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Impact of Temperature and Humidity on the Persistence of Vaccinia Virus and Ricin Toxin on Indoor Surfaces

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Investigation Report

Impact of Temperature and Humidity on the Persistence of Ricin Toxin and Vaccinia Virus on Indoor Surfaces

By

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Notice

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Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development (ORD) provides data and scientific support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

In September 2002, EPA announced the formation of the National Homeland Security Research Center (NHSRC). The NHSRC is part of the Office of Research and Development; it manages, coordinates, supports, and conducts a variety of research and technical assistance efforts. These efforts are designed to provide appropriate, affordable, effective, and validated technologies and methods for addressing risks posed by chemical, biological, and radiological terrorist attacks. Research focuses on enhancing our ability to detect, contain, and decontaminate in the event of such attacks.

NHSRC's team of world renowned scientists and engineers is dedicated to understanding the terrorist threat, communicating the risks, and mitigating the results of attacks. Guided by the roadmap set forth in EPA's Strategic Plan for Homeland Security, NHSRC ensures rapid production and distribution of security related products.

The NHSRC has developed the Technology Testing and Evaluation Program (TTEP) in an effort to provide reliable information regarding the performance of homeland security related technologies. TTEP provides independent, quality assured performance information that is useful to decision makers in purchasing or applying the tested technologies. It provides potential users with unbiased, third-party information that can supplement vendor-provided information. Stakeholder involvement ensures that user needs and perspectives are incorporated into the test design so that useful performance information is produced for each of the tested technologies. The technology categories of interest include detection and monitoring, water treatment, air purification, decontamination, and computer modeling tools for use by those responsible for protecting buildings, drinking water supplies and infrastructure and for decontaminating structures and the outdoor environment. Additionally, environmental persistence information is also important for containment and decontamination decisions.

The U.S. EPA, through its Office of Research and Development's NHSRC, funded and managed this investigation through a Blanket Purchase Agreement (BPA) under General Services Administration contract number GS23F0011L-3 with Battelle. Information on NHSRC and TTEP can be found at http://www.epa.gov/ordnhsrc/index.htm.

Acknowledgments

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Abbreviations/Acronyms

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BSC II	Class II biological safety cabinet
^{o}C	degrees Celsius
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
ClO_2	chlorine dioxide
cm	centimeter
CO_2	carbon dioxide
d	depth
EPA	U.S. Environmental Protection Agency
h	height
HVAC	heating, ventilation, and air conditioning
l	length
L	liter
mL	milliliter
µg	microgram
µL	microliter
mm	millimeter
mg	milligram
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5,-diphenyl tetrazolium bromide
n	number of observation (or replicate samples)
nm	nanometer
NHSRC	National Homeland Security Research Center
ORD	Office of Research and Development
PBS	phosphate buffer solution
PFU	plaque-forming unit
QA	quality assurance
QC	quality control
QMP	quality management plan
RH	relative humidity
SD	standard deviation
TSA	technical systems audit
TTEP	Technology Testing and Evaluation Program
T0	Time zero
µg	microgram
vs.	versus
w	width

Executive Summary

The U.S. EPA's NHSRC TTEP is helping to protect human health and the environment from adverse impacts resulting from acts of terror by carrying out performance tests on homeland security technologies. Under TTEP[1], the persistence of biological agents on indoor building materials under various temperature and humidity conditions, consistent with conditions that might be achieved using a heating, ventilation, and air conditioning (HVAC) system, was investigated. The objective of the study was to determine the attenuation of vaccinia virus viability, a surrogate for smallpox virus (*variola*), and the cytotoxicity of ricin toxin on indoor building materials due to prolonged exposure to ambient conditions [20°C, relative humidity (RH) 40 - 70%], or at 30°C with high (>70%) or low (<40%) RH. It is important to know whether environmental conditions can impact the persistence of biological agents in order to properly structure first-responder assessment and plan and interpret results from use of fumigant, liquid or foam decontamination technologies. Further, decontamination of indoor surfaces following intentional release of biological agents may potentially be enhanced by manipulating environmental conditions, such as temperature or RH, if an effect on persistence of biological organisms or toxicity of toxins is observed.

Ricin toxin and vaccinia virus were selected based upon a review of available information and other on-going research and assessment efforts; the selection represent biological agents with a range of reported environmental persistence. Both vaccinia and *variola* viruses are species in the genus *Orthopoxvirus*. There is a 96% identity at the nucleotide level between vaccinia and *variola*.[1] Given the high levels of similarity between these viruses, decontamination of *variola* is expected to be similar to that observed for vaccinia.

Coupons of galvanized metal or painted concrete were inoculated with the respective agent being investigated and placed in test conditions of either ambient (~20^{o}C and 40%-70% RH), or at 30^{o}C at either high (>70%) or low RH (<40%). Thus, persistence of the agents on the materials was determined at the three environmental conditions as a function of time up to 14 days. Persistence of a biological agent exposed to known environmental conditions can be evaluated by comparing the temporal reduction in toxin bioactivity or organism viability following inoculation onto indoor building material coupons. The attenuation, or reduction in persistence, can be represented as a log reduction (for vaccinia virus) or percent reduction (for ricin toxin) in the amount of viable organisms or bioactive toxin extracted from coupons after a treatment (environmental conditions) compared to extractions one hour after inoculating the coupons.

The results showed that the bioactivity of both ricin and vaccinia persisted on galvanized metal ductwork and painted concrete for up to 14 days under low RH conditions. The results also indicated that RH impacts the persistence of both ricin and vaccinia virus; both agents were more persistent when maintained at low RH rather than at high RH. In each case, the persistence was dependent upon the type of material onto which the biological organisms or toxin was applied. Furthermore, the results indicate that, depending on the type of contaminated substrate, elevated RH may be useful for inactivating ricin or vaccinia virus before the application of a decontamination technology. Because of the close relationship between vaccinia and *variola*, the results will likely be applicable to *variola;* however, because of differences between *variola* and *vaccinia*, in the event of *variola* contamination, the persistence should be verified, rather than assumed.

The Q-fever causative agent, *Coxiella burnetii*, was also selected for this study. However, the *C. burnetii* strain used in this testing resulted in quantification challenges that could not be addressed within the scope of the project, while still achieving the overall project objectives for ricin toxin and vaccinia virus. Therefore, *C. burnetii* was removed from the test matrix; details of the challenges and attempts to overcome them are provided in Appendix B to this report.

1.0 Introduction

The U.S. EPA's NHSRC is helping to protect human health and the environment from adverse impacts resulting from acts of terror. With an emphasis on decontamination and consequence management, water infrastructure protection, and threat and consequence assessment, NHRSC is working to develop tools and information that will help detect the introduction of chemical, biological, or radiological contaminants in buildings or water systems, contain these agents, decontaminate buildings and/or water systems, and dispose of materials resulting from cleanups.

NHSRC's TTEP works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, scientists, and government regulators; and with participation of individual technology developers in carrying out performance tests of homeland security technologies. In response to the needs of stakeholders, TTEP evaluates the performance of innovative homeland security technologies by developing test plans, conducting evaluations, collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure the generation of high quality data and defensible results. TTEP provides unbiased, third-party information supplementary to vendor-provided information that is useful to decision makers in purchasing or applying the evaluated technologies. Stakeholder involvement ensures that user needs and perspectives are incorporated into the testing design to produce useful performance information for each technology investigated.

The objective of the study was to determine the persistence of biological organisms or toxin on indoor building materials under various temperature and RH conditions, consistent with conditions that might be achieved using an HVAC system. The attenuation of the persistence was investigated as a function of biological agent type, building material surface, and environmental conditions. The work was motivated by the need to assess the impact of environmental conditions on the persistence (or attenuation) of biological agents in order to properly structure testing and interpret results from use of decontamination technologies, in addition to developing an understanding of the persistence of biological agents on surfaces in indoor environments, Further, decontamination of indoor surfaces following intentional release of biological agents may potentially be enhanced by manipulating environmental conditions, such as raising the temperature and raising or lowering RH, if an effect on persistence of biological organisms or toxicity of toxins is observed.

C. burnetii, ricin toxin, and vaccinia virus were selected to represent a broad range of biological agents with reported environmental persistence. *C. burnetii*, the causative agent of Q-Fever, was selected as a representative of the bacterial order Rickettsiae that is reported to be persistent. Rickettsiae include a number of important human pathogens that cause diseases such as Rocky Mountain Spotted Fever and Typhus. However, the *C. burnetii* strain used in this testing resulted in quantification challenges that could not be addressed within the scope of the project, while still achieving the overall project objectives for ricin toxin and vaccinia virus. Therefore, *C. burnetii* was removed from the test matrix; details of the challenges and attempts to overcome them are provided in Appendix B to this report. Ricin toxin was selected as a high-profile protein toxin with a history of terrorist use. Vaccinia virus was selected as a

surrogate for *variola* virus, the causative agent for smallpox. Smallpox is a potential biological agent of concern because of the extremely grave consequences that would be expected from its use.

The investigation of the persistence of the biological agents or surrogates on indoor building materials was conducted according to a peer-reviewed test/QA plan [2] that was developed according to the requirements of the quality management plan (QMP)[3] for the TTEP program. The test plan outlines an investigation of the persistence of the selected agents on various materials. Bioactivity indicating persistence was measured as the number of viable vaccinia virus determined as plaque-forming units (PFU) or the cytotoxicity of ricin toxin remaining on the coupons after exposure to the specified test conditions. In this investigation, coupons of galvanized metal ductwork or painted concrete were inoculated with the ricin toxin or vaccinia virus and placed in test conditions of either ambient (~20^{o}C and 40%-70% RH), or at 30^{o}C at either high (>70%) or low RH (<40%). Mean attenuation in persistence at the treatment conditions was measured as the percent reduction in ricin cytotoxicity or log reduction in viral viability.

2.0 Investigation Procedures

2.1 Experimental Design

This report provides results for the investigation of the persistence of biological organisms or toxin exposed to various temperature and RH conditions that might be observed or easily achieved in a building. Persistence was measured on two indoor surfaces, galvanized metal ductwork and painted concrete, typical of those that might require decontamination after a deliberate release of biological agent inside of a public building or subway system. The experimental treatments performed are shown in Table 2-1. The independent test parameters measured included temperature, RH, and time. The dependent variable was the extracted residual PFU or mass of cytotoxic ricin.

Statistical comparisons were conducted to determine whether the treatment mean reduction in viable virus or cytotoxic ricin exceeded the control mean reduction by an amount that was statistically significant at a 95% confidence level.

Table 2-1. Test Matrix and Measured Parameters

Agent	Indoor Surface Material	Environmental Conditions	Time (Days)	Dependent Variable
Ricin toxin	Galvanized metal ductwork	30°C; <40% RH	0, 1, 3, 9, and 14	Cytotoxic mass
Ricin toxin	Galvanized metal ductwork	30°C; >70% RH	0, 1, 3, 9, and 14	Cytotoxic mass
Ricin toxin	Galvanized metal ductwork	~20°C; ~40% RH (ambient)	0, 1, 3, 9, and 14	Cytotoxic mass
Ricin toxin	Painted concrete	30°C; <40% RH	0, 1, 3, 9, and 14	Cytotoxic mass
Ricin toxin	Painted concrete	30°C; >70% RH	0, 1, 3, 9, and 14	Cytotoxic mass
Ricin toxin	Painted concrete	~20°C; ~40% RH (ambient)	0, 1, 3, 9, and 14	Cytotoxic mass
Vaccinia virus	Galvanized metal ductwork	30°C; <40% RH	0, 1, 3, 9, and 14	PFU
Vaccinia virus	Galvanized metal ductwork	30°C; >70% RH	0, 1, 3, 9, and 14	PFU
Vaccinia virus	Galvanized metal ductwork	~20°C; ~40% RH (ambient)	0, 1, 3, 9, and 14	PFU
Vaccinia virus	Painted concrete	30°C; <40% RH	0, 1, 3, 9, and 14	PFU
Vaccinia virus	Painted concrete	30°C; >70% RH	0, 1, 3, 9, and 14	PFU
Vaccinia virus	Painted concrete	~20°C; ~40% RH (ambient)	0, 1, 3, 9, and 14	PFU

2.2 Test Chamber

The persistence of biological agent on various indoor building materials was investigated at three chamber temperature and RH conditions: "ambient" (40%-70% RH at ~20ºC), "low RH" (<40% RH at 30ºC), and "high RH" (>70% RH at 30ºC). Test coupons (described in Section 2.3) were transferred into the test chamber for experimental treatment after being inoculated with biological agent as described in Section 2.5, below. Plastic Lock&Lock™ containers (Target®) were used as test chambers as shown in Figure 2-1. The Lock&Lock plastic storage container had dimensions of 25 cm width (w) x 18 cm depth (d) x 17 cm height (h).

