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Determination of the Persistence of Non-Spore-Forming Biological Threat Agents in the Environment

REPORT

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Determination of the Persistence of Non-Spore-Forming Biological Threat Agents in the Environment

U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

Disclaimer

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Abbreviations/Acronyms

B. mallei	*Burkholderia mallei*
BSC	biological safety cabinet
°C	degrees Celsius
CFU	colony forming unit(s)
CI	confidence interval
CMAD	Consequence Management Advisory Division
EPA	U.S. Environmental Protection Agency
F. tularensis	*Francisella tularensis*
g	gravity
g	gram(s)
HSRP	Homeland Security Research Program
kg	Kilogram(s)
kGy	kilogray(s)
mg	milligram(s)
mL	milliliter(s)
mm	millimeter(s)
µL	microliter(s)
MFDB	microbial freeze dry buffer
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OEM	Office of Emergency Management
ORD	Office of Research and Development
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
QA	quality assurance
QAPP	Quality Assurance Project Plan
QC	quality control
QMP	quality management plan
RH	relative humidity
rpm	revolution(s) per minute
SD	standard deviation
SE	standard error
TSA	tryptic soy agar
TSB	tryptic soy broth
Y. pestis	*Yersinia pestis*

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Executive Summary

The persistence of biological agents outside a host is influenced by environmental conditions and the materials with which these biological agents are in contact. This generation of scientifically defensible persistence data is useful for the proper planning of decontamination efficacy tests and for formulation of response or remediation plans in preparation for possible natural occurrences or intentional releases of biological agents. This report presents the results of an investigation to evaluate the persistence (or natural attenuation) of *Yersinia pestis* (*Y. pestis*), *Francisella tularensis* (*F. tularensis*), and *Burkholderia mallei* (*B. mallei*) on glass and soil under multiple environmental conditions and time points.

Persistence (recovery of viable organisms) was assessed for *Y. pestis*, *F. tularensis*, and *B. mallei* inoculated onto glass coupons and soil materials. The cell suspensions deposited onto the surfaces were allowed to dry under ambient conditions for approximately one hour or were lyophilized for approximately two hours, prior to being placed into environmental chambers for up to 99 days.

The target environmental conditions were low temperature (5 degrees Celsius [°C]) and low relative humidity (RH; 30%), moderate temperature (22 °C) and moderate RH (40%), and high temperature (35 °C) and high RH (65%) conditions.

Summary of Results

When inoculated coupons were lyophilized and maintained at a low temperature and RH, all three organisms survived on glass at a higher concentration than when inoculated and dried under ambient conditions (non-lyophilized). A similar effect was observed at the moderate temperature conditions, but with fewer organisms recovered at each time point. *F. tularensis* survived ≥63 days (<99 days), and *B. mallei* survived ≥6 day (<21 days) when using a non-lyophilized inoculum, while viable cells were recovered for all three organisms after 99 days when lyophilized. Less than 1 log (log_{10}) reduction was observed for all three organisms on glass when lyophilized and held at the low temperature and RH condition. At the high temperature condition, *F. tularensis* survived ≥6 days (<21 days) and *B. mallei* ≥ 1 day (<6 days) using a non-lyophilized inoculum, while no viable *Y. pestis* was recovered at any time point. When lyophilized, all three organisms survived ≥1 day (<6 days) at the high temperature/RH condition. Tables ES-1 and ES-2 summarize the quantitative recoveries of *Y. pestis*, *F. tularensis*, and *B. mallei* applied to 5 × 5 millimeter (mm) glass coupons. Similar results were observed with 100 milligram (mg) soil samples, but these samples were only qualitatively assessed due to indigenous flora present in the soil samples that interfered with organism enumeration (Tables ES-3 and ES-4).

Table ES-1. Summary of Persistence Testing on Glass (Non-Lyophilized)

Test Organism	Temperature $^{0}C^{\dagger}$	% RH†	Starting Inoculum*	Mean Recovered Organisms (log survivorship) by Duration in Days*					
				0	1	6	21	63	99
5 ± 3 ^{0}C; 30 ± 15% RH									
Y. pestis	6	41	7.50	5.80	4.88	4.55	4.15	3.71	3.46
F. tularensis	6	34	8.17	6.93	7.93	7.16	6.75	5.10	4.62
B. mallei	6	38	8.06	8.06	5.82	5.93	4.77	1.74	0.10
22 ± 3 ^{0}C; 40 ± 15% RH									
Y. pestis	23	46	7.50	5.80	3.88	1.26	ND	ND	ND
F. tularensis	22	48	8.17	6.93	7.24	4.74	2.34	0.16	ND
B. mallei	23	47	8.06	8.06	5.92	0.36	ND	ND	ND
35 ± 3 ^{0}C; 65 ± 15% RH									
Y. pestis	35	70	7.50	5.80	ND	ND	ND	ND	ND
F. tularensis	35	67	8.17	6.93	1.16	0.16	ND	--	--
B. mallei	35	65	8.06	8.06	3.19	ND	--	--	--

*Data are expressed as mean logs colony forming units (CFU) recovered.
"--" indicates no data collected at indicated time point.
†Mean temperature and RH values based on continuous monitoring at six minute intervals over the entire 99 days.
"ND" indicates that no viable organisms were recovered from any of the replicate coupons.

Table ES-2. Summary of Persistence Testing on Glass (Lyophilized)

Test Organism	Temperature $^{0}C^{\dagger}$	% RH†	Starting Inoculum*	Mean Recovered Organisms (log survivorship) by Duration in Days*					
				0	1	6	21	63	99
5 ± 3 ^{0}C; 30 ± 15% RH									
Y. pestis	6	41	8.35	8.59	8.66	8.13	8.31	8.51	7.97
F. tularensis	6	34	8.46	7.40	7.63	7.48	7.52	7.29	7.63
B. mallei	6	38	7.98	7.70	7.41	7.51	7.27	7.12	7.16
22 ± 3 ^{0}C; 40 ± 15% RH									
Y. pestis	23	46	8.35	8.59	8.48	8.01	7.01	4.92	2.69
F. tularensis	22	48	8.46	7.40	7.27	6.95	6.51	6.82	6.02
B. mallei	23	47	7.98	7.70	6.63	5.91	6.41	6.01	5.53
35 ± 3 ^{0}C; 65 ± 15% RH									
Y. pestis	35	70	8.35	8.59	1.82	ND	ND	ND	ND
F. tularensis	35	67	8.46	7.40	4.99	ND	--	--	--
B. mallei	35	65	7.98	7.70	5.64	ND	--	--	--

*Data are expressed as mean logs CFU recovered.
"--" indicates no data collected at indicated time point.
†Mean temperature and RH values based on continuous monitoring at six minute intervals over the entire 99 days.
"ND" indicates that no viable organisms were recovered from any of the replicate coupons.

Table ES-3. Summary of Persistence Testing on Soil (Non-Lyophilized)

Test Organism	Temperature $^{0}C^{\dagger}$	% RH†	Starting Inoculum*	Recovered Organisms by Duration in Days					
				0	1	6	21	63	99
5 ± 3 ^{0}C; 30 ± 15% RH									
Y. pestis	6	41	7.50	✓	✓	✓	--	✓	✓
F. tularensis	6	34	8.17	✓	✓	✓	✓	✓	✓
B. mallei	6	38	8.06	✓	✓	✓	✓	✓	✓
22 ± 3 ^{0}C; 40 ± 15% RH									
Y. pestis	23	46	7.50	✓	✓	✓	--	ND	ND
F. tularensis	22	48	8.17	✓	✓	✓	✓	ND	ND
B. mallei	23	47	8.06	✓	✓	✓	ND	ND	ND
35 ± 3 ^{0}C; 65 ± 15% RH									
Y. pestis	35	70	7.50	✓	✓	ND	--	ND	ND
F. tularensis	35	67	8.17	✓	✓	ND	--	--	--
B. mallei	35	65	8.06	✓	ND	ND	--	--	--

*Data are expressed as mean logs CFU recovered.
†Mean temperature and RH values based on continuous monitoring at six minute intervals over the entire 99 days.
"--" indicates no data collected at indicated time point.
"✓" indicates that viable organisms were recovered from at least one of the replicate samples and the target test organism confirmed.
"ND" indicates that no viable organisms were recovered from any of the replicate samples.

