS is comprised of an insulated aircraft enclosure (AE), three heat-hydration modules (ADU-Hs); one cooling unit (ADU-C); and a control module (CM). JBADS is designed to generate an environment in and around the aircraft that is consistent with BTD – specifically 170 ± 5 °F and 90 ± 5% RH – and maintain these conditions for at least 96 hours. AeroClave is the JBADS prime contractor and conducts system operation and sustainment.

Under the JBADS acquisition program, similar interior-only decontamination demonstrations were conducted on C-130 and C-17 aircraft using a subset of the system components in a configuration known as JBADS Lite. In those demonstrations, JBADS ADU-Hs were used to generate the proper temperature and humidity to induce disinfection. The number of ADU-Hs needed is dependent primarily on two factors, the volume of the vehicle interior and the capability of the vehicle to hold heat (the insulation factor). In the full JBADS configuration, 3 ADU-Hs are used to create and maintain decontamination conditions in the 291,300 cubic feet (ft^3) AE. One ADU-H can condition a large volume, but it is oversized for most public transport vehicles: buses, railcars, etc. with interior volume of approximately 5,000 cubic feet (ft^3). Therefore, a scaled-down version of the JBADS system, shown in Figure 1, was created for the decontamination of public transport vehicles. The new JBADS Lite System consists of a Vehicle Decontamination Unit (VDU – heat, fans, steam dispersion), water tank, reverse osmosis system, generator, fuel tank, and four bulk containers for duct work and miscellaneous support items. All subcomponents are mounted to a flatbed trailer for ease of transport and maneuverability. A mounting skid for rail transport was also added to allow access of the system to the end of a railcar. The new JBADS Lite system is utilized by connecting ductwork to the vehicle being decontaminated with air ducts and plenums inside the vehicle to distribute the air evenly. Dew point, temperature, and relative humidity are measured by sensors inside the vehicle, and the system is programmed to maintain the desired dew point for decontamination.

Figure 1. Current JBADS Lite configuration

(from left to right) Vehicle Decontamination Unit (Heat, Fans, Steam Dispersion), Water Tank, Reverse Osmosis System, Generator, Fuel Tank, 4 Bulk Containers (hidden by Conestoga fabric) for duct work and misc. items

During the development of JBADS Lite, field demonstrations were performed to assess the operational and decontamination performance of the system. When feasible, BIs were placed within the interior of the unit (aircraft, railcar, etc.) that was being decontaminated. The concentration of culturable spores on the BIs were measured before, during (if feasible), and after the decontamination cycle. The results were used to determine the level of disinfection/decontamination, expressed as LR versus time, achieved by the treatment.

3.0 OBJECTIVE

The goal of this study was to evaluate microorganisms with varying levels of resistance to disinfection and provide data to expand the utility of the JBADS process. To meet this goal, protocols were established for preparing BIs containing various microorganisms to compliment JBADS field testing efforts. The BIs were qualified in terms of concentration, stability, and resistance to a range of BTD treatments under controlled laboratory conditions. Thereafter, BIs were incorporated in JBADS field demonstrations to assess the effectiveness of BTD treatment.

The primary technical objectives of the study were to:

1. Establish methods to prepare BIs with representative types of microorganisms.
2. Establish kill rates (LR vs. time) of the BIs against a panel of BTD conditions at bench-scale.
3. Identify BTD conditions that can achieve a 1 LR within 2 hours (or 3-4 LR in 6-8 hours) per organism.
4. Assess kill rates of BIs prepared with Phi6 bacteriophage, a surrogate to SARS-CoV-2 virus, when placed in public transport vehicle and treated to a JBADS Lite decontamination cycles (Guardian Center Demonstration).
5. Assess kill rates of the diverse types of BIs when treated via JBADS Lite in public transport vehicle and compare these rates to those generated corresponding lab BTD efficacy tests (Orlando Demonstration).

This study was intended to provide initial estimates for BTD conditions and efficacy against a range of microorganisms. Ideally, these estimates will be refined with future studies consisting of more microorganisms from each category, more replicates of each BTD condition, and more refined timelines for decontamination.

4.0 SCOPE

4.1 Organism Selections

Microorganisms for this study were chosen from each of the five tiers of organisms identified by common “hierarchy of susceptibility” pyramids such as the one shown in Figure 2. Therefore, the organisms chosen for study are representative surrogates for pathogens in their respective category. The selected organisms listed in Table 2 are Biosafety Level 1 (BSL-1) organisms that pose no threat to human health, making them ideal for field testing with respect to safe transport, storage, and handling.

In this study, METSS developed protocols for preparing BIs of the organisms on uncoated aluminum coupons and established the stability of these organisms for field testing. In addition, the resistance of each organism to disinfection was established for BTD conditions that are currently used for other pathogens (e.g., SARS-COV-2); referenced in the literature for similar classes of organisms; and readily achievable with the current BTD systems.

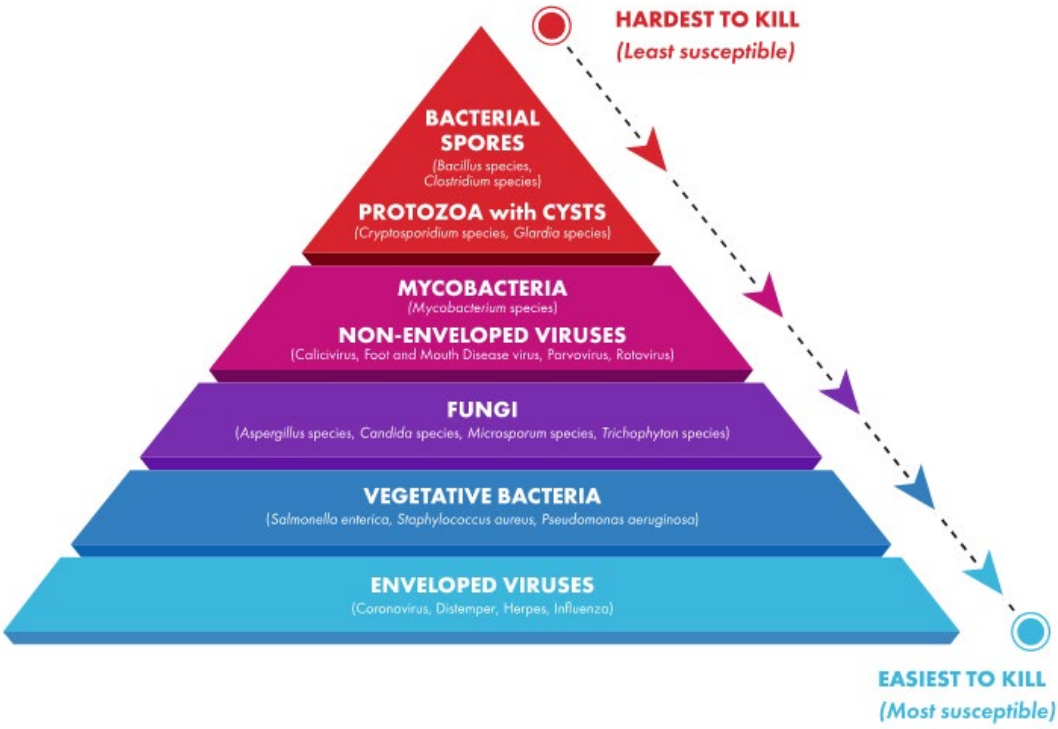


Figure 2. Hierarchy of Susceptibility

(https://www.bigred.com.sg/blogs/hierarchy-of-microbial-susceptibility-to-disinfection-and-sterilisation-processes/)

Table 2. Organism Selection

Organism Type	Organism	Surrogate Of	Comments
Virus, enveloped	Phi6 bacteriophage	SARS-CoV-2, Ebola, Herpes, Influenza, Reovirus, Picornavirus, Retrovirus, Rhabdovirus	Host bacteria is *Pseudomonas syringae*
Virus, non-enveloped	MS2 bacteriophage ATCC 15597	Smallpox, Adenovirus, Parvovirus, Rotovirus	More resistant to disinfection than bacteria and enveloped virus
Gram positive vegetative bacteria	*Staphylococcus epidermidis* ATCC 12228	Methicillin-Resistant *Staphylococcus aureus* (MRSA)	Quality control strain for food and media testing
Gram negative vegetative bacteria	Escherichia coli ATCC 11229	*E. coli* 0157, Tuberculosis sp, *Yersinia pestis*, etc.	Common strain used for standard disinfection testing
Fungus	*Aureobasdium pullulans* ATCC 15233	Common environmental fungi such as black mold (Stachybotrys)	AFRL reported most prevalent in previous C-5 study (Westover AFB)[1]
Fungus	*Cladosporium cladospoiodes* ATCC 16022	Pathogenic Cladosporium sp.; common indoor and outdoor mold in areas with humidity, moisture, and water damage	AFRL reported most prevalent in prior C-130 study (Peterson AFB)[1]
Fungus	*Penicillium crysogenum* ATCC 10106	Pathogenic *Penicillium* species	Common in the environment
Bacterial Spore	*Bacillus thuringiensis (from Tech-Grade DiPel)* *Bacillus atrophaeus sub. globigii* ATCC 9372	*Bacillus anthracis*	Common surrogates for *B. anthracis* spores

The bacteriophage listed are common surrogates for enveloped and non-enveloped viruses and frequently used for air and surface decontamination studies. Although Phi6 is recognized as the surrogate for SARS-CoV-2 virus, MS2 was included here and evaluated under a subset of BTD conditions to compare its stability and heat resistance to Phi6. The Gram positive and negative bacteria were selected based phenotype and genotype similarities with common bacterial pathogens. The three fungal strains listed were selected based on their prevalence in the environment and were shown be predominant contaminates recovered by AFRL during several aircraft inspection studies. The spore-forming bacteria *Bacillus thuringiensis* variety kurstaki (Btk) and *Bacillus atrophaeus* variety globigii (Bg) were selected as both have been used historically as surrogates for *B. anthracis*. Since bacterial spores are known to be very heat resistant, no inactivation was expected at the BTD conditions evaluated in this study. Therefore, only limited stability and efficacy testing was performed with these two organisms in this study to demonstrate that point.

4.2 BI Preparation and Characterization

BIs for each of the selected organisms were prepared following a standardized protocol (described in detail in section 5.1.1) and then characterized by measuring concentration and assessing stability during simulated field test storage and transport conditions. A main objective of this task was to down-select one organism from each category based on results of stability and lab-scale BTD efficacy testing. The first field test was performed to assess the ability of JBADS Lite to decontaminate a railcar contaminated with SARS-CoV-2; therefore, only Phi6 BIs were used. A second field study was performed to assess the kill rates of diverse types of pathogens; therefore, a panel of down-selected organism representing enveloped virus, non-spore forming bacteria (Gram positive and Gram negative, fungal spores, and bacterial spores were included in the field study.

Since bacteriophage and vegetative bacterial cells are far less robust (i.e., stable) than bacterial spores and fungal spores, the stability of organisms prepared in three organic test soils (aqueous organic solutions) was evaluated. Organisms prepared in organic soils are more stable post-drying than when prepared in an inorganic solution such as water or phosphate buffered saline (PBS). The stabilizing property of the test soil also generally makes organisms more resistant to disinfection. Screening a variety of virus and cell preparations afforded the ability to downselect the test soil that provided the best stability for each organism. Fungal and bacterial spores were not prepared in test soils as the organic constituents of the test soils would likely induce spore germination. The details of the BI preparation, analysis, and characterization are described in Section 4.

For the BIs to be useful for field testing, the organisms needed to remain stable (i.e., remain viable with minimal die off; ideally < 1LR) during the period of intended use for field testing. A period of 2 weeks was selected based on the following assumptions:

1. The BIs would be prepared and transported to a field test site ≤7 days prior to the test and stored refrigerated prior to use.
2. The field test would span several consecutive days.
3. The BIs would be assayed for viability immediately following field testing.

Stability testing was performed for BIs of each organism prepared in various test soils except for the fungal and bacterial spores. The stability results were then used to downselect the test soil that provided the best stability per organism type and use the test soil for preparation of the BIs that were used in lab and field efficacy tests. The stability results are discussed in Section 5.

4.3 Laboratory BTD Testing

A panel of six BTD conditions (Table 3) were evaluated against the selected organisms, excluding the bacterial spores. These conditions were chosen based on prior studies of the JBADS Lite system and similar BTD systems for interior-only decontamination without an insulated enclosure.[2,3,4] The target conditions were based on temperatures of 143 °F or 120 °F at 90%, 50%, or 10% RH; representing a broad range of dew points from 50 to 139 °F. Time-to-kill efficacy tests were performed at the bench-scale for each organism (excluding bacterial spores) at each condition using an environmental chamber where replicate BIs were removed at designated time points and analyzed for viability. The goal was to establish kill curves for each organism at each BTD condition. The kill curves, in turn, were then used to calculate the D-values (the time at a given condition to achieve a 90% reduction (or 1 LR)). The D-values were then used to calculate the approximate time to achieve a 4 LR at a given BTD condition. The results are discussed in Section 6.

Table 3. JBABS Lite Decontamination Conditions

BTD Condition No.	Temp. (°F)	Temp. (°C)	Relative Humidity (%)	Dew Point (°F)
1	143	62	90	139
2	143	62	50	118
3	143	62	10	67
4	120	49	90	117
5	120	49	50	97
6	120	49	10	50

4.4 Field Demonstrations

Two JBADS Lite field demonstrations were performed during this study, one at Guardian Center in Georgia using a retired Washington D.C. metropolitan subway railcar and the other at AeroClave in Florida using retired New York City railcar.

Guardian Center Demo - The goal of this demonstration was to assess the effectiveness of the JBADS Lite for decontamination of public transportation vehicles that are contaminated with a surrogate for infectious viruses. During the demonstration, hot/humid air was circulated throughout the interior of a subway railcar, and the environment within the vehicle was monitored continuously with temperature and humidity sensors. In addition, Phi6 bacteriophage

BIs, a surrogate for various enveloped viruses including SARS-CoV-2, were placed throughout the railcar to verify that the decontamination conditions neutralized corresponding viruses that pose a threat to human health. Three decontamination trials were performed.

Orlando Field Demo - The main goals of this demonstration were to provide data to (1) measure the rate of kill of various pathogens using surrogates of infectious virus, bacteria, and fungi in a railcar treated by JBADS Lite, (2) compare the data with kill rates generated in controlled laboratory tests using the same hot, humid air conditions, and (3) compare the kill rates of two decontamination treatments at the same dew point but achieved under different temperature and RH conditions. Three decontamination trials were performed.

The results and observations from the Guardian Center and AeroClave demonstrations are presented in Sections 7 and 8, respectively.

5.0 METHODS AND MATERIALS

5.1 BI Preparation

5.1.1. Organism Preparation

The first step in preparing the BI was to make dose suspensions at ≥ 1x 10^6 organisms per milliliter in test soils listed in Table 4. Fetal bovine serum (FBS), 3-Part,[5] and Tryptic Soy Broth (TSB) all contain proteins that provide stability to organisms and are commonly used in disinfection research. The target concentration for the BIs was a minimum of 10^5 organisms per BI (or 5 Log_{10} organisms per BI). This inoculation level would allow for at least a 5 LR to be measured over time during stability and efficacy testing. These suspensions were then used to dose sterile, non-painted, aluminum coupons (½" x ¾"). The method of preparation for the various types of dose suspensions are described:

Bacteriophage – Frozen concentrated stock suspensions (>10^{10} virions/milliliter (mL)) of each bacteriophage were diluted 1:10 in 100% FBS, 100% TSB, or 100% 3-Part test soil resulting in a final concentration of 90% of each test soil.

Vegetative Bacteria – Broth cultures were grown overnight in TSB at 35-37 degrees Celsius (°C) at 200 rotations per minute (rpm). The cultures were then washed by centrifuging the cells at 9,000 times gravity (xg) for 5 minutes, decanting the supernatant and resuspending the cell pellets with PBS. The centrifugation process was repeated resulting in a suspension of washed cells at >10^8 Colony Forming Unit CFU/mL. This suspension was then diluted 1:10 in 100% FBS, 100% TSB, or 100% 3-Part test soil resulting in a final concentration of 90% of each test soil.

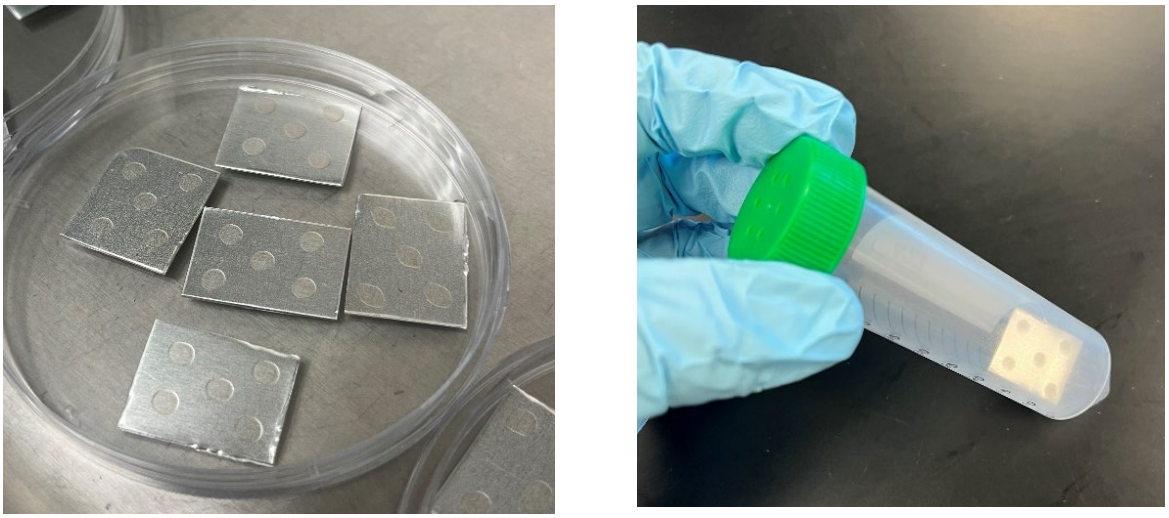
Fungi – Cultures acquired from ATCC were spread plated onto approximately 30 Potato Dextrose Agar (PDA) plates and incubated for 1-2 weeks at 27 °C to allow the fungi to grow and sporulate. The biomass was then harvested from the agar by adding 3-5 mL PBS to each plate and then scraping off the biomass with a spreader. The biomass was transferred to a sterile bottle with porcelain beads and shaken vigorously to separate spores from mycelium. The suspension was then filtered through a layer of sterile glass wool. The wool captures the mycelium but allows the spores to pass. The resulting filtrate

was enumerated to determine the titer. The concentration was adjusted accordingly by either concentrating or diluting the suspension with distilled water (dH_2O).

