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Neutralization of Ricin Toxin on Surfaces using Low Concentration Hydrogen Peroxide Vapor

Office of Research and Development
Center For Environmental Solutions and Emergency Response

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REPORT

Neutralization of Ricin Toxin on Surfaces using Low Concentration Hydrogen Peroxide Vapor

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

Disclaimer

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Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the Nation's land, air, and water 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, EPA's research program is providing data and technical support for solving environmental problems today and building a science knowledge base necessary to manage our ecological resources wisely, understand how pollutants affect our health, and prevent or reduce environmental risks in the future.

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This report describes a study to evaluate an easy-to-use decontamination technique for materials that have become contaminated with ricin. The findings from the study can inform plans for remediation following a ricin contamination incident.

Gregory Sayles, Director
Center for Environmental Solutions and Emergency Response

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Executive Summary

This report documents a study that investigated the efficacy of low concentration hydrogen peroxide vapor (LCHPV) for decontaminating materials contaminated with crude ricin preparations. Ricin is a highly toxic protein derived from castor beans historically considered a bioweapon agent and that has been used in several bioterrorism incidents in recent years.

This study focused on the neutralization of crude ricin preparations on four materials representative of common indoor building materials: pine wood, ceramic tile, industrial carpet, and acrylonitrile butadiene styrene (ABS) plastic. Crude ricin preparations were applied to the positive control (not exposed to the LCHPV) and test material coupons as a liquid and allowed to dry. Test coupons were then exposed to either 25 or 50 parts per million LCHPV, for contact times that ranged from 24-96 hours. Following the decontamination treatment, an extraction procedure was used to remove the remaining toxin from the test coupons and the positive controls. The ricin toxin was quantified using a cytotoxicity assay. Decontamination efficacy was determined as the percent reduction, based on the mass of active ricin toxin recovered from test coupons compared to the mass of active toxin recovered from the positive controls.

Summary of Major Findings

For most test conditions, the use of LCHPV was an effective method to neutralize crude ricin toxin preparations contaminating evaluated materials. Greater than 90% reduction in toxin cytotoxicity was achieved using 25 and 50 parts per million (ppm) hydrogen peroxide vapor for all materials tested at exposure times of 96 and 48 hours, respectively. The LCHPV can be generated using low-tech methods such as an off-the-shelf humidifier filled with hydrogen peroxide solution, providing sufficient efficacy for situations where higher concentrations of hydrogen peroxide vapor may not be readily obtainable in the field or an appropriate option for use on certain contaminated materials. Variability within the percent reduction values of the crude ricin may be due to the presence of additional proteins and other organic materials in the crude suspension. The use of a biological system (a cell-based assay) to quantitate ricin toxicity, regardless of ricin type, may also have contributed to variability in results.

Statistical analyses indicate that increasing either the LCHPV concentration or exposure contact time resulted in a significant increase in neutralization efficacy against the crude ricin toxin preparations. A regression model fitted to the entire study dataset indicated no statistical differences were observed when assessing by material type. Average values for percent toxin cytotoxicity reduction by test material and LCHPV concentration are provided in Figure ES-1.