Table ES-4. Summary of Persistence Testing on Soil (Lyophilized)

Test Organism	Temperature $^{0}C^{\dagger}$	% RH†	Starting Inoculum*	Recovered Organisms by Duration in Days					
				0	1	6	21	63	99
5 ± 3 ^{0}C; 30 ± 15% RH									
Y. pestis	6	41	8.35	✓	✓	✓	--	✓	✓
F. tularensis	6	34	8.46	✓	✓	✓	✓	✓	✓
B. mallei	6	38	7.98	✓	✓	✓	✓	✓	✓
22 ± 3 ^{0}C; 40 ± 15% RH									
Y. pestis	23	46	8.35	✓	✓	✓	--	ND	ND
F. tularensis	22	48	8.46	✓	✓	✓	✓	✓	ND
B. mallei	23	47	7.98	✓	✓	✓	ND	ND	ND
35 ± 3 ^{0}C; 65 ± 15% RH									
Y. pestis	35	70	8.35	✓	ND	✓	--	ND	ND
F. tularensis	35	67	8.46	✓	✓	ND	--	--	--
B. mallei	35	65	7.98	✓	ND	ND	--	--	--

*Data are expressed as mean logs CFU recovered.
†Mean temperature and RH values based on continuous monitoring at six minute intervals over the entire 99 days.
"--" indicates no data collected at indicated time point.
"✓" indicates that viable organisms were recovered from at least one of the replicate samples and the target test organism confirmed.
"ND" indicates that no viable organisms were recovered from any of the replicate samples.

Conclusions

The data presented here are intended to help responders develop effective remediation strategies following an environmental contamination incident involving *Y. pestis*, *F. tularensis*, or *B. mallei*. The primary conclusion is that lyophilized or dried organisms applied to a surface pose a more significant threat as the persistence of these organisms increases. As expected, lower temperature and relative humidity is correlated with increased persistence.

1.0 Introduction

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program (HSRP) is helping to protect human health and the environment from adverse impacts resulting from the release of chemical, biological, or radiological agents. With an emphasis on decontamination and consequence management, water infrastructure protection, and threat and consequence assessment, the HSRP is working to develop tools and information that will help detect the intentional introduction of chemical or biological contaminants into buildings or water systems; contain these contaminants; decontaminate buildings, outdoor environments, or water systems; and facilitate the disposal of material resulting from restoration activities.

In this work, the persistence of three biological threat agents (*Yersinia pestis* [*Y. pestis*], *Francisella tularensis* [*F. tularensis*], and *Burkholderia mallei* [*B. mallei*]) was evaluated under varying environmental conditions to assist in determining conditions under which further decontamination may likely be needed. The results of this investigation provide technology users and stakeholders with high quality, peer-reviewed data on the effectiveness of various temperature and relative humidity (RH) combinations on indoor and outdoor materials (glass and soil) contaminated with these biological agents. Soil and glass were chosen to represent two common yet vastly different surface types common within the environment.

2.0 Procedures

Glass (5 × 5 millimeter [mm]) and soil (100 milligrams [mg]) were inoculated with either *Y. pestis*, *F. tularensis*, or *B. mallei* (~3.17×10^9 to 2.87×10^{10} colony forming units/milliliter [CFU/mL]), allowed to air dry or were lyophilized on the material, and then exposed to three environmental conditions (5 ± 3 °C and 30 ± 15% RH, 22 ± 3 ^{0}C and 40 ± 15% RH, or 35 ± 3 ^{0}C and 65 ± 15% RH) for controlled exposure durations up to 99 days. Persistence was determined quantitatively for glass and qualitatively for soil (due to the abundance of indigenous flora observed in the soil samples, making it difficult to enumerate) for each environmental condition at 0, 1, 6, 21, 63, and 99 days. Persistence was assessed by measuring the recovery of *Y. pestis*, *F. tularensis*, and *B. mallei* as CFU from each tested combination of environmental condition and exposure duration. All testing was performed in accordance with the peer-reviewed and EPA-approved *Quality Assurance Project Plan (QAPP).*

2.1 Biological Agents

Testing was conducted with virulent *Y. pestis* CO92 (BEI Resources, Manassas, VA, NR-641), *F. tularensis* SCHU S4 (BEI Resources, NR-10492), and *B. mallei* China 7 (BEI Resources, NR-23). All three are Gram-negative, non-spore-forming coccobacilli. All samples were maintained in pure culture for this investigation. Ribonucleic acid from stock cultures was isolated using a NucliSens® easyMAG™ (bioMérieux, St. Louis, MO) and were identified by polymerase chain reaction (PCR) using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY) using internal standard operating procedures with specialized primers and probes. All cultures were confirmed to be either *Y. pestis* CO92, *F. tularensis* SCHU S4, or *B. mallei* China 7 by PCR as well as Gram stain.

2.2 Test Materials

Information on the materials and sterilization approaches used for testing are presented in Table 2-1. Persistence testing was conducted on two materials: glass and soil. These materials were selected to represent common nonporous (glass) and porous (soil) materials or matrices. Glass materials were supplied by the manufacturer at a uniform length and width (5 × 5 mm) from stock material. Soil was supplied by a commercial vendor and approximately 100 mg aliquotted as needed into 1.5 mL microcentrifuge tubes. The selected sterilization approach, shown in Table 2-1, was based on cost-effectiveness and minimization of the physical alterations of the materials. Autoclaving was conducted at 121 °C for 15 minutes and electron-beam sterilization was conducted at ~200 kilograys (kGy) by E-Beam Services, Inc. (Lebanon, OH) using a 5.0 MeV E-beam accelerator. The intent of sterilization was to eliminate the probability of bacterial background interference. Approximately 6 kilograms (kg) of soil were sent to E-Beam Services, Inc., for irradiation in ~500 gram (g) aliquots. Following receipt of soil samples after irradiation, a representative set of samples (two 100 mg samples from each 500 g lot) was extracted, plated onto tryptic soy agar (TSA) and confirmed to be sterile. However, for the t=0 time point during the *Y. pestis* testing, contamination (indigenous flora, not the test organisms) was noted in soil samples during enumerations. As result, subsequent soil samples were assessed only qualitatively.

Table 2-1. Test Materials

Material	Lot or Batch No.	Supplier	Approximate Size	Material Preparation
Glass	EPA-1101	Erie Scientific (Portsmouth, NH)	5 × 5 × 1 mm	Autoclave
Soil	Mollisol, HCB-SL-PF, collected from Easter, ND	Agvise Laboratories (Northwood, ND)	100 mg in 1.5 mL microcentrifuge tubes	E-Beam

Soil samples (500 g, pre- and post-sterilization via E-beam at ~200 kGy) were sent to an independent laboratory for analysis of moisture, organic content and other characteristics. An approximately 500 g sample was analyzed in compliance with 40 CFR Part 160 for Good Laboratory Practices for the parameters listed in Table 2-2.

Table 2-2. Soil Sample Analysis

% Sand		% Silt		% Clay		USDA Textural Class		Bulk Density (g/cc[3])	
Pre[1]	E[2]	Pre	E	Pre	E	Pre	E	Pre	E
33	31	38	34	29	35	Clay Loam	Clay Loam	0.81	0.86

Cation Exchange Capacity (meq[4]/100 g)		% Moisture at 1/3 Bar (Field Capacity)		% Organic Matter		pH	
Pre	E	Pre	E	Pre	E	Pre	E
22.0	24.3	42.0	36.9	7.22	7.21	7.6	7.4

Calcium				Magnesium				Sodium			
Pre		E		Pre		E		Pre		E	
%	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%	ppm
71.7	3150	70.7	3440	15.3	402	15.6	456	0.3	13	0.3	19

Potassium				Hydrogen			
Pre		E		Pre		E	
%	ppm	%	ppm	%	ppm	%	ppm
4.4	374	4.4	418	8.4	18	8.9	22

[1]Pre = Pre-Sterilization
[2]E = E-beam irradiation @ 200 kGy
[3]cc = cubic centimeters
[4]meq = milliequivalents

2.3 Inoculation

Fresh cultures were prepared in advance of each day that coupons were inoculated by transferring colonies from a streak plate (freshly growing or stored less than 2 weeks at 2 °C to 8 °C) into 100 mL of the appropriate liquid culture media (Table 2-3). This culture was then incubated overnight at the appropriate temperature (Table 2-3) on an orbital shaker set to 200 revolutions per minute (rpm). The bacterial culture was then centrifuged at 10,000 x gravity (g) for 10 minutes, the supernatant discarded, and the pellet resuspended into 10 mL of either the appropriate buffer (Table 2-3) for the non-lyophilized inoculum or into microbial freeze dry buffer (MFDB; OPS Diagnostics, Lebanon, NJ). All samples were inoculated with one 10 microliter (µL) drop of concentrated agent. The non-lyophilized samples were allowed to dry under ambient conditions for one hour prior to extraction (Time 0 control) or placed into the respective environmental

chambers. The lyophilized samples were placed at ≤-70 °C overnight prior to being placed in the lyophilizer (Labconco 77530, Kansas City, MO) for approximately two hours. These samples were then extracted (Time 0 control) or placed into their respective environmental chambers. All inoculated and dried glass coupons were kept in 1.5 mL microcentrifuge tubes for ease of handling.