Bacterial Spores – A stock suspension of *B. thuringiensis* spores derived from Technical Grade DiPel (lot 256890-V9; September 7, 2016) was used as the dose suspension and prepared in sterile dH_2O. *Bacillus atrophaeus subsp. globigii* (Bg), acquired from Microbiologics, was grown to sporulation in Sporulation media[6] for 7 days at 30 °C and 300 rpm. Cultures were then filtered and pelleted at 16,000 xg for 10 minutes. The pellets were then resuspended in sterile water, enumerated to determine the titer, and stored refrigerated. The concentrated spore stock was then diluted accordingly with dH_2O to make a dose suspension at the desired concentration.

After preparing the dose suspension, the desired number of coupons to be inoculated were placed in Petri dishes (5-6 coupons per dish) within a biosafety cabinet (BSC). For each organism, five, 10-microliter (µl) aliquots of dose suspension were dispensed onto individual coupons using a calibrated pipet and allowed to dry in the BSC. [Note: BIs for one organism were prepared at a time to avoid cross-contamination.] The appearance of BIs post-drying is depicted in Figure 3. Once dry (1-2 hours), the coupons were placed into individual, 50-mL bioreactor tubes (i.e., tubes with vented caps; Figure 3) and stored refrigerated until used. The vented conical vials contain a nylon filter membrane in the cap with 0.22 micrometer (µm) nominal pore size that acts as a barrier to microbiology while allowing heat and humidity within the vial to equilibrate with the surrounding environment. The vented conical membrane ensures that the known quantity of test organisms remain within the vial and native environmental microbes do not contaminate the sample. Although there is no safety risk associated with the organisms used to prepare the BIs, the sealed samples can be manipulated easily while minimizing the potential for mishandling the BIs and/or cross-contamination.

The dose suspensions were enumerated using standard microbiology spread plating methods for bacteria and fungi, and a standard Double Agar Overlay (DAL) method for bacteriophage. *Pseudomonas syringae* and *E. coli* were used as host cultures for Phi6 and MS2, respectively. Bacteria and fungi concentrations were reported at CFU/mL and bacteriophage concentrations were reported at plaque forming units/milliliter (PFU/mL). For each batch of BIs, replicate BIs were also extracted and enumerated the same day they were prepared to establish a baseline concentration.



(A) (B)

Figure 3. Biological Indicators

(A) aluminum coupons inoculated with five, 10-µl droplets of dose suspension and dried; (B) BI in 50-mL bioreactor tube.

Table 4. Preparation of BI Inoculum

Organism	Organic or Inorganic Test Soils
Phi6 and MS2 bacteriophage	90% FBS 90% 3-Part[a] 90% TSB[b]
Staphylococcus epidermidis	
E. coli	
Aureobasdium pullulans	Sterile dH_2O
Cladosporium cladospoiodes	
Penicillium crysogenum	
Bacillus thuringiensis	
Bacillus atrophaeus	

[a]3-Part test soil is a mixture of high molecular weight peptides, low molecular weight peptides, and mucous material consisting of 0.5 g tryptone, 0.5 g bovine serum albumin, and 0.04 g bovine mucin per 10 mL.

[b]TSB is a common bacterial growth medium comprised of pancreatic digest of casein, papaic digest of soybean, and sodium chloride.

5.1.2. BI Analysis

BIs were analyzed for viability using the following extraction/enumeration procedure:

Ten milliliters (10 mL) of PBS were added to the 50-ml tube containing the BI and allowed to sit static for 5 minutes. Then, both sides of the BI surface were swabbed with

a nylon FLOQ Swab (Copan Diagnostics; part 502CS01). The swab was retained in the tube and agitated in an incubator shaker set at 200 rpm at ambient temperature for 10 minutes, then vortexed for 30 seconds. The extract was then serial diluted with PBS.

For bacteriophage, the concentration of infective virions in the extracts was measured using the DAL method in which the following constituents were combined and overlayed onto Tryptic Soy Agar (TSA): 0.2 mL of an overnight *Pseudomonas syringae* TSB culture, 0.1 mL of diluted extract, and 5 mL of molten TSA top agar supplemented with calcium chloride ($CaCl_2$). Depending on the anticipated level of kill for a given sample, duplicate aliquots of neat and diluted extracts were plated to achieve 0-250 PFU per plate. If complete kill was anticipated, the entire extract was plated (2-mL aliquots per plate x 5 plates). The plated samples were incubated overnight at 25-27 °C. PFUs were then counted and recorded. Plates with 25-250 PFUs were used to calculate total PFU recovered. The mean PFU recovered was then calculated as mean Log_{10} PFU recovered.

For vegetative bacteria, the concentration of culturable cells in the extracts were measured using a standard Drop Plate enumeration method in which triplicate 10-µl aliquots of neat (i.e., undiluted) and diluted extracts were dispensed onto TSA plates and allowed to dry. If near complete or complete kill was anticipated, standard spread plate and membrane filtration methods were used. All plated samples were incubated for 1-2 days at 35-37 °C. CFU were then counted and recorded. Plates with 25-250 CFU were used to calculate total CFU recovered. The mean CFU recovered was then calculated as mean Log_{10} CFU recovered.

For bacterial spores and fungi, standard spread plate and membrane filtration methods were used. Fungal samples were incubated at 25-27 °C until colonies reached a distinguishable size (2-3 days). Bacterial spore samples were incubated 35-37 °C for 1 day. If no colonies were visible, plates were incubated for additional 2 days.

The concentration of recovered organisms per BI was expressed as PFU or CFU/mL. This concentration was multiplied by a factor of 10 to account for the extract volume to determine the total PFU or CFU recovered, expressed at Log_{10} PFU or CFU recovered per BI.

5.1.3. Data Interpretation

For a given test, the mean Log_{10} recovered per replicate BI at each time point was plotted as Log_{10} PFU or CFU recovered versus time (minutes). The slope of the data during which linear decay was observed was used to determine the D-value (defined as the time required at a given condition to achieve a 90% reduction or 1 LR and calculated as the negative inverse of the slope). The D-value was then used to calculate the time at the given condition to achieve 4 LRs (the D-value multiplied by 4 hours).

6.0 STABILITY TESTING

The stability of the BIs over a 2-week storage period was assessed. BIs for each organism were prepared using test soils listed in Table 4. The BIs were incubated following simulated conditions of a common field test. Here, BIs would be prepared, transported and stored refrigerated for 7 days (representing the time the BIs would be stored at the test site until ready for use) at which time they were allowed to come to room temperature for 8 hours (representing the general period of time BIs would be removed from storage to conduct a decontamination

test), and then returned to refrigerated storage for another 7 days (simulating time for transport and storage prior to analysis).

6.1 Bacteriophage BI Stability

As shown in Figure 4, Phi6 (lot 090922P6; prep. 9/9/22) was equally stable in each of the three test soils evaluated with <1 LR observed over 2 weeks. MS2, however, was less stable in all three test soils, decreasing 2-4 logs within 7 days and > 6 logs by day 14 (Figure 5). MS2 was most stable in FBS. The poor stability of MS2 was unexpected since MS2 (a non-enveloped bacteriophage) is generally known to be more stable than Phi6 (an enveloped bacteriophage) as indicated in Figure 2. MS2's poor stability may be attributed to the age (approx. 7 months) of the MS2 stock (lot 1MS206; prep. 3/3/2022) used for testing. A new stock of MS2 was prepared (lot MS209; prep. 10/31/22) and evaluated again in FBS. Although still not as stable as Phi6, the newer lot of MS2 was moderately stable over two weeks as shown in Figure 6. A second possible cause of poor stability for MS2, however unlikely, may have been the potential of Hydrogen (pH) of the test soils. All three test soils evaluated were neutral ranging from pH 6.5 to 8.0. The FBS used in the study measured pH 8. The slightly alkaline pH may be more suitable to Phi6 than MS2. A study by Woods, et al.[7] demonstrated that blood (having similar organic constituents as FBS) provided better stability of Phi6 than MS2, which contradicted the general thinking behind the disinfection hierarchy that the nonenveloped viruses (such as MS2) should be more resistant than the enveloped viruses. This same study also showed MS2 was more stable in PBS than Phi6, which was consistent with the general thinking that enveloped viruses are less resistant than nonenveloped viruses to disinfectants.

In this study, Phi6 was more stable than MS2 and is generally recognized as an appropriate surrogate of SARS-CoV-2; therefore, Phi6 BIs were selected as the bacteriophage of choice for the Guardian Center and Orlando field demonstrations.

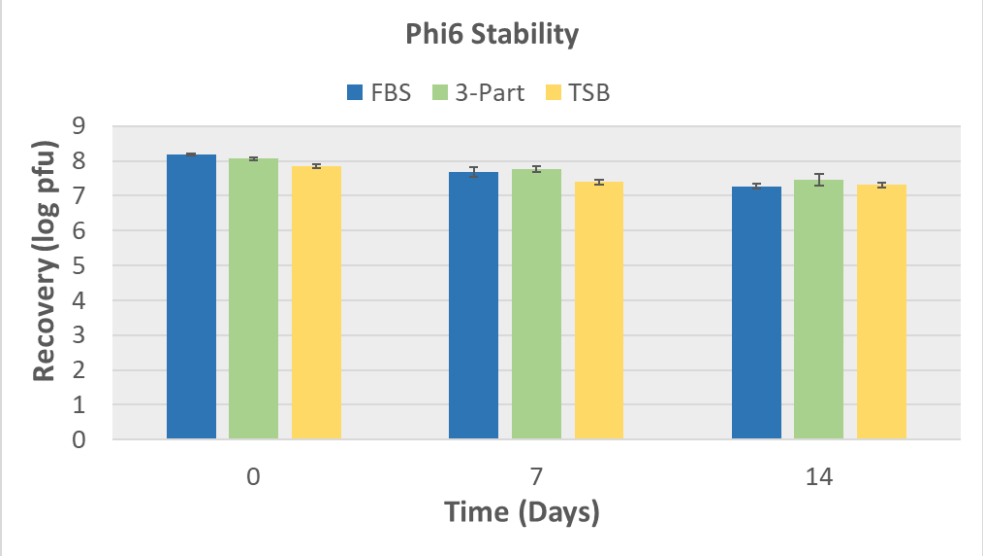


Figure 4. Stability of Phi6 Bacteriophage BI

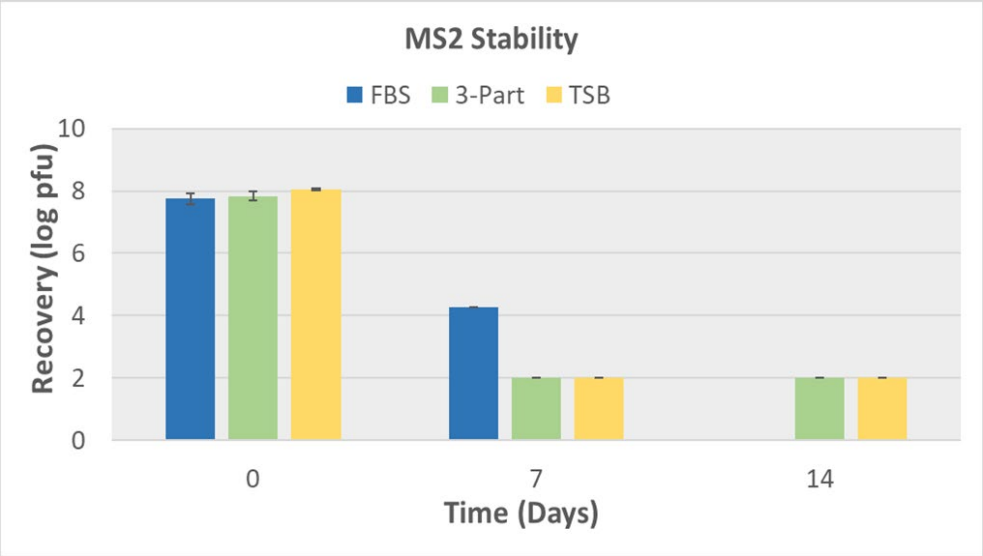


Figure 5. Stability of MS2 Bacteriophage BI

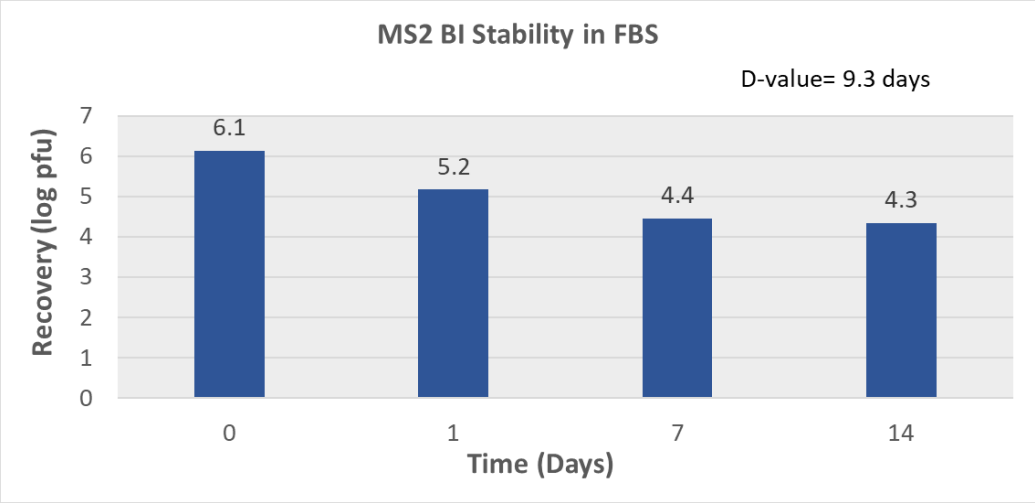


Figure 6. Stability of MS2 BI Prepared in FBS.

6.2 Bacterial Cell BI Stability

The stability of Gram positive bacterial (*S. epidermidis*) and Gram negative bacterial (*E. coli*) BIs in the three types of organic tests soils are shown in Figure 7 and Figure 8, respectively. *S. epidermidis* was very stable in FBS and TSB showing no die-off over 2 weeks, and nearly as stable in 3-Part test soil with approximately 1 LR reduction by week 2. *E. coli* was clearly far more stable in FBS (<0.5 LR over 2 weeks) than in the other two test soils. FBS became the test soil of choice for all subsequent testing.

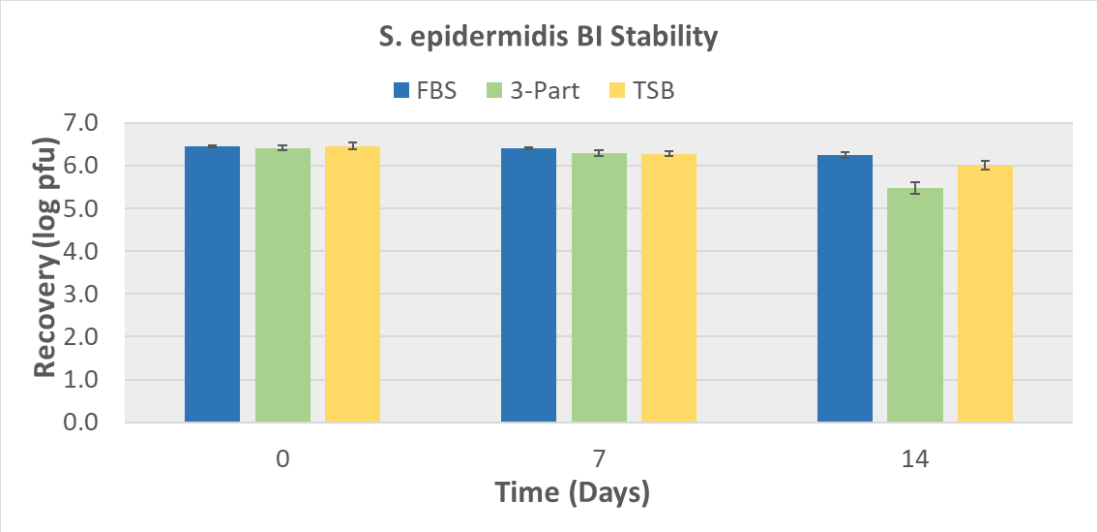


Figure 7. Stability of S. epidermidis BIs

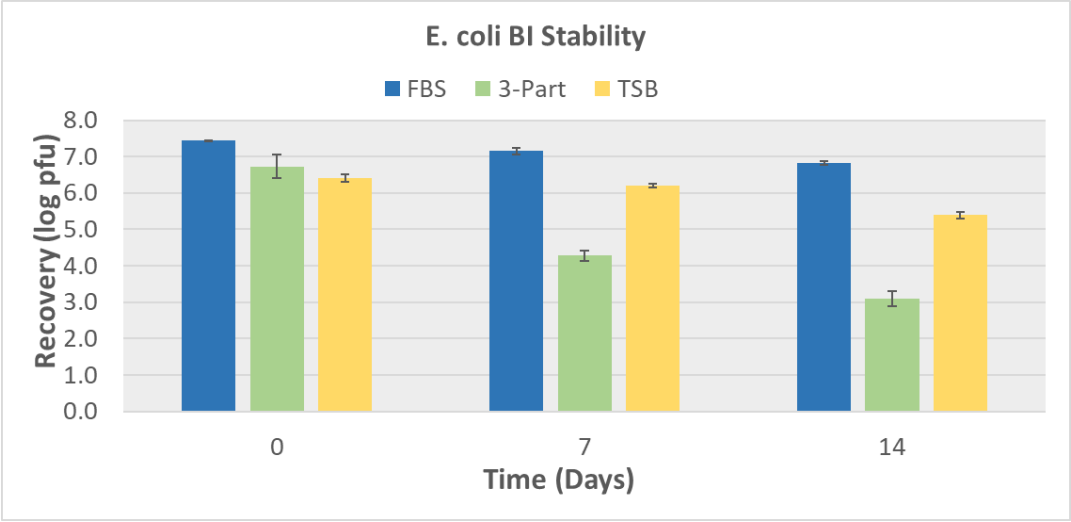


Figure 8. Stability of E. coli BIs

6.3 Fungal Spore BI Stability

The stability results for *Cladosporium*, *Penicillium*, and *Aureobasidium* fungal spores are shown in Figure 9, Figure 10, and Figure 11, respectively. *Cladosporium* and *Penicillium* showed moderately good stability with <1.5 LR and <0.5 LR over two weeks. Although the starting concentration of the *Aureobasidium* BIs was lower than desired, the spores were very stable with virtually no decay over 2 weeks. Since *Aureobasidium* was the most stable and was found to be predominant fungi present in a C-5 aircraft from a previous AFRL study,[1] this strain was selected to represent the fungi family in the Orlando Field test. The spore stock concentration was increased as much as possible via centrifugation/resuspension allowing for the BIs to be dosed at

a higher concentration. As forementioned, fungal and bacterial spores were not tested in organic test soils as these soils are not required to achieve the desired BI stability for these organisms.