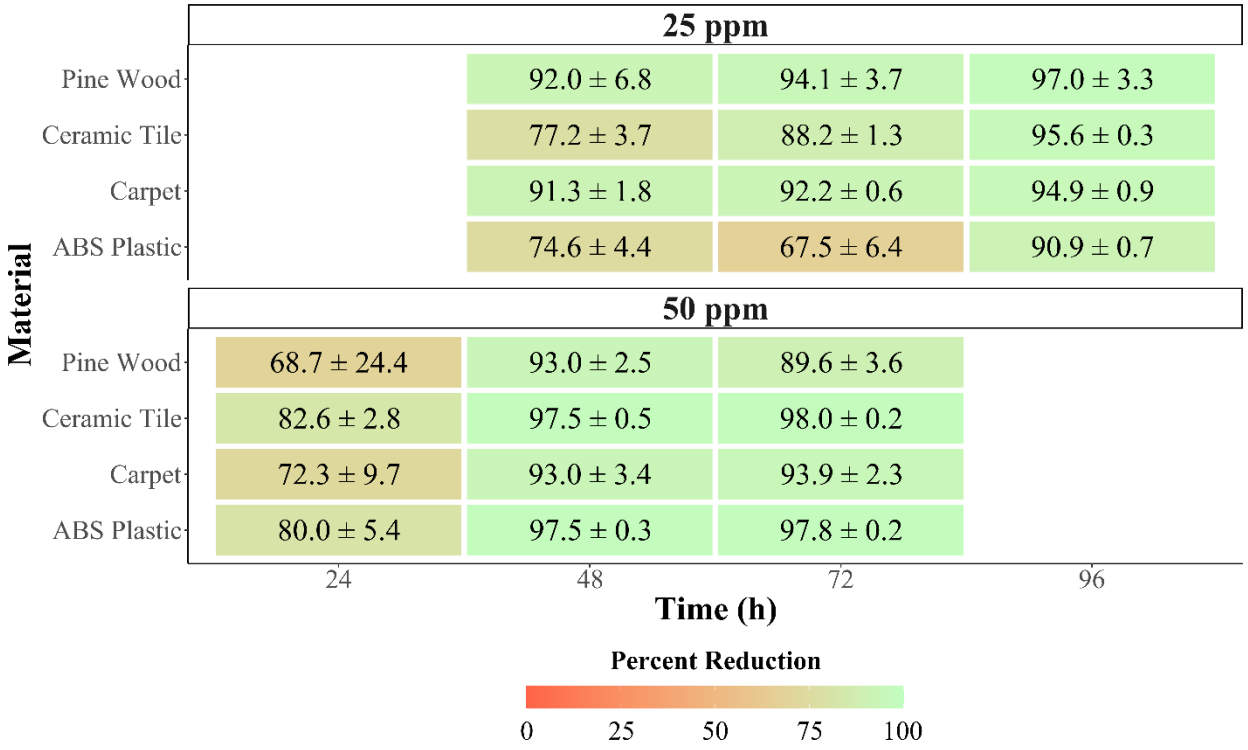

Figure ES-1. Summary of Average Percent Toxin Cytotoxicity Reduction ± 95% Confidence Interval Results, by LCHPV Concentration, Time, and Material

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Abbreviations/Acronyms

4-PL	four-parameter logistic
ABS	acrylonitrile butadiene styrene
ASTM	American Society of Testing and Materials
BSC	biological safety cabinet
CI	confidence interval
cm	centimeter(s)
°C	degree(s) Celsius
CT	concentration x time
E-beam	electron beam
EPA	U.S. Environmental Protection Agency
h	hour
HPV	hydrogen peroxide vapor
HSRP	Homeland Security Research Program
IV	intravenous
kg	kilogram(s)
kGy	kilogray(s)
L	liter(s)
LCHPV	low concentration hydrogen peroxide vapor
LD_{50}	median lethal dose; individual dose required to kill 50 percent of a population of test animals
LOD	limit of detection
µg	microgram(s)
µL	microliter(s)
mg	milligram(s)
mL	milliliter(s)
mil	thousandth of an inch
min	minute(s)
MTT	3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	not applicable
ng	nanogram(s)
nm	nanometer(s)
ORD	Office of Research and Development
PBS	phosphate buffered saline
ppm	parts per million
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
STREAMS	Scientific, Technology, Research, Engineering, and Modeling Support
TSA	technical systems audit

1.0 Introduction

Ricin is a highly toxic, ribosome inactivating protein, capable of inhibiting protein synthesis within cells, and is produced from the beans of the castor bean plant, *Ricinus communis*. The median lethal dose (LD_{50}) in mice is 5 micrograms per kilogram (µg/kg) via intravenous (IV) injection [(1)]. Extrapolations have been made that indicate a human LD_{50} exposure could be ~1 to 5 milligrams per kg (mg/kg) IV. The ricin aerosol LD_{50} for nonhuman primates is estimated to be 10-15 µg/kg [(1)]. Previous studies have shown that ricin toxin can persist for more than 28 days when deposited onto building materials, dried, and held at 20 °C [(2)].

