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DAHLGREN DIVISION
SCIENCE AND TECHNOLOGY - RESEARCH AND DEVELOPMENT - TEST AND EVALUATION

ELECTROMAGNETIC & SENSOR SYSTEMS
DEPARTMENT

Combining Spore Germination and Hot Air Treatment to Reduce the Costs and Time and Lower the Temperature for Hot Air Decontamination

EPA Decontamination Conference
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Presented by

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Objective

Buhr et al. 2012, 2014, 2015; Herzberg 2012; Koch et al. 2001

Objective: The objective is to add a spore germination step prior to hot air decontamination in order 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.

Need for Decontamination:

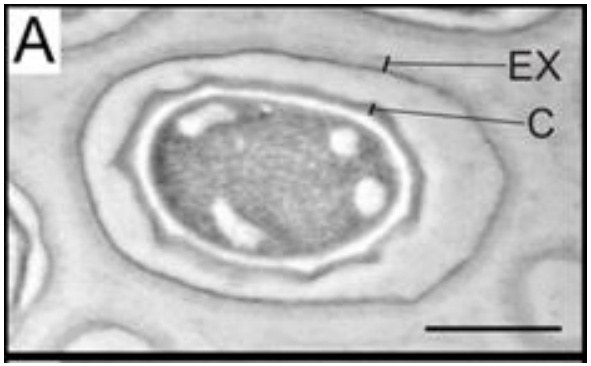
Commercial aircraft temperature materials are typically tested at 140˚F (60˚C). This does not imply that materials won't survive higher temperature, but the cost of changing the test temperature for qualifying all materials is high. DoD accepts risk of higher temperatures.

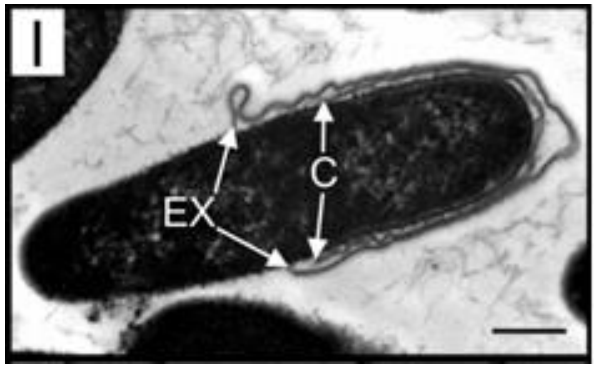
Lower decontamination temperature and times translates to lower costs, higher practicality, increased applications

B. anthracis Spore, Germination and Outgrowth

Buhr et al. (2012) – Dr. Derrell McPherson; *B. anthracis* germinated spore TEM courtesy Dr. Chris Cote (USAMRIID)

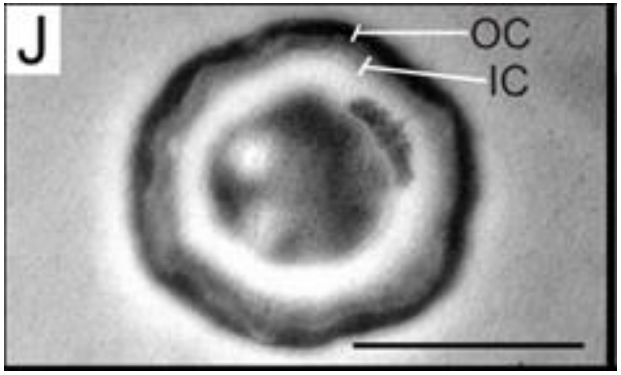
B. anthracis – Dormant spore, germinated spore, outgrown spore





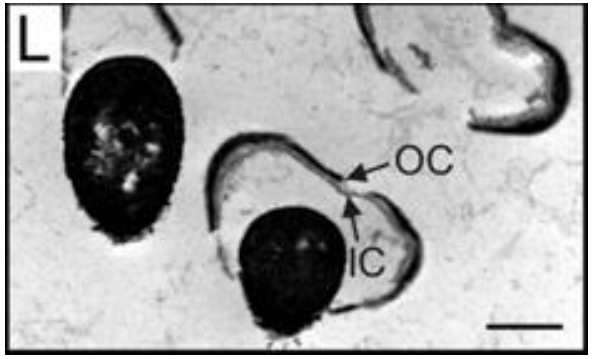
B. atrophaeus (Bg)