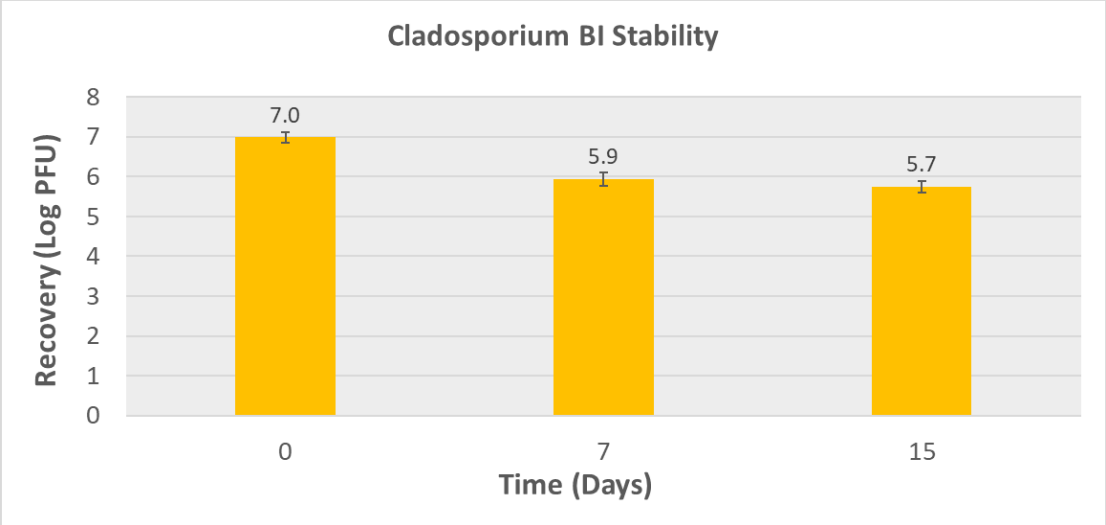


Figure 9. Stability of Cladosporium BIs

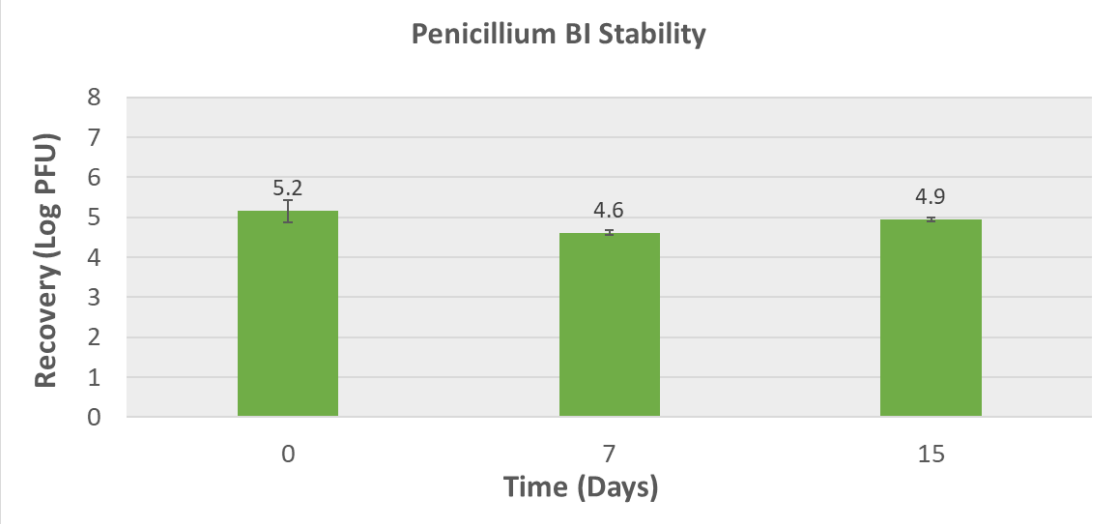


Figure 10. Stability of Penicillium BIs

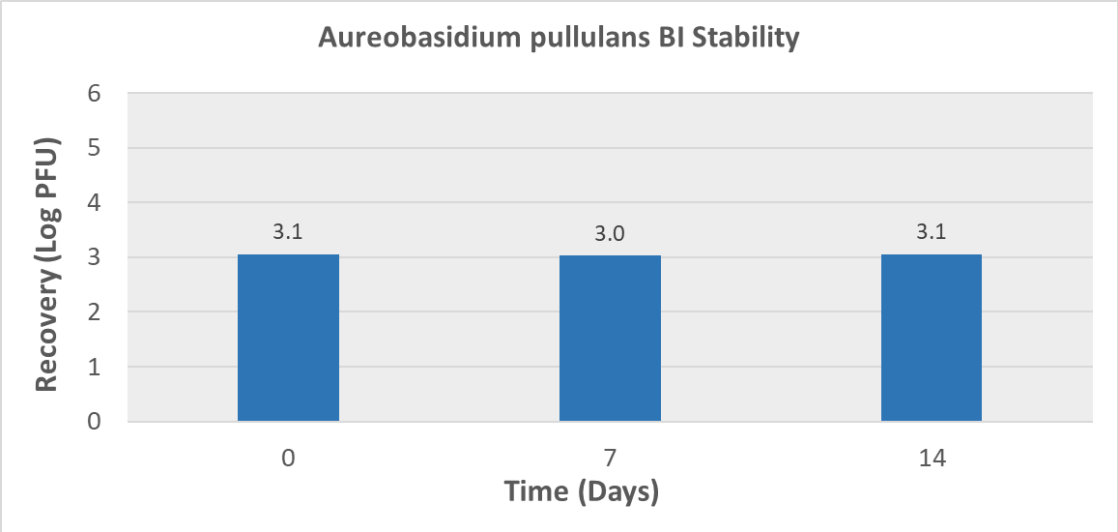


Figure 11. Stability of Aureobasidium BIs

6.4 Bacterial Spore BI Stability

The Btk and Bg spores were very stable in the BIs prepared as shown in Figure 12 and Figure 13, respectively. Since these spores are known to be very heat resistant, the BIs were only evaluated in one of the three trials performed during the Orlando Field test. The intent was to demonstrate the spores would be unaffected by the less harsh BTD conditions performed in the Field Test. One BTD efficacy test was conducted in the laboratory at the harshest of the six BTD conditions selected for this study (Condition 1 at 143 °F and 90% RH; 139 °F Dew Point) was also performed with Btk and Bg spore BIs.

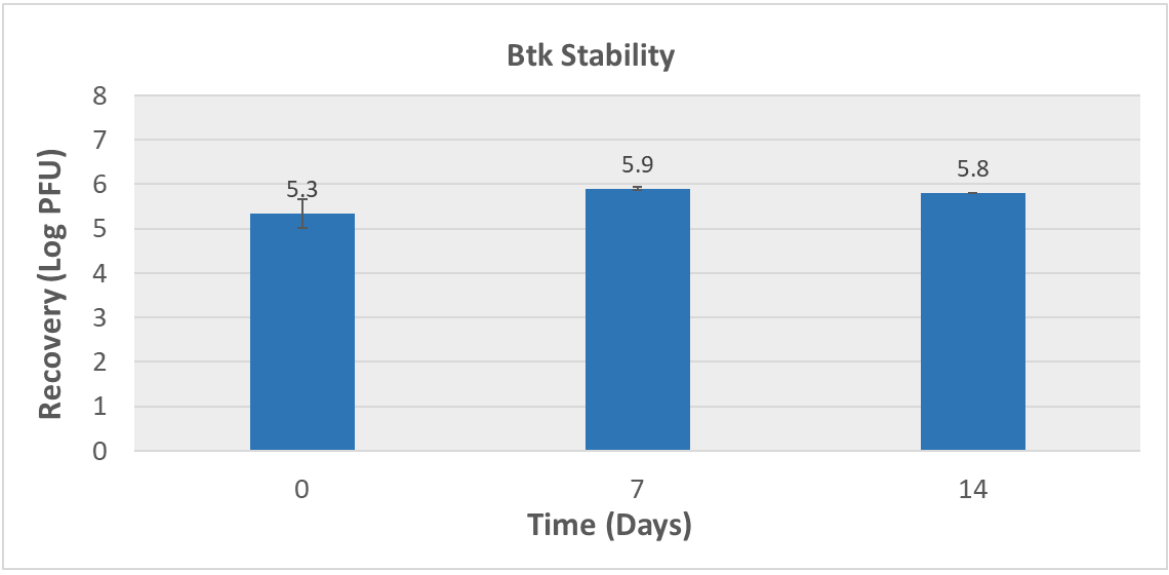


Figure 12. Stability of Btk Spore BIs

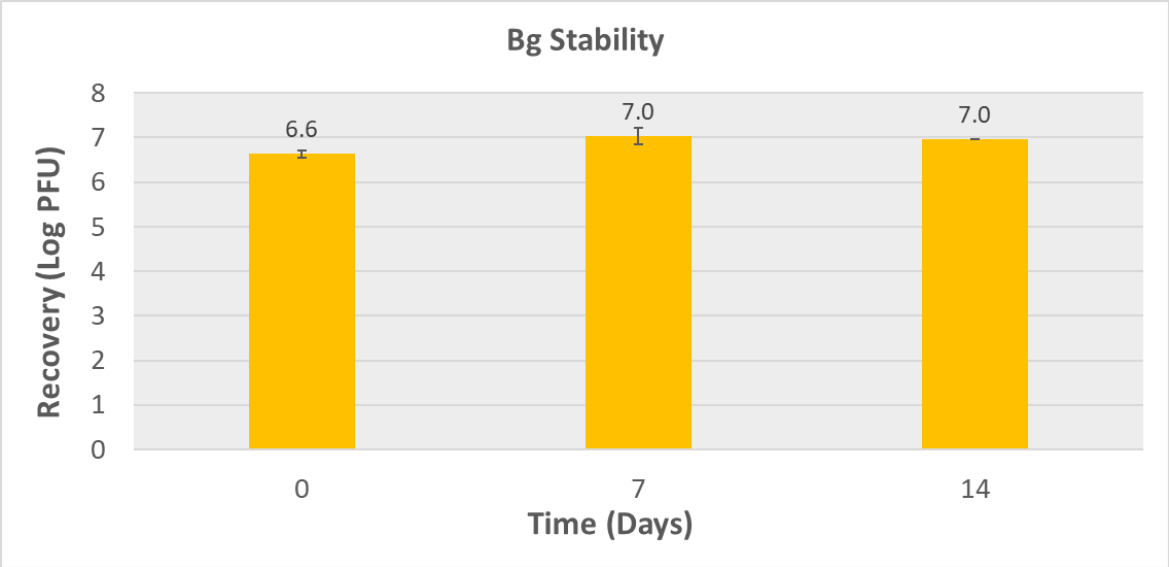


Figure 13. Stability of Bg Spore BIs

6.5 Discussion

The stability tests determined that that FBS at a final concentration of 90% of the dose suspension achieved the best stability for the bacteriophage and vegetative bacteria. Therefore, FBS was selected as the test soil of choice to prepare these types of BIs. The fungal and bacterial spores were stable in water. BIs were prepared from stocks that were <3 months old for all organisms tested, other than Btk spores. It is recommended that future lab or field disinfection tests use organisms from freshly prepared stocks (i.e., <3 months) to prepare the BIs. The titer of the stocks should be measured periodically during storage and just prior to use to confirm viability or infectivity has not diminished. Spores should be evaluated microscopically under phase-contrast to ensure the spores appear dormant, as indicated by a phase-bright appearance.

7.0 LAB BTD TESTING

7.1 Test Matrix and Test System

Laboratory BTD efficacy tests were performed using a Thermotron 8800 environmental chamber operated at the six BTD conditions (Table 3). The BIs were prepared the day prior to testing and stored in individual bioreactor tubes in the refrigerator. The day of testing, the BIs were allowed to acclimate to room temperature for at least 30 minutes during which time the Thermotron was set to operate at the desired decontamination test condition. Three to four sets of triplicate BIs were placed vertically in a test tube rack and then placed within the Thermotron, marking the start of the decontamination cycle. Triplicate BIs were removed at discrete time points during the cycle and analyzed for viability. The goal of the timed sampling events was to remove BIs throughout the range of linear decay such that kill curve could be generated from at three time points including T_0 with viable organisms. The duration of each test and associated sampling schemes were determined based on the type of organisms being testing and their general level of resistance to disinfection. The sample schemes used were the following:

- 0, 10, 20, and 30 minutes
- 0, 30, 60, 90 and 120 minutes
- 0, 60, 120, and 180 minutes

One test at each condition was performed for each BI type (excluding MS2, which was tested under Conditions 2 and 4, and Btk and Bg BIs which were tested under Condition 1). Tests were repeated as necessary to obtain accurate kill curves. For example, if a test was initially performed using the 0 to 180 minute regime and no viable organisms were recovered from the first time point, the test was repeated using a shorter scheme such as 0 to 30 minutes or 0 to 120 minutes to gather data points as the viability decreases.

7.2 Results and Discussion

The D-values (the time required to achieve a 1 LR) calculated from the kill curves generated in the lab efficacy test (Section 11; Figure 35 to Figure 41) are presented in Table 5. The bolded D-values shown in the table denote conditions that achieved 1 LR (or 90% reduction) in ≤2 hours. All values were generated from one test per organism per condition. The D-values were then used to estimate the time (hours) required to achieve a 4 LR. Figure 14 provides a snap-shot comparison of the time needed to achieve 4 LR at each of the six test conditions for Phi6 bacteriophage, the two bacteria, and the three fungi. The data illustrates Conditions 1 and 2 achieved the most rapid kill rates, followed by Conditions 4 and 5, then Condition 3, and lastly by Condition 6. Since the MS2 had poor stability, only Conditions 2 and 4 were evaluated with MS2 to compare kill rates with Phi6. The bacterial spore BIs were only evaluated against Condition 1 which had the highest dew point of the six conditions. The results (time to achieve 4 LR) per organism type are discussed.

Table 5. D-values Generated from Lab BTD Efficacy Tests

Organism	D-value (hours to achieve 1 LR or 90% reduction)					
	Condition 1 (143°F & 90% RH)	**Condition 2 (143°F & 50% RH)**	**Condition 3 (143°F & 10% RH)**	**Condition 4 (120°F & 90% RH)**	**Condition 5 (120°F & 50% RH)**	**Condition 6 (120°F & 10% RH)**
Phi6	**0.07**	**0.12**	**1.08**	**0.10**	**0.27**	6.7
MS2	nd*	**0.09**	nd	0.20	nd	nd
S. epidermidis	**0.08**	**0.14**	8.1	**1.4**	2.6	4.9
E. coli	**0.04**	**0.07**	4.1	**0.2**	**0.8**	6.3
Aureobasidium	**0.06**	**0.12**	3.8	**0.2**	**0.9**	4.0
Cladosporium	**0.16**	**0.25**	3.5	**0.3**	**1.2**	10.4
Penicillium	**0.16**	**0.22**	6.7	**0.3**	**1.6**	83
Bt	3,068	nd	nd	nd	nd	nd
Bg	22,430	nd	nd	nd	nd	nd

*nd Not determined

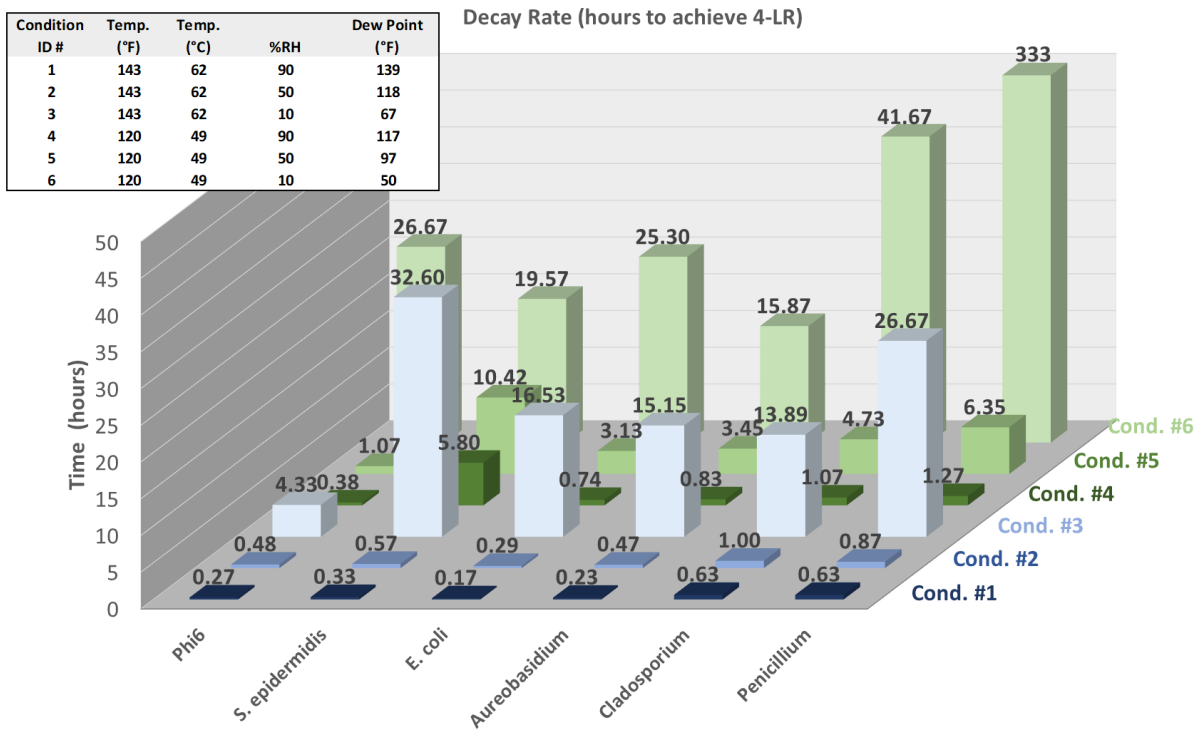


Figure 14. Lab-Scale BTD Efficacy Results

Bacteriophage

Phi6 – Conditions 1, 2, and 4 killed Phi6 rapidly with 4 LR occurring within 30 minutes. These conditions had the highest dew point set points ranging from 117 to 139 °F. Condition 5 achieved 4 LR in just over 1 hour. Under Conditions 3 and 6, the slowest kill rates were observed at 4.3 hours and 27 hours.

MS2 – The results of MS2 BIs treated to Conditions 2 and 4 are shown in Figure 15. The Phi6 results at these same conditions have been included in the figure for comparison. Both MS2 and Phi6 were killed rapidly with 4 LR achieved in < 1 hour under both conditions. The kill rate of MS2 and Phi6 under Condition 2 were comparable achieving 4 LR within 0.5 and 0.4 hours, respectively. Under Condition 4, the time to achieve 4 LR for MS2 and Phi6 were 0.8 and 0.4 hours, respectively.

Although MS2 was less stable over a 2-week cold storage period than Phi6, MS2 and Phi6 had comparable kill rates under two BTD conditions tested. MS2, a non-enveloped virus, was expected to be more resistant to disinfection than the enveloped Phi6 virus as indicated in hierarchy depicted in Figure 2. Additional replicate testing at these and other conditions would be recommended to generate a more substantial data set from which to compare the two viruses.