Table 2-3. Media Types and Incubation Parameters

Organism (strain)	Solid Media	Liquid Media	Incubation Temperature (°C)	Incubation Time (hours)
Y. pestis (CO92)	Tryptic Soy Agar (G60, Hardy Diagnostics, Santa Maria, CA)	Tryptic Soy Broth (T1550, Teknova, Hollister, CA)	26 ± 2	36-96
F. tularensis (SCHU S4)	Chocolate II (221169, BD Biosciences, Franklin Lakes, NJ)	Mueller Hinton Broth (90922, Sigma Aldrich, St. Louis, MO)	37 ± 2	36-96
B. mallei (China 7)	Chocolate II	Nutrient Broth (233000, BD Biosciences)	37 ± 2	48-96

2.4 Environmental Conditions and Persistence Testing Procedures

Persistence testing was conducted under three sets of target environmental conditions:

- Low temperature and relative humidity: 5 ± 3 °C and 30 ± 15% RH
- Moderate temperature and relative humidity: 22 ± 3 °C and 40 ± 15% RH
- High temperature and relative humidity: 35 ± 3 °C and 65 ± 15% RH.

All actual values for environmental conditions are presented in Table 3-2. The test coupons were held at the required temperature for the required time period (up to 99 days) in a closed, airtight persistence testing chamber (Lock & Lock, HPL838P, Farmers Branch, TX). All lids to the 1.5 mL microcentrifuge tubes were left open for the duration of the required time period. After the coupons were removed from their respective persistence testing chambers, all were moved to a biological safety cabinet (BSC) II for extraction (refer to Section 2.5).

For all testing conditions, fixed humidity-point salt solutions (magnesium chloride for the low temperature condition, potassium carbonate for the moderate temperature condition, and potassium iodide for the high temperature condition) were used to maintain the RH in the respective chambers. All salts were mixed with water and added as a slurry to separate containers and placed in the bottom of the appropriate persistence test chambers following ASTM E104.[(1)] The coupons were never allowed to come into direct contact with the salt or the salt containers. For the low temperature and RH (5 ± 3 °C and 30 ± 15% RH) test, persistence coupons were kept in a refrigerator; chambers containing the moderate and high temperature and RH (22 ± 3 °C and 40 ± 15% RH, and 35 ± 3 °C and 65 ± 15% RH) were kept in an incubator set to the appropriate temperature. The temperature and RH for all conditions were recorded approximately every six minutes using a HOBO data logger (Onset Computer Corporation, Bourne, MA). The actual temperature and RH levels observed during testing are documented in Section 3.

2.5 Recovery

For sample extraction, glass materials were placed into 1.5 mL microcentrifuge tubes containing 1 mL sterile phosphate buffered saline (PBS), and 1 mL of sterile PBS was added to each 100 mg soil sample. All vials were vortexed for approximately two minutes at room temperature and then sonicated (Bransonic 1200, Danbury, CT) for approximately two minutes at room temperature. Following extraction, a series of dilutions (serial 1:10 dilutions) was prepared using PBS. Aliquots (0.1 mL) of the undiluted extract and dilutions were plated as appropriate in triplicate onto the specified solid agar for each organism. The cultures were incubated for the appropriate time and temperature according to Table 2-3. The colonies were counted manually, and the concentration of viable organisms determined in CFU/mL. Typically, plates having colony counts between 25 and 250 are used for calculating the CFU/mL. Under certain circumstances (i.e., poor recovery, reduced persistence over time), there were fewer than 25 colonies per plate from the undiluted extract. In these cases, the number of colonies was counted and recorded even if there were fewer than 25 colonies per plate. The total CFU/coupon was calculated as:

Total CFU/coupon = [(mean CFU plate count x 1/dilution factor)/plated volume] (1)

where:

Mean CFU plate count	= average number of colonies counted on three replicate plates
Plated volume	= 0.1 mL
Dilution factor	= portion of the total extraction buffer that was used to prepare the dilutions

A single viable bacterium present in a plated aliquot of sample under ideal growth conditions would be expected to be observed as one CFU. Therefore, the individual coupon detection limit is approximately 3.3 CFU/coupon if one CFU was observed on one of three plates of the undiluted extract. Since only a portion (i.e., 0.1 mL aliquot per plate) of undiluted extract is cultured, viable bacteria could be present in the extract that were not used for plating (approximately 0.9 mL). However, given the number of replicate coupons (five) and replicate plates (three) per undiluted coupon extract, there is a low probability the presence of viable bacteria would go undetected.

The recovery of bacteria (quantified as mean CFU/coupon ± standard deviation [SD]) was calculated for each environmental condition and exposure duration combination by dividing the total number of viable organisms extracted from all five test coupons by the number of replicate coupons (i.e., five). Attenuation refers to the log reduction (as calculated by Equation 2) of persistence test coupons held at their respective environmental conditions for up to 99 days. Reduction for a test organism on the i^{th} coupon material was calculated as the difference between those mean log values:

$$Attenuation = \overline{(\log CFUc_{ij})} - \overline{(\log CFUt_{ij})} \quad (2)$$

where log $CFUc_{ij}$ refers to the j individual logarithm values obtained from the positive control coupons and log $CFUt_{ij}$ refers to the j individual logarithm values obtained from the corresponding test coupons, and the overbar designates a mean value. In tests conducted under this plan, there were five control and five corresponding test coupons (i.e., $j = 5$). In the case where no CFU were

found in a coupon extract, a CFU value of 1 was assigned, resulting in a log CFU of zero for that coupon.

The variances (i.e., the square of the SD) of the log $CFUc_{ij}$ and log $CFUt_{ij}$ values were also calculated for both the control and test coupons (i.e., S^2c_{ij} and S^2t_{ij}), and were used to calculate the pooled standard error (SE) for the efficacy value calculated in Equation 3, as follows:

$$SE = \sqrt{\frac{S^2c_{ij}}{5} + \frac{S^2t_{ij}}{5}} \quad (3)$$

where the number 5 again represents the number *j* of coupons in both the control and test data sets. Thus, each result is reported as a log reduction value with an associated SE value.

The significance of differences in attenuation across the different environmental conditions and inoculation procedures was assessed based on the 95% confidence interval (CI) as:

$$95\%\ CI = Efficacy \pm (1.96 \times SE) \quad (4)$$

For extraction of soil samples, 1 mL of PBS was added to each microcentrifuge tube and extracted as stated above. However, these samples were plated on selective media (undiluted sample only) to assess the presence of the target organism. *Y. pestis* samples were plated on CIN agar (BD Biosciences; Cat # 221848), *F. tularensis* samples were plated on Modified Thayer Martin agar (BD Biosciences; Cat #221567), and *B. mallei* samples were plated on Burkholderia Cepacia agar (Remel Cat #R01709). Target organisms were visually confirmed, and no suspect organisms were noted.

3.0 Quality Assurance/Quality Control

Quality assurance (QA)/quality control (QC) procedures were performed in accordance with the *Battelle Quality Management Plan* (QMP) and the QAPP.[1] The QA/QC procedures and results are summarized below.

3.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, biological safety cabinets) and monitoring devices (e.g., thermometer, hygrometer) used at the time of evaluation were verified as being certified, calibrated, or validated.

3.2 QC Results

QC efforts conducted during testing included test material method demonstration (i.e., extraction efficiency), positive control samples (inoculated and extracted at Time 0) and procedural blanks (not inoculated and extracted at each time point).

Results for all positive control samples were within the target recovery range of 0.1% to 120% of the inoculated organisms, except where indicated in Table 3-1. Unfortunately, with the nature of vegetative bacteria, the counts are not available for up to 96 hours post-inoculation, precluding a restart of the test.

Table 3-1. Positive Control Percent Recoveries (Glass only)

Organism	Inoculation Method	Mean Logs Observed (CFU)	Mean % Recovery
Y. pestis	Non-lyophilized	5.80	2.02
	Lyophilized	8.59	189.42*
F. tularensis	Non-lyophilized	6.93	9.58
	Lyophilized	7.40	10.29
B. mallei	Non-lyophilized	8.06	98.97
	Lyophilized	7.70	51.88

*Positive control sample outside the allowable 0.1 to 120% of inoculated levels.

All procedural blanks met the criterion of no observed CFU for all organisms tested.

3.2.1 Operational Parameters

The temperature and RH during each test were controlled and recorded, as described in Section 2.4. Readings were taken once every six minutes for the duration of the contact time. The actual operational parameters for each test are shown in Table 3-2 and reported as the average value ± SD. A rise and/or fall of temperature and RH was observed when extracting coupons from the persistence testing chambers at the respective time points. However, all coupons returned to the appropriate target testing condition within one hour of opening the chambers, and any change in temperature or RH is expected to have no impact on the results as the time-weighted average conditions were well within the target ranges. In some instances, samples were not collected at later time points as earlier time points showed complete inactivation of the organism.