Low RH (<40%) was maintained in the test chambers using calcium sulfate desiccants (W.A. Hammond Drierite Co.). The W.A. Hammond Drierite Co. web site (www.Drierite.com) reports that the National Bureau of Standards has verified that the moisture remaining in gases dried with Drierite at 25°C-30°C is 0.005 mg/L (<1% RH). The desiccant could therefore reduce the %RH below the upper limit of 40% RH. High RH (>70%) was generated by enclosing wet paper towels in the test chamber. The elevated temperature (30ºC) was maintained by placing the test chamber inside of an incubator.

Temperature and RH were recorded using a HOBO U10 Data Logger (in Figure 2-1, note cable coming out of the sealed test chamber). Manufacturer's specifications indicate time accuracy of 61 seconds/month at 25°C (77°F), temperature accuracy of ± 0.4°C @ 25°C (± 0.7°F @ 77°F) and RH accuracy of ±3.5% from 25% to 85% over the range of 15°C to 45°C (59°F to 113°F). A VWR® Traceable™ hygrometer/thermometer with a temperature accuracy of ±1°C and RH accuracy of ±4% (between 20% and 80%) was included in the test chamber to enable environmental conditions to be independently verified.

Figure 2-1. Test Chamber (left) and Test Chamber in Incubator (right)

2.3 Test Surfaces

The indoor surface materials, shown in Table 2-2, included both nonporous (galvanized metal ductwork) and porous (painted concrete) surfaces. Test coupons were cut to 1.9 cm length (l) x 7.5 cm w in size from a larger piece of test material. Edges and damaged areas were avoided in cutting test coupons. The galvanized metal ductwork coupons were 0.6 mm thick. The painted concrete coupons were 0.7 cm thick. The test coupons were visually inspected prior to being inoculated with the biological agents. Coupons with anomalies on the test (application) surface were rejected from use.

On each day of testing, each coupon was assigned and marked with a unique identifier code for traceability. Prior to the application of the biological agent or surrogate, the coupons were autoclaved and the surface of each coupon was wiped with 70% isopropanol or ethanol. Principles of aseptic technique following Battelle guidance [1, 4-6] was exercised during all phases of handling the test coupons.

Table 2-2. Material Characteristics

Material	Lot, Batch, or ASTM No., or Observation	Manufacturer/ Supplier Name	Approximate Coupon Size, l x w, cm	Material Preparation
Galvanized metal ductwork	Industry HVAC standard 24-gauge galvanized steel	Accurate Fabrication	1.9 cm x 7.5 cm	Cleaned with acetone; autoclaved
Painted concrete, cinder block	ASTM C90	Wellnitz	1.9 cm x 7.5 cm	Brush and roller painted all sides. One coat Martin Senour latex primer (#71-1185) and one coat Porter Paints latex semi-gloss finish (#919); autoclaved

2.4 Biological Agent and Surrogate

The biological agent and surrogate used in the testing were ricin toxin (Vector Laboratories L-1090, product specification: *Ricin communis* agglutinin II, 5 mg/mL protein concentration) and vaccinia virus (American Type Culture Collection (ATCC) VR119, an orthopox virus closely related to *Variola major*, the causative agent for smallpox). The biological agent and surrogate were selected based on an evaluation of potential threats to buildings and discussions with and approval by EPA. Biological agent was used according to the Centers for Disease Control and Prevention (CDC) Select Agents Program (42 Code of Federal Regulations (CFR) Part 73) and the Biological Defense Research Program (32 CFR 626 and 627) in adherence with the Battelle Medical Research and Evaluation Facility Safety Plan.[7]

A ricin stock solution was prepared by transferring a 100 µL aliquot of ricin toxin (5 mg/mL), as described below in Section 2.7, into a sterile 50 mL conical vial to which 10.0 mL of sterile phosphate-buffered saline (PBS) was added and agitated on an orbital shaker for 15 minutes at approximately 200 rpm at room temperature (approximately 22°C). The cytotoxicity of the stock solution was determined using the bioassay methods for the respective biological agents specified in Section 2.7.

The vaccinia stock used in this study was propagated by inoculating a monolayer of Vero cells with vaccinia virus at a multiplicity of infection ranging from 0.01 to 1.0%. Cultures were maintained in an

incubator at 37°C ± 2°C under a 95% air, 5% CO_2 mixture until 90-100% cytopathic effects were observed. Infected cells were harvested and subjected to a rapid freeze/thaw cycle. Cellular debris was removed by centrifugation at 800 to 1,000 x g. The resulting supernatant (containing virus) was harvested and stored in 1.0 mL aliquots, approximately 1.0 x 10^8 PFU/mL, at ≤ -70ºC until used. The resulting supernatant, vaccinia in complete cell culture medium containing 5-10% fetal bovine serum, was not filter sterilized because the virus is propagated in sterile tissue culture. All manipulations are performed with sterile technique. The PFU/mL is determined after the virus has been aliquoted by randomly selecting and assaying vials of the stock samples. The samples are assayed using a standard plaque assay (described in Section B2.7) to determine the viral titer of the entire lot.

2.5 Application of Biological Agent to Test Coupons

The functional types of coupons included in this investigation are test coupons (five at each non-zero time point), control coupons (five at time zero [T0]), laboratory blank coupons (one at T0) and procedural blank coupons (one at each non-zero time point). Test coupons are inoculated with biological agent and subjected to specified temperature and RH conditions for the specified time; the test coupons are then extracted and analyzed. Positive control coupons are inoculated with biological agent and extracted and analyzed at T0. Laboratory blank coupons are not inoculated and are extracted and analyzed at T0. Procedural blank coupons are not inoculated and are extracted and analyzed along with test coupons at each time point.

The ricin toxin stock solution was applied onto the test coupons, lying flat, at approximately 25 µg per test and positive control coupon. A 5 µL aliquot of a stock suspension (5 mg/mL of ricin) was dispensed using a micropipette as a streak across the surface of the test coupon. The ricin toxin application was performed in a Class II Biological Safety Cabinet (BSC II).[8]

Application of vaccinia virus to the test coupons was performed in a BSC II in accordance with internal safety guidelines).[8] Test and positive control coupons were placed lying flat in the cabinet and inoculated at challenge levels of vaccinia virus at approximately 1 x 10^8 PFU per coupon. A 100 µL aliquot of a stock suspension (approximately 1 x 10^9 PFU/mL) of virus was dispensed using a multichannel micropipette (shown in Figure 2-2) as two rows of five 10 µL droplets on the surface of the test coupon.

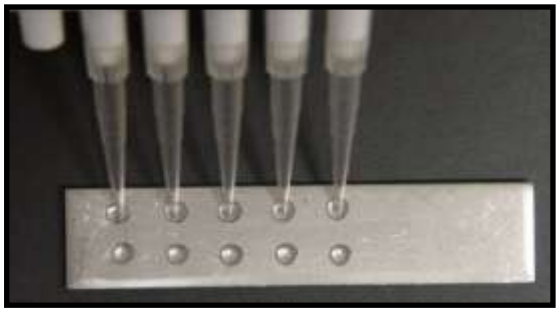

Figure 2-2. Multichannel Micropipette

After being inoculated with ricin toxin or vaccinia virus, the test coupons were left undisturbed for one hour at ambient laboratory (or BSC II) temperature and RH and then placed into test conditions of controlled temperature and RH, in accordance with the test matrix shown in Table 2-1.

2.6 Test Procedure

Two 14-day trial runs were used for each of the two agents investigated to complete the study at the three environmental conditions. In the first run for each agent, the ambient and high RH (at 30°C) conditions were investigated. In the second run for each agent, the coupons were treated at the ambient and low RH (at 30°C) conditions. Therefore, for each run, two test chambers were used concurrently (one for the ambient condition and one for the high or low RH condition). A total of five replicate coupons of each type treated at the same conditions were used for each time point investigated at each condition. The galvanized metal and concrete replicate coupons treated at the ambient conditions were split between the first and second runs. Two galvanized metal coupons and three concrete coupons were included for each time point in the first run at the ambient conditions. Three galvanized metal and two concrete coupons for each time point were treated at the ambient conditions in the second run. One blank coupon (inoculated only with PBS) of each type per time point at each condition was used in each of the runs. Five positive controls, extracted after the one hour drying period at Day 0, of each coupon type were used for each test condition. At the desired time points within each trial run, the appropriate coupons (test and blank) of each material type were removed from each test chamber. The brief opening and closing of the test chamber was observed to have minimal impact on the internal temperature and RH.

2.7 Determination of Percent Recovery and Persistence

The testing investigated persistence of biological agent by measuring the amount of residual biological agent on test coupons over time. The percent recovery, shown in Section 4, was determined by measuring and comparing the amount of viable or cytotoxic biological agent recovered from control coupons one hour after being inoculated to the amount of biological agent applied based upon analysis of the stock suspension or solution. Persistence was determined by measuring the amount of residual viable or cytotoxic biological agent on inoculated coupons over time. Attenuation in persistence was calculated by determining the reduction in persistence at each time point compared to the T0 measurement (positive control coupons). For extraction of ricin toxin and vaccinia virus, the test, control, and blank coupons were placed individually in a sterile 50 mL conical vial to which 10.0 mL of sterile PBS was added. The tubes were agitated on an orbital shaker for 15 minutes at approximately 200 rpm at room temperature. This extraction method has been used in previous work.[9-11]

The amount of cytotoxic ricin persisting on test coupons over time was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5,-diphenyltetrazolium bromide) bioassay developed by Mosmann.[12] The mechanism of action by which ricin exerts its toxic effect is through inhibition of protein synthesis within cells and activation of enzymes inducing apoptosis. Such inhibition of protein production and apoptosis lead to cell death. Therefore, an *in vitro* cytotoxicity assay, described below, was used to evaluate the mass of cytotoxic ricin in the applied volume of extraction fluid. The MTT assay is an indirect measure of ricin bioactivity. The MTT assay measures loss of mitochondrial activity arising from cell death as a result of direct impacts of ricin. Other cytotoxic substances or those toxic to mitochondrial activity could create false positives, result in an overestimate of ricin concentration, or

reduce the sensitivity of the assay. The extracts of negative control coupons were used to calculate background cytotoxicity. Dilutions, that reduce assay sensitivity, are used to "zero-out" background cytotoxicity.

Vero (African Green Monkey kidney) cells were seeded in wells of a 96-well microplate at a density of approximately 2 x 10^4 cells/well. Cells were cultured for 18-30 hours at 37ºC ± 2ºC under 95% air and 5% CO_2 and exposed to the coupon extracts. Following extraction of ricin from coupons, 1.0 mL of the PBS extracts of the ricin test and control coupons were removed and an 8-point series of two-fold dilutions were prepared in complete cell culture medium. An aliquot (100 µL) of the undiluted extract and each serial dilution was plated onto confluent Vero cells in the 96-well plate. For quantitation, a parallel standard curve was prepared using three-fold serial dilutions starting from 10 ng/mL and plated onto confluent Vero cells in the 96-well plate. Following exposure to extracts for 48-72 hours at 37ºC ± 2ºC in a humidified 5% CO_2 atmosphere, the cells were incubated in the presence of MTT (Promega, Madison, WI) for four hours, where mitochondrial enzymes within cells convert the yellow MTT to a purple formazan salt. The reaction was terminated at the end of the four-hour exposure to MTT by adding 100 µL of a Solubilization/Stop Solution (Promega). The plate was incubated overnight (for up to 24 hours) at 37ºC ± 2ºC in a humidified atmosphere to solublilize the formazan crystals as specified by Promega Technical Bulletin: CellTiter 96™ non-Radioactive Cell Proliferation Assay.[13] A Molecular Devices SPECTRAmax microplate reader was used to measure the absorbance at 570 nm wavelength using a reference wavelength of 630-750 nm. For each standard and test sample, absorbance values of the reference wavelength (630-750 nm) are subtracted from the absorbance values at 570 nm for each well. Absorbance values are directly proportional to the number of viable cells present in the sample well. For each standard, the mean absorbance values (Y-axis) are plotted against the concentration in ng/mL, and a four-parameter logistic curve is generated by the software included in the SPECTRAmax microplate reader using the equation:

Equation 1.
$$Y = \min + \frac{(\max - \min)}{1 + (X / C)^B}$$

where:

Y = absorbance %;
X = concentration of ricin ng/mL;
max = Y-value of the asymptote at the low values of X % absorbance;
min = Y-value of the asymptote at the high values of X % absorbance;
B = value related to the slope of the curve between the asymptotes;
C = X-value of the midpoint between max and min ng/mL

The amount of formazan produced is inversely proportional to the cytotoxic potential of ricin (Figures 2-3 and 2-4). To determine the concentration of ricin toxin from each test sample (i.e., the mass of ricin toxin extracted in a specified volume of extraction fluid), the ricin toxin stock solution (purified; purchased from Vector Laboratories, Burlingame, CA) was assayed in parallel and used to prepare a standard curve of absorbance versus mass of ricin protein. The absorbance of samples of extracts were plotted onto the standard curve to determine the mass of cytotoxic ricin in the sample. In cases where samples were outside of the linear range, appropriate dilutions were made and the assay was repeated. The limits of quantitation were dependent on the specific dilution and standard curve, but at the highest dilutions were in the range of 0.041 to 0.062 ug. The mass of cytotoxic ricin and percent recovery were calculated.