Dormant spore

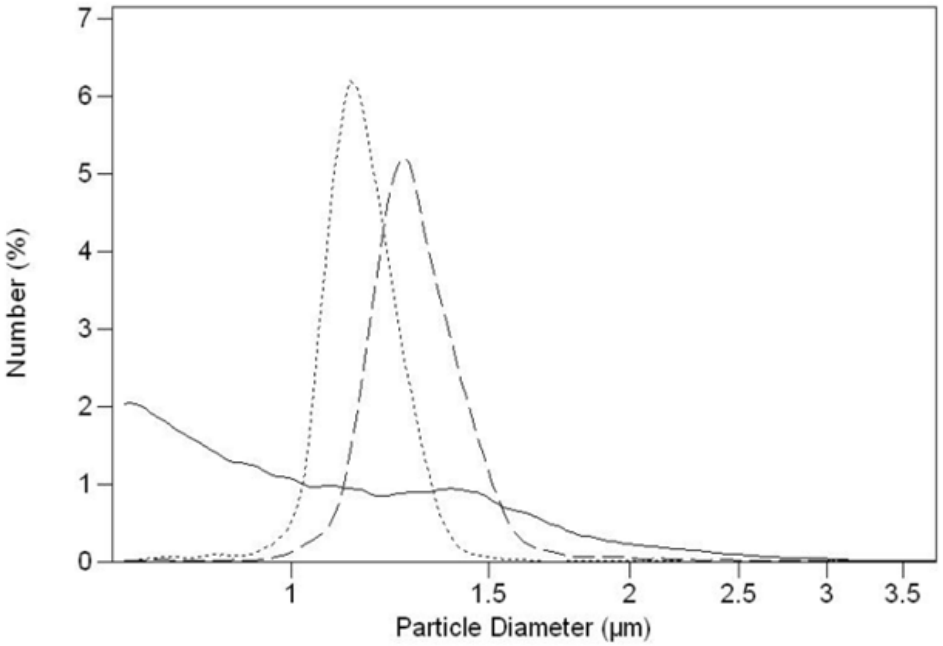


B. atrophaeus (Bg)

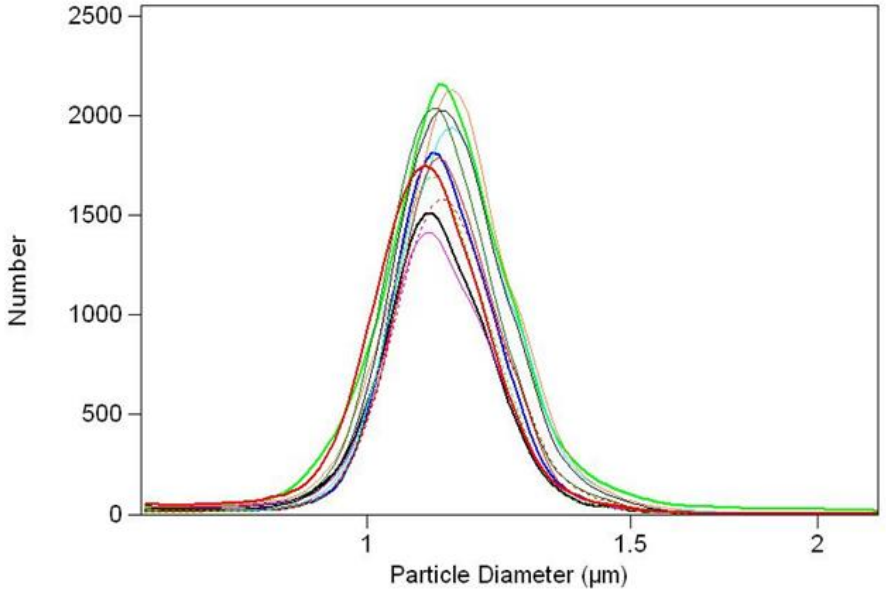
Outgrown spore



Standardized *Bacillus* Spore Preparation for Macrobacillus Spores Containing Exosporia