Numerous bioterrorism incidents using ricin toxin have occurred since the 1978 assassination of Georgi Markov using a pellet of ricin toxin disguised in the tip of an umbrella [(3)]. In 2013, several letters that contained ricin toxin were sent to various locations, including the White House and the office of the New York City mayor [(4)].

In 2018, ricin was detected in mail sent to the Pentagon [(5)]. More recently in 2020, ricin was discovered in a package sent to former President Donald Trump [(6)]. These contaminated letters and packages had the potential to also contaminate the corresponding mail-sorting facilities, equipment, and the associated buildings, creating an exposure hazard and subsequent health risk for those working in the area. Other ricin contamination incidents have been described elsewhere [(2)]; ricin incidents responded to by the US EPA may be found here [(7)].

Multiple studies have examined the decontamination efficacy of liquid disinfectants against ricin toxin [(8-12)]. However, implementing decontamination strategies using liquid-based disinfectants in large complex buildings can be challenging in terms of distribution as well as material degradation from the potentially harsh liquid disinfectants. Fumigants offer ease of distribution within large complex spaces; however, few studies have examined the effects of these types of decontamination methods against ricin toxin on building-relevant surfaces. One such study examined the effects of vapor phase hydrogen peroxide and found that when a pure or crude (a less refined product containing more extraneous material from the seeds) preparation of ricin was exposed to 400 ppm vapor phase hydrogen peroxide for 14 hours, greater than 99% reduction in ricin activity was achieved [(4)]. Achieving 400 parts per million (ppm) or greater of hydrogen peroxide vapor within a large building can be challenging, requiring methods such as tarping to subdivide a space into smaller areas [(13)]. One potential solution to simplify implementation is the potential use of low concentration hydrogen peroxide vapor (LCHPV) such as 25-50 ppm, which has recently been studied for the inactivation of an Ebola surrogate virus [(14)], as well as for the inactivation of anthrax simulant spores within the interior of a vehicle [(15)]. The LCHPV can be simply generated using off-the-shelf aqueous solutions of hydrogen peroxide disseminated with low-cost humidifiers. [(14)]

This study examined the use of LCHPV against a crude preparation of ricin toxin applied to common building materials (pine wood, ceramic tile, acrylonitrile butadiene styrene (ABS) plastic, and industrial carpet) to provide efficacy data assessing the suitability of LCHPV as part of a decontamination strategy for ricin toxin contamination. Neutralization efficacy was quantified as percent reduction in the mass of active ricin toxin recovered from test coupons compared to the mass of active toxin recovered from positive control coupons.

2.0 Procedures

This section provides an overview of the procedures used for the evaluation of LCHPV to inactivate a crude ricin toxin preparation on four different materials. Procedures were consistent with previous testing that examined the efficacy of hydrogen peroxide vapor to neutralize ricin [4].

2.1 Test Matrix

The test matrix for the study is shown in Table 2-1. All tests utilized material coupons made from pine wood, ABS plastic, ceramic tile, and carpet, using crude ricin with a target inoculation of 250 micrograms for each coupon.

The LCHPV concentration used for tests was either 25 or 50 ppm. Tests 1 and 2 used a 48-hour contact time, and then Tests 3 and 4 used two contact time points based on the results achieved from the 48-hour contact time. Note: The Test 3 contact time was extended, due to the LCHPV generator malfunction, to achieve the targeted LCHPV concentration x time equivalent dose. (This is further discussed below in Section 3.3.3.)

Table 2-1. Test Matrix

Test Number	Materials	LCHPV Concentration (ppm)	Target Ricin Mass (µg)	Target Temp °C	Target %RH	Contact Time (Hours)
1	Pine Wood ABS Plastic Ceramic Tile Carpet	50	Crude 250	Ambient		48
2		25				48
3		25				72/96*
4		50				24/72

*LCHPV generator malfunction. Extended time 3 h 16 min to obtain same dosage (concentration x time) exposure.

