NAVSEA
WARFARE CENTERS
DAHLGREN

NAVAL SURFACE WARFARE CENTER
DAHLGREN DIVISION
SCIENCE AND TECHNOLOGY - RESEARCH AND DEVELOPMENT - TEST AND EVALUATION

ELECTROMAGNETIC & SENSOR SYSTEMS
DEPARTMENT

Inactivation of Spores, Vegetative Bacteria and Virus on Surfaces Exposed to Hot, Humid Air

EPA
Norfolk, VA
November 19, 2019

Presented by

Tony Buhr, Ph.D.

Background

Need for Decontamination:

Commercial aircraft temperature materials are typically tested at 140˚F (60˚C). This does not imply that materials won't survive higher temperature, but the cost of changing the test temperature for qualifying all materials is high. Department of Defense accepts risk of higher temperatures. Lower decontamination temperature and times translates to lower costs, higher practicality, increased applications. This may drive commercial marketing and economies of scale production for public health. This will lower costs and increase capability.

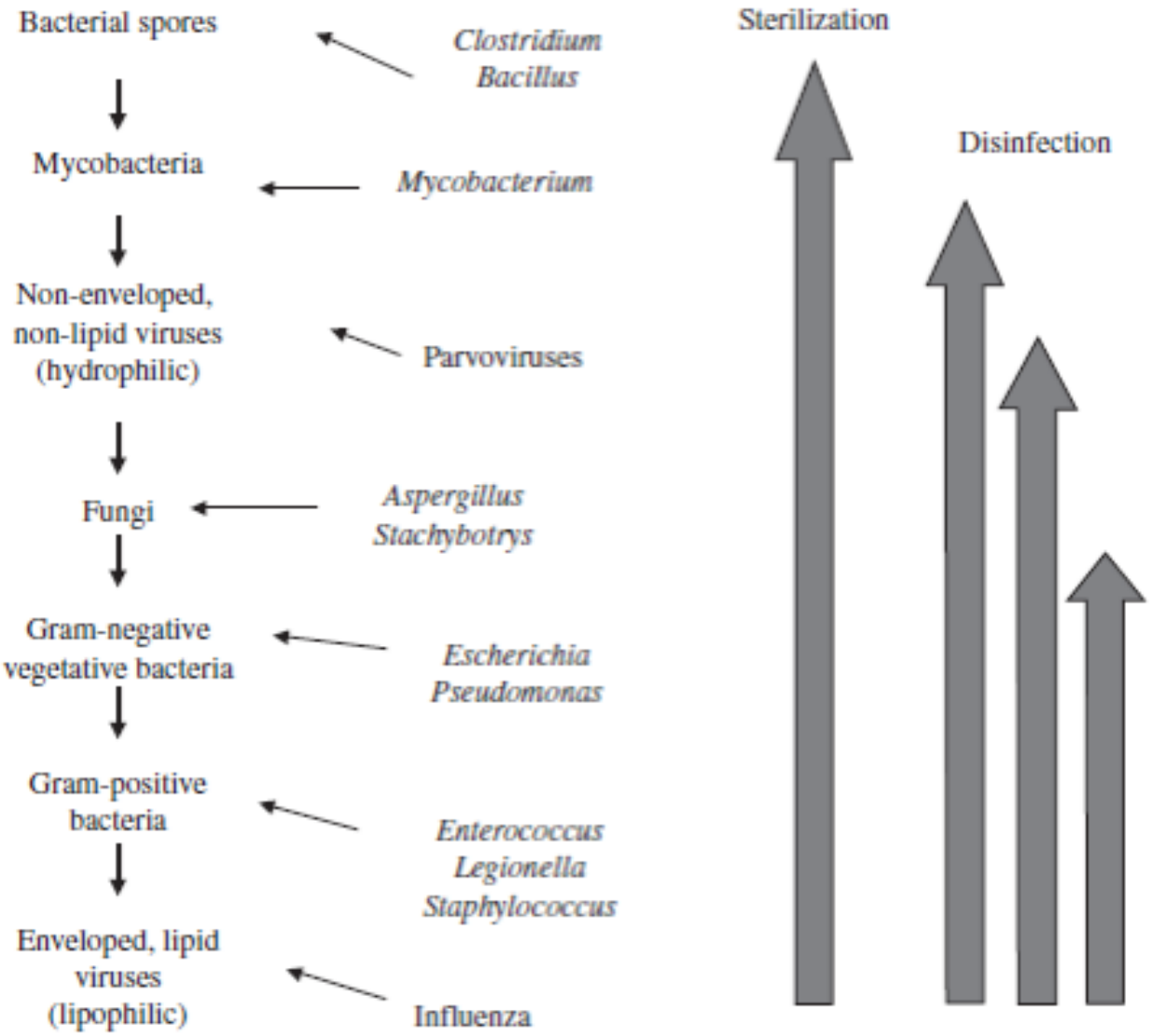
Objective:

Test microbial inactivation of virus to assess if inactivation/survival is at or below the hot, humid air parameters used to decontaminate spores. The objective is to reduce decontamination temperature and time requirements from the current Joint Biological Agent Decontamination System (JBADS) requirements, which are ≥75˚C, ≥72h, 70-90% relative humidity (RH), down to ≤60˚C for ≤24h. Manufacturer accepts risk of 60˚C exposure.

BLUF: Lab tests show ≥7 $\log_{10}$ virus inactivation with a single heat treatment of 60˚C for 9 h at 80–90% RH.

Spaulding Hierarchy of Disinfection (1957)

Spaulding, E.H. 1957. Chemical disinfection and antisepsis in the hospital. *J Hosp Res* 9, 5-31.
McDonnell, G. and Burke, P. 2011. Disinfection: is it time to reconsider Spaulding. *J Hosp Inf* 78, 163-170.



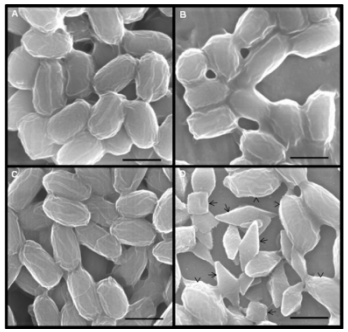
Select Agent Virus Surrogate Selection

Ebola- Morphology

- Pleomorphic (altering its size and shape in response to environmental conditions)

- Can exist in 3 forms
  1 Single virion (1 µm x .1 µm)
  2 Continuous
  3 Linked (> 20 µm in length !!)

  > 50% of virions from cell culture are thought to be polyploidy, polyploidy virions are also found in infected animal and human specimens