NSWC *B. anthracis* ΔSterne preparation (····)
NSWC *B. thuringiensis* Al Hakam preparation (– – –)
Independent laboratory *B. thuringiensis* preparation (—)



12 independent NSWC *B. anthracis* ΔSterne spore preparations

Anonymous. 2018. Standard Practice for Evaluating Efficacy of Vaporous Decontaminants Against *Bacillus* Spores Contained Within 0.2µm Filter-capped Tubes. ASTM Standard Practice E3092.

Cote, C., Buhr, T., Bernhards, C.B., Fetterer, D.P., Welkos, S.L., Bozue, J.A., McPherson, D., Fountain, A.W. III, Gibbons, H.S. and the *Bacillus anthracis* Spore Irradiation Consortium. (2018) A Standard Method to Inactivate *Bacillus anthracis* Spores to Sterility Using Irradiation. Submitted to Applied and Environmental Microbiology.

Plaut, R., Staab, A., Munson, M., Gebhardt, J., Klimko, C., Quirk, A., Cote, C., Buhr, T., Rossmaier, R., Bernhards, R., Love, C., Berk, K., Abshire, T., Rozak, D., Beck, L., Stibitz, S., Goodwin, B., Smith, M., Sozhamannan, S. (2018) Construction and Characterization of an Avirulent *Bacillus anthracis* Strain Carrying Molecular Assay Targets as a Surrogate for Irradiation-inactivated Virulent Spores. Emerging and Infectious Diseases, in review: Emerging Infectious Diseases 17-1646-R1.

Original Test Design Including Unanswered Test Parameters

Buhr et al. Annual Report (2017)

Original Test Design was based on a single germinant application with an estimated germination temperature of 35˚C, unknown time, followed by 60˚C, 1 h

- **What is the optimized germinant solution?**
- **Is germination efficiency sufficient to meet decontamination test requirements?**
  - **≥7 $\log_{10}$ spores/test to show at least a 6 $\log_{10}$ spore inactivation**
  - **≥8 $\log_{10}$ spores/m^2 (coupon tests are 2.5e10 $\log_{10}$ spores/m^2)**
- **Is heat inactivation needed?**
- **What are the germination limits (Temperature, RH, Time)?**
  - **Target temperature is 35˚C. Is germination efficient at >35˚C?**
  - **Target time is unknown, but short duration (<20h is a target).**
  - **Target RH is unknown.**
- **What are the heat inactivation limits (Temperature, RH, Time)?**
  - **Target temperature is 60˚C with a range of 50-60˚C.**
  - **Target time is 1 h. This is short duration.**
  - **Target RH is unknown.**
- **Can a single germinant application be used?**
- **Can a fogger be used?**

Germination for Optimized Solution Testing with KOH. Results: Heat Inactivation is Required; Quantitative Detection in Field is a Capability Gap

Buhr et al. Quarterly Report (2016)

Spore germination assayed by serial dilution and plating shows total $\log_{10}$ colony forming unit (CFU) ml^{-1} and heat-resistant CFU ml^{-1} survival of *B. anthracis* ΔSterne, *B. thuringiensis* Al Hakam or *B. thuringiensis* kurstaki cry- HD 1 after incubation at 35ºC and 200 rpm for 0, 1, 2, 4 and 24 hours in the 0.1M L-Alanine + 1mM Inosine + 40mM CaCl2 + 40mM DPA + 20mM Tris Base + 70mM KOH germination solution. Data is the mean of three independent experiments.