Table 3-2. Persistence Testing Operational Parameters

Organism	Contact Time (Days)	Target Environmental Conditions		Actual Environmental Conditions	
		Temperature (^{0}C)	RH (%)	Temperature (^{0}C)	RH (%)
Y. pestis	1	5 ± 3	30 ± 15	6.18 ± 2.38	43.11 ± 1.78
	6			5.46 ± 1.07	39.32 ± 3.28
	21			5.64 ± 0.81	42.76 ± 2.86
	63			5.65 ± 0.52	39.67 ± 3.19
	99			5.75 ± 0.54	40.61 ± 3.28
	1	22 ± 3	40 ± 15	21.88 ± 0.12	54.31 ± 2.01
	6			22.38 ± 0.67	49.98 ± 2.30
	21			22.59 ± 0.38	49.32 ± 1.31
	63			22.51 ± 0.57	45.88 ± 1.72
	99			22.52 ± 0.50	45.76 ± 1.53
	1	35 ± 3	65 ± 15	34.57 ± 2.66	70.67 ± 5.67
	6			35.26 ± 1.19	69.43 ± 2.32
	21			32.25 ± 1.23	69.38 ± 2.54
	63			35.41 ± 0.42	69.74 ± 0.68
	99			35.42 ± 0.40	69.77 ± 0.66
F. tularensis	1	5 ± 3	30 ± 15	6.30 ± 2.63	39.89 ± 5.58
	6			6.43 ± 1.21	34.64 ± 3.39
	21			6.23 ± 0.76	34.15 ± 2.37
	63			5.91 ± 0.75	33.73 ± 1.59
	99			6.09 ± 0.67	33.77 ± 1.37
	1	22 ± 3	40 ± 15	22.71 ± 0.07	47.48 ± 0.82
	6			22.63 ± 0.07	47.33 ± 0.65
	21			22.38 ± 0.45	47.56 ± 0.78
	63			22.47 ± 0.27	47.74 ± 0.53
	99			22.47 ± 0.22	47.93 ± 0.61
	1	35 ± 3	65 ± 15	35.14 ± 0.69	65.84 ± 3.31
	6			35.26 ± 0.72	66.92 ± 2.18
	21			35.36 ± 0.55	66.92 ± 1.42
	63			--	--
	99			--	--
B. mallei	1	5 ± 3	30 ± 15	6.54 ± 3.15	44.64 ± 3.86
	6			6.82 ± 1.55	39.99 ± 2.62
	21			5.92 ± 1.14	37.47 ± 2.44
	63			6.49 ± 0.83	38.96 ± 1.80
	99			6.25 ± 0.7	37.75 ± 1.18
	1	22 ± 3	40 ± 15	22.46 ± 0.47	47.80 ± 0.99
	6			22.59 ± 0.20	46.78 ± 0.64
	21			22.71 ± 0.14	46.99 ± 0.40
	63			22.69 ± 0.29	47.01 ± 0.39
	99			22.67 ± 0.24	47.05 ± 0.34
	1	35 ± 3	65 ± 15	34.65 ± 3.03	64.58 ± 5.13
	6			35.28 ± 1.42	64.64 ± 2.29
	21			--	--
	63			--	--
	99			--	--

-- denotes that samples were not collected; therefore, no temperature and RH data are available.

3.3 Audits

3.3.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during these tests. Table 3-3 summarizes the performance evaluation audits that were performed.

Table 3-3. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume	Micropipettes checked by gravimetric evaluation	±10%	14 pipettes checked, all <8%
Temperature	Compared to independent National Institute of Standards and Technology (NIST)-traceable thermometer value	±2 ^{0}C	187 temperature comparisons, all differences were ≤0.2 ^{0}C
RH	Compared to independent NIST-traceable hygrometer value	±10%	187 RH comparisons, all differences were ≤2%
Time	Compared to independent NIST-traceable timer value	2 seconds/hour	0 seconds/hour for 5 instances

3.3.2 Technical Systems Audit

Observations and findings from the technical systems audit were documented and submitted to the laboratory staff lead for response. Audits were conducted by the Battelle Quality Assurance Officer on January 14, 2015, to ensure the tests were being conducted in accordance with the QAPP and Battelle QMP. As part of the audit, test procedures were compared to those specified in the QAPP, and data acquisition and handling procedures were reviewed. None of the findings of the technical systems audit required corrective action.

3.3.3 Data Quality Audit

All transcribed data (100%) and 10% of all calculations and electronically transferred data acquired during the evaluation were audited. A QA auditor traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

3.4 QA/QC Reporting

Each assessment and audit was documented in accordance with the QAPP and Battelle QMP. For these tests, findings were noted (none significant) in the data quality audit, but no follow-up corrective action was necessary. The findings were mostly minor data transcription errors requiring some recalculation of efficacy results, but none were gross errors in recording. Copies of the assessment reports were distributed to the Battelle Contract Manager and laboratory staff. QA/QC procedures were performed in accordance with the QAPP.

3.5 Data Review

Records and data generated in the evaluation received a QC/technical review before they were utilized in calculating or evaluating results and prior to incorporation in reports. This process consists of review of all data transcribed from laboratory worksheets into Excel spreadsheets as well as embedded calculations for reportable values. The process also consists of reviewing general laboratory documentation according to Battelle standard operating procedures.

4.0 Persistence Test Results

For this investigation, persistence data were generated for *Y. pestis*, *F. tularensis*, and *B. mallei* in contact with glass and soil materials exposed to three environmental conditions for controlled exposure durations up to 99 days. Persistence curves were generated, where applicable, by graphing the log survivorship of each material against time in days for each set of environmental condition and organism combination. The following sections summarize the results of the method demonstration (tests conducted initially to ensure sufficient organism recovery) and the persistence investigation.

4.1 Method Demonstration – Organism Recovery

A method demonstration was performed with each organism to confirm the methods used for extraction were sufficient for this application. Additionally, three buffers [tryptic soy broth (TSB), microbial freeze dry buffer (MFDB), and 6% lactose] were tested to ensure the buffer chosen yielded sufficient recovery for each organism following the lyophilization process. TSB was chosen to compare as it is the buffer used in wet inoculum, and the latter two chosen as routinely used lyophilization buffers.[2, 3] Briefly, samples were prepared as described in Section 2.3, except the inoculation material was prepared in three preparations: TSB, MFDB, and 6% lactose. Materials were inoculated and allowed to dry for one hour (control) or frozen and lyophilized in each of the three preparations (n = 5). Samples were extracted as described in Section 2.5, and the results are summarized in Table 4-1. MFDB was chosen for all three organisms for the preparation of the samples for lyophilization based on these data and for consistency among the three organisms.

Table 4-1. Recovery Data from Method Demonstration (Glass only)

Organism	Test Method/Buffer	Inoculation Control, CFU/coupon	Recovered Organisms	
			CFU/coupon*	%*
Y. pestis	Non-Lyophilized/TSB‡	5.13×10^6	$1.89 \pm 0.81 \times 10^5$	3.69 ± 1.57
	Lyophilized/TSB	1.34×10^7	$2.45 \pm 1.41 \times 10^3$	0.018 ± 0.011
	Lyophilized/MFDB†	2.11×10^7	$4.83 \pm 4.89 \times 10^7$	228.70 ± 231.86
	Lyophilized/6% Lactose	2.29×10^7	$3.75 \pm 3.85 \times 10^6$	16.39 ± 16.81
F. tularensis	Non-Lyophilized/TSB	3.13×10^7	$4.20 \pm 3.39 \times 10^6$	13.42 ± 10.82
	Lyophilized/TSB	3.73×10^8	$9.77 \pm 3.83 \times 10^6$	2.62 ± 1.03
	Lyophilized/MFDB	3.07×10^8	$1.08 \pm 0.36 \times 10^8$	35.33 ± 11.86
	Lyophilized/6% Lactose	1.60×10^8	$4.43 \pm 1.42 \times 10^7$	27.71 ± 8.87
B. mallei	Non-Lyophilized/TSB	1.35×10^8	$8.61 \pm 2.28 \times 10^4$	0.064 ± 0.017
	Lyophilized/TSB	1.40×10^9	$1.15 \pm 0.24 \times 10^6$	0.082 ± 0.017
	Lyophilized/MFDB	1.37×10^9	$7.23 \pm 1.16 \times 10^6$	0.53 ± 0.08
	Lyophilized/6% Lactose	9.40×10^8	$7.18 \pm 4.05 \times 10^6$	0.76 ± 0.43

*Data are expressed as mean ± standard deviation of five replicate coupons.
‡TSB = tryptic soy broth
† MFDB = microbial freeze dry buffer

4.2 Persistence Results

Persistence results for each organism/environmental condition combination are summarized in Tables 4-2 through 4-10 and Figures 4-1 through 4-9.

4.2.2 Y. pestis

The results obtained for *Y. pestis* persistence on glass are summarized in Tables 4-2 and 4-3 and Figures 4-1 through 4-3. The qualitative persistence in soil is summarized in Table 4-4. When lyophilized (Table 4-3), *Y. pestis* persisted on glass for at least 99 days under both low and moderate temperature and RH conditions (>7 logs and >2 $\log_{10}$ recovery, respectively). For non-lyophilized samples (Table 4-2), *Y. pestis* persisted for at least 99 days at the low temperature/RH conditions, but with lower recoveries (3.46 logs viable organisms recovered). At the moderate temperature/RH condition, the non-lyophilized *Y. pestis* was recoverable after six days. Additionally, no *Y. pestis* was recovered from glass for any time point when not lyophilized and only at day one when lyophilized *Y. pestis* was tested at the high temperature/RH condition.