2.2 Ricin Toxin

Testing was conducted using two preparations of ricin toxin, a highly purified preparation to facilitate the generation of an assay standard curve (pure) and a crude preparation as the inoculum for test coupons (crude). The first form was a commercially available preparation of pure ricin toxin (Cat. No. L-1090C: *Ricin communis* agglutinin II, 20 mg per milliliter [mg/mL] protein concentration, Vector Laboratories, Burlingame, CA) and was kept at ≤ -70 degrees Celsius (°C) for long-term storage. This pure stock material was diluted from its initial concentration to a working stock concentration of 1,950 nanogram (ng)/mL and was stored at 2 to 8 °C during the study. This pure ricin stock was used to generate the 7-point standard curve for each 96-well test plate used in the cytotoxicity assay (discussed below).

The crude preparation of the toxin was extracted from whole castor beans obtained from Sheffield's Seed Company (*Ricinus communis*). The crude ricin preparation was prepared in the laboratory using methods derived from the scientific literature,[16] and this batch was used

throughout the study. Briefly, whole castor beans were de-husked and homogenized into a slurry. Whole protein was precipitated from the solution, dialyzed, and rinsed with sterile phosphate buffered saline (PBS [Cat #D8537 Sigma-Aldrich, St. Louis, MO]). The final crude ricin toxin was prepared in sterile PBS and stored at 2-8 °C. The initial titer of the crude protein extract generated for this study was ~12 milligrams/milliliter (mg/mL).

2.3 Test Materials

The test materials included pine wood, ABS plastic, ceramic tile and industrial carpet. Information on these materials is presented in Table 2-2, and representative samples of each test coupon are presented in Figure 2-1. Material coupons were cut to uniform length and width (Table 2-2) from larger pieces of stock material. Materials were prepared for testing by sterilization via electron beam (E-beam) irradiation at ~200 kilograys (kGy); E-beam Services Inc., Lebanon, OH). E-beam-irradiated material coupons were sealed in 6 mil (0.006 inch) thickness Uline Poly Tubing (Cat. No. S-2940, Uline, Chicago, IL) to preserve sterility until the coupons were ready for use. Sterilization was intended to eliminate contamination by microorganisms that might interfere with the cell-based assay used to assess ricin bioactivity.

Table 2-2. Test Materials

Material	Lot, Batch, ASTM No., or Observation	Manufacturer/ Supplier Name Location	Approximate Coupon Size, Width x Length x Thickness	Material Preparation
Pine Wood	Item #: 3542 Model #: 142 8PINE	Lowes, Hilliard, OH	1.9 centimeter (cm) x 7.5 cm x 0.3 cm	E-Beam
Carpet	Shaw Swizzle EcoWorx, Style: 10401 Color: Jacks	Shaw Industries Dalton, GA	1.9 cm x 7.5 cm x 0.7 cm	E-Beam
ABS Plastic	8586K551	McMaster Carr	1.9 cm x 7.5 cm x 0.2 cm	E-Beam
Ceramic Tile	PWHITW91L01	Lowes, Hilliard, OH	1.9 cm x 7.5 cm x 0.2 cm	E-Beam

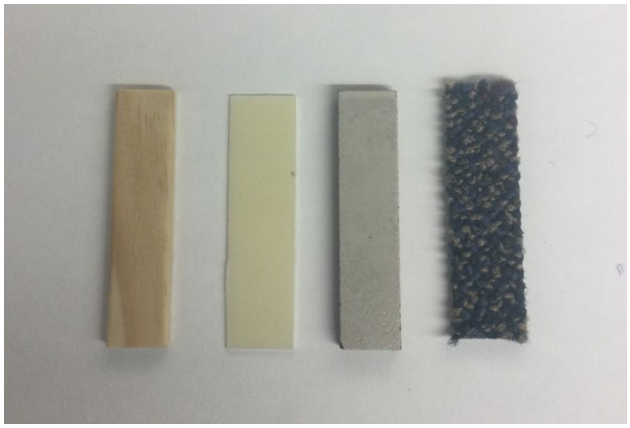

Figure 2-1. Coupon Types from Left to Right: Pine Wood, ABS Plastic, Ceramic Tile, Carpet.