Species	Inoculation Titer log10 (Spores ml^{-1})	30 min, 65˚C Heat	Time points (h)				
			0	1	2	4	24
BaΔSt	5.2	no	5.2	4.9	4.7	4.4	0.6
		yes	5.1	2.2	1.0	0.1	0.2
	6.1	no	6.1	5.7	6.0	5.6	1.4
		yes	6.0	2.7	1.7	0.7	0.2
	7.2	no	7.2	6.6	6.9	6.8	3.0
		yes	**6.7**	**3.5**	**2.8**	**2.3**	**1.6**
BtAH	5.2	no	5.2	5.2	5.2	5.0	1.3
		yes	5.3	0.4	0.0	0.0	0.0
	6.1	no	6.1	6.2	6.1	5.7	4.2
		yes	6.2	1.2	1.2	0.6	1.1
	7.3	no	7.3	7.2	7.0	6.8	5.8
		yes	7.4	2.2	1.4	1.7	4.9
Btk cry-HD1	5.2	no	5.2	5.2	5.3	4.5	0.0
		yes	5.2	0.7	0.2	0.0	0.0
	6.2	no	6.2	6.2	6.2	5.6	2.7
		yes	6.2	1.8	1.0	0.6	0.2
	7.2	no	7.2	7.3	7.2	7.0	4.3
		yes	6.9	2.4	1.9	1.5	2.2

Repeat Application of Germination/Hot Humid Air: RESULTS for spores on Nylon

Two Cycles of Germination and Heat Inactivation shows total $\log_{10}$ CFU survival of *B. anthracis* ΔSterne spores dried on to nylon webbing. Germination occurred at 35˚C, 90% RH in 0.1M L-Alanine + 1mM Inosine + 40mM CaCl2 + 40mM DPA + 20mM Tris Base + 70mM KOH and heat inactivation was at 60˚C, 90% RH. Five independent replicates were tested.

Sample	Single Germination and Decontamination Step	Repeat Germination and Decontamination Steps						
	Test A	**Test B**	**Test C**	**Test D**	**Test E**	**Test F**	**Test G**	**Test H**
	2h Germination	2h Germination 1h Decon;	2h Germination	2h Germination	2h Germination	2h Germination	2h Germination	2h Germination
			1h Decon;	1h Decon;	1h Decon;	1h Decon;	1h Decon;	1h Decon;
	1h Decon	2h Germination 1h Decon	16h Germination	16h Germination	20h Germination	20h Germination	24h Germination	24h Germination
			1h Decon	No Decon	1h Decon	No Decon	1h Decon	No Decon
Test - Nylon	2.4 ± 0.5	1.4 ± 0.5	0.6 ± 0.3	0.8 ± 0.9	0.5 ± 0.6	0.6 ± 1.0	0.2 ± 0.3	0.5 ± 0.7
Dry Extraction Control - Nylon	6.7 ± 0.0	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
Wet Extraction Control - Nylon	6.5 ± 0.1	6.6 ± 0.1	6.4 ± 0.1	6.4 ± 0.2	6.3 ± 0.2	6.5 ± 0.1	6.6 ± 0.1	6.5 ± 0.2
Test - Solution Control	7.3 ± 0.3	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.2	7.3 ± 0.2	7.2 ± 0.1	7.3 ± 0.0	7.3 ± 0.1
Ca-DPA Media 1st Germination	6.0 ± 0.1	6.0 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.1 ± 0.1	6.1 ± 0.1
Ca-DPA Media 2nd Germination	NA	0.8 ± 0.5	0.7 ± 1.0	0.0 ± 0.0	0.4 ± 0.6	0.0 ± 0.0	0.4 ± 1.0	0.0 ± 0.0
RT - Nylon	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1
RT - Solution Control	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1

Formulating for Corrosion Mitigation

Germinant combinations with lower salt concentrations.