B. anthracis: 5,227,293 base pairs (Read et al., 2003)

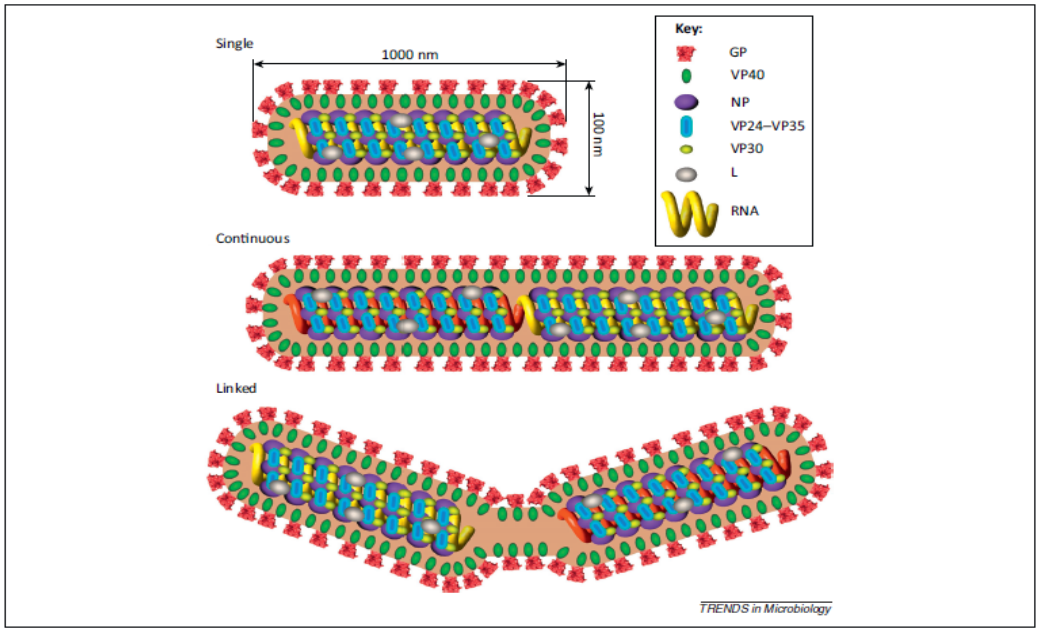


Figure 1. Schematic of Ebola virus structure. The three different types of Ebola virus and location of the viral proteins and RNA genome. Single virions (top) have one copy of the genome, whereas the continuous (centre) and linked (bottom) are polyploid and have two or more copies of the genome. Virions of length more than 20 µm from cell culture have been measured [6]. The nucleocapsid consists of NP (nucleoprotein), L ('large', polymerase), and viral proteins VP35, VP30, and VP24, and the envelope has two integral membrane proteins, the VP40 matrix protein on the cytoplasmic side and the externally exposed glycoprotein (GP) spike. Particles are not drawn to scale for clarity. In addition, comma-shaped and toroidal virions exist (not shown), and these are usually variants of the single virion.

19,000 bases (9,500 base pairs)

Ebola Virus Environmental Stability

(Schuit et al. pLOS One 2016; NBACC study)

Ebola stability – temperatures of 22°C/17%, 22°C/41% and 28°C/90% were tested.

Blood stabilizes the virus. Virus lasts at least a week in blood.

Feces and Vomit destabilize the virus.

Longest survival at 28°C/90%. Lowest survival at 22°C/17%.

Data Gaps – Doesn't meet decontamination requirements

Plaque assays not used to measure (microtiter assay was used, which loses 0.7 $\log_{10}$ of assay sensitivity). We incubate the entire virus-contaminated coupons with host cells.

Assay sensitivity for Environmental Stability ~ 4.3 $\log_{10}$ test^{-1}.

DoD test requirement for Decontamination is ≥8 $\log_{10}$ PFU test^{-1}.

High test titers required for decontamination confidence

Hamilton et al. (2013) JAOAC Int 96, 1138–1151.

Decontamination was not tested, just environmental persistence

Extraction efficiency not tested in this publication.

Virus Surrogate Selection – Bibby et al. 2015

Properties	Ebola	Marburg	Attenuated Ebola	Influenza[2]	Vaccinia	Phage,[3] Plant
Enveloped	Yes	Yes	Yes	Yes	Yes (multi-layered)	Yes- Φ6, Cauliflower Mosaic Virus No- MS2, Tobacco Mosaic Virus
Nucleic Acid	Single stranded-ribonucleic acid (RNA) (18.9 kilobases)[1]	Single stranded-ribonucleic acid (RNA) (19.1 kilobases)[1]	Single stranded-ribonucleic acid (RNA) (≈ 19 kilobases)	Single stranded-ribonucleic acid (RNA) (13.6 kilobases)	Double stranded-deoxyribonucleic acid (DNA) (190 kilobases)	Double stranded-ribonucleic acid (RNA) (13.5 kilobases)
Morphology						
Single Virion	Yes	Yes	Yes	Yes	Yes	Yes - Φ6, MS2
Filamentous	Yes	Yes	Yes	Yes	No	Yes- Tobacco Mosaic Virus
Clinical Form	Filamentous	Filamentous		Filamentous	Spherical	
Biosafety (BSL) Level	Biosafety Level (BSL)-4	Biosafety Level (BSL)-4	Biosafety Level (BSL)-3	Biosafety Level (BSL)-2 Biosafety Level (BSL)-1 (?)	Biosafety Level (BSL)-1 Biosafety Level (BSL)-2	Biosafety Level (BSL)-1 Field

Φ6 (bacteriophage) was selected as a surrogate for Ebola because it is an enveloped, ribonucleic acid (RNA) virus that can be tested in a Biosafety (BSL)-1 at a much lower cost than direct testing on Biosafety (BSL)-4 Ebola.

Plaque assays can be used to fulfill Joint requirements of a ≥ 8 $\log_{10}$ limit of detection. Enveloped viruses, are challenging to purify and test.