2.4 Inoculation of Coupons

Test and positive control coupons were placed on a flat surface within a Class II biological safety cabinet (BSC) and inoculated individually with a target mass of approximately 250 µg of crude ricin toxin. Actual delivered mass of crude ricin toxin per coupon material was determined each day of testing using a cell-based bioassay (see Section 2.6) and averaged approximately 1316 ± 197 µg per coupon for the study. While this average actual quantity of crude ricin toxin applied to each coupon was much higher than our target of 250 µg, it does not impact ricin inactivation/reduction calculations, since reduction on test coupons was determined relative to recovery of ricin from the positive controls from each test.

The higher-than-expected crude ricin inoculum levels are most likely due to the degradation of the pure ricin standard prior to and during testing, when it was removed from long-term storage at -70 °C and then stored at 2-8 °C. The titer of the crude ricin preparation at the start of the project was 11.77 mg/mL but increased to nearly 76 mg/mL by the time of the last test conducted, which occurred several months later. The degradation of the pure ricin standard would make the titer of the crude ricin appear to increase.

At the end of the study, a new working stock of the pure ricin standard was produced for the cytotoxicity assay standard curve, and this resulted in the titer measurement of the crude material diminishing back to 15.0 mg/mL. This titer would have resulted in an inoculum level of ~319 µg and would more closely align with previous testing [4].

Based on the initial crude ricin titer determination of 11.77 mg/mL, the volume inoculated onto each coupon was 21.25 microliters (µL), to achieve the targeted 250 µg of crude ricin toxin per test coupon. This inoculum volume was used throughout the study to maintain consistency. The inoculum of the crude ricin toxin stock suspension was dispensed using a micropipette and applied as a single streak across the coupon surface (Figure 2-2). This technique provided decreased drying times and enabled greater distribution of toxin across the coupon surface as compared to a single drop of the suspension. After inoculation, the coupons were left undisturbed to dry for approximately one hour (h) (or until visually dry) under ambient conditions, ~22 °C and 40% relative humidity (RH).

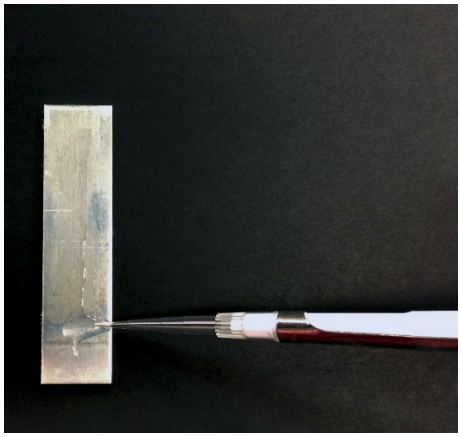

Figure 2-2. Liquid Inoculation of Coupon Using a Micropipette

The number and type of replicate coupons used for each combination of material and environmental condition included were (N=9 total):

- Three test coupons (inoculated with crude ricin toxin and exposed to LCHPV for the test duration);
- Three positive controls (inoculated with crude ricin toxin and exposed to ambient environmental conditions for each time point of the experiment);
- One laboratory blank (not inoculated and exposed to ambient environmental conditions for the test duration); and
- One procedural blank (not inoculated and exposed to experimental LCHPV).

Approximately 1 h post-inoculation (or until materials were visibly dry), coupons intended for LCHPV testing (including appropriate blanks) were transferred into the test chamber and exposed to the LCHPV conditions using the custom test chamber and application conditions specified in Section 2.5. Positive controls (including appropriate blanks) remained in a small LocknLock (LocknLock Co., Seoul, Republic of Korea) enclosure within the BSC II.