Germination Medium	Description
0.1M L-Alanine + 1mM Inosine + 40mM CaCl2 + 40mM DPA + 20mM Tris Base + 70mM KOH, pH 8.3	Optimized germination medium
0.1M L-Alanine + 1mM Inosine + 20mM CaCl2 + 20mM DPA + 10mM Tris Base + 35mM KOH, pH 8.3	Optimized germination medium with salts reduced to 50%.
0.1M L-Alanine + 1mM Inosine + 40mM CaCl2 + 40mM DPA, pH 8.3 w/ Tris Base	Optimized germination medium with no KOH added. Tris base was used to achieve pH of 8.3.
0.1M L-Alanine + 1mM Inosine, pH 8.3 w/ Tris Base	0.1M L-Alanine + 1mM Inosine only with Tris Base used to achieve pH of 8.3.
0.1M L-Alanine + 1mM Inosine, pH 6.1	0.1M L-Alanine + 1mM Inosine only with no pH adjustment performed.

Single Application – No K+ (Address Corrosion) 60°C maximum temperature; < 24 h total time

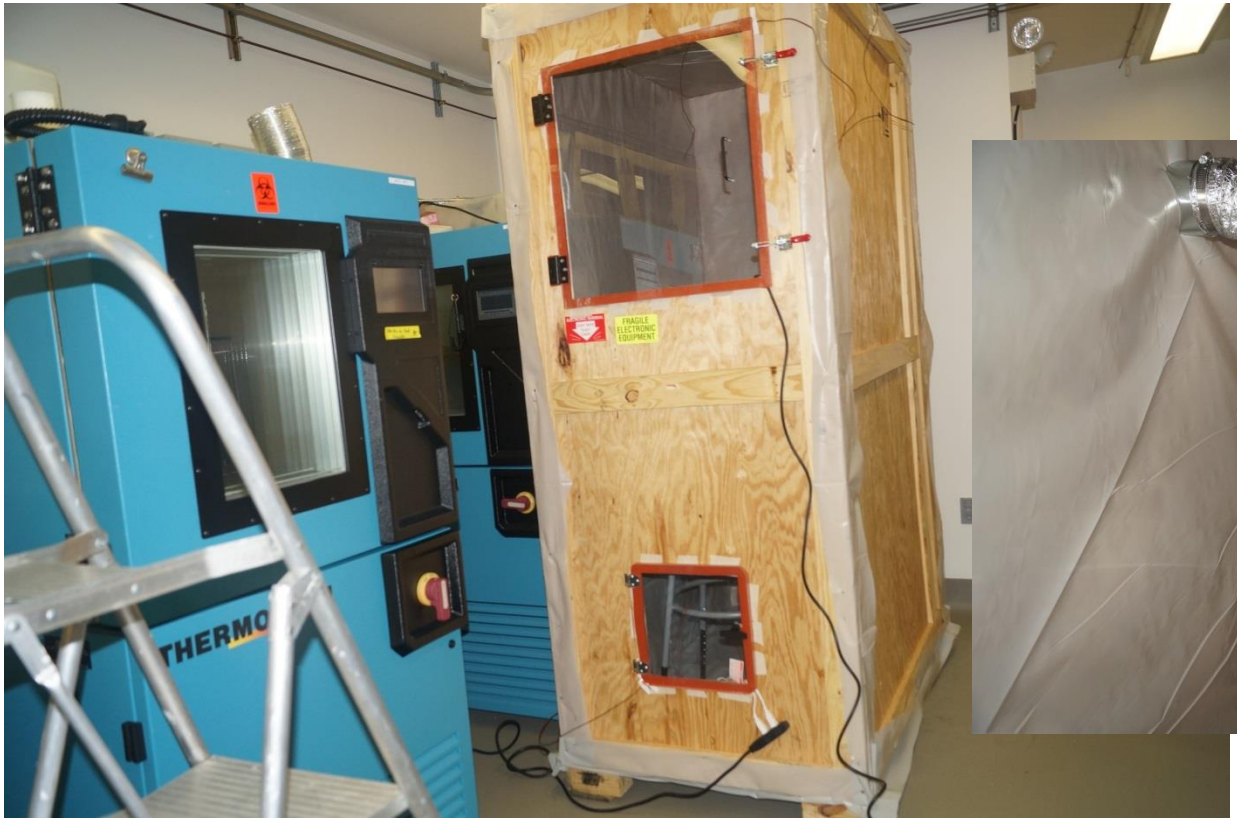
Sample	2 h germination; 1 h decontamination			
	Test A	**Test B**	**Test C**	**Test D**
	Optimized germination medium with KOH	Optimized germination medium 50% salts	Optimized germination medium w/o KOH	0.1M L-Alanine + 1mM Inosine pH 8.3
Test - Nylon	3.2 ± 0.7	3.9 ± 0.5	3.1 ± 0.8	4.7 ± 0.9
Dry Extraction Control - Nylon	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
Wet Extraction Control - Nylon	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
Test - Solution Control	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1
RT - Nylon	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
RT - Solution Control	7.2 ± 0.0	7.2 ± 0.0	7.2 ± 0.0	7.2 ± 0.0