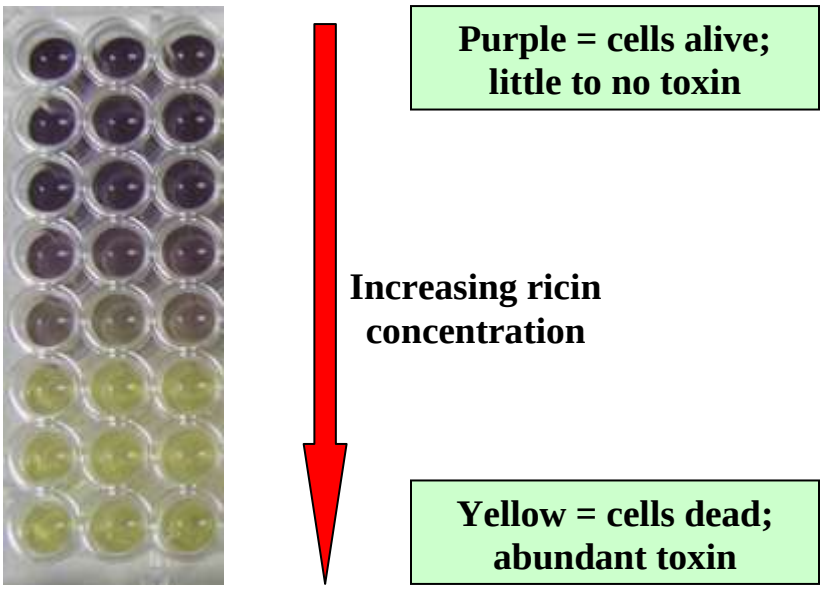

Figure 2-3. Visual Demonstration of MTT Assay on a Microplate

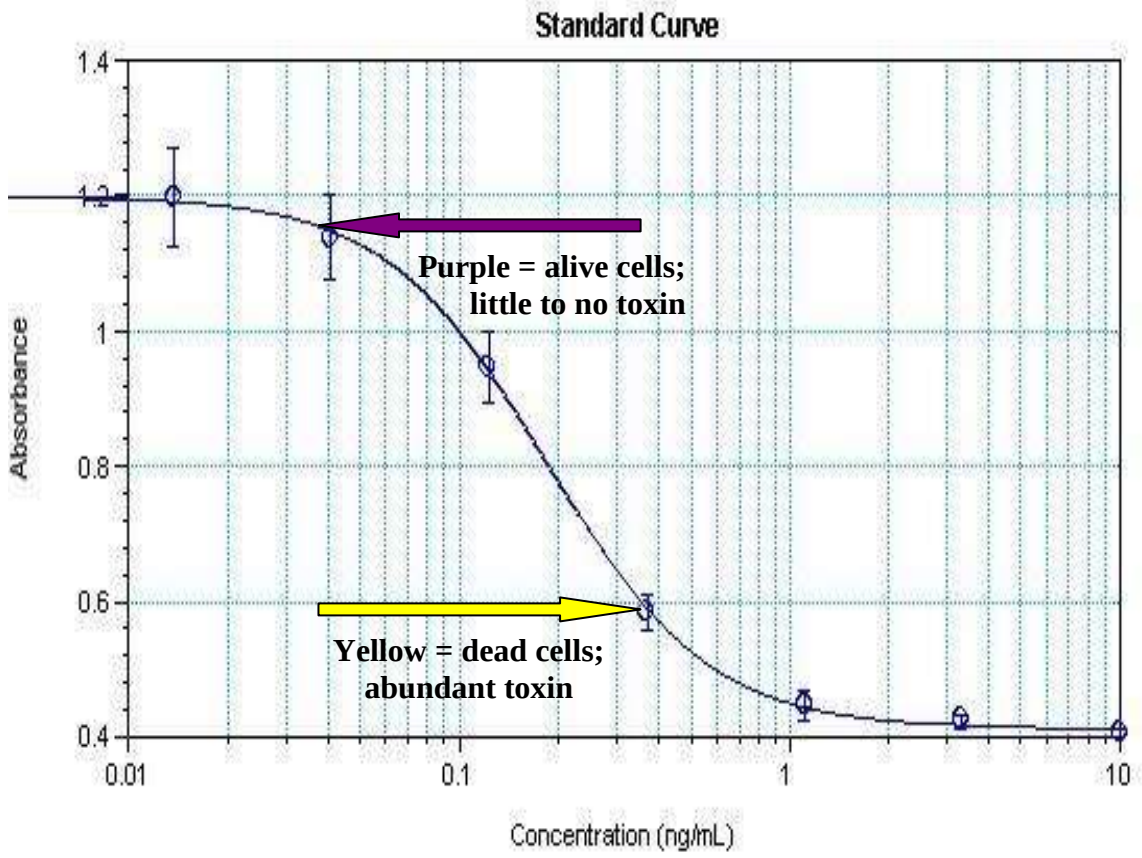

Figure 2-4. Example of Ricin Standard Curve from this Investigation

The amount of vaccinia virus persisting on test coupons over time was determined using a plaque assay approach. Following extraction, 1.0 mL of the PBS extract was removed and a series of dilutions up to 10^{-7} were prepared in sterile water. A 100-µL volume of the undiluted and each serial dilution was plated onto Vero (African Green Monkey kidney) cell monolayers, prepared as described above, and allowed to adsorb for one hour. Following inoculation and adsorption of virus to the Vero cells, 1.0 mL of 0.7% methylcellulose (containing fetal bovine serum and antibiotics) was added to each well of the six-well plate. Plates were incubated for 44-48 hours at 37 ± 2ºC under 95% air and 5% CO_2. The methylcellulose was removed and 2.0 mL of 0.13% crystal violet was added and cells incubated for 30 minutes. The crystal violet was removed, cells washed with PBS, and plaques visualized and counted.

Percent recovery for each biological agent on each test material, shown in Section 4, was determined prior to investigating persistence. Percent recovery (mean ± standard deviation [SD]) was calculated for each type of test material inoculated with each biological agent by dividing the mass of cytotoxic ricin extracted from control coupons or number of viable vaccinia PFU by the number of biological organisms or ricin mass applied. The quantity applied is determined during the confirmation of initial titer described in Section 2.5.

Percent recovery (% R) is calculated as:

Equation 2. $$\%R = \left(\frac{x}{A}\right) \times 100$$

where x is the number of viable vaccinia PFU or mass of cytotoxic ricin recovered from the control coupons, and A is the number of viable vaccinia virus or mass of cytotoxic ricin applied to the control coupons.

Persistence was the measurement of the amount of vaccinia virus (as PFUs) or cytotoxicity of ricin recovered from each coupon at each time point. Attenuation of the biological agent was calculated as the log reduction (mean ± SD) in viable vaccinia virus or percent reduction in cytotoxic ricin over the observed time course for each testing scenario. The higher the attenuation value, the less persistent is the biological agent on the test material with a given treatment. Attenuation was calculated for the *j*th test coupon within the *i*th combination of treatment factors, e.g., high RH on Day 3 of treatment. For vaccinia virus, attenuation was calculated as a log reduction according to the equation:

Equation 3. $$Attenuation_{ij} = \log_{10}\left(\bar{x}_0\right) - \log_{10}\left(x_{ij}\right)$$

where:

$\bar{x}_0$ = arithmetic mean of the number of viable virus extracted from positive control coupons;
x_{ij} = number of viable virus extracted from the *j*th replicate coupon of the *i*th treatment.

For ricin toxin, attenuation was calculated as a percent reduction according to the equation:

Equation 4. $$Attenuation_{ij} = \frac{x_{ij}}{\bar{x}_0} \times 100\% \quad .$$

where:

$\bar{x}_0$ = arithmetic mean of the mass (mg) of cytotoxic ricin extracted from positive control coupons;

x_{ij} = mass (mg) of cytotoxic ricin extracted from the *j*th replicate coupon of the *i*th treatment.

A total of five replicate coupons of each material type at each time point were analyzed for each agent. The attenuation data for each agent and type of material, calculated as shown in Equation 3 or 4, were fit to a one-way analysis of variance (ANOVA) model of form:

Equation 5. $$Attenuation_{ij} = \mu + t_i + \varepsilon_{ij}$$

where:

μ = overall mean attenuation;

t_i = average effect on the mean attenuation due to the *i*th treatment g11roup/day combination;

$\varepsilon_{i,j}$ = error terms for the *j*th replicate of the *i*th treatment group; the errors are assumed to be normally distributed.

After fitting each model, residuals were examined to assess normality and the adequacy of assuming a common variance. These model diagnostics supported the appropriateness of the statistical models. The modeling and analysis were carried out with PROC Mixed in SAS v9.1.

A mean and range of the attenuation values was determined for each material, agent, and environmental condition combination at each time period. Persistence curves were developed by graphing reduction in viable organisms (PFU) or toxin mass over time. Persistence curves were developed for the two controlled temperature and RH conditions and for ambient conditions for each agent and material.

Statistical analysis consisted of performing a set of pre-planned comparisons. These included:

- Comparing whether the attenuation at a particular RH and day was statistically significantly different from zero
- For the high and low RH treatments, comparing the estimated mean attenuation on each day to the corresponding attenuation estimate for the ambient RH treatment to determine if there was a statistically significant difference between RH treatments within each day.

The overall error rate for the set of comparisons was controlled to be no more than 5% for each material and agent.

For the evaluation of ricin on galvanized metal, there are separate comparisons of ambient to zero for each run. Also, the comparison of high and low RH to ambient can only be done within each run so that the high RH to ambient comparison is based on the results of the ambient treatment coupons tested in

the same run as the high RH coupons and the low RH to ambient comparison is based on the results of the other set of ambient treatment coupons tested in the same run as the low RH coupons.

In some of the treatment groups for vaccinia, no virus was observed on any of the replicate coupons. Since a zero value for a virus count leads to an undefined attenuation value, these treatment groups and their data were excluded from the statistical model. However, it was still possible to report attenuation for these treatment groups based on the following observations:

- A conservative estimate of the mean attenuation can be formed by replacing the value of zero PFUs for each coupon with the value one.
- A discrete distribution analysis indicated the average true number of virus PFUs in the five separate coupon extracts is less than 5% likely to be over 10 PFUs. Therefore, a lower 95% bound on attenuation was determined for these treatments by replacing the observed number of PFUs for the coupons (i.e., zero) with the value 10.

2.8 Determination of Ricin Renaturation

Ricin, a protein, is known to denature at high temperatures. Denaturation is associated with a loss of biological activity. It is less certain whether ricin can renature and, thereby, regain biological activity when returned to ambient temperature and RH. If bioactivity loss is observed under test, but not under ambient conditions, an additional test would be run to evaluate the potential for ricin renaturation.

For the investigation of potential renaturation, ricin would be applied to test coupons as described in Section B2.5. These coupons would be incubated at 30ºC and controlled RH for a period of time shown to result in a loss of cytotoxicity. The coupons would then be returned to ambient temperature and RH for 24 hours. After the ambient 24-hour incubation, attenuation would be determined as described in Section 2.6. A decline in attenuation would suggest that renaturation may have occurred.

3.0 Quality Assurance/Quality Control

QA/quality control (QC) procedures were performed in accordance with the program QMP and the test/QA plan)[2] for this investigation. QA/QC procedures are summarized below.

3.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, biological safety cabinets) used at the time of investigation was verified as being certified, calibrated, or validated.

3.2 Audits

3.2.1 Performance Evaluation Audit

No performance evaluation audit was performed for biological agents and surrogates because quantitative standards for these biological materials do not exist. The confirmation procedure, controls, blanks, and method validation efforts support the biological evaluation results.

3.2.2 Technical Systems Audit

Battelle QA staff conducted a technical systems audit (TSA) on June 9, 2005 to ensure the investigation was being conducted in accordance with the test/QA plan[2] and the QMP.)[3] As part of the TSA, test procedures were compared to those specified in the test/QA plan; and data acquisition and handling procedures were reviewed. Observations and findings from the TSA were documented and submitted to the Battelle Task Order Leader for response. None of the findings of the TSA required corrective action. TSA records were permanently stored with the TTEP QA Manager.

3.2.3 Data Quality Audit

At least 10% of the data acquired during the investigation were audited. A Battelle 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.

3.3 QA/QC Reporting

Each audit was documented in accordance with the QMP.[3]. The results of the TSA were submitted to the EPA.

One deviation was documented, in compliance with the QMP,[3] , and reported to the EPA Task Order Project Officer. The test/QA plan specified that dilutions through 10^{-7} would be analyzed. In some cases, dilutions outside of the useful range, based on expected concentrations of biological agent, were not run. This saved time and money without reducing useful data.

3.4 Data Review

Records and data generated in the investigation received a QC technical review and a QA review before they were used to calculate, or report results. All data were recorded by Battelle staff. The person performing the review was involved in the experiments and added his/her initials and the date to a hard copy of the record being reviewed. This hard copy was returned to the Battelle staff member who stored the record.