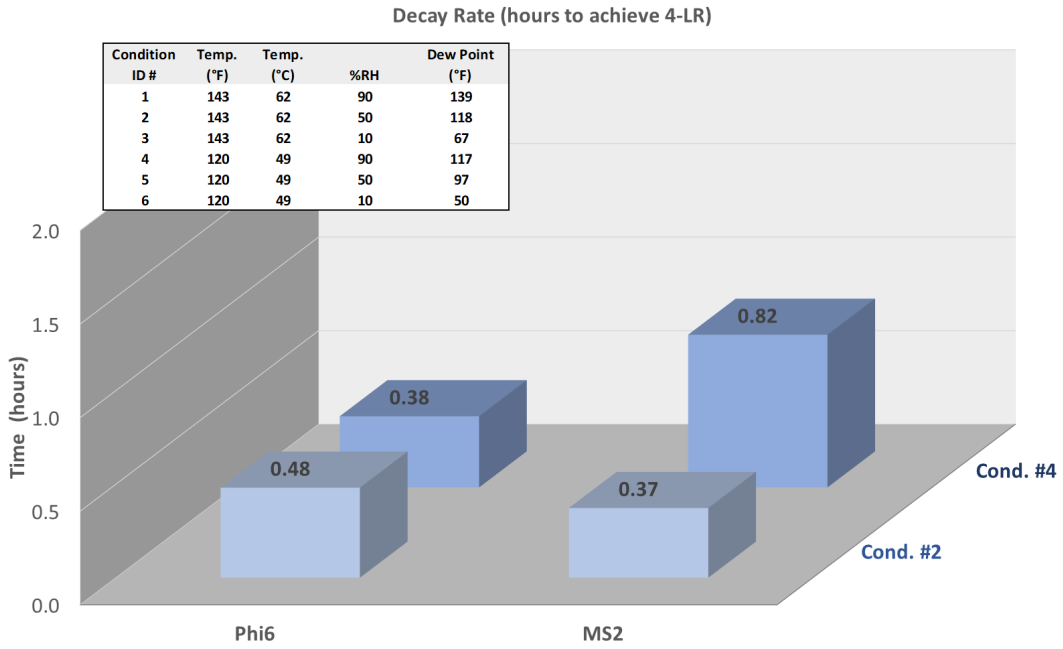


Figure 15. Comparison of Phi6 and MS2 BI Efficacy versus Conditions 2 and 4

Bacterial Cells

The kill rates and trends between the Gram positive bacteria (*S. epidermidis*) and Gram negative bacteria (*E. coli*) were comparable. The fastest kill rates occurred with Conditions 1 and 2 in which 4 LR was achieved in < 1 hour for both organisms. Other key observations were as follows:

S. epidermidis – After Conditions 1 and 2, the next fastest kill rate was achieved under Condition 4 at 5.8 hours to achieved 4 LR. Conditions 3, 5, and 6 resulted in poor kill rates requiring approximately 33, 10, and 20 hours to achieve 4 LR, respectively. These last three BTD conditions would not be recommended for rapid decontamination of Gram positive organisms.

E. coli – After Conditions 1 and 2, the next fasted kill rates were achieved under Conditions 3 and 5 achieved 4 LR in < 1 hour and < 4 hours, respectively. Conditions 4 and 6 achieved the slowest kill rates taking approximately 17 h and 25 hours to achieved 4 LR. Recommended BTD cycles for Gram negative bacteria are Conditions 1, 2, 4, and 5.

Fungal Spores

The rates of kill for all three fungi followed the same trend in which the kill rate from fastest to slowest were Conditions 1, 2, 4, 5, and 6. Condition 3 was not performed. Conditions 1, 2, and 4 all achieved 4 LR within < 1.5 hours. Condition 5 required 3.6 to 6.4 hours to achieve 4 LR. Condition 6 as not effective ranging from 16 hours for *Aureobasidium* to 80 hours for *Penicillium*. Conditions 1, 2, and 4 would be recommended to achieve rapid kill. Condition 5 would be acceptable requiring a slightly longer treatment ranging from 4 to 7 hours.

Bacterial Spores

The bacterial spore BIs were only evaluated against Condition 1 in the lab testing, since these spores are known to be very heat resistant. The BIs were treated over 7 days. The kill curve for the Bg spores showed the spores were unaffected by this decontamination cycle with a D-value of 16 days. The Btk spores were less resistant to the treatment with D-value of 1.1 days. Based on these D-values, the time required to achieve a 4 LR of the Bg and Btk was 64 days (or 9 weeks) and 4.4 days, respectively. The difference in kill rates between the two strains is unknown. The age of the Btk spores (approx. 6 years; acquired as powder from DiPel in August 2016) may be the reason as the Btk spores were significantly less heat resistant than the Bg spores, which were only months old. For both organisms, the spores appeared phase-bright under 1000x phase-contract magnification, indicating the spores were in a dormant state (note: a phase-dark appearance suggest the spores are germinating or dead). A study by Holwitt, et al.[8] suggested that Bg was perhaps the most heat resistant of three *B. anthracis* spore surrogates including *B. anthracis* va Sterne (BaS) and Btk, and that BaS and Btk resistance was comparable.

8.0 GUARDIAN CENTER DEMONSTRATION

8.1 Objective and Scope

The main goal of this demonstration was to provide data to support the application of JBADS Lite to public transport vehicles. A retired Washington D.C. subway system railcar, located in a mock subway station at Guardian Centers (Perry, GA), was used as the public transport vehicle for this study. Specifically, the demonstration was designed to gather data on viral disinfection (specifically SARS-CoV-2 virus) aimed at combating communicable diseases in public spaces. Phi6 bacteriophage was used as the surrogate to SARS-CoV-2 and other pathogenic enveloped virus. While there were no formal requirements for this demonstration, the following objectives were established:

- Objective 1: Maintain a dew point of at least 138.9 °F throughout the vehicle for 1 hour.[a]
- Objective 2: Demonstrate ≥ 4-$\log_{10}$ reduction of Phi6 BIs
- Objective 3: Document material degradation, if any.
- Objective 4: Determine the throughput for sequential decontamination cycles.

During the demonstration, hot/humid air was circulated throughout the interior of a subway car, and the environment within the vehicle was monitored continuously with temperature and humidity sensors. In addition, Phi6 bacteriophage BIs were placed throughout the railcar to verify that the decontamination conditions will neutralize corresponding viruses that pose a threat to human health.

One Washington DC Metro subway car was treated using JBADS Lite. Guardian Centers maintains several realistic training environments, including a dual rail mass transit subway system, modeled after the Foggy Bottom station in the Washington Metro system. JBADS Lite was integrated with existing doors of the subway car. Inside the vehicle, fabric ducts were used to distribute the conditioned air throughout the interior space. All adapters were dry fit to the vehicle without altering the vehicle in any permanent way. The day prior to disinfection testing, AeroClave balanced the air distribution system to achieve optimal, uniform heating of the vehicle. The railcar was also instrumented with temperature and humidity sensors to continuously monitor the environment throughout the decontamination cycle. The BIs were also positioned throughout the railcar to measure the efficacy of the decontamination cycle.

AeroClave LLC was solely responsible for operation of the decontamination system. Hot/humid air was supplied by the new trailer-mounted JBADS Lite. The target setpoint for decontamination was 138.9 °F; however, the maximum achieved during the demonstrations was 129.2 °F. With this limitation discovered prior to the introduction of BIs, the test team decided to run the system at the maximum dewpoint capacity for 4-6 hours to determine the efficacy of decontamination, rather than stopping at a predetermined dewpoint for shorter duration (e.g., 138.9 °F for 1 hour). At the conclusion of each decontamination demonstration the vehicle temperature and humidity were lowered in a controlled manner to avoid condensation.

[a] Since the temperature is expected to vary throughout the railcar, the humidity requirement is given in terms of dew point, which is more readily translatable to a range of temperature and humidity parameters.

8.2 Railcar Description and BI Layout

Four demonstration cycles were initially planned for this study, and each cycle was to include 25 BIs in the railcar during the JBADS Lite treatment. The plan was to position 5 replicate BIs in 5 locations throughout the railcar and then remove them after completion of the cycle. However, upon arrival at the test site, it was determined that BIs could be safely removed from the railcar during the cycle without adversely affecting the operation of the JBADS Lite system or the conditions within the railcar. Removing BIs intermittently during the cycle provided the ability to assess the efficacy of the treatment throughout the cycle. Three locations were selected from which BIs could be placed within arms-reach inside the railcar and removed quickly (within 30 seconds) as desired. These locations included the railcar driver windowsill (Position A), on the floor just inside the front-right passenger door (Position B), and on the floor just inside the left-rear passenger door (Position C). In each of these positions A, B, and C, nine replicate BIs were placed for three timepoints (three replicates for each timepoint). In addition, for two of the three demonstrations (Demo 1 and 2), three replicate BIs were placed on two passenger seats located near the front, right-side of the car (Position D) and near the back, left side of the car (Position E). The BIs at Positions D and E were removed at the end of the cycle once the temperature decreased to conditions deemed safe for personnel to enter the railcar. In Demo 3, the BIs at positions D and E were omitted in favor of an additional timepoint at locations A, B, and C. Figure 16 illustrates the general locations of these positions in relation to the sensors. Triplicate BIs were taped together for each time point and laid horizontally at positions A-C with the vented cap facing inward. The BIs were tapped together to make retrieval easy and rapid. One set BIs were retrieved per time point from Positions A, B, and C. Figure 17 shows how the BIs were oriented at each location. Since the BI positioning and collection times were changed from the original plan, the number of demonstration cycles for the field activity was reduced from four to three.

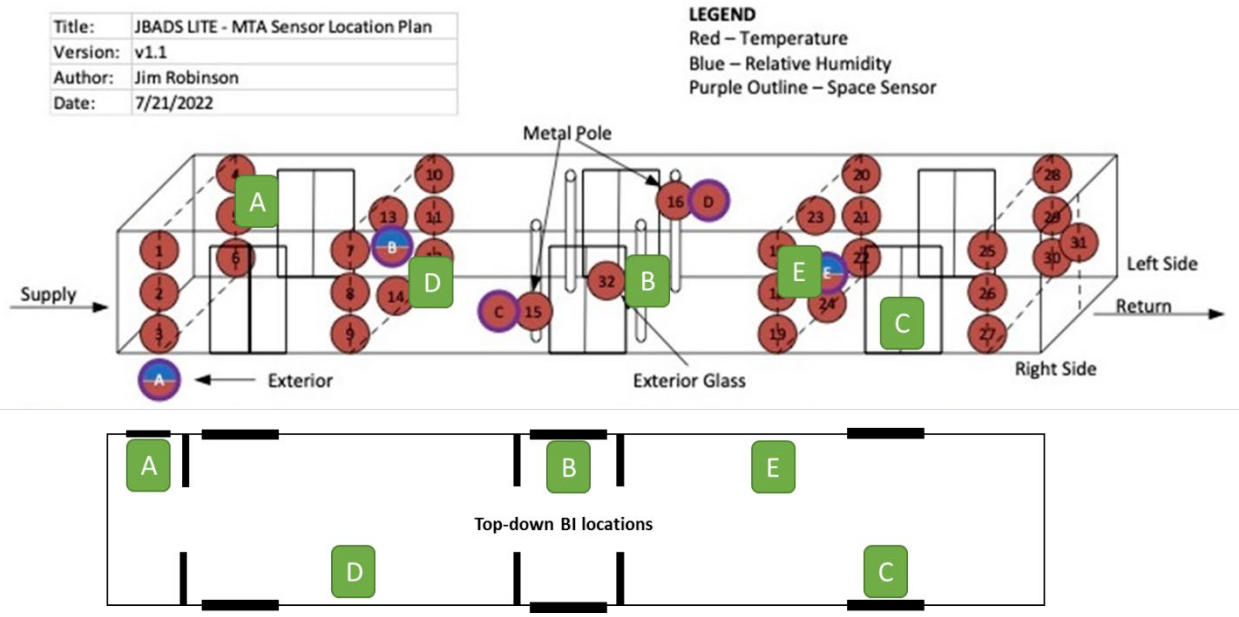


Figure 16. BI Positions Within the Railcar

The following control BIs were included in the demonstration:

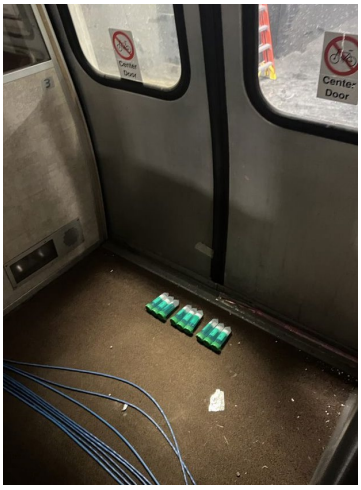
- Transport Controls (TCs): These BIs were transported to the test site with the test samples under cold storage. While the test samples were exposed to JBADS Lite conditions, the TCs were allowed to warm to ambient conditions for the same duration but were not exposed to the test conditions. At the conclusion of each demo, the TCs were transported with the test samples under cold storage for analysis. The purpose of the TCs was to capture the level of attrition from the time the samples were prepared, transported to the test site, and received back at the analysis lab for processing. Recovery from the TCs were used to determine the level of inactivation (i.e., the log reduction (LR)) achieved by the decontamination treatment.
- Laboratory Controls (LCs): These BIs were retained in the analysis laboratory under refrigerated conditions and analyzed with the other BIs when the demonstration was complete. These BIs were used to account for natural Phi6 attrition that is not caused by transportation or the decontamination cycle.
- Baseline Controls (BCs): Five (5) of these control BIs were processed on the day the BIs were prepared. These BIs were analyzed shortly after inoculation to accurately determine the number of viable organisms deposited on the coupons.

Any attrition in these controls was used to inform the results of decontaminated samples. On each day of samples analysis, negative controls (no Phi6) and positive controls (TCs, LCs, and BCs) were processed after the JBADS Lite-treated BIs were analyzed. The negative controls were included to demonstrate there was no environmental contamination or cross-contamination during transit and analysis. Positive controls were processed last to reduce the possibility of cross-contamination and false positives during sample analysis.

All BIs for the Guardian Center demonstration were prepared on the same day, and the test samples and TCs were shipped to the test site, on ice prior to the event and refrigerated. On each day testing, the appropriate number of BIs were transported in a cooler with ice packs to the test site. The BIs, including three TCs and one negative control BI, were allowed to acclimate to ambient conditions for at least 30 minutes during which time they were labeled with unique sample identification numbers. Sets of triplicate BIs were tapped together. One set of BIs were placed in each railcar position for each time point. The TCs remained at ambient conditions until the given demonstration cycle was completed and all BIs were retrieved. After each BI sample collection time point, the BIs were allowed to acclimate to ambient conditions for 30 minutes and then placed in a cooler with ice packs. Once all BIs had been collected, the cooler was shipped via express mail to METSS in Westerville, OH for analysis. At METSS, the condition of the BIs was documented upon receipt and then stored refrigerated. Onset HOBO temperature and relative humidity dataloggers were shipped with the BIs and used to monitor the shipping/storage conditions of the BIs throughout the duration of the study.

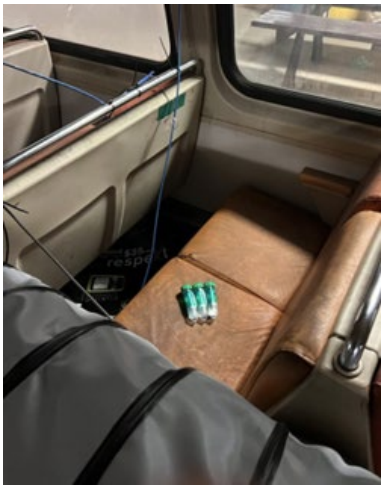
Position A. Railcar driver windowsill

Position B. On floor inside front-right passenger door



Position C. On floor inside rear-left passenger door

Position D. On passenger seat near front-right section of car



Position E. On passenger seat near rear-left section of car

Figure 17. Orientation and Position of BIs within Railcar

At least 37 temperature sensors and 3 humidity sensors were placed throughout the vehicle interior by AeroClave personnel. The sensors were divided into two groups: the operational array and the test array. The operational array contains 5 temperature sensors and 3 RH sensors that drive the JBADS Lite system. The test array contained 32 additional temperature sensors that were surface-mounted throughout the railcar to ensure that the operational array was truly representative of the railcar environment at large. If the test array revealed areas of the railcar that were not well represented by the operational array (e.g., cold spots or hot spots), the operational array sensors were relocated to best represent the dynamics of heating the railcar for all future uses.

Before and after the decontamination cycle, the vehicle was carefully inspected and photographed to ensure that there was no degradation to the vehicle because of the decontamination process. The final vehicle configuration was documented at the event and validated during the timed demonstration run.

8.3 Results and Discussion

8.3.1. BI Analysis

At the completion of the field demonstration, the BIs were shipped with ice packs to METSS for analysis. At METSS, the BIs were removed from cold storage and allowed to acclimate to room temperature (approximately 1 hour) before being analyzed as described in Section 5.1.2. Positive control BIs (BCs, TCs, and LCs) were plated in duplicate at dilutions $10^{-3, -4, \text{ and } -5}$. Five 2-ml aliquots of each negative control BI (uninoculated coupons) were plated. Since the level of kill of the JBADS Lite treated BIs was unknown, duplicate 2-ml aliquots of the neat (undiluted) extract were plated along with duplicate 0.1 ml aliquots of dilutions $10^{-1 \text{ to } -3}$ along with four 1.5-2-ml aliquots constituting the remaining volume of the extract. The plated samples were incubated overnight at 25-27 °C. PFUs were then counted and recorded. Plates with 25-250 PFUs were used to calculate total PFU recovered. The mean PFU recovered was then calculated as mean Log_{10} PFU recovered. The LR achieved by the disinfection treatment was then calculated by subtracting the mean PFU recovered of the HAD treated samples per location from the mean Log_{10} recovered from the corresponding TCs for the given test.

The BIs were prepared on August 22, 2022 (two days prior to the first demonstration). The Phi6 dose suspension and five BCs were also analyzed that day. TCs and LCs were processed along with each set of Demo BIs (those exposed to the JBADS Lite treatment in the railcar). The mean log recovery and LR of these controls as compared to the BCs (analyzed on Day 0) are shown in Table 6. The LR achieved by the JBADS Lite treatment was based on the recovery of the TCs versus recovery from the Demo samples. Recovery from the TCs for Demo 1, 2, and 3 were 6.7, 5.2, and 6.1 log PFU/coupon, respectively. These recoveries correspond to the BCs of 1.0, 2.5, and 1.6 for Demo 1, 2, and 3, respectively. The reason for the lower recovery from the Demo 2 TCs is unknown. Despite this difference, a LR of ≥ 5.2 could be reported for all tests. Recovery from the LCs (stored refrigerated at the analysis lab) ranged from 6.8 to 7.2 log PFU/BI, demonstrating the BIs were stable at this storage condition with LRs ranging only from 0.5 to 0.8 as compared to the BCs.