Repeat Application – No K+
≤60°C maximum temperature; ≤ 24 h total time

Two-cycle germination + heat inactivation comparing four germination media. Total $\log_{10}$ CFU survival of *B. anthracis* ΔSterne spores on nylon. Germination was tested at 35˚C, 90% RH and heat inactivation at 60˚C, 90% RH. Five independent replicates were tested per condition.

Sample	2 h germination; 1 h decontamination / 16 h germination, 1 h decontamination						
	Test A	**Test B**	**Test C**	**Test D**	**Test E**	**Test F**	**Test G**
	1st germination: Optimal germination medium; 2nd germination: Optimal germination medium	1st germination: Optimal germination medium 50% salts; 2nd germination: Optimal germination medium 50% salts	1st germination: Optimal germination medium w/o KOH; 2nd germination: Optimal germination medium w/o KOH	1st germination: 0.1M L-Alanine + 1mM Inosine pH 8.3; 2nd germination: 0.1M L-Alanine + 1mM Inosine pH 8.3	1st germination: Optimal germination medium; 2nd germination: 0.1M L-Alanine + 1mM Inosine pH 8.3	1st germination: Optimal germination medium 50% salts; 2nd germination: 0.1M L-Alanine + 1mM Inosine pH 8.3	1st germination: Optimal germination medium w/o KOH; 2nd germination: 0.1M L-Alanine + 1mM Inosine pH 8.3
Test - Nylon	1.6 ± 0.7	1.8 ± 0.4	**1.2 ± 0.9**	2.6 ± 0.8	1.3 ± 0.9	2.4 ± 0.7	1.8 ± 0.9
Dry Extraction Control - Nylon	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
Wet Extraction Control - Nylon	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1
Test - Solution Control	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1
RT - Nylon	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
RT - Solution Control	7.2 ± 0.1	7.2 ± 0.1	**7.2 ± 0.1**	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1

Fogging



Fog tests to date: No spore inactivation.

Conclusion after fog testing: Fogging is not a good application method. Can't get enough water into the system. Spraying is the only current option.