4.0 Test Results

Persistence of a biological agent exposed to known environmental conditions can be gauged by measuring the reduction in the cytotoxicity or viable organisms extracted from inoculated coupons over time. Attenuation is the reduction in amount of cytotoxicity or viable organisms extracted from coupons after a treatment (environmental conditions) compared to extractions of inoculated coupons (held at ambient conditions) one hour after inoculating the coupons. Thus, high attenuation indicates low persistence. The test results presented here show the impact of temperature and RH conditions on the persistence over time of ricin toxin or vaccinia virus extracted from inoculated galvanized metal or painted concrete. The environmental conditions investigated were ambient (~20^{o}C, 40% – 70% RH, high RH (30^{o}C, >70% RH), and low RH (30^{o}C, <40% RH). The high and low RH conditions also were investigated to assess the impact of higher temperature on persistence (or attenuation). The temperature and RH throughout the trial runs are reported in Appendix A.

4.1 Recovery of Ricin and Impact of Environmental Conditions on Persistence of Ricin

A 25-µg amount of total protein in the ricin stock solution was inoculated onto the coupons of indoor building materials and allowed to dry for one hour under ambient conditions. The ricin-inoculated coupons were then placed into controlled environmental conditions. After the one hour drying time and at specified intervals, ricin was extracted from the coupons and the bioactivity was measured using a cytotoxicity assay. The ricin bioactivity was reported as cytotoxicity in micrograms protein equivalent to the cytotoxicity of a given mass of protein in the ricin stock solution.

Table 4-1 summarizes the extraction efficiency from different test material coupons by calculating the recovery of cytotoxic ricin extracted after one hour drying time (T0 positive control coupons) as a percentage of the cytotoxic ricin applied (measured in the application control). The test matrix for ricin (see Table 2-1) was conducted using two experimental trials (or runs). In Run 1, ambient and high RH conditions were investigated. In Run 2, ambient and low RH conditions were run. Measurements at different times (replicates) are referenced as different runs (Run 1 or Run 2).

The recovery for ricin is generally high, although the results for galvanized metal in the first run were substantially lower than all other recovery averages. The variability in galvanized metal results between Runs 1 and 2 may be attributed to different RH conditions in the BSC II during the initial one hour of drying on the different days. There was high RH (~70%) experienced during drying in the BSC II on Day 0 of the first run. The RH condition during drying on the day of the second run was approximately 40-50%. High RH, as shown in Table 4-2 below, results in about a 78% attenuation of ricin on galvanized metal in the first day subsequent to drying. The lower recovery after high RH drying conditions in the BSC II may result from higher rates of loss occurring during the one hour drying time when RH during drying is moderate to low.

Table 4-1. Ricin Extraction Efficiencies

Material	Run	Observations (n)	Recovery (%)	
			Mean	SD
Galvanized metal	1	8	24.34	7.34
	2	7	79.10	18.32
Painted concrete	1	7	98.82	25.81
	2	8	81.84	10.16

4.1.1 Ricin on Galvanized Metal

Table 4-2 and Figure 4-1 summarize the cytotoxicity of ricin extracted from galvanized metal under the specified temperature and RH conditions. The average (and corresponding joint 95% intervals from the statistical model) of the replicate (n) positive control samples are reported under "Mean Ricin Cytotoxicity (μg) after Drying". The values are reported as the average (in units of μg) of cytotoxicity recovered from the replicate coupons at the specific time point indicated; the numbers in parentheses are the confidence interval endpoints for the averages as determined from the statistical model fit to the data. The confidence intervals are individually of 99.8% confidence to control the chance of error at 5% for the total number of statistical comparisons made. Cytotoxic ricin extracted from galvanized metal declined over the 14 day period at all RH and temperature conditions investigated. However, there were significant differences between the ricin from coupons maintained in the low RH condition compared to those at the ambient or high RH conditions. Under the low RH condition, recovery on Day 14 was 14% of the amount recovered on positive control coupons after drying. In ambient or high RH conditions, the cytotoxic ricin recovered on Day 14 was <0.3% of the amount recovered on positive control coupons after drying. A blank coupon was analyzed for each material at each time point and little cytotoxicity was measured (cytotoxicity equal to 0.001 – 0.009 μg of cytotoxic ricin was observed for galvanized metal).

The amount of ricin remaining on the coupons in Run 1 maintained at ambient conditions was significantly lower on Day 1 than the amount of ricin remaining on coupons on Day 1 in Run 2 maintained at ambient conditions. This unexpected difference is attributed to the difference in RH during the drying time. Specifically, after inoculation, coupons remained undisturbed at the ambient conditions in the BSC II for one hour ("drying time") before being transferred into controlled temperature and RH conditions. In the test/QA plan the RH during the drying time was not specified and controlled. The drying was to be done under ambient conditions. The RH at ambient conditions during drying on different days could be different. The ambient RH during the drying time was observed qualitatively to have been higher on the day of Run 1 than on the day of Run 2. This difference in RH during the drying time may have impacted ricin persistence on galvanized metal. The direction of change (higher RH is associated with increased attenuation of bioactive ricin) is consistent with our other observations.

Table 4-2. Mean Recovery of Ricin Cytotoxicity on Galvanized Metal[a]

Run	Mean Ricin Cytotoxicity (µg) after Drying[b]	Treatment	Mean Ricin Cytotoxicity (µg) after Treatment			
			Day 1	Day 3	Day 9	Day 14
1	6.086[c] (4.04,8.132) n=8	**Ambient RH** (20°C, RH 40%-70%)	1.672 (0,5.013) n=3	0.572 (0,3.913) n=3	0.007 (0,3.348) n=3	0.005 (0,3.346) n=3
1		**High RH** (30°C, RH >70%)	1.344 (0,3.932) n=5	1.963 (0,4.551) n=5	0.376 (0,2.963) n=5	0.014 (0,2.602) n=5
2	19.776 (17.589,21.963) n=7	**Ambient RH** (20°C, RH 40% - 70%)	7.615 (3.523,11.706) n=2	0.633 (0,4.725) n=2	0.006 (0,4.098) n=2	0.013 (0,4.105) n=2
2		**Low RH** (30°C, RH < 40%)	8.996 (6.408,11.584) n=5	4.750 (2.162,7.338) n=5	4.865 (2.277,7.453) n=5	2.802 (0.214,5.39) n=5

[a] Initial inoculation of 25 µg protein
[b] Ambient BSC II conditions for one hour
[c] High ambient RH conditions (~70%) were qualitatively observed during the one hour drying time before the coupons were placed into controlled conditions

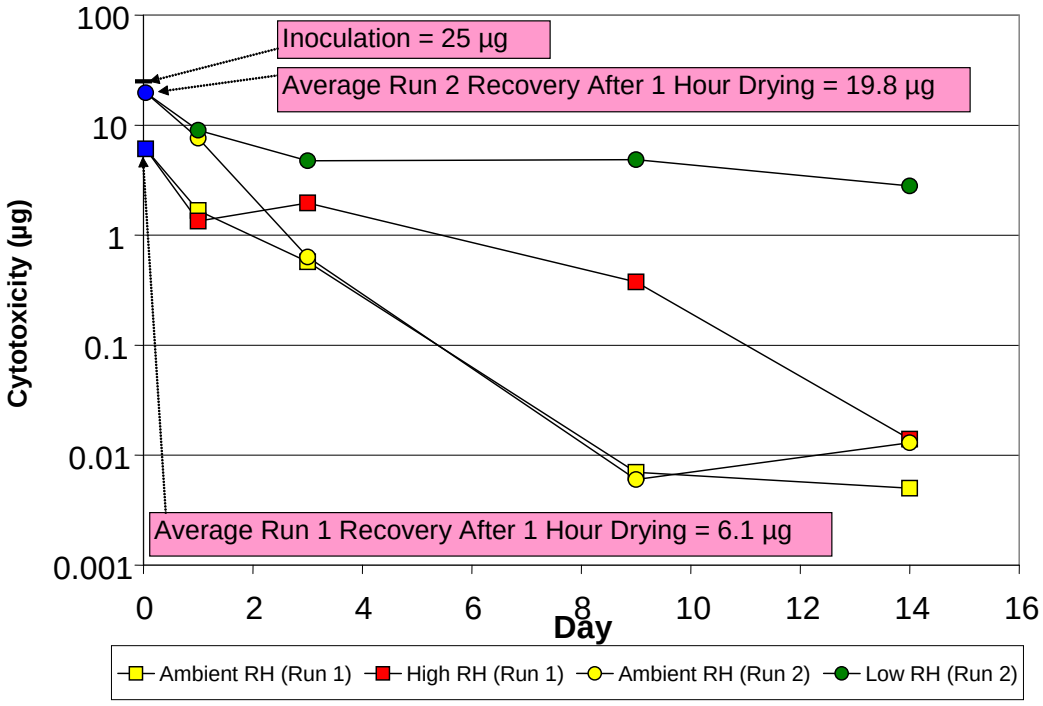

Figure 4-1. Persistence of Ricin on Galvanized Metal

Table 4-3 and Figure 4-2 show that at ambient to high RH condition, the decrease in cytotoxic ricin extracted from galvanized metal after Day 14 (compared to the ricin extracted after one hour drying) corresponds to a 99.77 to 99.93% reduction. In contrast, cytotoxic ricin exhibited less attenuation (85.83% reduction on Day 14) at the low RH condition. Because ricin cytotoxicity was lost at ambient conditions, no renaturation testing was performed.

Table 4-3. Mean Attenuation: Ricin on Galvanized Metal[a] (Shown as Percent Reduction)

		Mean Attenuation of Ricin Cytotoxicity (% Reduction)			
Run	**Treatment**	**Day 1**	**Day 3**	**Day 9**	**Day 14**
1	**Ambient RH (20°C, RH 40 - 70%)**	72.53 [b]	90.59 [b]	99.88 [b]	99.92 [b]
1	**High RH (30°C, RH >70%)**	77.91 [b]	67.75 [b]	93.83 [b]	99.77 [b]
2	**Ambient RH (20°C, RH 40 - 70%)**	61.50 [b]	96.80 [b]	99.97 [b]	99.93 [b]
2	**Low RH (30°C, RH < 40%)**	54.51 [b]	75.98 [b]	75.40 [bc]	85.83 [b]

[a] Initial inoculation of 25 µg protein; percent reduction was calculated from a basis of Day 0 (after one hour drying time)
[b] Attenuation significantly different from zero ($P \leq 0.0021$)
[c] Attenuation significantly different from ambient within same day and run ($P \leq 0.0021$)

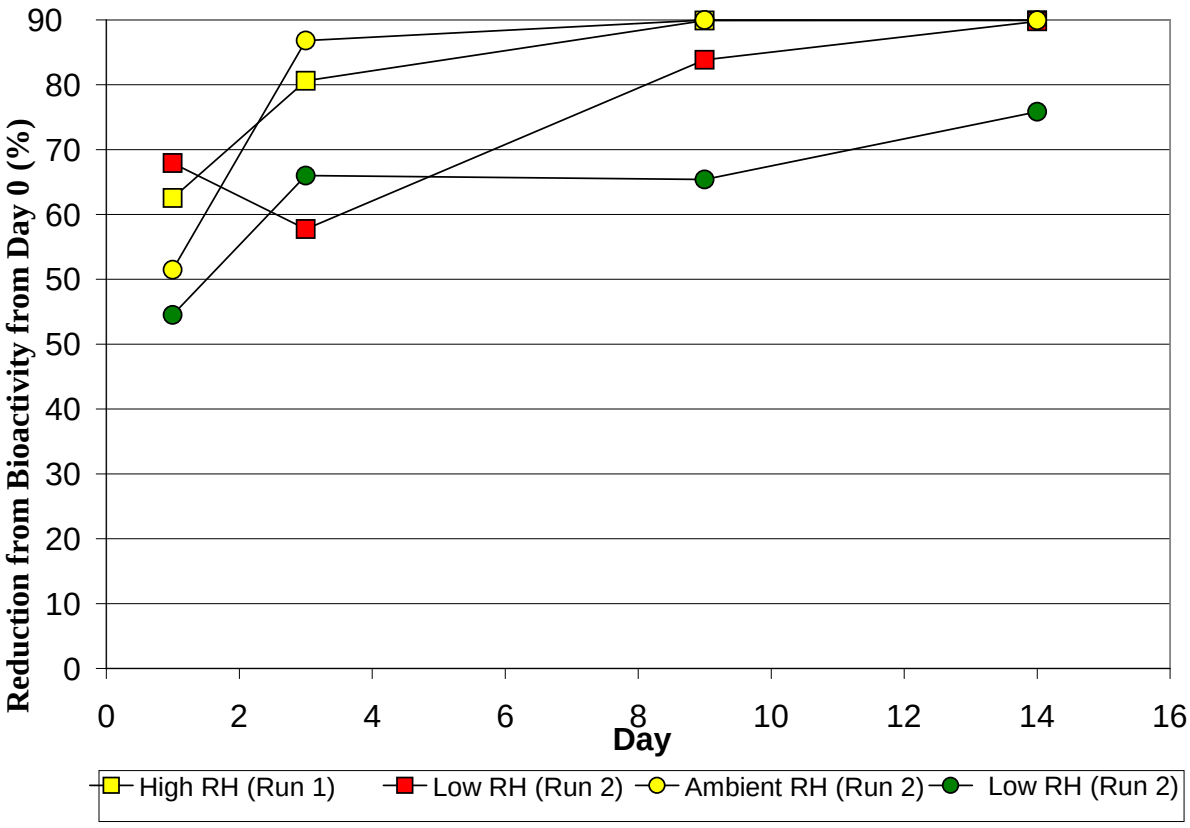

Figure 4-2. Mean Attenuation (Percent Reduction): Ricin on Galvanized Metal

4.1.2 Ricin on Painted Concrete

Table 4-4 and Figure 4-3 summarize the extractable cytotoxic ricin from painted concrete at the three treatment conditions. As for the galvanized metal, the values in the table and reported in the figure are averages (in units of µg) of cytotoxity recovered from the replicate coupons at the specific time point indicated; the numbers in parentheses in Table 4-4 are the statistical model based confidence intervals (joint 95% confidence for all intervals) of the replicates. After one hour drying there were no significant differences between extractable ricin in Run 1 and Run 2; therefore, these data were combined and are shown as results after one hour in Table 4-4. There was no significant loss of ricin (compared to ricin extracted after one hour drying) after Day 14 at ambient (20ºC) or low RH at 30ºC. However, there is a statistically significant reduction in extractable ricin from painted concrete incubated at high RH and 30ºC on Days 9 and 14.