Table 6. Control BI Results

Controls	Recovery (mean Log pfu)			Reduction (mean Log pfu compared to BC)		
	Demo 1	Demo 2	Demo 3	Demo 1	Demo 2	Demo 3
Baseline Controls (BC)	7.7 ± 0.1			n/a	n/a	n/a
Laboratory Controls (LC)	7.2 ± 0.0	6.8 ± 0.1	6.9 ± 0.1	0.5	0.9	0.8
Transport Controls (TC)	6.7 ± 0.4	5.2 ± 0.1	6.1 ± 0.1	1.0	2.5	1.6

The BI results for Demo 1, 2, and 3 are discussed in sections 8.3.1.1, 8.3.1.2, and 8.3.1.3, respectively. The results are expressed as LR of JBADS Lite treated BIs as compared to recovery of the TCs per demonstration.

The storage temperature and RH's profiles generated from the HOBO dataloggers for the LCs and the TCs per Demo were evaluated. In summary, the LCs remained refrigerated with average temperature of 10 °C. The TCs remained at 1-10 °C other than the time during Demos when the TCs were stored at ambient conditions at the test site. During this Demos, the TCs reached temperatures ranging from 26-29 °C (or 79-84 °F).

8.3.1.1 Demo 1

During the first demonstration, heat was introduced at 7:42 am, and steam was introduced at 7:55 am. Dew points measured during the cycle are shown in Figure 18. When the first set of BIs were removed after 2 hours (9:42 am), the average dewpoint was 115.4 °F. The dewpoint continued to rise throughout the cycle, reaching 124.7 °F after 4 hours, and a maximum of 129.2 °F at the end of the 6-hour cycle.

A LR of >6 was achieved at all time points (2, 4, and 7 hours; Figure 19). Complete kill (i.e., no recovery of Phi6) was observed for nearly all the BI as only a few virions (reported as plaque forming units or PFU) were recovered from just one BI at the 2-hour time point.

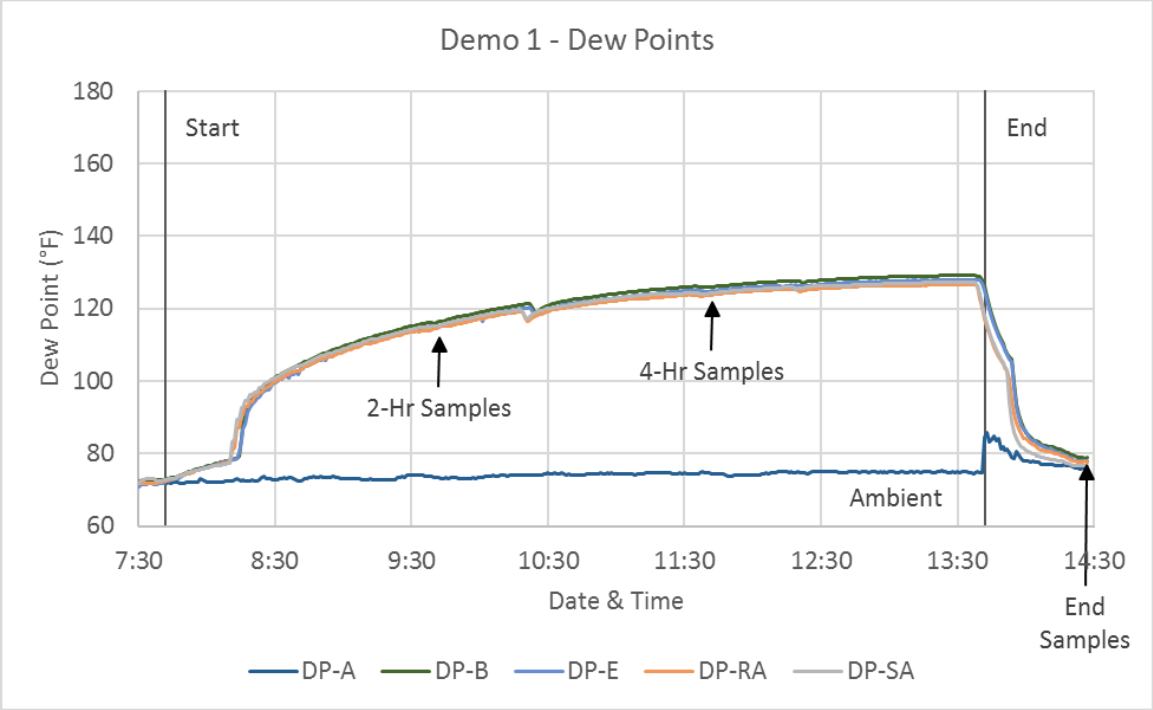


Figure 18. Dew Points Recorded During Demo 1.

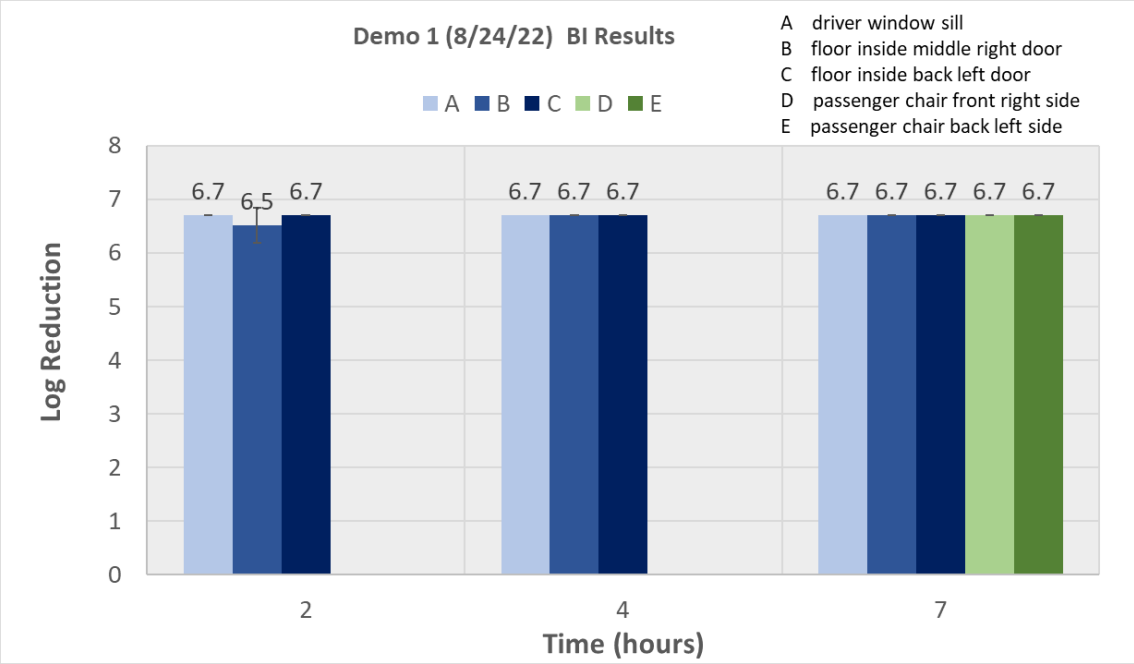


Figure 19. Demo 1 BI Results

8.3.1.2 Demo 2

This demonstration was a replicate of Demo 1 to assess reproducibility. Heat was introduced at 7:50 am, and steam was introduced at 8:14 am. Dew points measured during the cycle are shown in Figure 20. When the first set of BIs were removed after 2 hours (9:50 am), the average dewpoint was 116.0 °F. The dewpoint continued to rise throughout the cycle, reaching 123.9 °F after 4 hours, and a maximum of 128.2 °F at the end of the 6-hour cycle.

A LR of >5 was achieved at all time points (2, 4, and 7 hours; Figure 21). Again, complete kill (i.e., no recovery of Phi6) was observed for nearly all the BIs. A few PFU were recovered from two BIs at the 4-hour time point and two BIs at the 7-hour time point. As mentioned previously, the LR is based on recovery of the TCs. Since the recovery of the TCs for Demo 2 was lower than the other two Demos, the effectiveness of the treatment also appears lower; however, the disinfection again exceeded the target objective of at least a 4 LR.

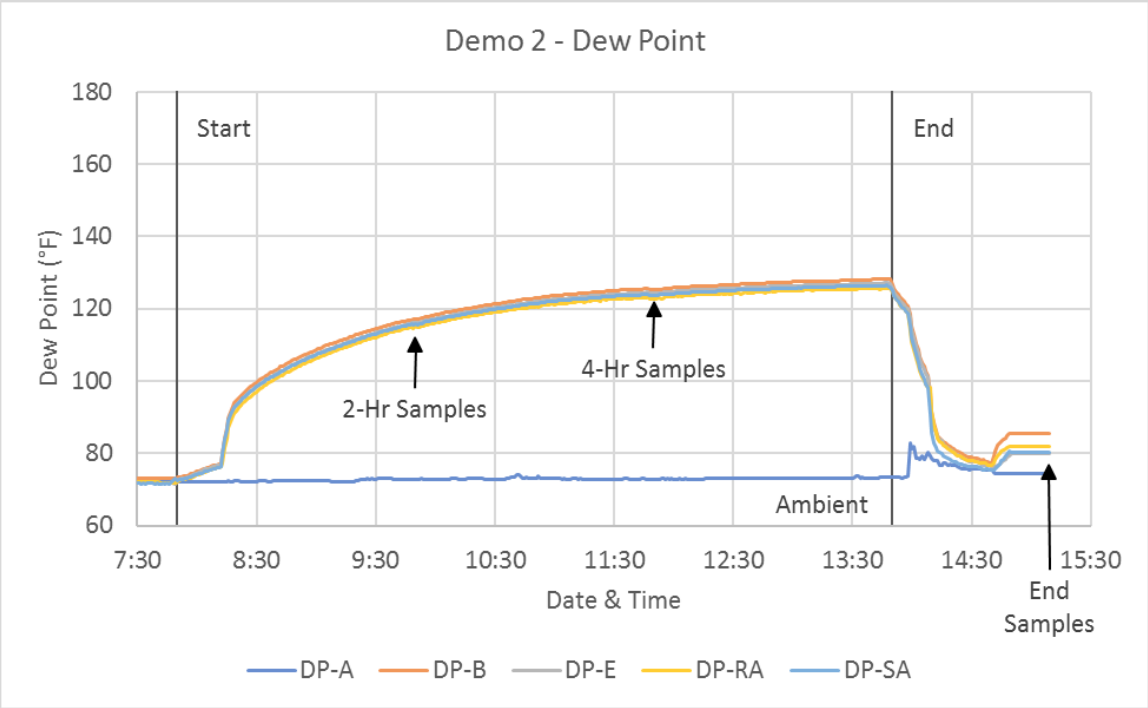


Figure 20. Dew Points Recorded During Demo 2

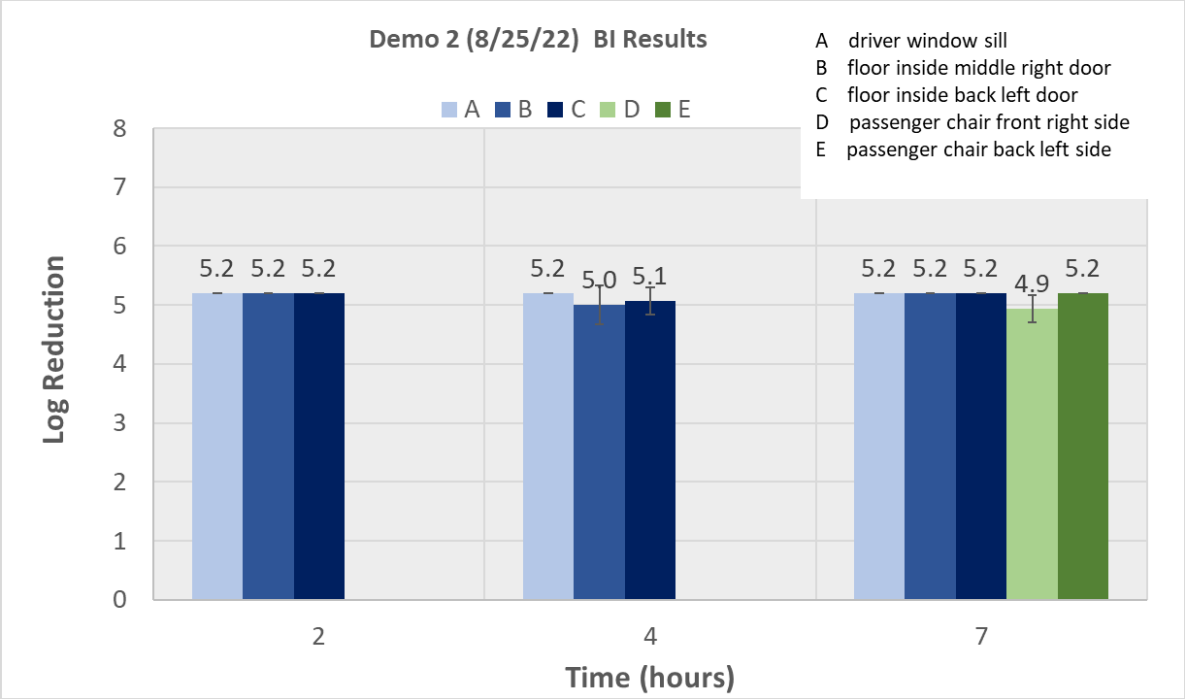


Figure 21. Demo 2 BI results.

8.3.1.3 Demo 3

In this demonstration, triplicate BI were removed from Positions A, B, and C after 1, 2, 3, and 4 hours from the time the cycle was started. Heat was introduced at 7:16 am, and steam was introduced approximately 20 minutes later. Dew points measured during the cycle are shown in Figure 22. After 1 hour (8:16 am), when the first set of BIs were removed, the average dewpoint was 104.6 °F. The dewpoint continued to rise throughout the cycle, reaching 115.4 °F after 2 hours, 120.8 °F after 3 hours, and a maximum of 124.8 °F by the end of the 4-hour cycle.

As shown in Figure 23, <1 LR was achieved after 1 hour, but > 5 LR was achieved after 2 hours and >6 LR after 3 and 4 hours. Therefore, most of the kill occurred between 1-2 hours in the dew point range of 104.6-115.4 °F.

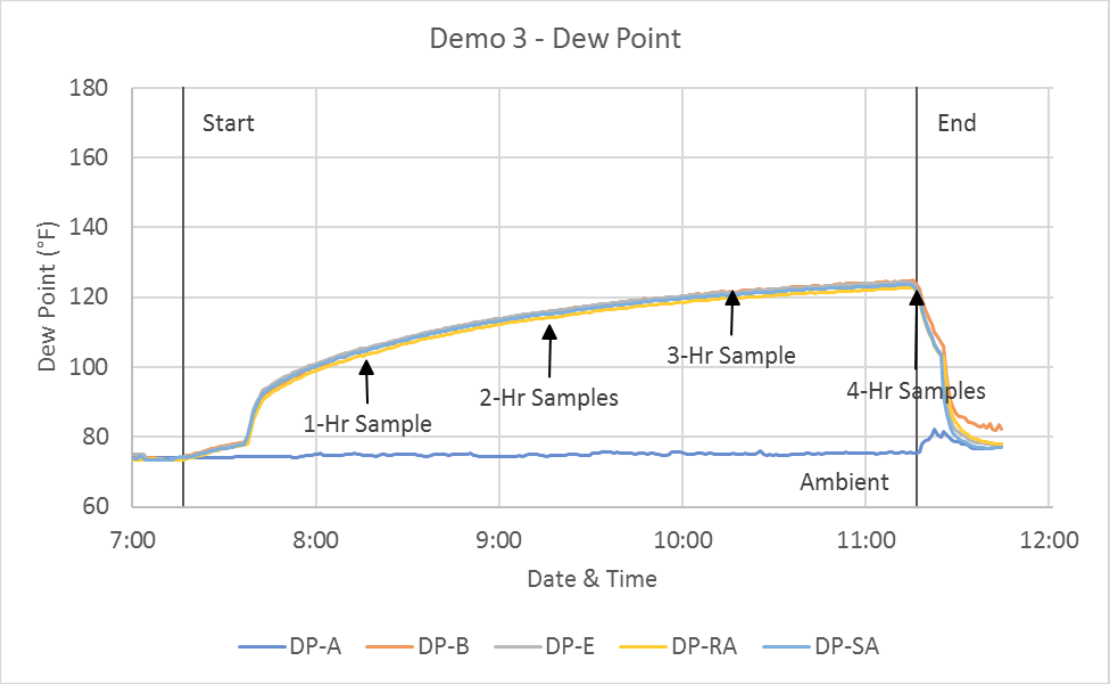


Figure 22. Dew Points Recorded During Demo 3

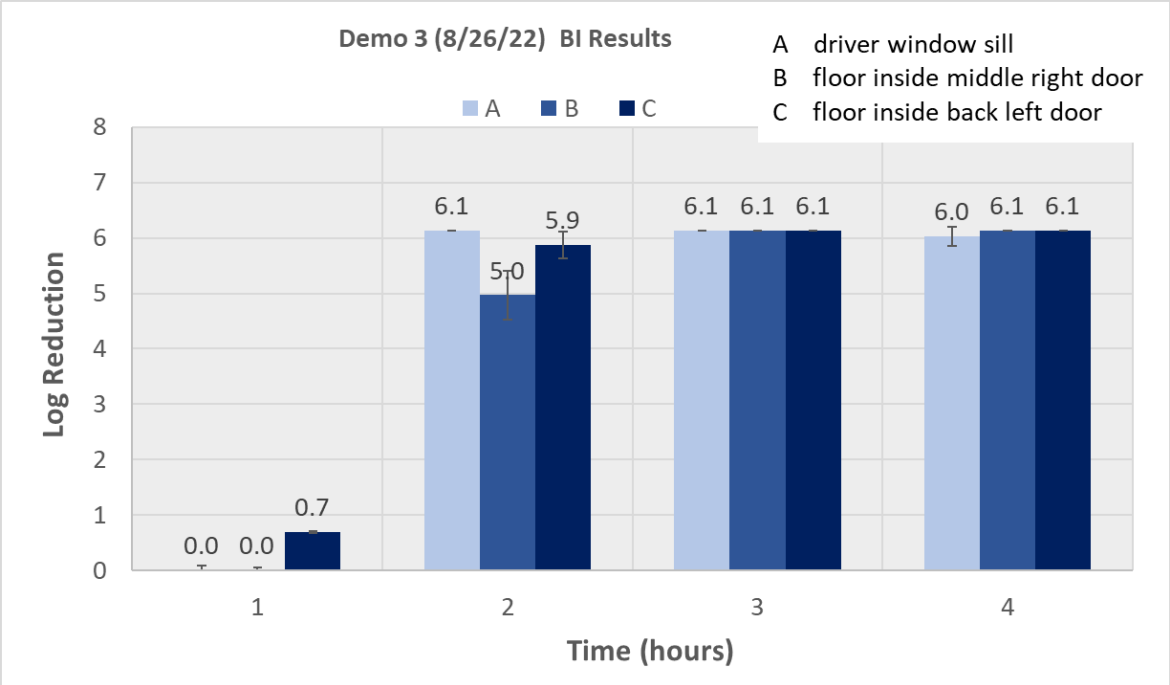


Figure 23. Demo 3 BI Results

8.3.2. Timed Transition

Following the Demo 2 decontamination cycle, JBADS Lite was disconnected from the railcar and reconnected to gauge how much time is required to transition between railcars for sequential decontamination. This throughput estimate is based on a scenario where JBADS Lite is stationary while ducting and sensors are moved to an adjacent railcar and/or track. The time required to move railcars was not captured. This was the first operationally representative installation and tear down exercise, conducted only a few days after the first full system installation for this event. The team was not specifically trained for this exercise, so there will be efficiencies gained with repetition and training in the future.