Similar to the results observed from glass, *Y. pestis* persisted up to 99 days under the low environmental conditions and ≥6 days under the moderate environmental conditions with both preparations in soil. No organisms were detected after six days in soil under the high temperature environmental conditions. Analysis of Day 21 samples was not completed, as contamination of soil samples with indigenous bacteria was detected, and revised methods (qualitative results only, exploration of selective media) were being developed. As a result, data from this time point were not collected.

Table 4-2. *Y. pestis* Persistence on Glass (Non-Lyophilized)

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *Y. pestis*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	3.17×10^7	5.80 ± 0.06	4.88 ± 0.15	0.92 ± 0.14
6			4.55 ± 0.32	1.26 ± 0.28
21			4.15 ± 0.19	1.65 ± 0.17
63			3.71 ± 2.01	2.09 ± 1.76
99			3.46 ± 0.43	2.34 ± 0.38
22 ± 3 °C; 40 ± 15% RH				
1	3.17×10^7	5.80 ± 0.06	3.88 ± 0.17	1.92 ± 0.16
6			1.26 ± 0.82	4.54 ± 0.72
21			0.00 ± 0.00	5.80 ± 0.05
63			0.00 ± 0.00	5.80 ± 0.05
99			0.00 ± 0.00	5.80 ± 0.05
35 ± 3 °C; 65 ± 15% RH				
1	3.17×10^7	5.80 ± 0.06	0.00 ± 0.00	5.80 ± 0.05
6			0.00 ± 0.00	5.80 ± 0.05
21			0.00 ± 0.00	5.80 ± 0.05
63			0.00 ± 0.00	5.80 ± 0.05
99			0.00 ± 0.00	5.80 ± 0.05

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% confidence interval (CI).

Table 4-3. *Y. pestis* Persistence on Glass using a Lyophilized Inoculum

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *Y. pestis*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	2.25×10^8	8.59 ± 0.23	8.66 ± 0.11	-0.08 ± 0.22
6			8.13 ± 0.48	0.45 ± 0.47
21			8.31 ± 0.10	0.28 ± 0.22
63			8.51 ± 0.15	0.07 ± 0.24
99			7.97 ± 0.34	0.62 ± 0.36
22 ± 3 °C; 40 ± 15% RH				
1	2.25×10^8	8.59 ± 0.23	8.48 ± 0.23	0.11 ± 0.29
6			8.01 ± 0.13	0.58 ± 0.23
21			7.01 ± 0.16	1.57 ± 0.25
63			4.92 ± 1.21	3.67 ± 1.08
99			2.69 ± 0.91	5.90 ± 0.82
35 ± 3 °C; 65 ± 15% RH				
1	2.25×10^8	8.59 ± 0.23	1.82 ± 0.89	6.76 ± 0.80
6			0.00 ± 0.00	8.59 ± 0.20
21			0.00 ± 0.00	8.59 ± 0.20
63			0.00 ± 0.00	8.59 ± 0.20
99			0.00 ± 0.00	8.59 ± 0.20

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% CI.

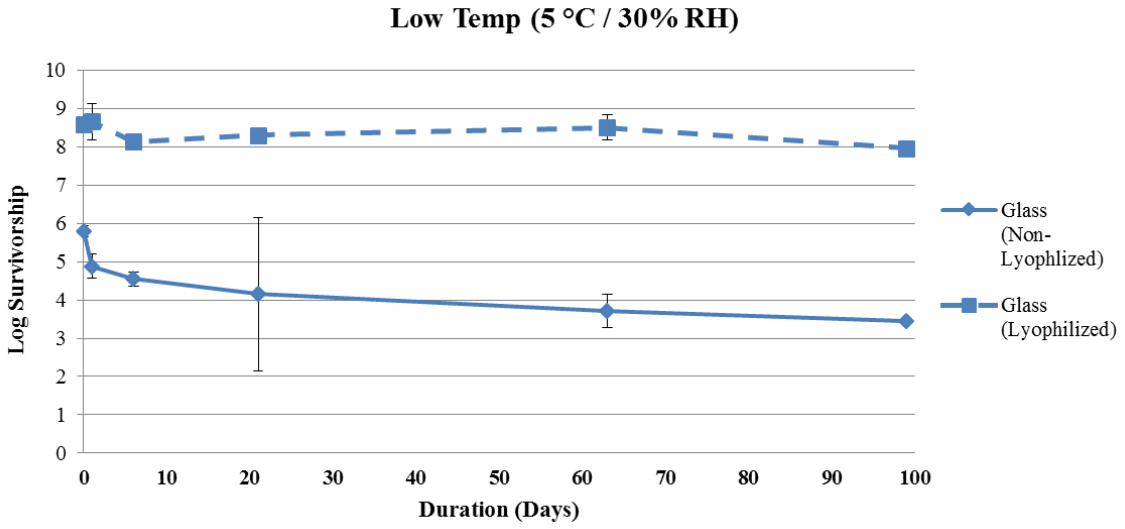

Figure 4-1. *Y. pestis* Persistence at 5 ± 2 °C and 30 ± 15% RH
Data are expressed as mean log survivorship.

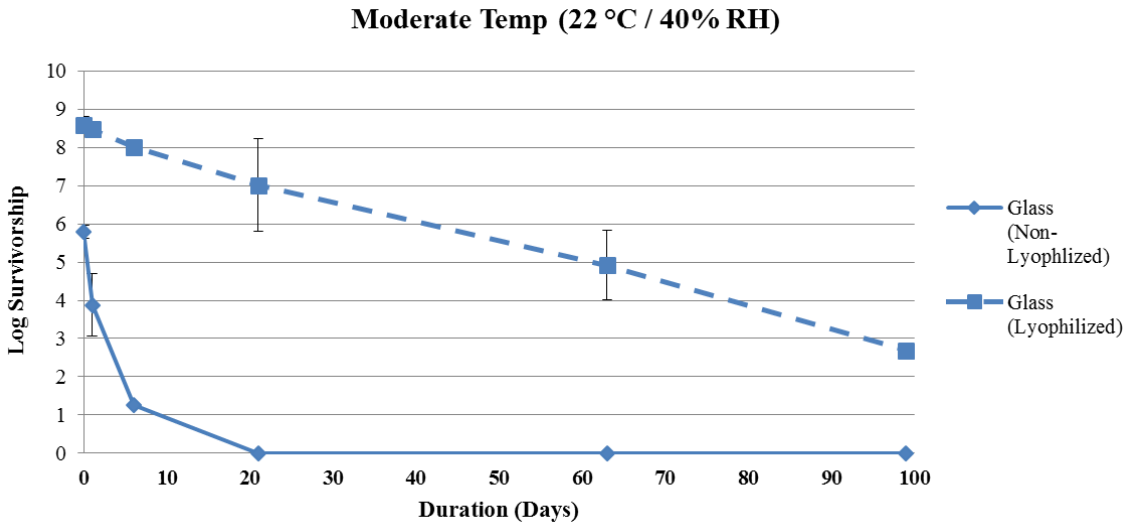

Figure 4-2. *Y. pestis* Persistence at 22 ± 2 °C and 40 ± 15% RH
Data are expressed as mean log survivorship.

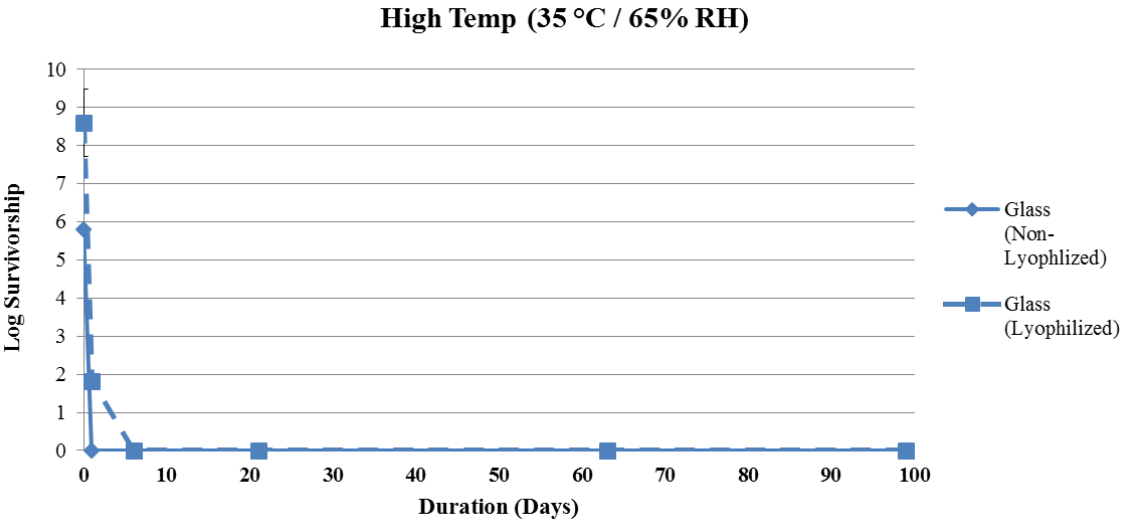

Figure 4-3. *Y. pestis* Persistence at 35 ± 2 °C and 65 ± 15% RH
Data are expressed as mean log survivorship.