A blank coupon was analyzed for each material at each time point and little cytotoxicity was measured (0.005 – 0.011 µg protein cytotoxicity for painted concrete).

Table 4-4. Mean Recovery of Ricin Cytotoxicity on Painted Concrete[a]

Run	Mean Ricin Cytotoxicity (µg) after Drying[b]	Treatment	Mean Ricin Cytotoxicity (µg) after Treatment			
			Day 1	Day 3	Day 9	Day 14
1, 2		**Ambient RH** (20°C, RH 40%-70%)	15.337 (9.898,20.776) n=5	16.033 (10.594,21.472) n=5	18.398 (12.959,23.837) n=5	19.017 (13.578,24.456) n=5
1	22.441 (19.301,25.582) n=15	**High RH** (30°C, RH >70%)	16.093 (10.654,21.532) n=5	19.264 (13.825,24.703) n=5	10.008 (4.569,15.447) n=5	6.339 (0.9,11.778) n=5
2		**Low RH** (30°C, RH <40%)	13.871 (8.432,19.310) n=5	17.950 (12.511,23.389) n=5	16.538 (11.099,21.977) n=5	16.375 (10.936,21.814) n=5

[a] Initial inoculation of 25 µg protein
[b] Ambient BSC II conditions for one hour

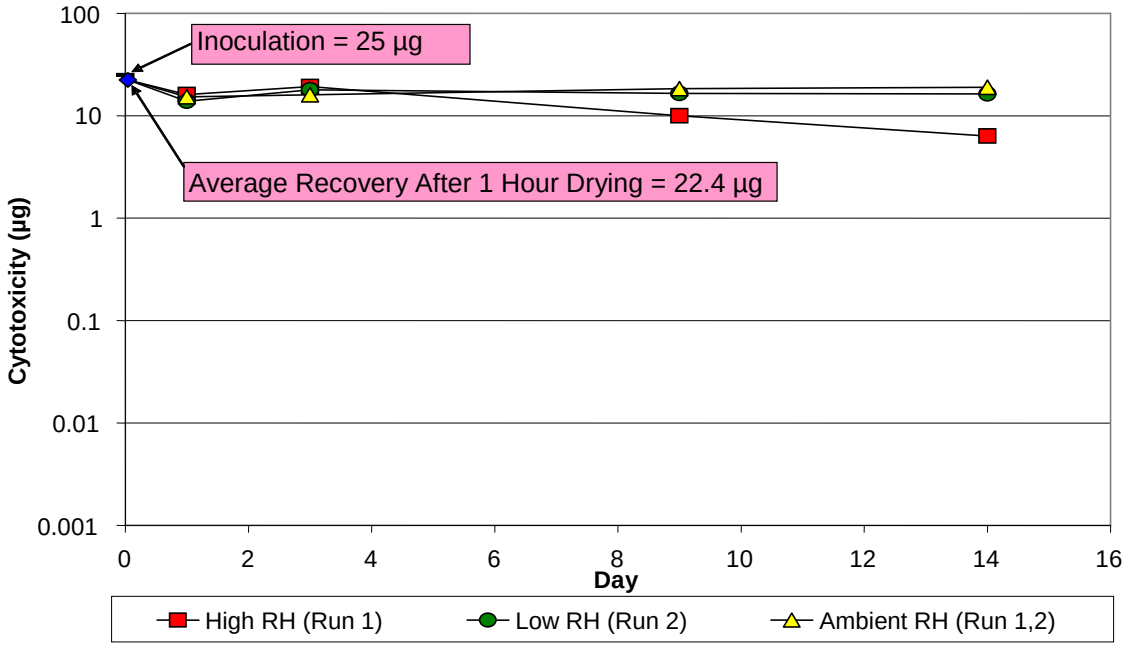

Figure 4-3. Persistence of Ricin on Painted Concrete

The percent reduction values for ricin extracted from painted concrete are shown in Table 4-5 and Figure 4-4. On Day 1, small but significant decreases in ricin extracted from painted concrete were observed for the ambient RH (31.7%), the high RH (28.3%) and the low RH (38.2%) conditions; on Day 3, the average extracted ricin continued to be less than that compared to the ricin extracted after one hour drying, but only the ambient RH reduction (28.6%) was statistically significant. For Days 9 and 14, the reduction at high RH increased to 55.4 and 71.8%, respectively; significantly higher than the baseline condition as well as significantly higher than the corresponding ambient RH reductions (18.0% at Day 9 and 15.3% at Day 14). The reductions at ambient RH and low RH on Days 9 and 14 were not statistically significant when compared to the ricin extracted after one hour drying. The statistical significance is determined from the ANOVA model fit to the extracted cytotoxicity data. A Bonferroni adjustment was employed to control the overall error rate for all statistical comparisons at 5%. With 20 total comparisons made for the ricin on painted concrete; this required that each comparison individually have a p-value of 0.05 divided by 20, or 0.0025, to be identified as statistically significant. There is no more than a 5% chance that the set of statistically significant comparisons contains an erroneous conclusion of significance by chance.

Table 4-5. Mean Attenuation: Ricin on Painted Concrete[a] (Shown as Percent Reduction)

		Mean Attenuation of Ricin Cytotoxicity (% Reduction)			
Run	**Treatment**	**Day 1**	**Day 3**	**Day 9**	**Day 14**
1, 2	**Ambient RH (20°C, RH 40 - 70%)**	31.66 [b]	28.56 [b]	18.02	15.26
1	**High RH (30°C, RH >70%)**	28.29 [b]	14.16	55.40 [bc]	71.75 [bc]
	Low RH (30°C, RH <40%)	38.19 [b]	20.02	26.31	27.03

[a] Initial inoculation of 25 µg protein; percent reduction was calculated from a basis of Day 0 (after one hour drying time)
[b] Attenuation significantly different from zero ($P \leq 0.0025$)

[c] Attenuation significantly different from ambient within this day ($P \leq 0.0025$)

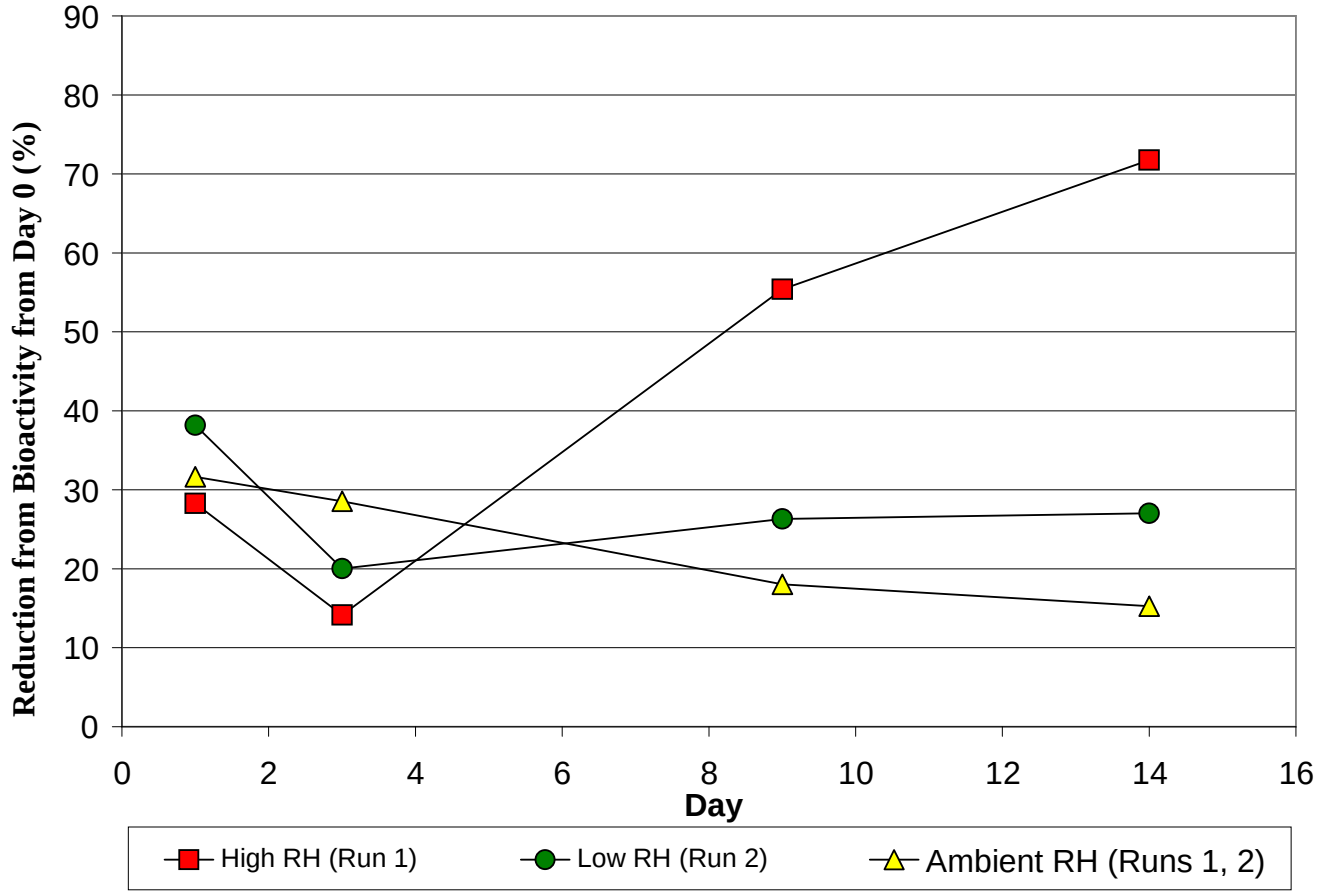

Figure 4-4. Mean Attenuation: Ricin on Painted Concrete

The attenuation results for ricin on painted concrete indicate that the impact of temperature and RH are much less than observed for galvanized metal. The Day 1 attenuation results for all conditions, as well as the results for Ambient RH at Day 3 are significantly greater than zero, but the Ambient RH and low RH attenuation falls to below significant levels at Days 9 and 14. By contrast, the high RH attenuation increases through Days 9 and 14, but still exhibits lower final attenuation than was seen on galvanized metal. Generally, cytotoxic ricin was persistent on painted concrete throughout the two week test period regardless of the treatment. Since the cytotoxicity of ricin remained high, no testing for renaturation was performed.

4.2 Recovery of Vaccinia and Impact of Environmental Conditions on Persistence of Vaccinia

Approximately 7 x 10^7 PFU vaccinia virus (vaccinia) were inoculated onto each test coupon of the indoor building materials and allowed to dry for one hour under ambient BSC II conditions. The vaccinia-inoculated coupons were then placed into the test chambers with controlled temperature and RH. After the one hour drying time and at specified intervals, vaccinia was extracted from the coupons, inoculated onto Vero (African Green Monkey kidney) cell monolayers, and quantified using a standard

plaque assay. A blank coupon was analyzed for each material at each time point. No viable vaccinia were detected on any of the galvanized metal or painted concrete blank coupons.

Table 4-6 summarizes the extraction efficiency from different test material coupons by calculating the recovery of number of viable virus extracted after one hour drying time (T0 positive control coupons) as a percentage of the viable virus applied (measured in the application control). Measurements at different times (replicates) are referenced as different runs (Run 1 or Run 2).

Less than 20% of the number of vaccinia inoculated onto the coupon were extracted and viable after one hour drying. The recovery percentages for vaccinia are low for galvanized metal but are consistent between the two runs. The percentages for painted concrete are higher than for galvanized metal but still are low on an absolute basis. To demonstrate this low recovery was not due to poor extraction, vaccinia was inoculated onto the coupons and immediately extracted. Recovery with immediate extraction of these samples ranged from 90-100% (data not shown). The low levels of viable virus after one hour drying are consistent with the continuing loss of viability observed in subsequent time periods at ambient or high RH. Under high RH or ambient conditions, viable vaccinia exhibited a 3 log reduction in the first day (see discussion below).