The original plan was to disconnect JBADS Lite from one railcar and reconnect to an adjacent railcar for a subsequent decontamination demonstration. Although Guardian Centers provided two railcars on neighboring rails near JBADS Lite, windows of the second railcar were broken and could not be sealed for decontamination. Therefore, the timed transition demonstration was conducted by disconnecting all ducting and sensors from the railcar and repositioning those elements in the same railcar moments later.

During the timed transition, there were six-seven members of the AeroClave team working simultaneously. The overall time for tear down and installation was approximately 1 hour and 22 minutes. Teardown started at 1452 and was completed at 1509, for a total of 17 minutes. Installation of the system started at 1525 and was completed at 1630, for a total of 65 minutes. Timing of individual tasks are noted in Table 7; some of these tasks were performed concurrently within the overall timing described above.

Table 7. Approximate Times for Tear Down and Installation Activities.

Tear Down Activity	**Approximate Time (minutes)**
Unseal doors and remove interior Ducting	17
Remove Operational Sensor Arrays (OSA)	15
Remove return and supply ducts from railcar and VDU	4
Remove right door plug	7
Remove front door plug	7
Remove exterior tape on vents	5
Installation Activity	**Approximate Time (minutes)**
Install ducts inside	25
Install right door plug	15
Install front door plug	9
Scaffolding, to support ducts, erected	2
Install supply and return ducts to railcar and VDU	20
Installed OSAs	47
Sealing of doors	5

8.4 Guardian Center Demo Conclusions

Overall, the demonstration was highly successful. Conclusions for each of the stated objectives are as follows:

- Objective 1: Maintain a dew point of at least 138.9 °F throughout the vehicle for 1 hour.
    - The system was not capable of reaching the desired dew point. The maximum dew points achieved were 129.2, 128.2, and 124.8 °F for Demos 1-3, respectively.
- Objective 2: Demonstrate ≥ 4-$\log_{10}$ reduction of Phi6 BIs
    - All three demonstrations achieved >4 LR within 2 hours from the time the cycle was initiated.
- Objective 3: Document material degradation, if any.
    - No significant material degradation was noted.
- Objective 4: Determine the throughput for sequential decontamination cycles.
    - The time required to transition from one railcar to another is approximately 45 minutes, based on the tear-down and reinstallation demonstrated.

9.0 ORLANDO DEMONSTRATION

9.1 Objective and Scope

The goal of this demonstration was to assess the effectiveness of the JBADS Lite for decontamination of a public transportation vehicle that was contaminated with diverse types of organisms including virus, vegetative bacteria, bacterial spores, and fungal spores. Surrogate organisms were used for safety reasons as they do not pose risks to human health. In this study, a retired New York City metropolitan subway railcar (located AeroClave's campus in Orlando, FL) was used to represent a public transportation vehicle. The organisms were inoculated onto aluminum coupons to create BIs. During the demonstration, hot/humid air was circulated throughout the interior of a railcar, and the environment within the railcar was monitored continuously with temperature and humidity sensors. In addition, replicate BIs inoculated with Phi6 bacteriophage (a surrogate for various enveloped viruses including SARS-CoV-2), *Staphylococcus epidermidis* (a surrogate for infectious Gram positive bacteria), *Escherichia coli* (a surrogate for infectious Gram negative bacteria), *Cladosporium sp*. (a surrogate for infectious fungi), and *Bacillus thuringiensis* and *Bacillus atrophaeus* spores (surrogates for Bacillus anthracis spores) were placed inside the railcar. Triplicate BIs for each organism were removed from the periodically throughout the cycle and analyzed for viability. Three hot/humid air conditions, deemed suitable and achievable for the JBADS Lite system with a railcar, were evaluated.

The overarching goal was to compare the rate of kill achieved by the JBADS Lite system in decontaminating a railcar versus kill rates achieved in controlled laboratory BTD efficacy tests with the same BIs. Ideally, the kill rates would be comparable such that results of this study could be used to the predict the duration of JBADS Lite treatment to achieve a desired level of kill (i.e., reduction of viability) for a particular type of infectious organism. A second goal was to assess the kill rates of from two decontamination cycles that had equivalent target dew points that were achieved at different temperatures and humidity.

The following three hot, humid air decontamination tests were conducted during the field test:

- Test 1: 143 °F and 50% RH with dew point of 118 °F (Condition 2)
- Test 2: 120 °F and 90% RH with dew point of 117 °F (Condition 4)
- Test 3: 120 °F and 50% RH with dew point of 97 °F (Condition 5)

These conditions that were deemed achievable by the JBADS Lite system on a railcar and were expected to achieve rapid kill of virus and bacteria. Slower rates of kill were expected for bacterial and fungal spores as they are far more heat resistant. While there were no formal requirements for this demonstration, the following objectives were established:

- Objective 1: Achieve and maintain target decontamination conditions.
- Objective 2: Obtain at least 3 data points from which kill rates could be calculated per organism.

9.2 Railcar Description and Sample Layout

One retired New York City Metro subway car (railcar), located outdoors on the campus of Aeroclave (Orlando, FL), was treated using JBADS Lite. JBADS Lite was integrated with existing doors of the subway car. Inside the vehicle, fabric ducts were used to distribute the conditioned air throughout the interior space. All adapters were dry fit to the vehicle without altering the vehicle in any permanent way. The day prior to disinfection testing, AeroClave balanced the air distribution system to achieve optimal, uniform heating of the vehicle. Figure 24 illustrates the JBADS Lite and railcar configuration.

(A)

(B)

(C)

(D)

Figure 24. JBADS Lite and Railcar

(A) heating duct, (B) ducts inside railcar, (C) steam generator, (D) heat and humidity controller and blower

Prior to demonstration, the railcar was instrumented with temperature and humidity sensors to continuously monitor the environment throughout the decontamination cycle. Once the desired absolute humidity conditions were reached based on controlled increase of temperature and relative humidity, replicate BIs of each organism type were positioned inside the railcar. To measure the effectiveness of the decontamination cycle over time, replicate BIs were removed periodically during the treatment and analyzed for viability. Post-demonstration analysis of the BIs was conducted at METSS.

The BIs evaluated in this demonstration are listed and described in Table 8 and include at least one strain for each type of microorganism. Since no kill was anticipated for bacterial spores from either of the three decontamination cycles chosen for this demonstration, a subset of Btk and Bg BIs were included in one of the three trials to demonstrate the stability and resistance of the bacterial spore BIs to a short, less aggressive BTD cycle (Condition 2).

All BIs were prepared a week prior to the demonstration, shipped to AeroClave with ice packs and stored refrigerated until used. As for the Guardian Center Demo, baseline controls (BC), transport controls (TC), and laboratory controls (LC) along with test BIs for Demo's 1, 2, and 3, were prepared for each type of BI.

Table 8. BIs Used for Orlando Demo

Organism Type	Organism	Preparation
Virus	*Phi6* bacteriophage	Suspension in 90% Fetal Bovine Serum (FBS)
Gram positive bacterial cells	*Staphylococcus epidermidis*	Suspension of washed cells in PBS prepared from an overnight broth culture
Gram negative bacterial cells	*Escherichia coli*	Suspension of washed cells in PBS prepared from an overnight broth culture
Fungal Spores	*Cladosporium cladospoiodes*	Suspension in sterile dH_2O
Bacterial Spores	*B. thuringiensis*	Suspension in sterile dH_2O
	B. atrophaeus	Suspension in sterile dH_2O

Just prior to each test, the appropriate number of BIs were removed from the refrigerator and allowed to warm to ambient temperature for 30 minutes. Once the desired BTD conditions in the railcar were reached and stabilized by the JBADS Lite, the BIs were placed vertically in racks and the promptly place inside the railcar onto a step latter positioned just inside the side-passenger door of the railcar (Figure 25). The time the BIs were placed inside the railcar represented that start of the decontamination cycle (T_0). At the specified time points listed below,

triplicate BIs were removed from the rail car (Figure 25). The BIs, in bioreactor tubes (Figure 26), were allowed to sit at ambient conditions before they were place under cold storage (1-9 °C) conditions.

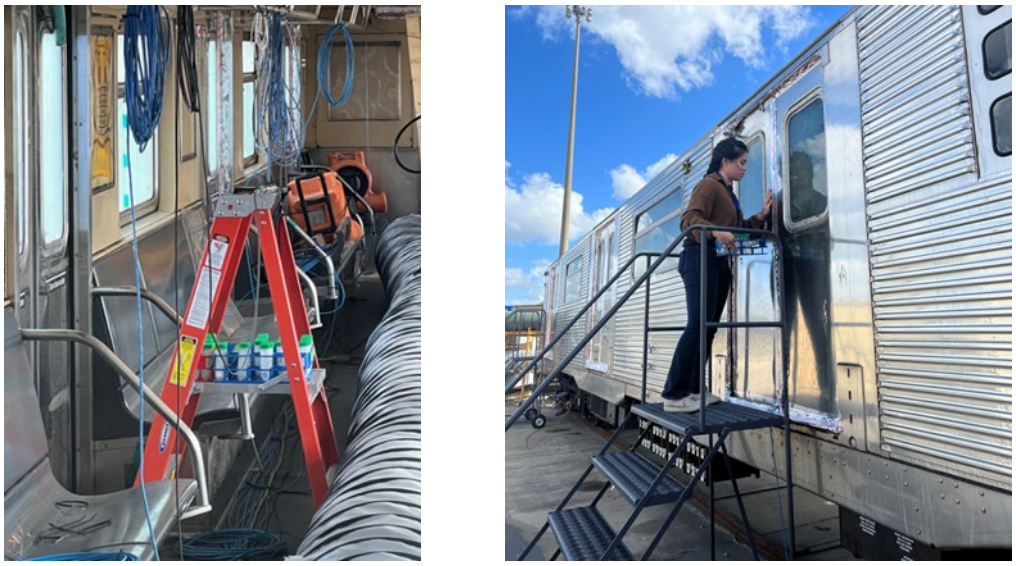


Figure 25. BI Placement and Collection

Racks of BIs in bioreactors on latter inside railcar (left); Side-door of railcar from which BIs were removed during decontamination cycle (right).

Figure 26. BI Inside Bioreactor Tube.

9.3 Results and Discussion

The field demonstration was successful as the objectives were met. The JBADS Lite was able to reach and maintain target decontamination cycle set points and kill rates were measured for each type of BI excluding bacterial spores which remained stable, as expected, for the decontamination cycle they were evaluated against.

The kill curves generated for each type of BI were used to calculate D-values, which in turn, were used to predict the time (hours) required for each of the decontamination cycles to achieve a 4 LR (Figure 27). Condition 2 (143 °F, 50% RH, 118 °F Dew Point) and Condition 4 (120 °F, 90% RH, 117 °F Dew Point) achieved 4 LR in less than 2 hours for the virus, bacterial cells, and fungal spore BIs. Condition 5 (120 °F, 50% RH, 97 °F Dew Point) also achieved 4 LR within 2 hours for the virus and Gram negative bacteria. Significantly longer times were required under Condition 5 to achieve 4 LR of the Gram positive bacterial cell BIs and fungal spores BIs at 5.1 hours and 48 hours, respectively. No kill was observed of the Btk and Bg BIs treated to Condition 2 (Figure 28).

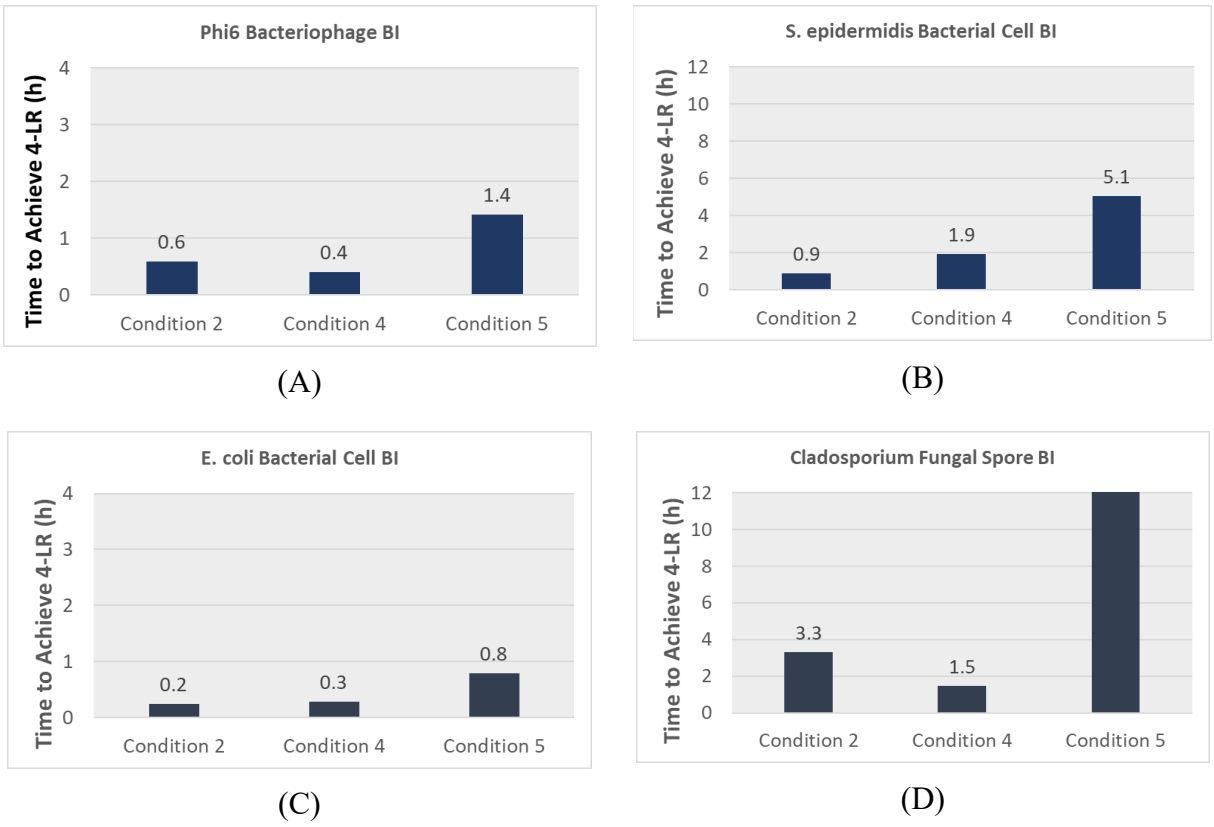


Figure 27. Orlando Demo BI Efficacy Results

(A) Phi6 bacteriophage, (B) S. epidermidis cells, (C) E. coli cells, and (D) Cladosporium spores.

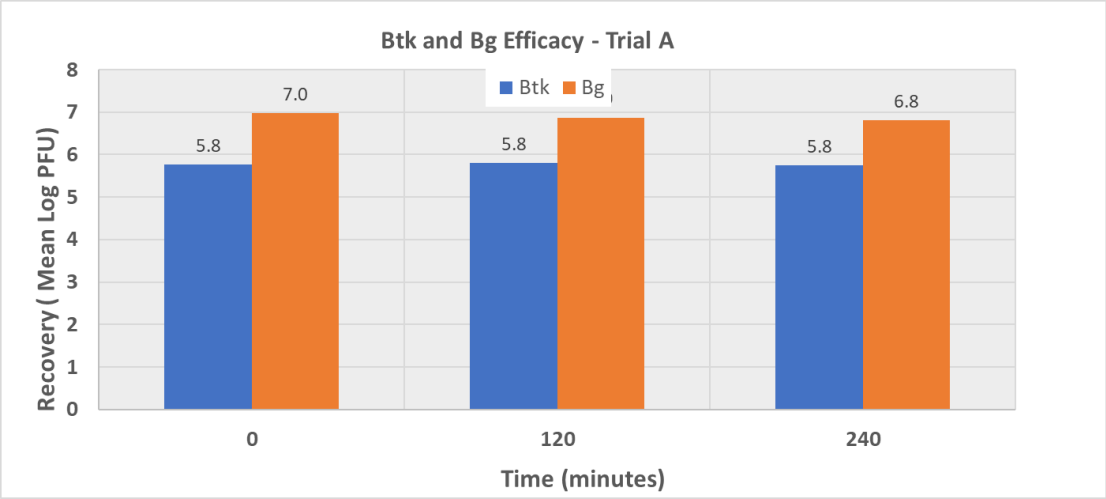


Figure 28. Btk and Bg BIs in Trial 1 (Condition 2)

All controls BIs (baseline, lab, and transport) for each organism remained stable (Figure 29).

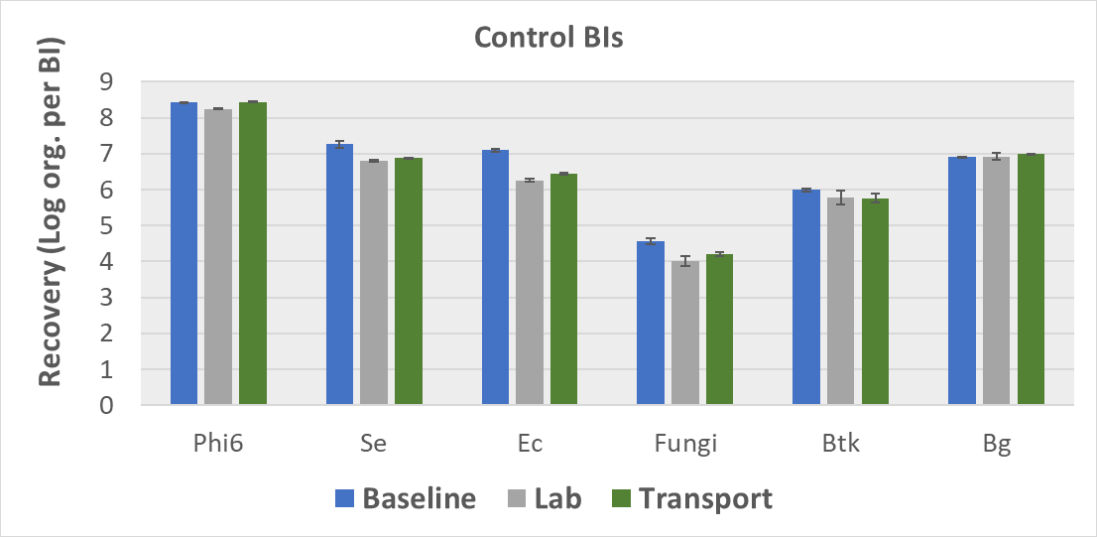


Figure 29. Orlando Demo Control BI Results

Trial 1 under Condition 2 was performed on January 24th, and Trial 2 under Condition 5 and Trial 3 under Condition 4 were performed on January 25th. The temperature, dew point and relative humidity conditions as measured by AeroClave's sensors for Trials 1, 2, and 3 are shown in Figure 30, Figure 31, Figure 32, respectively. The time points (minutes) when replicate BIs were removed from the railcar during the decontamination cycles are denoted by numbers and arrows below the blue temperature profiles in each figure.