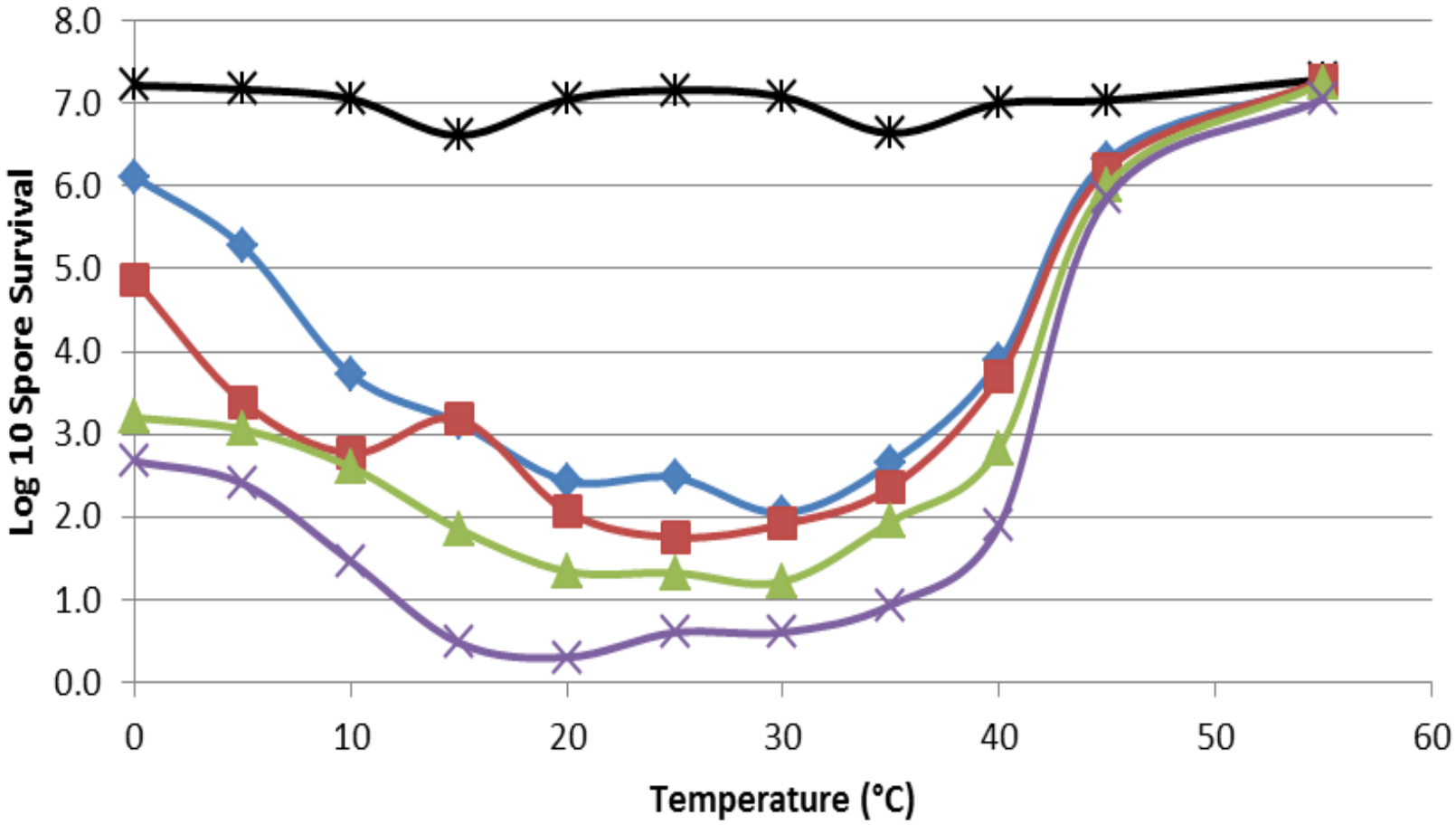
Estimated Sprayer Quantities

Estimated material quantity and costs for applying a single application of the optimized germinant solution without KOH to military equipment.
Quantities are based upon a 0.2 ml coupon^{-1} or 2 l m^{-2} from preliminary spray testing.
Note: these are with Sigma prices. Bulk wholesale prices should be significantly lower, probably 10x less.

Application	Est Surface Area (m^2)	Germinant Quantity for Single Application (Liters)	L-Ala (kg)	Inosine (kg)	DPA (kg)	$CaCl_2$ (kg)	Tris Base (kg)	Total Cost ($K)
C-130J (Interior)	250	500	4.45	0.13	3.34	4.38	7.57	3.285
C-130J (Exterior)	400	800	7.13	0.21	5.35	7.01	12.11	5.255
HMMWV (Exterior)	50	100	0.89	0.03	0.67	0.88	1.51	0.657
M1126 Skryker (Exterior)	100	200	1.78	0.05	1.34	1.75	3.03	1.314
Abrams Tank (Exterior)	125	250	2.23	0.07	1.67	2.19	3.79	1.642

Solution Testing: Limits for Germination Temperature and Time?

B. anthracis ΔSterne spore germination after incubation of spores over time and at different temperatures at spore concentrations of 7.0-7.3 $\log_{10}$ (1-2e7) spores ml^{-1}. The colored lines represent $\log_{10}$ survival of spores evaluated after mixing spores with germination medium and then heat treatment at 60°C, 1h or 65°C, 30 min at times of 0h (black), 1h (blue), 2h (red), 4h (green) and 24h (purple).