2.5 Test Chamber and Procedures

Decontamination testing was conducted inside a 498 liter (L) custom acrylic compact glove box (Plaslabs, Lansing, MI). The test chamber was outfitted with one low speed mixing fan and two patch panels on the walls to allow for the required plumbing and electrical connections. All testing was conducted at ambient uncontrolled laboratory temperature and relative humidity. Temperature and RH inside the test chamber were measured using an MX1101 temperature and humidity data logger (Onset, Bourne, MA), and data were recorded every minute for the duration of the experiment. LCHPV concentration was measured using an ATI B12 2-wire gas transmitter (Analytical Technology, Inc., Collegeville, PA) and was connected to a CNI-822 process controller (Omega Engineering, Norwalk, CT) which allowed for automatic control of hydrogen peroxide vapor concentration within the test chamber. Data were recorded every minute during the experiment using the associated Platinum software.

In lieu of using a low-tech approach to generate the LCHPV, such as with off-the-shelf humidifiers and aqueous solutions of hydrogen peroxide, generation of the LCHPV was achieved using a commercial Bioquell L4 generator (Bioquell, Horsham, PA) and a two-chamber control design. This design allowed for better control of the hydrogen peroxide vapor concentration used in the experiments. A stock solution of 35% aqueous H_2O_2 Cat # HPV-AQ was used as the starting

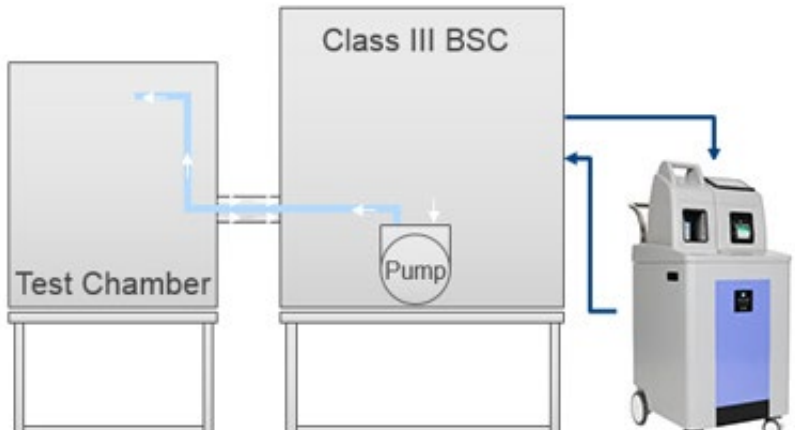

Figure 2-3. Schematic Diagram of LCHPV Exposure Chamber

material (Bioquell). Figure 2-3 illustrates a schematic diagram of the two-chamber LCHPV exposure method in which the generator was primarily connected to a class III BSC. A higher than target ppm (~350 ppm) was generated in this chamber. The test chamber used the process controller to turn the linear pump (Gast Mfg. Inc., Model DDL60, Benton Harbor, MI) placed inside this BSC III on and off to inject LCHPV from the primary BSC III chamber to the test chamber. This method allowed for precisely controlled concentrations of 25 and 50 ppm and was used for the duration of testing. (These are concentrations that can be achieved using a low-tech approach as previously described.) Once target LCHPV was achieved and stable, the inoculated coupons were placed into a sealed container and transferred to the test chamber and opened, starting the exposure. At the selected timepoints, a set of identified coupons was removed by placing them back into a sealed container and transferring them to a Class II BSC where they were opened to allow for offgassing of the peroxide vapor for 1 hour.

A representative graph of the environmental conditions (Test 2) data collection is presented in Figure 2-4.