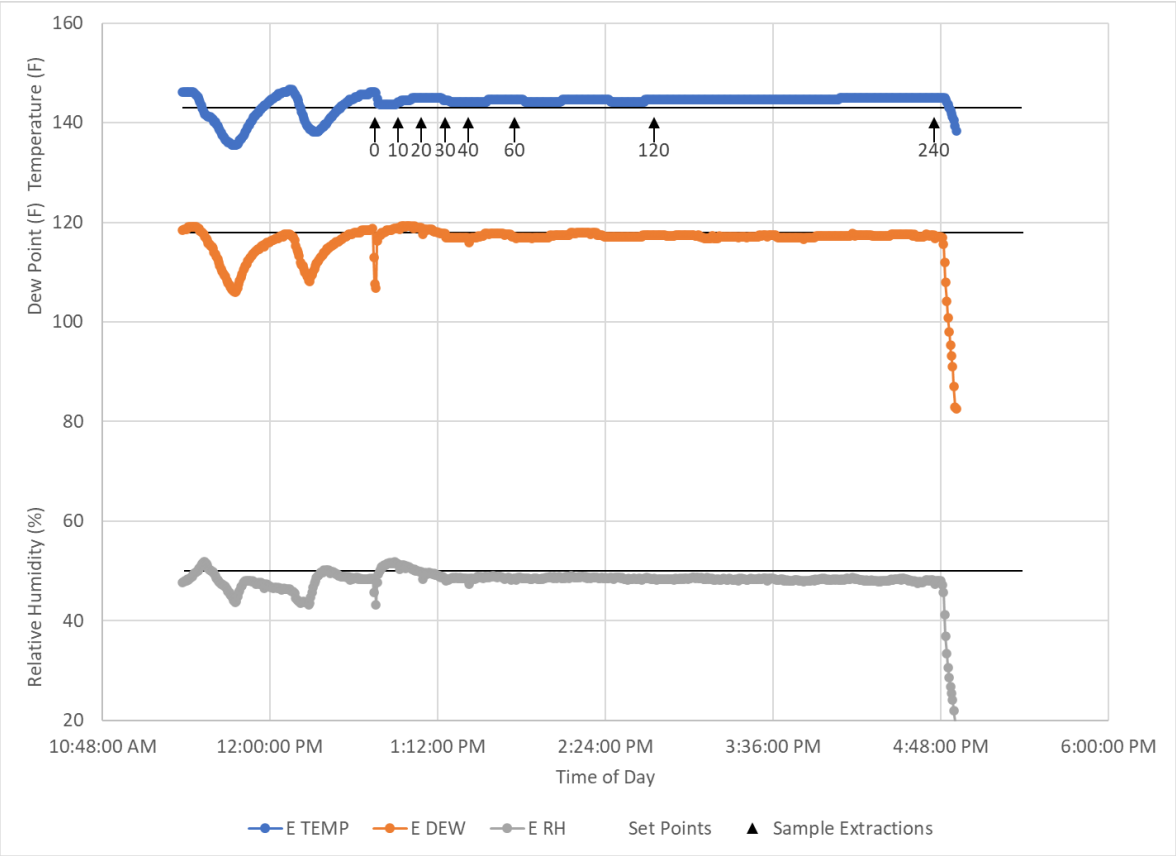


Figure 30. Orlando Demo Trial 1 (Condition 2) JBADS Lite Profiles

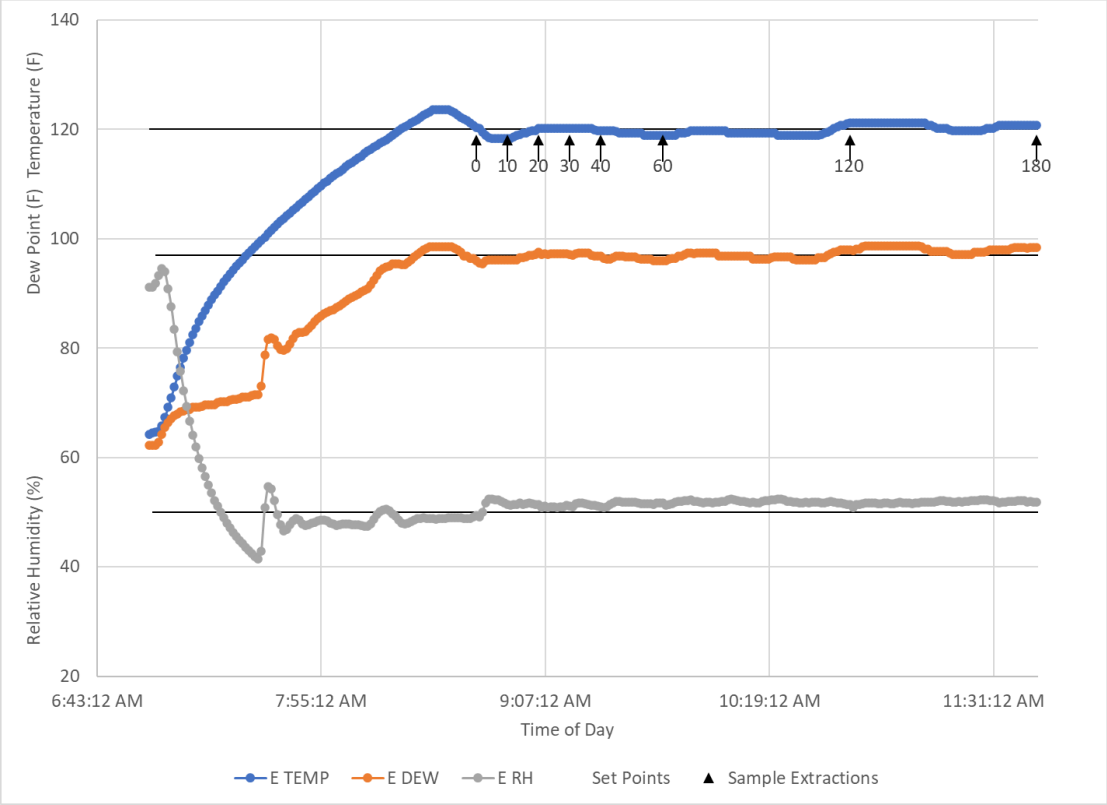


Figure 31. Orlando Demo Trial 2 (Condition 5) JBADS Lite Profiles

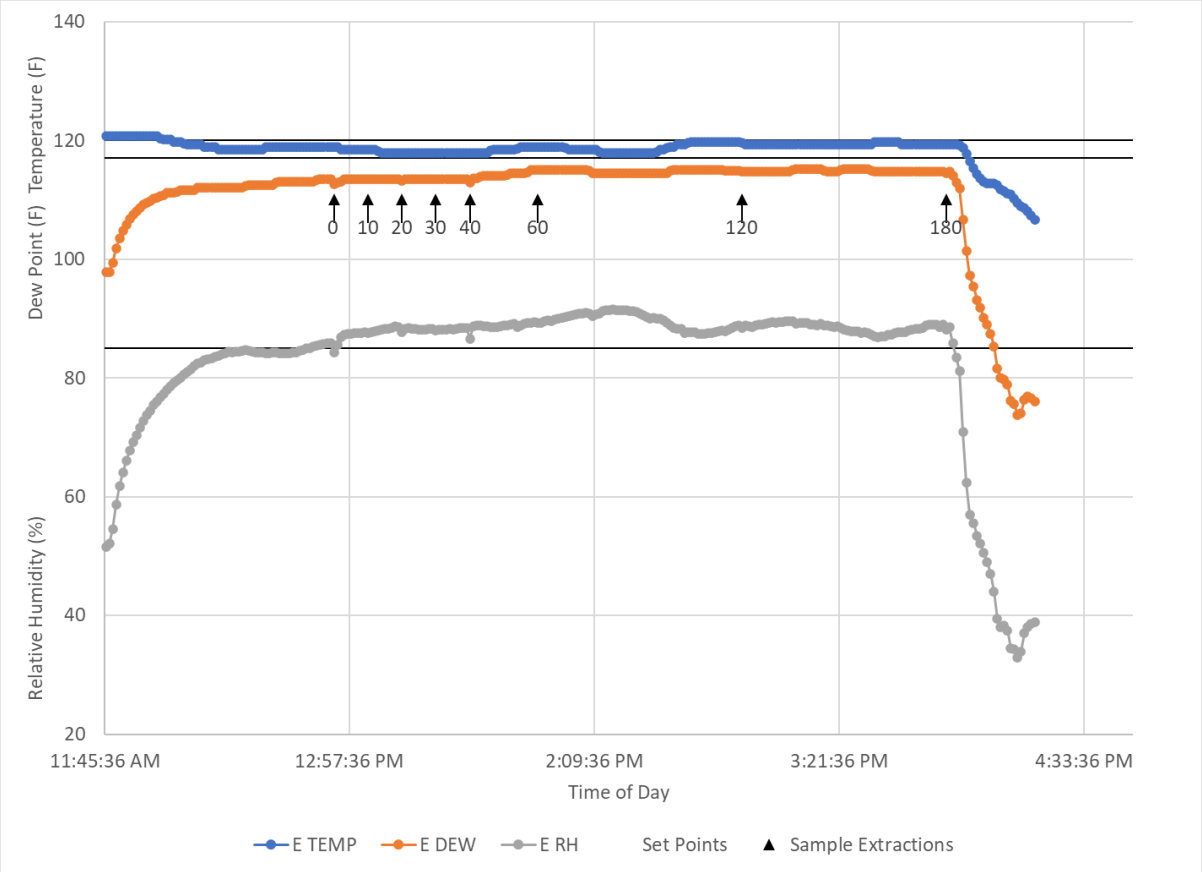


Figure 32. Orlando Demo Trial 3 (Condition 4) JBADS Lite Profiles

10.0 COMPARISON OF LAB AND FIELD RESULTS

One objective of the Orlando Demonstration was to compare the rates of kill measured in the field to those measured in the laboratory for Conditions 2, 4, and 5. These results are shown in Figure 33 with the rates of kill expressed as time (hours) to achieve a 4-LR.

Overall, the lab and demo (railcar) efficacy data were comparable, especially under Conditions 2 and 4 for *Phi6, E. coli*, and *Cladosporium*. For *S. epidermidis*, the lab and demo results were comparable under Condition 2; however, the rate of kill was 2 to 3 times slower in the lab under Conditions 4 and 5. The most dramatic difference between the lab and demo results were observed for Condition 5 in which the rate of kill in of the Gram positive and Gram negative cells in the lab was 2-3 times slower. Conversely, the kill rate for fungal spores was approximately 9 times faster in the lab than in the demo at 48 hours versus 5.2 hours, respectively.

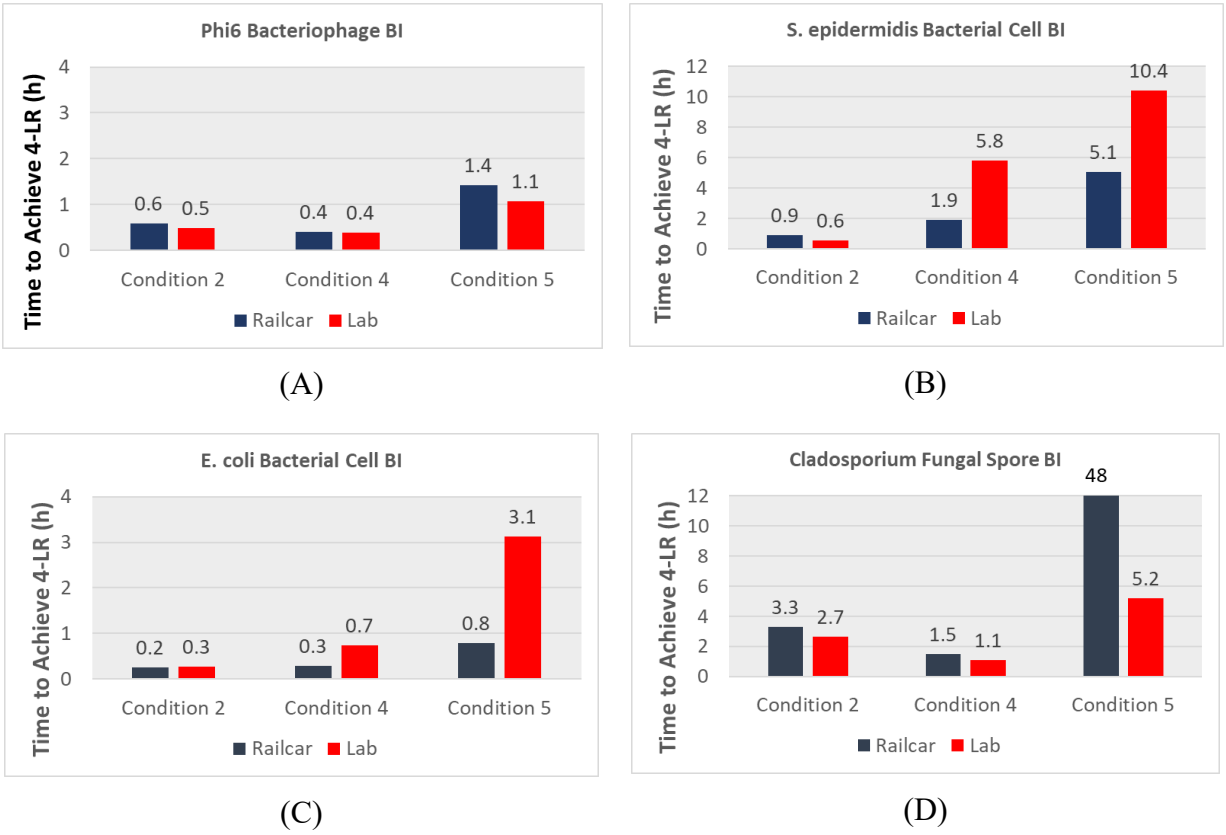


Figure 33. Kill rates of Lab Efficacy Tests versus Orlando Demo Tests

(A) Phi6 bacteriophage, (B) S. epidermidis cells, (C) E. coli cells, and (D) Cladosporium spores.

The kill rates were generally comparable at similar Dew Points (118 °F and 117 °F for Conditions 2 and 4, respectively) achieved under significantly different temperature and RH conditions during lab and field tests; however, some differences were observed as shown in

Figure 34. The variability observed may be attributed to the limited data set. Additional replicate tests would be warranted to better assess these trends. Key observations are provided below.

- For *Cladosporium* fungal spores, the kill rate was approximately 2x slower in the field at Condition 2 at 3.3 hours, but generally the same at Condition 2 in the lab and Condition 4 in the lab and field ranging from 1 to 1.5 hours.
- For *E. coli*, the rates were the same at Condition 2 in the lab and field and at Condition 4 in the field, but showed a much slower kill rate in the lab at Condition 4.
- *S. epidermidis* kill rates were comparable and faster under Condition 2 in the lab and field than Condition 4. At Condition 4, the kill rate in the lab was approximately 2x slower in the lab than in the field.
- For Phi6 bacteriophage, the kill rates were comparable in the lab and field at both conditions.

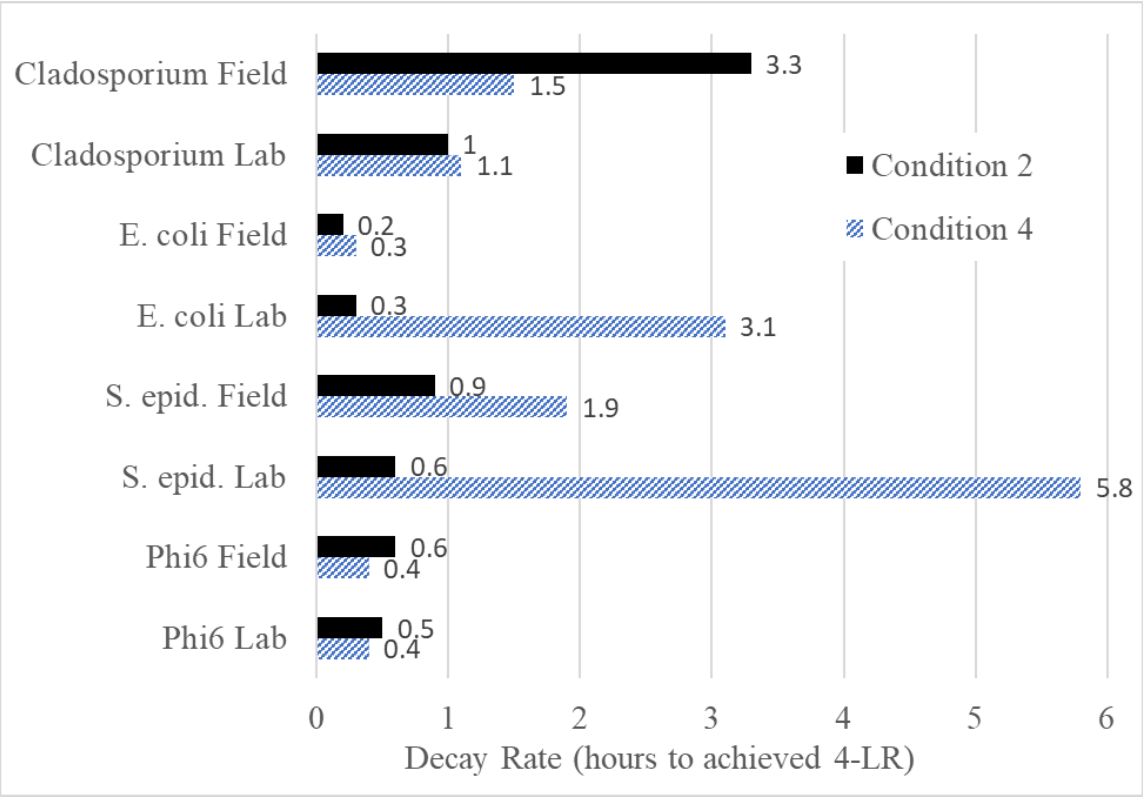


Figure 34. Comparing Kill Rates at Same Dew Points Achieved Under Two Different Temperature and RH Conditions

11.0 CONCLUSIONS

This study evaluated microorganisms with varying levels of resistance to disinfection and provided data to expand the utility of the JBADS process. Methods were established for preparing individual BIs consisting of non-traditional biowarfare agents. The surrogates represented enveloped and non-enveloped virus, Gram positive and Gram negative vegetative cells, fungal spores, and bacterial spores. The BIs were qualified in terms of concentration, stability, and resistance to a range of BTD treatments under controlled laboratory conditions. Thereafter, BIs were incorporated in JBADS field demonstrations to assess utility of the JBADS Lite to decontaminate a railcar potentially contaminated pathogens with varying levels of heat resistance.