Φ6 Overlay Plating Methods

- Objective was a ≥7 $\log_{10}$ PFU inactivation out of a ≥8 $\log_{10}$ PFU coupon^{-1} challenge at 60°C, ≤24 h
- Methods development was Highly Challenging. Φ6 Overlay Plating Method was developed with ≥8 $\log_{10}$ PFU coupon^{-1}.
- Coupon Materials were Nylon, Polypropylene, Wiring Insulation, APC, Plus a Solution Control
- Method Confidence was dependent on host cells
  - Clean enveloped virus is unstable. This is commonly known for Enveloped Virus but not always commonly observed in methods
  - Blood destabilized Φ6, Blood stabilizes Ebola
  - Humic acid destabilized Φ6, Humic acid "stabilizes" spores
  - *Pseudomonas syringae* pv *phaeolicola* cell debris stabilized Φ6

FINAL Design of Experiments (DOE) for Φ6 Virus + Host Cell with the Improved Test Methods: DOE #5

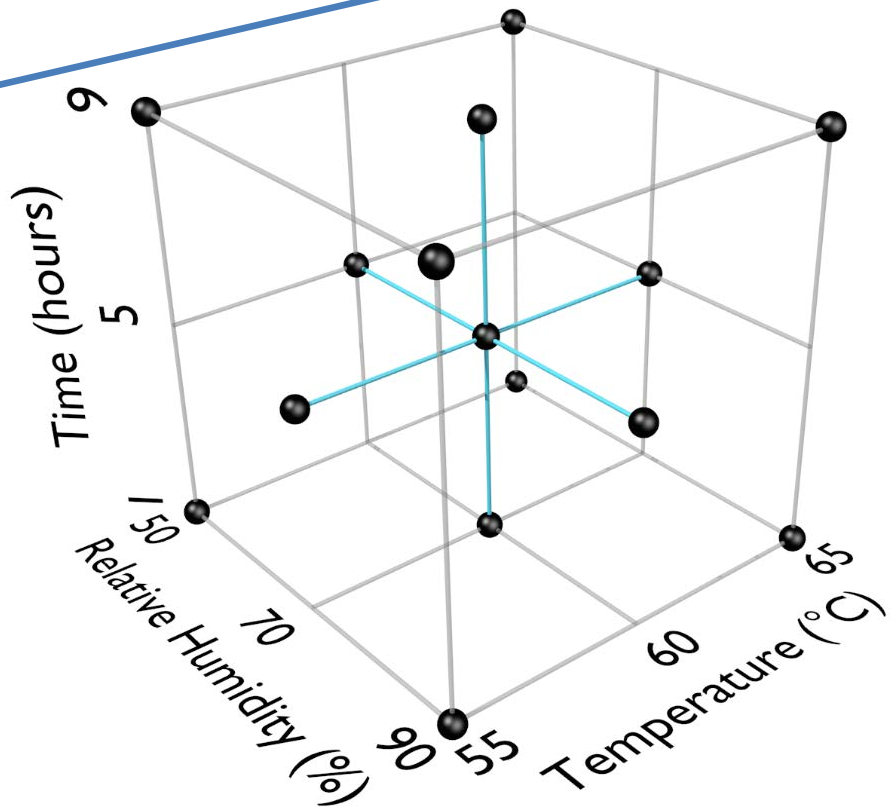
Critical Data to dictate the Final DOE are from the annual report: Table 20 and 21. High and Low RH Test: $\log_{10}$ virus survival of Φ6 mixed with *Pseudomonas syringae* HB10Y host cell debris.
Survival out of 8.3 ± 0.0 $\log_{10}$ virus inoculated onto coupons

Nylon	9h	11h	13h	15h
60°C, 90%	1.6 ± 1.5	0.5 ± 1.2	0.0 ± 0.0	0.0 ± 0.0

Nylon	11h	20h
60°C,5%	8.1 ± 0.2	8.0 ± 0.2

Φ6 Virus + Host Cell DOE #5

Temp: 55, 60, 65°C
RH: 50, 70, 90%
Time: 1, 5, 9 h



Design of Experiments (DOE) #5 Data Table for Φ6 Virus + Host Cell (Celsius (°C), Relative Humidity (RH)