Table 4-4. Qualitative *Y. pestis* Persistence in Soil

Duration (Days)	Non-Lyophilized Inoculum	Lyophilized Inoculum
	Detected	
5 ± 3 °C; 30 ± 15% RH		
1	✓	✓
6	✓	✓
21	--	--
63	✓	✓
99	✓	✓
22 ± 3 °C; 40 ± 15% RH		
1	✓	✓
6	✓	✓
21	--	--
63	ND	ND
99	ND	ND
35 ± 3 °C; 65 ± 15% RH		
1	✓	✓
6	ND	✓
21	--	--
63	ND	ND
99	ND	ND

✓ = *Y. pestis* was qualitatively detected in at least one replicate.
ND = *Y. pestis* was not detected in any replicate.
-- = samples not tested at that inoculum/time point combination.

4.2.3 F. tularensis

The results obtained for *F. tularensis* persistence on glass are summarized in Tables 4-5 and 4-6 and Figures 4-4 through 4-6. The qualitative persistence in soil is summarized in Table 4-7. When lyophilized, *F. tularensis* persisted on glass for at least 99 days at both low and moderate temperature and RH conditions (>7 logs and >6 $\log_{10}$ recovery, respectively). When not lyophilized, *F. tularensis* persisted for at least 99 days at the low temperature/RH conditions but with lower recoveries (4.62 $\log_{10}$ recovery). For the moderate temperature/RH condition, the non-lyophilized *F. tularensis* was recoverable after 63 days. Additionally, no *F. tularensis* was recovered from glass after 21 days for non-lyophilized or 6 day for lyophilized preparations, at the high temperature/RH condition.

F. tularensis persisted in soil up to 99 days at the low temperature/RH condition with both sample preparations. At the moderate temperature/RH condition, *F. tularensis* persisted longer when lyophilized compared to non-lyophilized in soil (≥63 vs ≥21 days, respectively). No organisms were detected after one day in soil at the high temperature/RH condition.

Table 4-5. *F. tularensis* Persistence on Glass Using a Non-Lyophilized Inoculum

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *F. tularensis*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	1.47×10^8	6.93 ± 0.43	7.93 ± 0.06	-1.00 ± 0.38
6			7.16 ± 0.29	-0.23 ± 0.46
21			6.75 ± 0.11	0.18 ± 0.39
63			5.10 ± 0.25	1.83 ± 0.44
99			4.62 ± 0.40	2.31 ± 0.52
22 ± 3 °C; 40 ± 15% RH				
1	1.47×10^8	6.93 ± 0.43	7.24 ± 0.08	-0.31 ± 0.39
6			4.74 ± 0.16	2.19 ± 0.41
21			2.34 ± 0.32	4.59 ± 0.47
63			0.16 ± 0.37	6.76 ± 0.50
99			0.00 ± 0.00	6.93 ± 0.38
35 ± 3 °C; 65 ± 15% RH				
1	1.47×10^8	6.93 ± 0.43	1.16 ± 0.86	5.76 ± 0.84
6			0.16 ± 0.37	6.76 ± 0.50
21			0.00 ± 0.00	6.93 ± 0.38

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% CI.

Table 4-6. *F. tularensis* Persistence on Glass Using a Lyophilized Inoculum

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *F. tularensis*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	2.87×10^8	7.40 ± 0.27	7.63 ± 0.20	-0.23 ± 0.30
6			7.48 ± 0.19	-0.08 ± 0.29
21			7.52 ± 0.06	-0.12 ± 0.24
63			7.29 ± 0.32	0.11 ± 0.37
99			7.63 ± 0.19	-0.23 ± 0.29
22 ± 3 °C; 40 ± 15% RH				
1	2.87×10^8	7.40 ± 0.27	7.27 ± 0.22	0.13 ± 0.31
6			6.95 ± 0.14	0.45 ± 0.27
21			6.51 ± 0.39	0.90 ± 0.42
63			6.82 ± 0.23	0.58 ± 0.31
99			6.02 ± 0.69	1.38 ± 0.65
35 ± 3 °C; 65 ± 15% RH				
1	2.87×10^8	7.40 ± 0.27	4.99 ± 0.13	2.41 ± 0.26
6			0.00 ± 0.00	7.40 ± 0.24

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% CI.

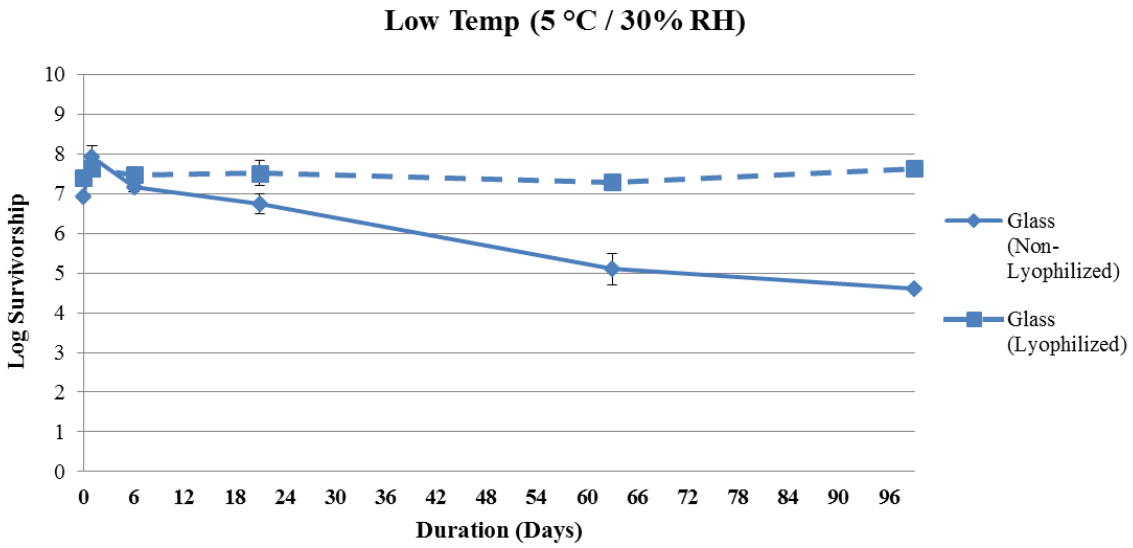

Figure 4-4. *F. tularensis* Persistence at 5 ± 2 °C and 30 ± 15% RH
Data are expressed as mean log survivorship.

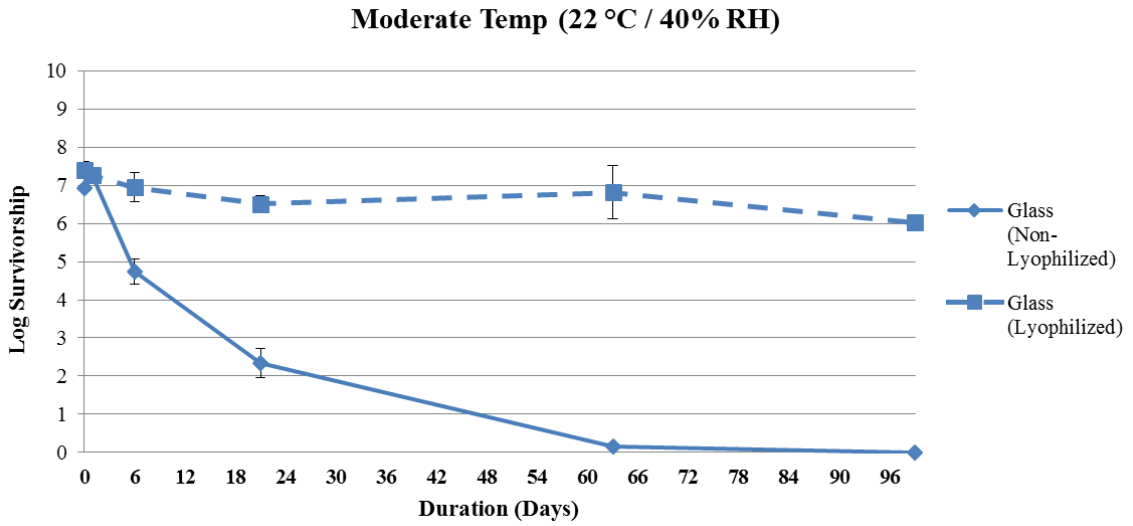

Figure 4-5. *F. tularensis* Persistence at 22 ± 2 °C and 40 ± 15% RH
Data are expressed as mean log survivorship.