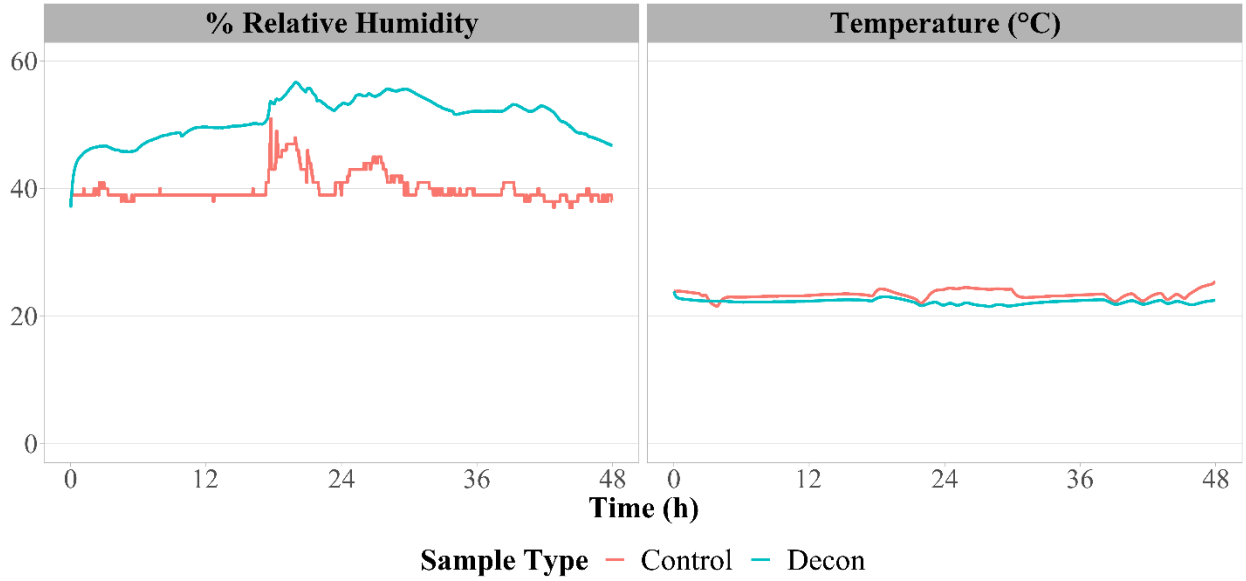

Figure 2-4. Representative Graph of Temperature and RH Stability (Test 2).

A representative graph of the LCHPV concentration (Test 2) data collection are presented in Figure 2-5. Note: a process control adjustment after ~ 26 hours of exposure resulted in a brief spike in concentration up to ~29 ppm but quickly recovered to the target of 25 ppm. The cause of this spike in concentration was unknown, and the spike was not reproducible.

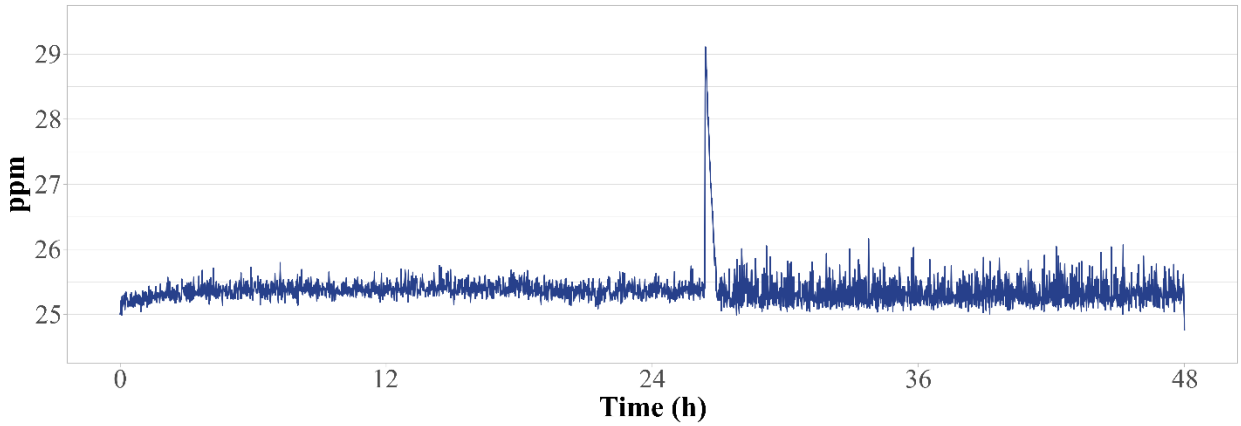

Figure 2-5. Representative Graph of LCHPV Stability (Test 2).