Bacteriophage Φ6 in Cell Debris										
Bacteriophage Φ6 Log Survival; 13 independent runs - 8.4±0.0 logs; 6 independent runs - 8.3±0.1 logs; 5 independent tests per material per run										
		50% RH	50% RH	50% RH	70% RH	70% RH	70% RH	90% RH	90% RH	90% RH
Material	Temp (°C)	1 h	5 h	9 h	1 h	5 h	9 h	1 h	5 h	9 h
Nylon	55	8.2 ± 0.2	NA	7.0 ± 0.2	NA	5.5 ± 0.1	NA	7.2 ± 0.1	NA	1.1 ± 1.5
Nylon	60	NA	7.0 ± 0.4	NA	6.7 ± 0.2	4.9 ± 0.6	3.4 ± 0.3	NA	0.0 ± 0.0	NA
Nylon	65	7.3 ± 0.2	NA	5.6 ± 0.1	NA	0.7 ± 0.9	NA	0.0 ± 0.0	NA	1.4 ± 1.3
Polypropylene	55	8.3 ± 0.3	NA	7.6 ± 0.2	NA	7.8 ± 0.3	NA	7.7 ± 0.2	NA	0.0 ± 0.0
Polypropylene	60	NA	6.9 ± 0.2	NA	8.1 ± 0.1	6.5 ± 0.7	4.9 ± 0.3	NA	0.0 ± 0.0	NA
Polypropylene	65	7.2 ± 0.2	NA	2.4 ± 0.4	NA	2.0 ± 0.3	NA	0.0 ± 0.0	NA	0.0 ± 0.0
Wiring	55	8.3 ± 0.1	NA	7.9 ± 0.3	NA	7.0 ± 0.8	NA	7.5 ± 0.1	NA	0.0 ± 0.0
Wiring	60	NA	6.9 ± 0.6	NA	7.9 ± 0.1	6.7 ± 0.5	4.3 ± 0.5	NA	0.0 ± 0.0	NA
Wiring	65	7.0 ± 0.4	NA	4.1 ± 0.6	NA	2.0 ± 1.4	NA	0.0 ± 0.0	NA	0.0 ± 0.0
APC	55	8.3 ± 0.1	NA	6.8 ± 0.4	NA	7.0 ± 0.6	NA	7.5 ± 0.2	NA	1.3 ± 1.2
APC	60	NA	3.8 ± 0.9	NA	7.8 ± 0.2	6.2 ± 0.7	4.6 ± 0.3	NA	0.0 ± 0.0	NA
APC	65	5.3 ± 0.7	NA	2.9 ± 1.6	NA	0.8 ± 0.8	NA	0.0 ± 0.0	NA	0.0 ± 0.0
Sol Cont	55	0.0 ± 0.0	NA	0.3 ± 0.6	NA	0.0 ± 0.0	NA	0.4 ± 0.9	NA	0.0 ± 0.0
Sol Cont	60	NA	0.0 ± 0.0	NA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA	0.0 ± 0.0	NA
Sol Cont	65	0.0 ± 0.0	NA	0.0 ± 0.0	NA	0.0 ± 0.0	NA	0.0 ± 0.0	NA	0.0 ± 0.0