Table 4-6. Vaccinia Extraction Efficiency

Material	Run	Observations	% Recovery	SD
Galvanized metal	1	7	4.52	2.40
	2	8	6.65	1.89
Painted concrete	1	8	10.64	0.83
	2	7	19.19	3.93

4.2.1 Vaccinia on Galvanized Metal

Table 4-7 and Figure 4-5 summarize the results for vaccinia recovered from galvanized metal maintained under the test temperature and RH conditions. The data are reported as mean PFUs recovered from the five replicate coupons (n = 5) at the specified time points; the SDs are reported in parentheses. At ambient and high RH conditions no viable virus was extracted from any of the five replicate coupons at Days 3, 9, and 14. At low RH, a comparatively high level of extractable virus ($>10^5$ PFU) was recovered from on the coupons after Day 14. The temperature and RH data for these runs are reported in Appendix A.

Table 4-7. Mean Vaccinia Recovery from Galvanized Metal[a]

Run	Mean Vaccinia Recovery (PFU) after Drying[b]	Treatment	Mean Vaccinia Recovery (PFU) after Treatment			
			Day 1	Day 3	Day 9	Day 14
1, 2		**Ambient RH** (20°C, RH 40 - 70%)	2.27E+03 (1.75E+03) n=5	0.00E+00[c] (0.00E+00) n=5	0.00E+00[c] (0.00E+00) n=5	0.00E+00[c] (0.00E+00) n=5
1	3.548E+06 (1.767E+06) n=15	**High RH** (30°C, RH >70%)	4.06E+04 (6.39E+04) n=5	0.00E+00[c] (0.00E+00) n=5	0.00E+00[c] (0.00E+00) n=5	0.00E+00[c] (0.00E+00) n=5
2		**Low RH** (30°C, RH <40%)	1.52E+06 (6.60E+05) n=5	8.67E+05 (3.00E+05) n=5	4.81E+05 (6.97E+04) n=5	5.10E+05 (1.29E+05) n=5

[a] Average initial inoculation of 6.76E+07
[b] Ambient BSC II conditions for one hour
[c] Zero organisms observed in extracts from all coupons

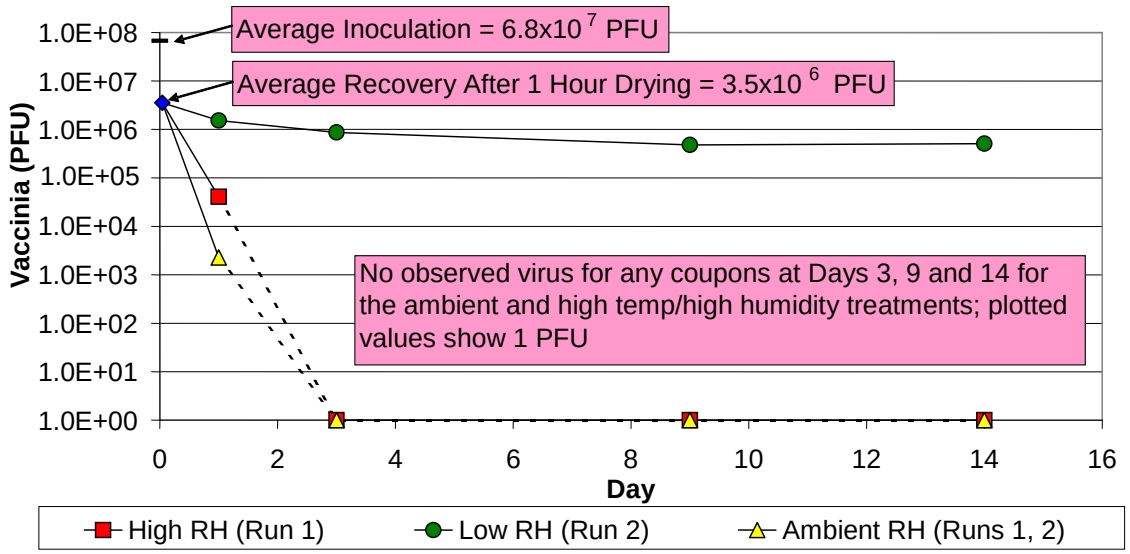

Figure 4-5. Persistence of Vaccinia on Galvanized Metal

The data in Table 4-8 and Figure 4-6 indicate that in ambient to high RH conditions the decrease in viable vaccinia extracted from galvanized metal at and after three days corresponds to a minimum 6.6 log reduction (conservative estimate based on replacing observation of no viable vaccinia PFU observed with a value of one vaccinia PFU). In contrast, vaccinia exhibits a much higher persistence (<1 log reduction at Day 14) at low RH conditions.

Table 4-8. Mean Attenuation: Vaccinia on Galvanized Metal[a] (Shown as Log Reduction)

Run	Treatment	Mean Attenuation after Treatment			
		Day 1	Day 3	Day 9	Day 14
1, 2	**Ambient RH** (20°C, RH 40 - 70%)	3.323[b]	>6.550[d]	>6.550[d]	>6.550[d]
1	**High RH** (30°C, RH >70%)	2.548[bc]	>6.550[d]	>6.550[d]	>6.550[d]
2	**Low RH** (30°C, RH <40%)	0.393[c]	0.635[b]	0.872[b]	0.857[b]

[a] Average initial inoculation of 6.76E+07; log reduction was calculated from a basis of Day 0 (after one hour drying time)
[b] Attenuation significantly different from zero ($P \leq 0.05$)
[c] Attenuation significantly different from ambient within this day ($P \leq 0.05$)
[d] Attenuation not calculable because all 5 replicate coupons had zero observed virus PFUs; minimum attenuation is estimated by assuming one organism observed; 95% confidence that attenuation is >5.550 (i.e., less than or equal to 10 PFU in coupon extract) and hence it is statistically significantly different from zero

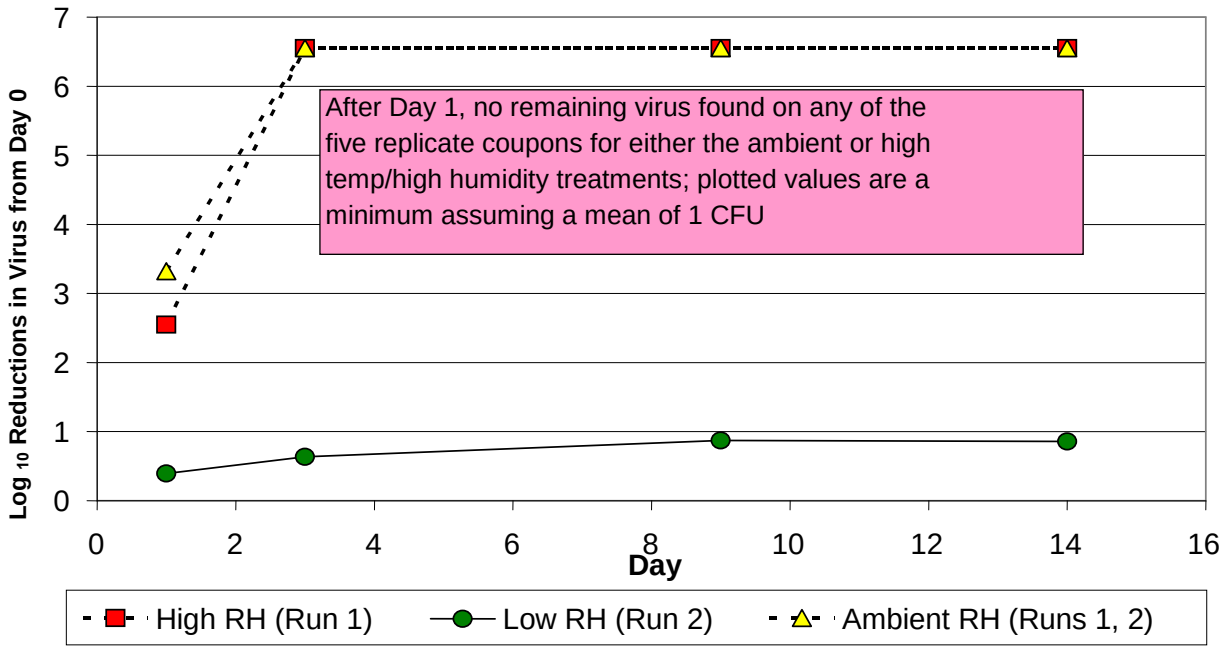

Figure 4-6. Mean Attenuation: Vaccinia on Galvanized Metal

The attenuation in the persistence of vaccinia on galvanized metal was significantly greater than zero at Day 1 for both the ambient and high temperature/high RH treatments. On Days 3, 9, and 14, no remaining viable virus (0 PFU) was found for either of these treatments. Because only a sample of the extract was tested for viable virus, the observed mean value of 0 PFU from every coupon was determined to indicate (at 95% confidence) at least a 5.5 log reduction of viable virus in the total extract. This suggests there may be a rapid decrease and complete loss of viable virus from galvanized metal upon exposure to ambient to high temperature and RH. Vaccinia persisted at low RH (at high

temperature), although the observed attenuation was statistically significantly than zero after Day 1. However, the mean attenuation at low RH was less than 1 log reduction at Day 14.

4.2.2 Vaccinia on Painted Concrete

Table 4-9 and Figure 4-7 summarize the results for vaccinia virus recovered from painted concrete under the test and control temperature and RH conditions. The extraction of vaccinia after one hour drying was replicated at times designated as Run 1 or Run 2. There were no significant differences in the recovery after the one hour drying period, as shown in Table 4-6, between these runs. Therefore, the overall average number of PFU is shown as the one hour mean value. Less than 15% of the PFU of vaccinia inoculated onto the coupons were extracted and viable after one hour drying. The low levels of viable virus after one hour drying are consistent with the continuing loss of viability that is observed in subsequent time periods at ambient or high RH. Under high RH conditions, a 3 log reduction in viable vaccinia was observed within the first day. To demonstrate this low recovery was not due to poor extraction, vaccinia was inoculated onto the coupons and immediately extracted. Recovery from these samples ranged from 90-100% (data not shown).

At low RH, $>10^6$ PFU of extractable virus was recovered from the coupons after Day 14. Approximately 10^3 PFU of viable vaccinia were extracted from coupons after being maintained at ambient conditions for Day 14. In contrast, at high temperature and RH no viable vaccinia was extracted from any of the five replicate coupons at Days 9 and 14. Thus, a trend in persistence with RH is evident.

Table 4-9. Mean Vaccinia Recovery from Painted Concrete[a]

Run	Mean Vaccinia Recovery (PFU) after Drying[b]	Treatment	Mean Vaccinia Recovery (PFU) after Treatment			
			Day 1	Day 3	Day 9	Day 14
1, 2		**Ambient RH** (20°C, RH 40 - 70%)	2.63E+06 (1.18E+06) n=5	6.00E+05 (5.28E+05) n=5	2.68E+04 (2.07E+04) n=5	1.712E+03 (9.092E+02) n=5
1	9.285E+06 (1.562E+06) n=15	**High RH** (30°C, RH >70%)	2.16E+03 (1.30E+03) n=5	2.40E+02 (1.67E+02) n=5	0.00E+00[c] (0.00E+00) n=5	0.00E+00[c] (0.00E+00) n=5
2		**Low RH** (30°C, RH <40%)	7.54E+06 (1.967E+06) n=5	6.27E+06 (2.24E+06) n=5	1.30E+06 (8.80E+05) n=5	3.19E+06 (1.35E+06) n=5

[a] Average initial inoculation of 7.103E+07
[b] Ambient BSC conditions for one hour
[c] Zero organisms observed in extracts from all coupons

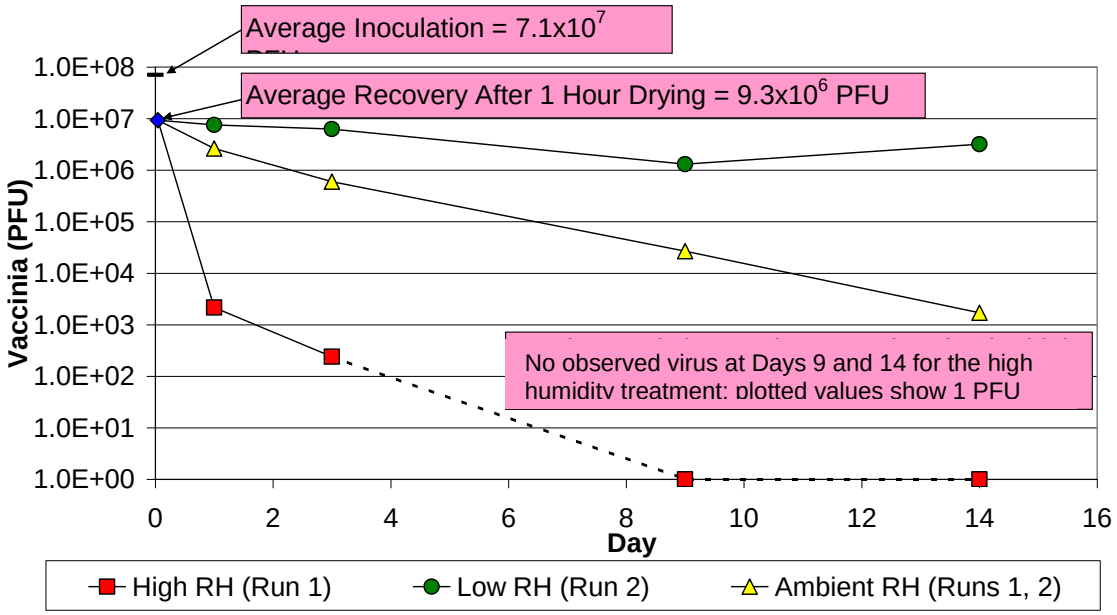

Figure 4-7. Persistence of Vaccinia on Painted Concrete

Table 4-10 and Figure 4-8 provide results for mean attenuation in the persistence of vaccinia virus on painted concrete at the three treatment conditions. Because only a sample of the extract was tested for viable virus, in cases where the observed mean value of 0 PFU was observed from every coupon, a lower 95% bound on attenuation was determined. At high temperature and RH conditions the decrease in viable vaccinia extracted from galvanized metal at Days 9 and 14 corresponds to a mean reduction of approximately 7 log (assumes 1 PFU rather than 0 actually observed) and a 95% confidence of at least a 6 log reduction. In contrast, at low RH conditions little viability of vaccinia is lost (~0.5 log reduction after Day 14).