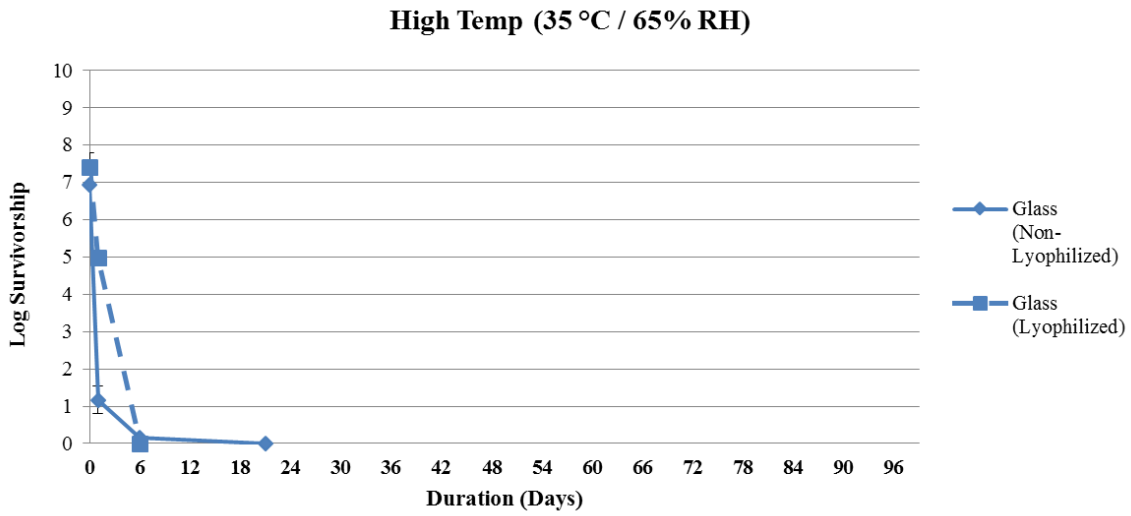

Figure 4-6. *F. tularensis* Persistence at 35 ± 2 °C and 65 ± 15% RH
Data are expressed as mean log survivorship.

Table 4-7. Qualitative *F. tularensis* Persistence in Soil

Duration (Days)	Non-Lyophilized Inoculum Detected	Lyophilized Inoculum Detected
5 ± 3 °C; 30 ± 15% RH		
1	✓	✓
6	✓	✓
21	✓	✓
63	✓	✓
99	✓	✓
22 ± 3 °C; 40 ± 15% RH		
1	✓	✓
6	✓	✓
21	✓	✓
63	ND	✓
99	ND	ND
35 ± 3 °C; 65 ± 15% RH		
1	✓	✓
6	ND	ND
21	--	--
63	--	--
99	--	--

✓ = *F. tularensis* was qualitatively detected in at least one replicate.
ND = *F. tularensis* was not detected in any replicate.
-- = samples not tested at that inoculum/time point combination.

4.2.4 B. mallei

The results obtained for *B. mallei* persistence on glass are summarized in Tables 4-8 and 4-9 and Figures 4-7 through 4-9. The qualitative persistence in soil is summarized in Table 4-10. Similar to the results obtained with *Y. pestis* and *F. tularensis*, *B. mallei* persisted on glass for at least 99

days at both low and moderate temperature and RH conditions (>7 logs and >5 $\log_{10}$ recovery, respectively), when lyophilized (Table 4-9). When not lyophilized (Table 4-8), *B. mallei* persisted for at least 99 days at the low temperature/RH condition (0.10 $\log_{10}$ recovery) and at least six days at the moderate temperature/RH condition (0.36 $\log_{10}$ recovery). No *B. mallei* was recovered from glass after six days for either sample preparation at the high temperature/RH condition.

B. mallei persisted in soil up to 99 days at the low temperature/RH condition and up to six days at the moderate temperature/RH condition with both sample preparations. No organisms were detected in soil after at any time point/preparation combination at the high temperature/RH condition.

Table 4-8. *B. mallei* Persistence on Glass Using a Non-Lyophilized Inoculum

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *B. mallei*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	1.16×10^8	8.06 ± 0.03	5.82 ± 0.07	2.23 ± 0.06
6			5.93 ± 0.12	2.13 ± 0.11
21			4.77 ± 0.12	3.29 ± 0.11
63			1.74 ± 0.92	6.31 ± 0.81
99			0.10 ± 0.23	7.95 ± 0.21
22 ± 3 °C; 40 ± 15% RH				
1	1.16×10^8	8.06 ± 0.03	5.92 ± 0.10	2.14 ± 0.09
6			0.36 ± 0.57	7.69 ± 0.50
21			0.00 ± 0.00	8.06 ± 0.03
63			0.00 ± 0.00	8.06 ± 0.03
99			0.00 ± 0.00	8.06 ± 0.03
35 ± 3 °C; 65 ± 15% RH				
1	1.16×10^8	8.06 ± 0.03	3.19 ± 0.35	4.87 ± 0.31
6			0.00 ± 0.00	8.06 ± 0.03

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% CI.

Table 4-9. *B. mallei* Persistence on Glass Using a Lyophilized Inoculum

Duration (Days)	Inoculation Control (CFU/coupon)	Mean Recovered *Y. pestis*, Log Survivorship*		Mean Log Reduction§
		Positive Control†	Test Coupon‡	
5 ± 3 °C; 30 ± 15% RH				
1	9.60×10^7	7.70 ± 0.05	7.41 ± 0.14	0.29 ± 0.13
6			7.51 ± 0.05	0.18 ± 0.06
21			7.27 ± 0.11	0.42 ± 0.11
63			7.12 ± 0.10	0.57 ± 0.10
99			7.16 ± 0.06	0.53 ± 0.07
22 ± 3 °C; 40 ± 15% RH				
1	9.60×10^7	7.70 ± 0.05	6.63 ± 0.61	1.07 ± 0.53
6			5.91 ± 0.94	1.78 ± 0.83
21			6.41 ± 0.18	1.29 ± 0.17
63			6.01 ± 0.02	1.68 ± 0.05
99			5.53 ± 0.28	2.16 ± 0.25
35 ± 3 °C; 65 ± 15% RH				
1	9.60×10^7	7.70 ± 0.05	5.64 ± 0.94	2.06 ± 0.83
6			0.00 ± 0.00	7.70 ± 0.04

*Data are expressed as mean log survivorship ± standard deviation.
†Positive control coupons were inoculated and extracted at time zero (i.e., one hour after inoculation); one set of positive controls was used for all test durations.
‡Test coupons were inoculated and exposed to the environmental condition for the exposure duration.
§Data are expressed as mean log reduction ± 95% CI.

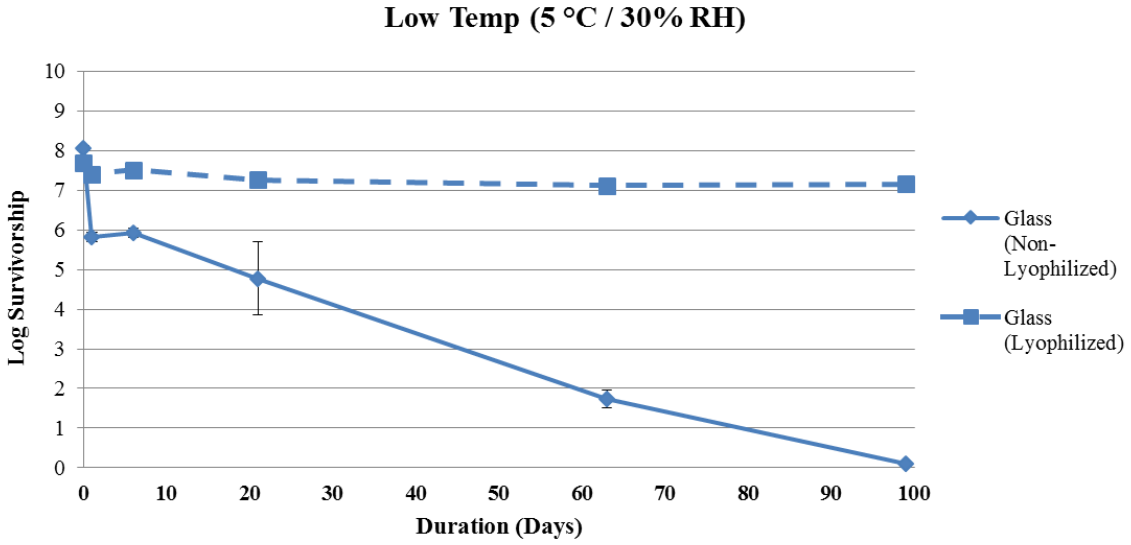

Figure 4-7. *B. mallei* Persistence at 5 ± 2 °C and 30 ± 15% RH
Data are expressed as mean log survivorship.