APC - Aircraft Performance Coating

NA - not available, not tested for DOE

h – Hours

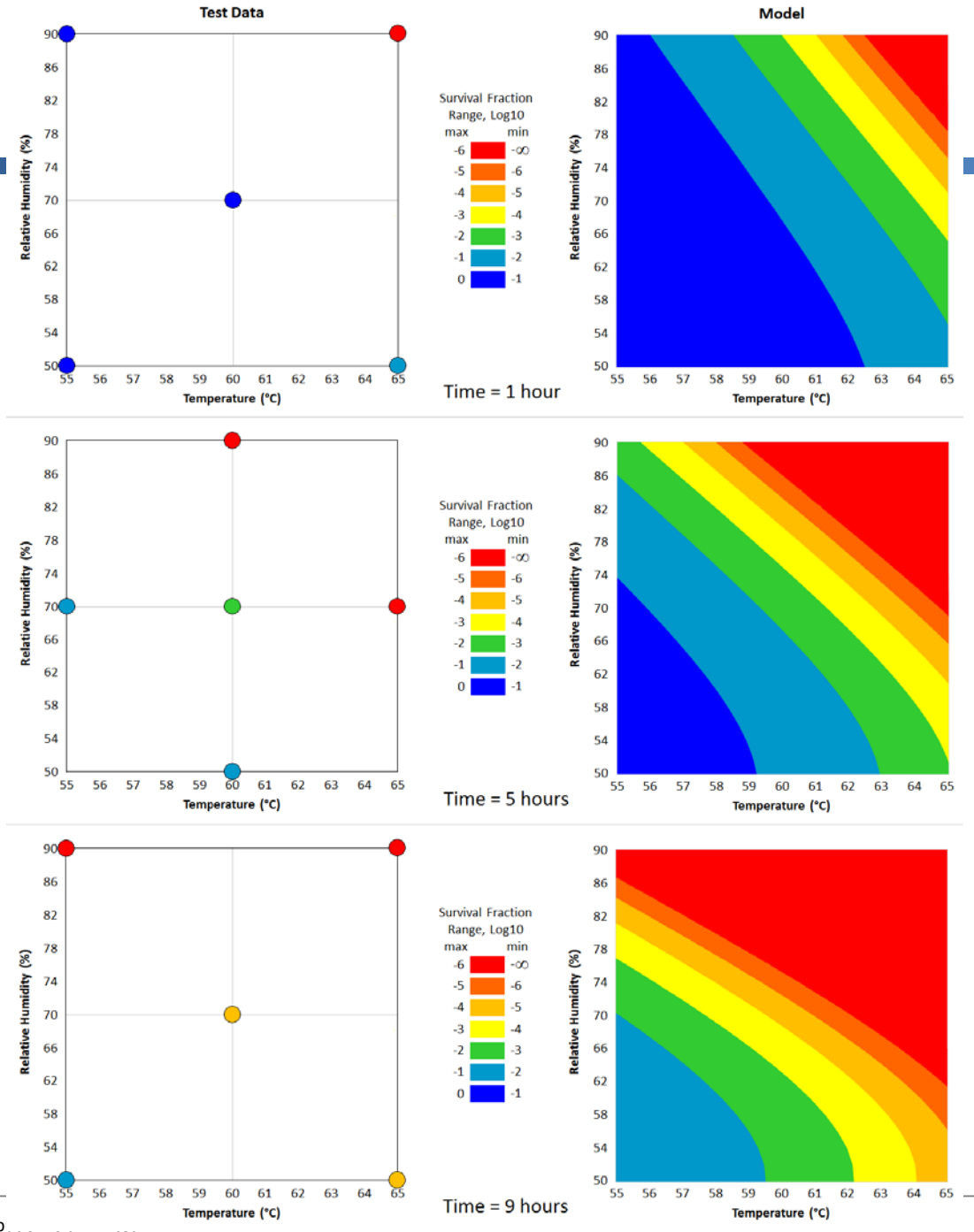
Polypropylene is the same as the TPP tube

Design of Experiments (DOE) Models (Geometric Means) for Decontamination of Composite Samples, ie All Samples Combined Contaminated with Φ6 Virus + Host Cell

1, 5, 9 hours

50-90% Relative Humidity (Y axis)

55-65°Celsius (X axis)



Summary of Results

- Hot, humid air decontamination of dirty spores at 75–80°C, 70–90% relative humidity for >3 days, preferably 4-5 days for a ≥6 $\log_{10}$ inactivation according to the dirty spore models
- Extensive Methods development to increase practical and statistical confidence for Φ6 (ENVELOPED VIRUS) is now completed. Virus tests with ≥8 $\log_{10}$ coupon^{-1}.
  - Enveloped virus stability is affected by host cells
  - All data indicated that dirt debris, other than host cells, reduced virus stability
- DOE with Φ6 + host cells required extensive iterations to find DOE parameters. Final DOE was DOE #5. Test parameters moving forward to other virus are ~10% of original test. The cost and schedule of this trial and error justified the selection of Φ6 for these original first models of enveloped virus decontamination.
- Hot, humid air decontamination of dirty virus at 60°C, 80–90% relative humidity for 9 h. One could extend decontamination to 60°C, 80–90% relative humidity for 12 h to mitigate risk of any unknown. Significant reduction in time and temperature compared to spores.
- 60°C should be applicable to airframes other than C-130 without impacting aircraft warranties. Hence 60°C is a risk mitigation strategy for warranties and maintenance.

Acknowledgements

- JPM-P – Brant Lagoon, LtCol Holl, LtCol Bordner, Bill Washington, Carrie Dager and many more
- DTRA – Dr. Charles Bass, Dr. Glenn Lawson, Dr. Mark Morgan
- NSWC – Dahlgren – Dr. Tony L. Buhr*, Zachary A. Minter, Alice A. Young, Neil L. Kennihan, Matthew D. Bohmke, Harold K. Barnette, Emily Osborn, Shelia Hamilton, Monique Kimani, Erica Borgers-Klonkowski, Mark Hammon, Charles Miller, Ryan Mackie, Jennifer Innocenti, Misty Bensman, Dr. Bradford W. Gutting