Table 4-10. Mean Attenuation: Vaccinia on Painted Concrete[a] (Shown as Log Reduction)

Run	Treatment	Mean Attenuation after Treatment			
		Day 1	Day 3	Day 9	Day 14
1, 2	**Ambient RH** (20°C, RH 40 - 70%)	0.587[b]	1.360[b]	2.814[b]	3.828[b]
1	**High RH** (30°C, RH >70%)	3.836[bc]	4.736[bc]	> 6.968[d]	> 6.968[d]
2	**Low RH** (30°C, RH <40%)	0.103[e]	0.189[c]	0.909[bc]	0.504[bc]

[a] Average initial inoculation of 7.103E+07
[b] Attenuation significantly different from zero ($P \leq 0.05$)
[c] Attenuation significantly different from ambient within this day ($P \leq 0.05$)

[d] Attenuation not calculable because all five replicate coupons had zero observed virus PFUs; minimum log reduction is estimated by assuming one organism observed; 95% confidence that attenuation is >5.968 and hence it is statistically significantly different from zero

[e] Attenuation not significantly different from zero (P > 0.05)

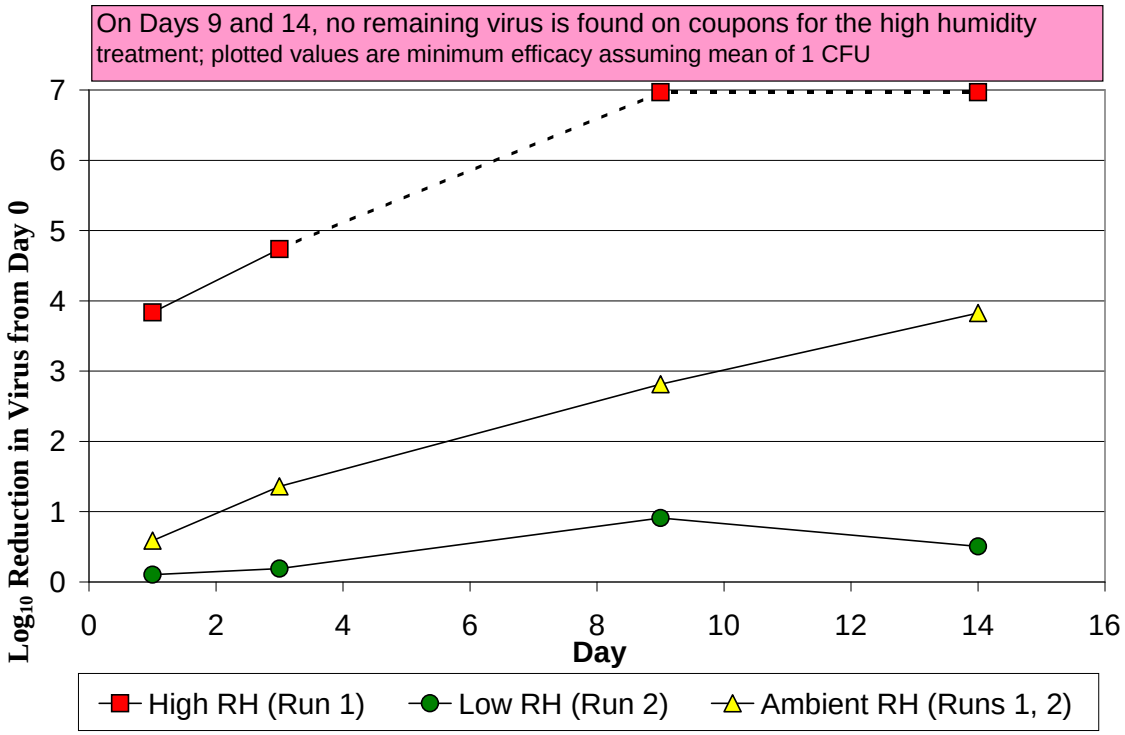

Figure 4-8. Mean Attenuation: Vaccinia on Painted Concrete

The attenuation in the persistence of viable vaccinia on painted concrete subjected to alternate temperature and RH conditions were similar to those for galvanized metal. The high temperature/high RH treatment promoted a decrease in viable vaccinia, with no extractable virus (PFU) observed at Days 9 or 14. At the ambient treatment conditions, significant reductions in vaccinia were observed increasing from Day 1; however, detectable quantity of viable virus was extracted at Day 14. Viable vaccinia was more persistent at low RH conditions with less than a 1 log attenuation throughout the test. At low RH, the attenuation at Days 9 and 14 were significantly lower than the ambient treatment.

4.2.3 Effect of Temperature on Persistence of Ricin and Vaccinia

This investigation did not show, but cannot rule out, modest effects from temperature differences that are masked by the large effects of RH.

5.0 Summary

The results show that RH, indoor material type, and time after application of the agent to the indoor material affect the persistence of cytotoxic ricin and viable vaccinia virus. The results summarized in Table 5-1 show that the RH affects the persistence of cytotoxic ricin and viable vaccinia virus. Both ricin toxin and viable vaccinia virus have greater persistence on galvanized metal and painted concrete for up to Day 14 under low RH conditions than high RH conditions.

Table 5-1. Ricin or Vaccinia Recovery from Different Materials under Varying RH Conditions at Constant Temperature (Temperature = 30°C)

Biological Agent or Surrogate	Building Material	RH	Amount Inoculated onto Coupons	Day 14 after Inoculation onto Coupons
Ricin toxin	Galvanized metal	>70%	25 µg cytotoxic protein	0.0 µg cytotoxic protein
		<40%	25 µg cytotoxic protein	2.8 µg cytotoxic protein
	Painted concrete	>70%	25 µg cytotoxic protein	6.3 µg cytotoxic protein
		<40%	25 µg cytotoxic protein	16.4 µg cytotoxic protein
Vaccinia virus	Galvanized metal	>70%	7×10^7 PFU	0 PFU
		<40%	7×10^7 PFU	5×10^5 PFU
	Painted concrete	>70%	7×10^7 PFU	0 PFU
		<40%	7×10^7 PFU	3×10^6 PFU

The results also suggest that the rate of loss of cytotoxic ricin and viable vaccinia virus (or, stated differently, persistence at a given point in time) depends on the type of material onto which the biological agent is applied. Table 5-2, for example, shows that under ambient conditions residual amounts of both ricin and vaccinia measured on Day 3 are very different for the different building materials.

Table 5-2. Day 3 Ricin or Vaccinia Recovery from Different Materials, Ambient Conditions

Biological Agent or Surrogate	Building Material	Day 3 after Inoculation onto Coupons
Ricin toxin	Galvanized metal	0.6 µg cytotoxic protein
	Painted concrete	16.0 µg cytotoxic protein
Vaccinia virus	Galvanized metal	0 PFU
	Painted concrete	6×10^5 PFU

Table 5-3 shows that, depending on the type of indoor material, elevated RH may be useful for lowering the cytotoxicity of ricin before the application of a decontamination technology. Ricin is most persistent, for both galvanized metal and painted concrete, at low RH conditions. Ricin was persistent on painted concrete retaining substantial extractable cytotoxicity after Day 14, even at high RH. For ricin on galvanized metal, little or no ricin cytotoxicity (above the background coupon cytotoxicity) was observed by Day 14 at high RH.

Elevated RH may be useful for inactivating vaccinia virus before the application of a decontamination technology. Vaccinia virus persisted on painted concrete or galvanized metal at Day 14 at low RH. However at high RH, no viable vaccinia virus was extracted on Day 3 or after from galvanized metal, or on Day 9 or 14 from painted concrete. Based on the results of this investigation, it is possible that elevated RH may have a comparable impact on the viability of *Variola* or other pox viruses on metal and painted concrete substrates.

Table 5-3. Mean Attenuation: Ricin or Vaccinia on Different Building Materials under Varying RH Conditions

Bioagent	Material	Treatment Providing Highest Attenuation	Day 14 Mean Reduction
Ricin	Galvanized metal	Ambient (20°C) or high RH (30°C)	99.9% (ambient RH), 99.8% (high RH)
Ricin	Painted concrete	High RH (30°C)	71.8%
Vaccinia	Galvanized metal	Ambient (20°C) or high RH (30°C)	>6.5 (no viable virus)
Vaccinia	Painted concrete	High RH (30°C)	>6.9 (no viable virus)

Because substrates and environmental conditions investigated here show differential impact on the cytotoxicity of the ricin or viability of the vaccinia, it is clear that these factors must be considered and controlled in evaluation of decontamination technologies (e.g., for control conditions).

This investigation did not show, but cannot rule out, modest effects on persistence from temperature differences that could be masked by the large effects of RH.

No results are presented for *C. burnetii.* Although *C. burnetii* were observed microscopically to be present in the Vero (African Green Monkey) kidney cells used for the plaque assay, plaques were not observed after inoculating either Vero kidney cell monolayer or a mouse fibroblast monolayer and incubation under appropriate culture conditions for ten days. Therefore, the quantitative impact of environmental conditions on *C. burnetii* persistence and the attenuation of controlling RH to kill *C. burnetii* could not be determined.

6.0 References

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Appendix A

Report of Temperature and RH Measurement Data

The temperature and RH for the each of the test chambers used in all four trial runs are reported in this appendix. For each trial run, two test chambers were used; one chamber for the ambient treatment and one for the alternate condition. A total of two runs were made for each of the two agents investigated. In Run 1 for each agent, the ambient (~20°C, 40%-70% RH) and high RH (30°C, >70% RH) conditions were investigated. In the second runs, the ambient (~20°C, 40%-70% RH) and low RH (30°C, <40% RH) treatments were performed. The temperature and RH data for each chamber in each of the four runs are presented graphically below for the entire 14 day run durations.

Ricin Run 1:
For the ambient condition, the mean temperature and RH were 21.2°C and 59.7%, respectively. The SDs were 0.6 and 0.7, respectively. The ranges were 18.2°C-22.1°C and 55.4%-61.3% RH. For the High RH condition, the mean temperature and RH were 31.7°C and 89.4%, respectively. The SDs were 0.6 and 7.9, respectively. The ranges were 27.4°C-33.4°C and 59.0%-99.6% RH.