The key elements successfully completed in this study include the following:

1. Established methods to prepare BIs with representative types of microorganisms.
2. Established kill rates (LR vs. time) of the BIs against a panel of BTD conditions at bench-scale.
3. Identified BTD conditions that can achieve a 1 LR within 2 hours (or 3-4 LR in 6-8 hours) per organism.
4. Assessed kill rates of BIs prepared with Phi6 bacteriophage, a surrogate to SARS-CoV-2 virus, when placed in public transport vehicle (railcar) and treated to a JBADS Lite decontamination cycles.
5. Assessed kill rates of the diverse types of BIs when treated via JBADS Lite in public transport vehicle (railcar) and compared these rates to those generated corresponding bench top BTD efficacy tests.

The BIs were shown to be stable for at least two weeks demonstrating they could be used to support field decontamination activities and can be used assess the effectiveness of a decontamination cycle for any given platform. Kill rates of each type of BI were determined for six BTD decontamination conditions that represented a range of target dew points achieved under different combinations of temperature and relative humidity. The conditions were deemed achievable using the JBADS Lite on a railcar with the exception of 143 °F @ 90% RH.

The Guardian Center Demo was successful in that all three demonstration trials achieved >4 LR within 2 hours from the time the cycle was initiated despite the JBADS Lite did not reach the desired dew point of 139 °F. The maximum dew points achieved were 129.2, 128.2, and 124.8 °F for Demos 1-3, respectively. Also, the time required to transition from one railcar to another was rapid at 45 minutes. The results from the demonstration can be used as an aid in determining decontamination cycle conditions and duration to achieve a desired level of kill for a particular infectious agent and that BIs can be used for future BTD or alternative decontamination lab-scale or field testing.

The Orlando Demo was also successful in that it demonstrated the utility of JBADS Lite to decontaminate a subway railcar that was contaminated with diverse types of organisms including virus, vegetative bacteria, and fungal spores. All three BTD conditions evaluated [Condition 2 (143 °F @ 50% RH), Condition 4 (120 °F @ 90% RH), and Condition 5 ((120 °F @ 50% RH),] achieved 4 LR for the virus and bacterial cell BIs in <6 hours. Conditions 2 and 4 also achieved 4

LR in <6 hours for the fungal BIs. As expected, the harshest of the conditions tested did not affect the viability of the bacterial spore BIs. Overall, the field efficacy data were comparable to the laboratory efficacy data, particularly at the higher temp/RH conditions (Conditions 2 and 4). While it is difficult to make conclusions based on a limited data set (one test per condition), reasonable correlation and reasonable kill times (<6 hours) for a variety of organisms were observed.

This study determined estimated kill rates of various BTD conditions ranging in dew points from 50 to 139 °F. Based on the laboratory and field demonstration efficacy results, the following estimated minimum target dew points are recommended as guide to achieve a 4 LR within 8 hours for each type of pathogen:

Microorganism	**Minimum Dew Point***	**Minimum Combination of Temperature and %RH**
Virus (enveloped or non-enveloped)	100 °F	120 °F and 50% RH
Gram positive Bacterial Cells	120 °F	143 °F and 50% RH 120 °F and 90% RH
Gram negative Vegetative Cells	100 °F	120 °F and 50% RH
Fungal Spores	100 °F	120 °F and 50% RH
Bacterial Spores	Significantly higher temperatures with elevated RH than those evaluated in this study	

*Dew point is defined as the atmospheric temperature (varying according to pressure and humidity) below which water droplets begin to condense and dew can form.

The D-values generated for the diverse types of BIs in this study can be used as an aid in selecting the decontamination cycle (time, temperature, and RH) to achieve a desired LR for a given type of pathogen using the JBADS Lite on a public transportation vehicle such as a subway railcar. This study was intended to provide initial estimates for BTD conditions and efficacy against a range of microorganisms. Ideally, these estimates will be refined with future studies consisting of more microorganisms from each category, more replicates of each BTD condition, and more refined timelines for decontamination.

12.0 RECOMMENDATIONS

Although this study did identify the kill rates for diverse types of microbiological BI to various BTD conditions, the data set was limited. Additional testing is recommended to expand the data set and subsequently strengthen the accuracy of the recommended BTD conditions for various types of pathogens and enhance the protocol for preparing the biological BIs. Recommended follow-on activities include (1) additional replicate tests of conditions evaluated in this study to increase accuracy of kill rates, (2) conduct more tests with MS2 bacteriophage to improve comparison of kill rates with Phi6 bacteriophage (this would include assessing stability and heat resistance of Phi6 and MS2 in other organic test soils as well as inorganic test soils such as PBS, (3) conduct more tests with other surrogate Gram positive bacteria, Gram negative bacteria, and fungi to ensure the surrogates chosen here are representative of each family of organisms, (4) assess heat resistance of fungal spores BIs prepared from fresh (>2 weeks) and aged (3 months) spores, and (5) assess stability and heat resistance of BIs prepared with of old and new bacteriophage and fungal stocks. Also recommended is field testing with JBADS Lite in an enclosed, insulated space to achieve higher dewpoints and address more robust organisms, like bacterial endospores.

13.0 SUPPORT DATA (LAB KILL CURVES)

The kill curves generated during lab BTD efficacy testing used to calculate the D-values at the for the panel of test organisms are shown from Figure 35 to Figure 41.

Figure 35. Phi6 BI Kill Curves

Figure 36. MS2 BI Kill Curves

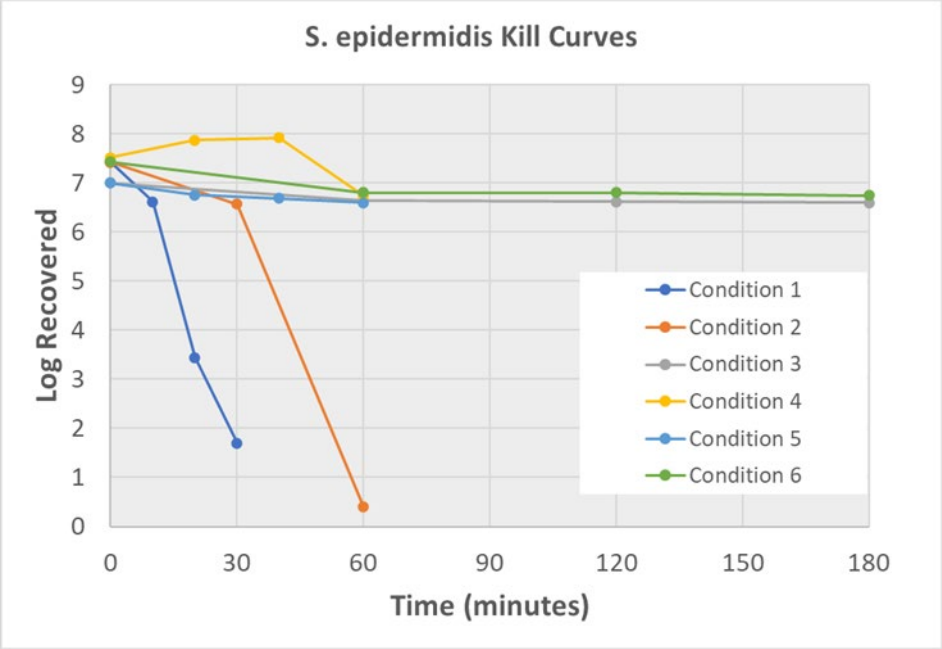


Figure 37. S. epidermidis BI Kill Curves

Figure 38. E. coli BI Kill Curves

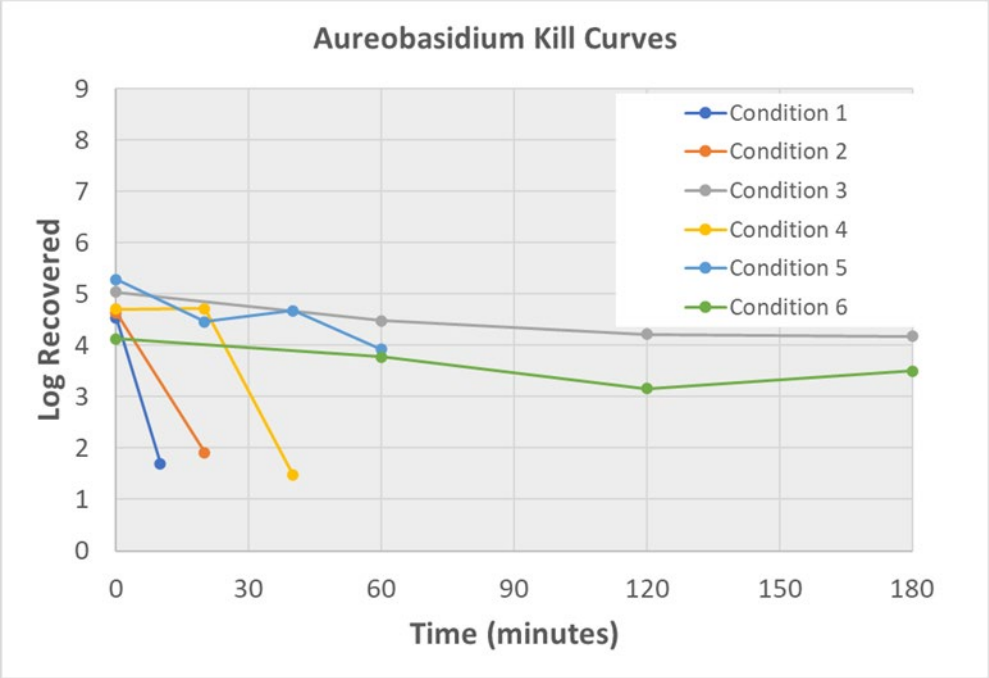


Figure 39. Aureobasidium BI Kill Curves

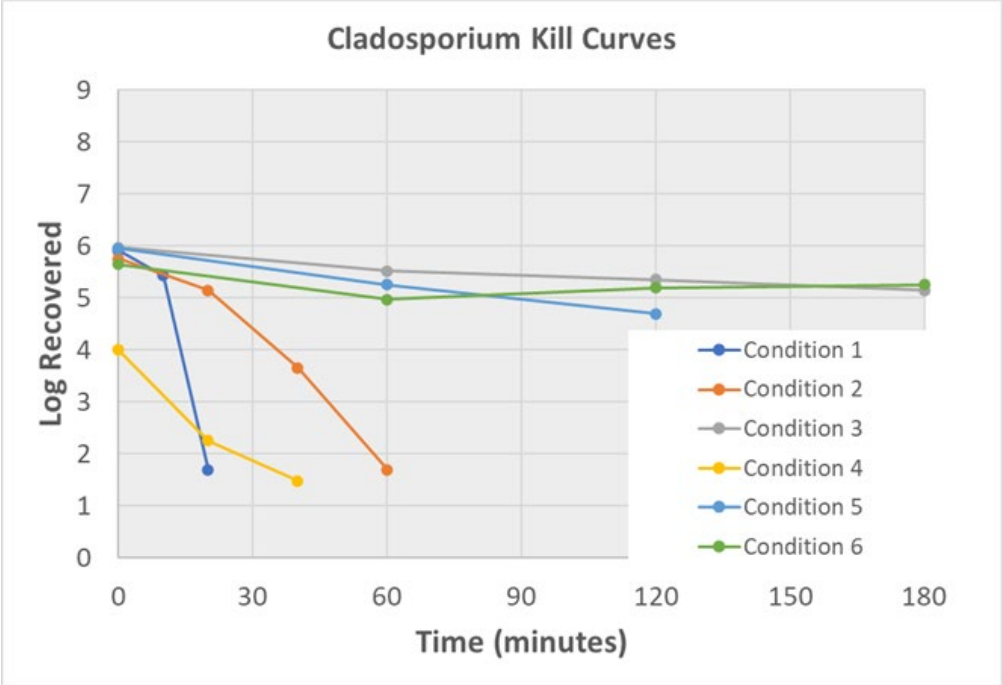


Figure 40. Cladosporium BI Kill Curves

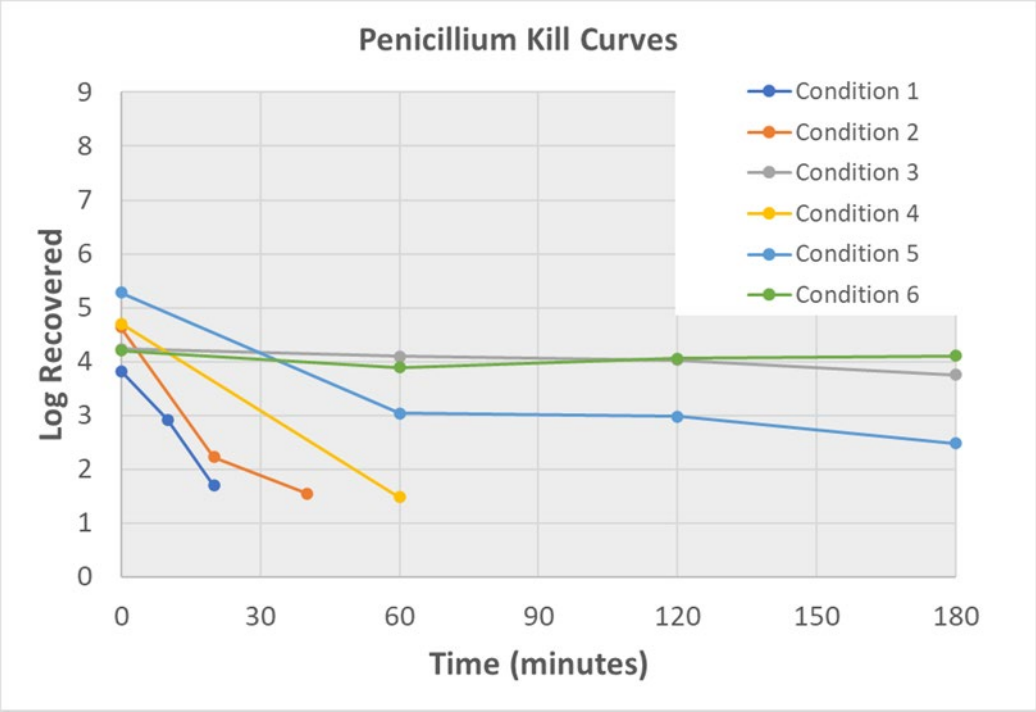


Figure 41. Penicillium BI Kill Curves

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

°F.....degrees Fahrenheit
ADU-H.....Aircraft Decontamination Unit - Heating
AE .....Aircraft Enclosure
AFB.....Air Force Base
AFRL .....Air Force Research Laboratory
AMC .....Air Mobility Command
BaS .....*B. anthracis* va Sterne
BC .....Baseline Controls
Bg.....*Bacillus atrophaeus* variety globigii
BI.....Biological Indicator
BSC.....Biosafety Cabinet
BTD.....BioThermal Decontamination
Btk.....*Bacillus thuringiensis* variety kurstaki
$CaCl_2$.....Calcium Chloride
CFU.....Colony Forming Unit
CONOP.....Concepts of Operation
COVID-19..... Coronavirus
DAL .....Double Agar Overlay
FBS .....Fetal Bovine Serum
GFE.....Government Furnished Equipment
HAD.....Hot Air Decontamination
JBADS .....Joint Biological Agent Decontamination System
LC .....Laboratory Controls
LR .....Log Reduction
mL.....milliliter
µL.....microliter
µm .....micrometer
OSA.....Operational Sensor Array
PBS .....Phosphate Buffered Saline
PFU .....Plaque Forming Unit
pH.....Potential of Hydrogen

RH .....Relative Humidity

rpm .....Rotations Per Minute

SARS-CoV-2 .....Severe Acute Respiratory Syndrome – Coronavirus 2

TC .....Transport Controls

TSA .....Tryptic Soy Agar

TSB .....Tryptic Soy Broth

USAF .....United States Air Force

VDU.....Vehicle Decontamination Unit

xg.....times gravity

REFERENCES

[1] Goodson, W. J., C. Drake. *Decontamination of Microbial Contaminants on Aircraft using Biothermal Decontamination*. September 26, 2016. Draft Report. Soft Matter Materials Branch, Air Force Research Laboratory, Wright-Patterson AFB, OH 45433.

[2] *Technical Assessment Report for Joint Biological Agent Decontamination System Lite (JBADS Lite) C-17 Interior Decontamination Capability Demonstration*. JPEO-CBRND. June 2020.

[3] A. Theys, D. Lorch, B. Davis, D. Lewis, W. Greer, H. Horenziak. *Hot Air Decontamination of a C-17 using Diesel-Powered Heaters at McKinley Climatic Laboratory*. USAF Contract No. FA8650-19-D-6990. AD1144604, AFRL-RH-WP-TR-2020-0138. Dec 2020.

[4] A. Theys, D. Lorch, M. Docter, D. Lewis, W. Greer, H. Horenziak. *Hot Air Decontamination of a C-17 using Diesel-Powered Heaters at Dover Air Force Base.* USAF Contract No. FA8650-21-D-6282. AD1145937, AFRL-RH-WP-TR-2021-0038. July 2021.

[5] ASTM E 2197-02. *Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Germicides*.

[6] Leighton T.J., and R. H. Doi. *The Stability of Messenger Ribonucleic Acid during Sporulation in Bacillus subtilis*. Control Mechanisms and Biochemical Genetics. 246(10) p. 3189-3196. May, 1971. DOI: https://doi.org/10.1016/S0021-9258(18)62213-6

[7] Wood, J.P, W. Richter, M. Sunderman, M. W. Calfee, S. Serre, and L. Mickelson. *Evaluating the Environmental Persistence and Inactivation of MS2 Bacteriophage and the Presumed Ebola Virus Surrogate Phi6 Using Low Concentration Hydrogen Peroxide Vapor*. Environmental Science & Technology 2020 54 (6), 3581-3590. DOI: 10.1021/acs.est.9b06034

[8] Holwitt, E., J.L. Kiel, J. L. Alls, P.J. Morales, and H. Gifford. *Thermal Sensitivity of Biowarfare Simulants*. Chem. And Biol. Sensing. 2000. Proceedings of SPIE Vol. 4036.