What are the Limits for Germination Temperature, Time, and Spore Concentration?

Temp	Inoculation Titer log_{10}	Heatshock 60°C for 60 min	Time points (h)			
			1	2	4	24
15°C	5.3	no	5.4	5.3	5.4	4.7
		yes	1.3	0.8	0.0	0.0
	6.3	no	6.3	6.4	6.3	6.0
		yes	2.2	1.6	1.1	0.0
	7.4	no	7.3	7.3	7.3	7.3
		yes	3.1	2.7	2.0	1.3
	8.4	no	8.4	8.3	8.3	8.4
		yes	4.6	3.4	2.5	1.6
	9.4	no	9.4	9.3	9.3	9.4
		yes	6.3	4.7	3.7	2.1
25°C	5.2	no	5.1	5.1	5.1	2.4
		yes	1.0	0.7	0.0	0.0
	6.1	no	6.1	6.1	6.2	4.4
		yes	1.9	1.4	0.5	0.0
	7.2	no	7.1	7.2	7.2	6.3
		yes	3.0	2.6	1.8	2.0
	8.2	no	8.1	8.2	8.2	8.1
		yes	4.2	3.5	2.8	2.6
	9.2	no	9.2	9.2	9.2	9.1
		yes	6.6	5.1	4.3	4.1
35°C	5.2	no	5.2	5.1	3.3	0.5
		yes	0.9	0.6	0.0	0.0
	6.2	no	6.2	6.2	4.7	1.5
		yes	1.8	1.5	0.5	0.0
	7.1	no	7.2	7.2	6.1	2.6
		yes	2.8	2.4	2.0	1.5
	8.2	no	8.2	8.3	7.9	4.3
		yes	4.3	4.1	4.1	4.2
	9.2	no	9.2	9.2	9.1	8.0
		yes	7.9	8.0	8.0	8.1

What are the Limits for Heat Inactivation Temperature, ie 50°C versus 60°C?

Temp	Inoculation Titer	60°C for 60 min	1	2	4	24	48	7 d
BaΔSt 35°C	5.2	Yes	3.2	2.6	1.7	0.3	0.0	0.0
	6.0	Yes	3.5	3.0	2.8	1.8	1.3	0.0
	7.1	Yes	5.0	4.7	4.2	3.1	2.6	1.6
	8.2	Yes	4.2	4.0	4.0	3.9	3.8	3.8
	9.3	yes	7.7	7.7	7.8	7.8	7.7	7.8
Temp	Inoculation Titer	50°C for 60 min	1	2	4	24	48	7 d
BaΔSt 35°C	5.1	yes	3.2	3.0	2.3	0.8	0.0	0.0
	6.0	yes	3.9	4.2	3.6	2.0	1.3	0.0
	7.0	yes	6.6	5.3	4.6	3.3	2.6	1.7
	8.3	yes	6.9	7.0	7.2	4.6	3.8	3.7
	9.3	yes	9.1	8.9	8.3	7.8	7.7	7.7

Germination/Hot Air Summary

- Optimal Germination solution was established
- Goal of ≤ 60°C and ≤ 24 hours should be achievable
- Repeat application of germinant, ie. germination for 0.5-2 hours followed by 60°C for 1 hour (No RH) followed by germination and heat treatment is the most reliable approach
- Spray application appears to be the best approach
- Test results for Spore Concentration, Germination Temperature, and Germination Time suggests that Germination has wider applicability than originally thought

- **What is needed?**
  - **Statistics**
  - **Repeat application on coupons at lower germination temperatures**
  - **Addition of humic acid to system**
  - **Modeling of germination temperature, time and spore concentration**
  - **Identifying Heat Susceptible Molecular Targets may be Helpful**
  - **Field testing**

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- Numerous Academians and Government Agencies have worked on germination
- Numerous Wide Area Decontamination collaborators have worked on Germination