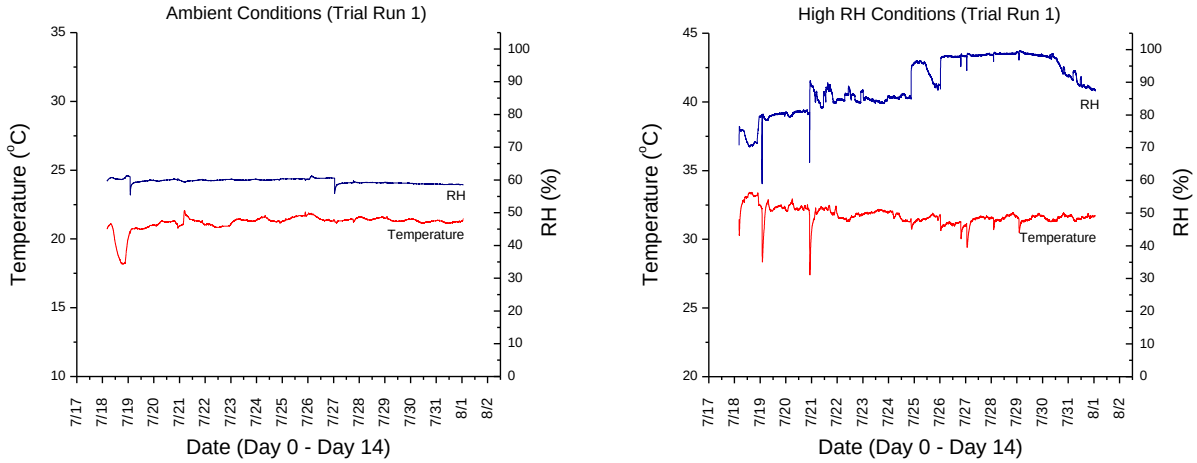

Figure A-1: Temperature and RH Measurements for Ricin Trial 1 Test Chambers: Ambient (Left) and High RH (Right) Conditions

Ricin Run 2:
For the ambient condition, the mean temperature and RH were 21.1°C and 54.7%, respectively. The SDs were 0.4 and 1.1, respectively. The ranges were 20.4°C-24.2°C and 51.2%-57.5% RH. For the Low RH condition, the mean temperature and RH were 32.7°C and 11.8%, respectively. The SDs were 1.2 and 3.7, respectively. The ranges were 21.0°C-34.3°C and 10.0%-66.9% RH.

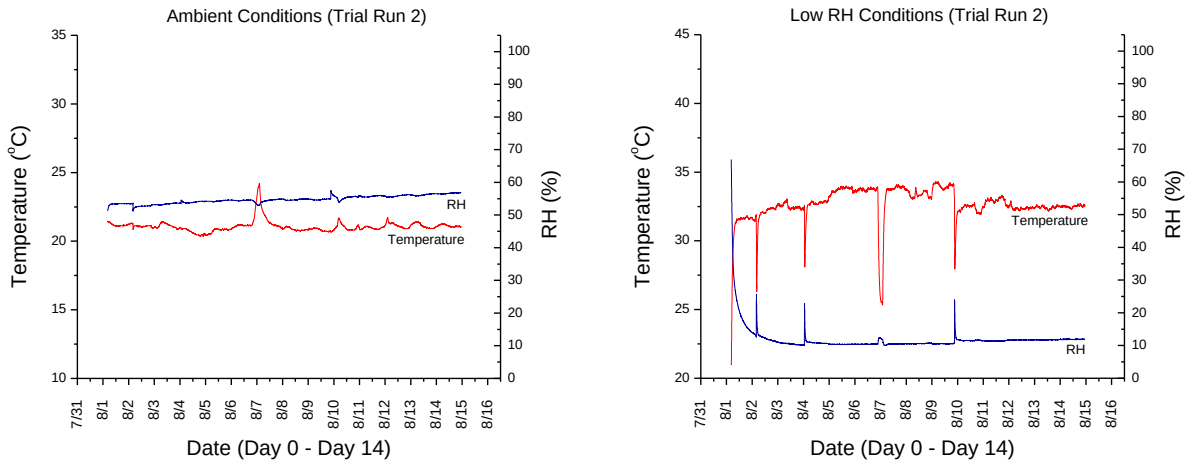

Figure A-2: Temperature and RH Measurements for Ricin Trial 2 Test Chambers: Ambient (Left) and LowRH (Right) Conditions

Vaccinia Run 1:

For the ambient condition, the mean temperature and RH were 20.8°C and 61.6%, respectively. The SDs were 0.2 and 1.2, respectively. The ranges were 19.8°C-21.7°C and 51.8%-64.5% RH. For the High RH condition, the mean temperature and RH were 30.8°C and 90.8%, respectively. The SDs were 2.9 and 3.8, respectively. The ranges were 20.5°C-32.6°C and 75.0%-99.8% RH.

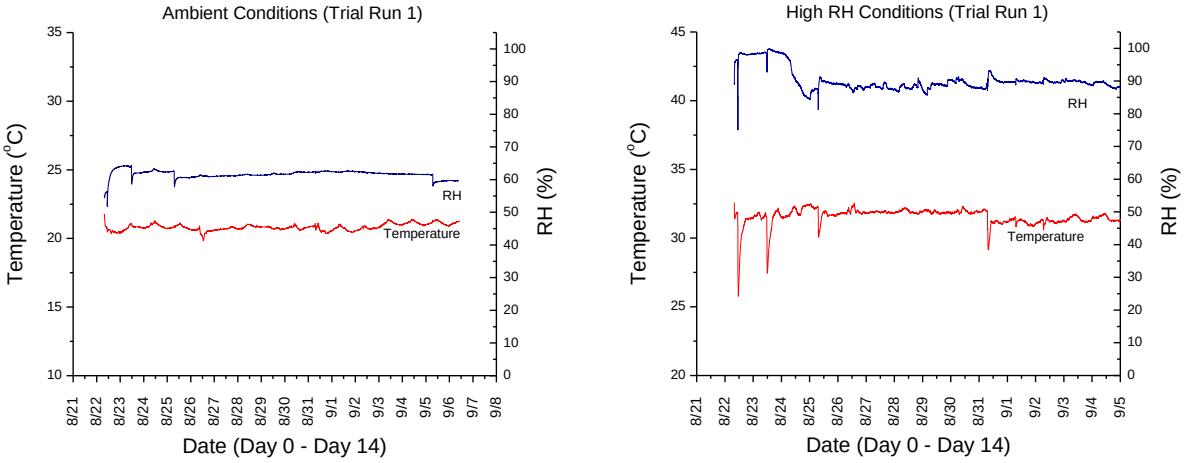

Figure A-3: Temperature and RH Measurements for Vaccinia Trial 1 Test Chambers: Ambient (Left) and High RH (Right) Conditions

Vaccinia Run 2:

For the ambient condition, the mean temperature and RH were 21.0°C and 62.2%, respectively. The SDs were 0.3 and 0.5, respectively. The ranges were 20.7°C – 23.5°C and 57.3%-64.0% RH. For the Low RH condition, the mean temperature and RH were 31.7°C and 19.8%, respectively. The SDs were 0.8 and 2.3, respectively. The ranges were 24.7°C-32.5°C and 18.5%-33.2% RH.

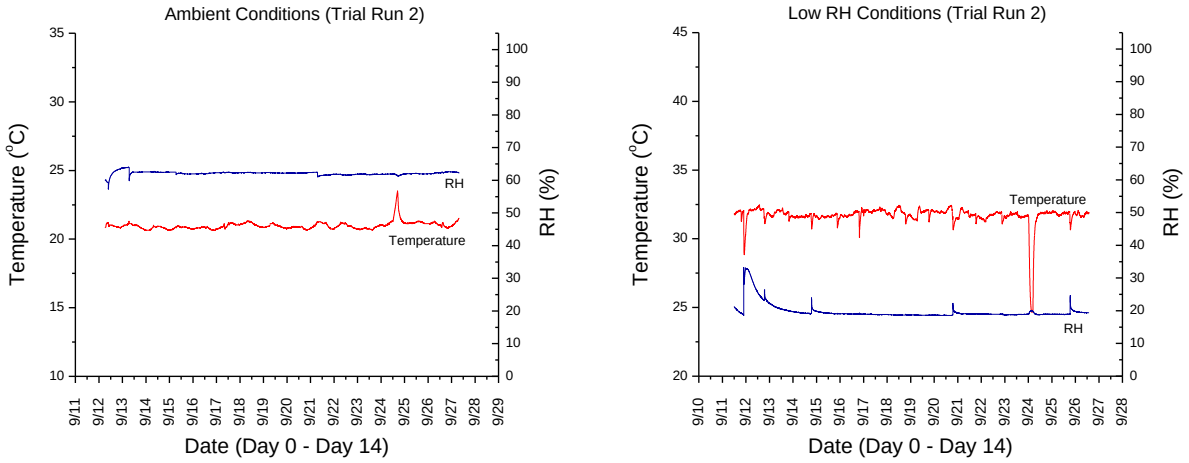

Figure A-4: Temperature and RH Measurements for Vaccinia Trial 2 Test Chambers: Ambient (Left) and Low RH (Right) Conditions

Appendix B

Report of Methods Development and Demonstration

Method development and demonstration performed under the test/QA plan[1], but not described in the report are summarized in this appendix.

Demonstration of Methods for Measuring Biological Activity of *C. burnetii*

A method was needed in order to quantify *C. burnetii*. The method of Gutierrez et al. (2002)[8] was tried. A dilution series of *C. burnetii* (Nine Mile strain, phase II, plaque-purified clone 4) was inoculated onto Vero (African Green Monkey) kidney cell, *C. burnetii* were observed microscopically to be present in the cells of the monolayers. However, plaques, necessary for quantitation of the organisms, were not observed at any dilution after 10-day incubation.

The plaque assay that was done with the Vero cells was repeated with a slower growing normal fibroblast culture. The idea was that the slower-growing fibroblasts would enable resolution of plaques caused by *C. burnetii* infection. Repeating the approach using normal mouse fibroblast monolayers yielded similar results to those observed with Vero cells; although *C. burnetii* were observed microscopically to be present in the cells of the monolayers, plaques were not observed.

Flow cytometry was considered as another approach for quantifying *C. burnetii*. The conceptual approach was to use flow cytometry, with or without sorting, as a means to quantify cells discriminated with the Baclight™ (Invitrogen) Live/Dead® stain. Cell sorting was dropped from consideration because of the amount of method development and validation that would be required.

The proposed use of the Baclight stain was dropped from consideration because of reported over-estimation of viability. Additional fluorescent probes were considered that assess viability in a variety of organisms by measuring glucose uptake in live cells. For example, live/dead discrimination might be done with 2-NBDG [2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] and propidium iodide, or just live cells could be indicated with 2-NBDG. A rapid method for using the fluorescent probes was envisioned for quantifying living cells. The approach would use a florescence microplate reader to measure the fluorescence from the living cells extracted from a sample. The fluorescence would be calibrated against flow cytometer data to allow the number of viable cells to be quantified. However, the fluorescent probe method had not been tested with *C. burnetii* and the use of a calibrated microplate reader was a novel and untested idea. Limited method development showed that the fluorescent probe worked, however the dynamic range was too small. Substantial method development might be required.

In summary, a variety of approaches for quantifying *C. burnetii* were considered and some tried. but none were found sufficient without development beyond the scope of this investigation. Therefore, the quantitative impact of environmental conditions on persistence of *C. burnetii* could not be determined.

Demonstration of Methods for Improving Recovery from Coupons

Recoveries of ricin from galvanized metal coupons, shown in Table 4-1, after one hour were consistently low (6 – 20 µg of cytotoxic protein). The coupons used in this testing were more than a year old. To attempt to improve recoveries, coupons of new galvanized metal were tested. Three galvanized metal and three painted concrete coupons were inoculated with 25 µg cytotoxic ricin and handled in the same manner as described in the methods section of this report. Extractions were performed

immediately after inoculating the coupon (<10 seconds) or after setting undisturbed for one hour. Analysis was performed as described in the report. As shown in Table A-1, all or nearly all of the ricin was extracted from the painted concrete at <10 seconds and at one hour. In contrast, 1.3% or less of the cytotoxic ricin applied was extracted from galvanized metal at <10 seconds or at one hour. The galvanized metal used in this method development was new (in contrast to aged galvanized metal that was used in the persistence test. Recoveries of ricin were not improved by immediate recovery from new galvanized metal. Recoveries from the new galvanized metal, shown in Table B-1, (1.3% or less) were lower than from aged galvanized metal after one hour (24.4% and 79.2% from Run 1 and Run 2, respectively, shown in Section 4.1.1).

The mechanism of loss of cytotoxic ricin, whether from reaction with the galvanized metal or adsorption to the galvanized metal, was not determined. A possible explanation is that the reactive zinc in the new galvanized metal is having a greater deactivation effect on the ricin than the aged metal with a zinc carbonate patina. Zinc (the coating of galvanized metal) is highly reactive. It goes through a series of reactions as it ages, first reacting with oxygen in the air to form zinc oxide; then reacting with moisture to form zinc hydroxide; and finally the zinc oxides and hydroxides react with carbon dioxide to form zinc carbonate. Zinc oxide has been shown to inhibit enzymes and Zn(+2) has been shown to inhibit ricin cytotoxic effects, e.g., apoptosis. See, for example, Tamura, T., Sadakata, N., Oda, T., and Muramatsu, T. (2002) Role of zinc ions in ricin-induced apoptosis in U937 cells. Toxicol. Lett. 132, 141–151. Therefore, the new galvanized metal with more of the zinc and zinc oxide may deactivate ricin more readily than zinc carbonate.

Table B-1. Ricin Recoveries (µg) upon Immediate or Delayed Extraction

	Trial 1		Trial 2		Trial 3	
Dry time	**Galvanized metal, µg (n=3)**	**Concrete, µg (n=3)**	**Galvanized metal, µg (n=3)**	**Concrete, µg (n=3)**	**Galvanized metal, µg (n=3)**	**Concrete, µg (n=3)**
<10 sec	0.002	25.864	0.053	29.383	0.255	29.987
One hour	0	25.211	0	24.553	0.317	23.492

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