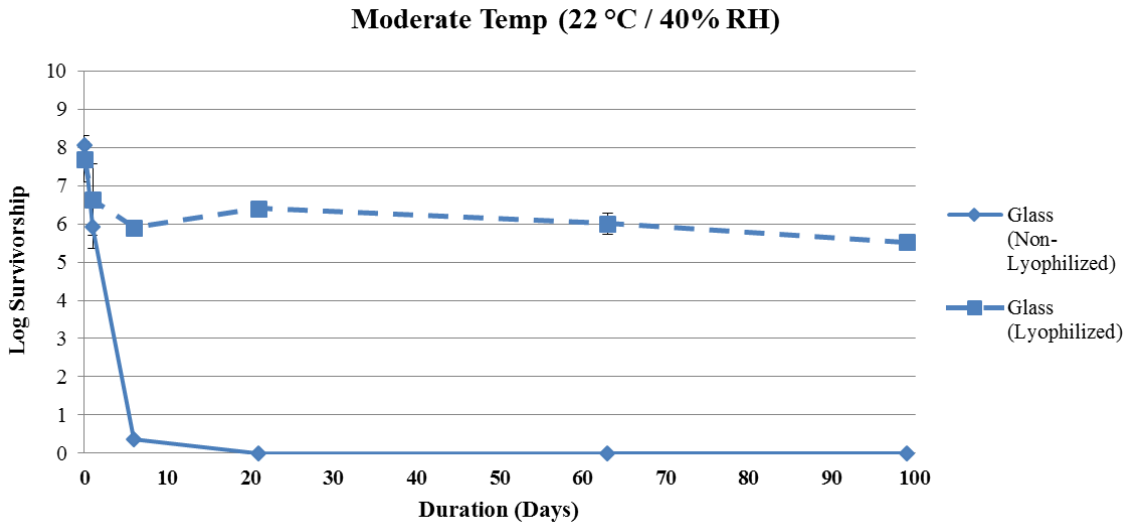

Figure 4-8. *B. mallei* Persistence at 22 ± 2 °C and 40 ± 15% RH
Data are expressed as mean log survivorship.

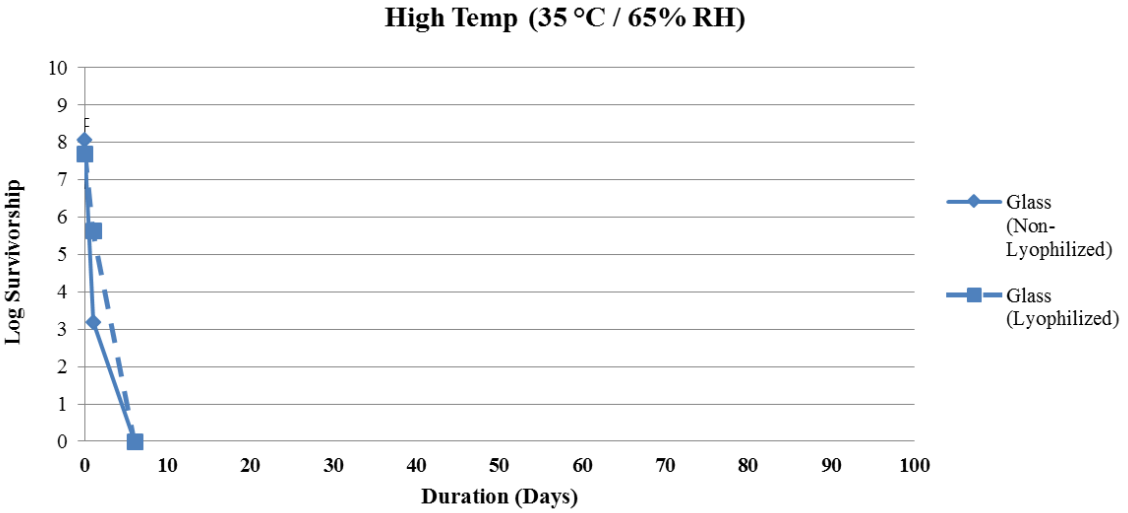

Figure 4-9. *B. mallei* Persistence at 35 ± 2 °C and 65 ± 15% RH
Data are expressed as mean log survivorship.

Table 4-10. Qualitative *B. mallei* Persistence in Soil

Duration (Days)	Non-Lyophilized Inoculum Detected	Lyophilized Inoculum
5 ± 3 °C; 30 ± 15% RH		
1	✓	✓
6	✓	✓
21	✓	✓
63	✓	✓
99	✓	✓
22 ± 3 °C; 40 ± 15% RH		
1	✓	✓
6	✓	✓
21	ND	ND
63	ND	ND
99	ND	ND
35 ± 3 °C; 65 ± 15% RH		
1	ND	ND
6	ND	ND
21	--	--
63	--	--
99	--	--

✓ = *B. mallei* was qualitatively detected in at least one replicate.
ND = *B. mallei* was not detected in any replicate.
-- = samples not tested at that inoculum/time point combination.

5.0 Summary

When exposed to 5 ± 3 °C and 30 ± 15% RH, *Y. pestis*, *F. tularensis*, and *B. mallei* all persisted on glass at a higher degree when lyophilized rather than non-lyophilized. Once lyophilized, little to no reduction in viability was observed on glass over the 99 days. In comparison, organisms from non-lyophilized samples were still recoverable, but to a lesser degree (log_{10} recovery ranged from 0.10 to 4.62). Although not quantitatively evaluated, organisms were also detected in soil up to 99 days at the low temperature/RH combination for all three organisms tested, regardless of the preparation.

When exposed to 22 ± 3 °C and 40 ± 15% RH, all organisms again persisted on glass at a higher degree when lyophilized rather than air dried. Once lyophilized, 2.69 (*Y. pestis*), 6.02 (*F. tularensis*), and 5.53 (*B. mallei*) log_{10} recovery was obtained after a 99-day contact time. When not lyophilized, no organisms were recovered after six days for *Y. pestis* and *B. mallei*, or after 63 days for *F. tularensis*. In soil, the same pattern was observed with organisms generally persisting at least as long, if not longer when lyophilized (*Y. pestis*: six days for both preparations; *F. tularensis*: 63 days versus 21 days; *B. mallei*: six days for both preparations).

Lastly, all organisms were inactivated by exposure to 35 ± 3 °C and 65 ± 15% RH sooner than the low and moderate temperature/RH conditions, regardless of inoculation method. No *Y. pestis* was recovered from glass after one day using the non-lyophilized inoculum or after six days using the lyophilized inoculum (1.82 log_{10} recovery after one day). No *F. tularensis* was recovered from glass after 21 days for the non-lyophilized preparations (0.16 log_{10} recovery after six days) or after six days using the lyophilized inoculum (4.99 log_{10} recovery after one day). Lastly, no *B. mallei* was recovered from glass after six days for either preparation technique (3.19 and 5.64 logs, for non-lyophilized, respectively). No *Y. pestis* or *F. tularensis* was detected in soil after six days, and no *B. mallei* was detected after one day when not lyophilized. When lyophilized, *Y. pestis* was detected out to six days, *F. tularensis* out to one day, and no *B. mallei* was detected after only one day.

The minimum exposure time where no organisms were recovered on either glass or soil are summarized in Table 5-1.

Table 5-1. Minimum Exposure Times for Organism Recovery on Glass or Soil

Y. pestis		*F. tularensis*		*B. mallei*	
Non-Lyophilized	**Lyophilized**	**Non-Lyophilized**	**Lyophilized**	**Non-Lyophilized**	**Lyophilized**
5 ± 3 °C; 30 ± 15% RH					
≥99 days	≥99 days	≥99 days	≥99 days	≥99 days	≥99 days
22 ± 3 °C; 40 ± 15% RH					
≥6 days	≥99 days	≥63 days	≥99 days	≥6 days	≥99 days
35 ± 3 °C; 65 ± 15% RH					
≥1 day	≥6 days	≥6 days	≥1 day	≥1 day	≥1 day

These data suggest that temperature and RH, as well as the preparation of the organism, significantly impact the persistence of *Y. pestis*, *F. tularensis*, and *B. mallei* on glass and in soil. All three organisms will apparently survive for extended periods of time (potentially greater than 99 days) when lyophilized and released into the environment, which may warrant additional decontamination investigations. However, the use of elevated temperature may be a viable

decontaminant option for some situations, as all three organisms were inactivated to a point of non-detect after six days in most instances (lyophilized *Y. pestis* was detected in soil after six days at 35 °C and 65% RH). These persistence data are useful for making response or remediation decisions following natural occurrences or intentional releases of biological agents.

6.0 References

1. ASTM E104. Standard Practice for Maintaining Constant Relative Humidity by Means of Aqueous Solutions. ASTM International, 2012.
2. Heckly RJ, Blank H. Virulence and viability of *Yersinia pestis* 25 years after lyophilization. *Applied and Environmental Microbiology*. 1980;39(3):541-543.
3. http://opsdiagnostics.com/applications/lyophilization/ecoli_lyophilization_stability.html, last accessed October 16, 2015.

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