2.6 Coupon Extraction and Ricin Toxin Quantification

At each predetermined timepoint, the test coupons, positive controls, and associated blanks were removed from the test chamber and allowed to offgas the hydrogen peroxide vapor for 1 hour. Next, each test coupon was individually placed in 50 mL conical tubes containing 10 mL of complete growth medium (Dulbecco's Modified Eagle's Medium, Gibco Cat. No. 10566016, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco Cat. No. 10082147) and penicillin-streptomycin (Gibco Cat. No. 15140122) for ricin extraction. The vials were capped, placed on their sides and agitated on an orbital shaker for 15 min at approximately 200 revolutions per minute (rpm) at room temperature. The presence of residual active toxin from the test and control coupon extracts was determined using the bioassay described below.

The mechanism of action by which ricin toxin exerts its toxic effect is through inhibition of protein synthesis within cells via ribosome inactivation. Such inhibition of protein production leads to cell death. Other assays exist that have the ability to detect ricin toxin through the use of antibodies such as enhanced chemiluminescence (ECL) or enzyme-linked immunosorbent assay (ELISA)[18]. However, these do not measure the ability of the toxin to biologically exert its toxic effect on the living cell. Cytotoxicity assays are highly accurate in controlled laboratory experiments in which all cytotoxins are known and controlled. These assays, however, may not be suitable for environmental samples where this may not be the case. Therefore, an *in vitro* cytotoxicity assay was used to evaluate the level of bioactive ricin toxin extracted from both the decontaminated and positive control material coupons. The bioassay used in this evaluation for determining the cytotoxicity (concentration) of bioactive ricin toxin is based on the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay developed by Mosmann[17]. Cytotoxicity is reported as mass of bioactive toxin as determined using a reference standard prepared from the purified form of ricin toxin.

To conduct this MTT assay, Vero cells (ATCC; Manassas, VA; kidney epithelial cells from the African green monkey) were seeded in wells of a 96-well microplate at a density of approximately 2×10^4 cells/well. Cells were then incubated for approximately 18 to 30 h at 37 ± 2 ºC under 95% air and 5% carbon dioxide and exposed to the coupon extracts by adding 100 µL neat extract or test dilution to each well and performing a series of two-fold dilutions down each plate. Following 48 to 72 h exposure to the sample extracts, the cells were incubated in the presence of MTT, where mitochondrial enzymes convert the yellow MTT to a purple formazan salt. The absorbance of this

purple reaction product, read at 570 nanometers (nm) using a BioTek Synergy HTX Multi-Mode microplate reader (Agilent, Santa Clara, CA), is directly proportional to the number of living cells and inversely proportional to the cytotoxic potential of ricin toxin (Figures 2-6 and 2-7). For all dilutions and sample transfers into the individual wells of a 96-well plate (Fisher Scientific; Pittsburgh, PA), a micropipette (Mettler-Toledo Rainin; Oakland, CA) was used with the pipette tip replaced between wells to ensure that cross contamination did not occur.

To determine the concentration of ricin toxin from each test sample, a pure ricin toxin standard (Vector Laboratories, Inc.) was prepared from the commercially available stock solution and assayed in parallel on each test plate. The pure ricin toxin stock solution was used to prepare a seven point-standard curve of absorbance versus calculated mass of active ricin toxin protein. For each standard and test sample, absorbance values of the reference wavelength (630 nm) were subtracted from the absorbance values at 570 nm for each well. For each point used in generating the standard curve, the mean absorbance values (Y-axis) were plotted against the concentration in ng/mL, and a four-parameter logistic (4-PL) curve was generated by the SoftMax Pro Version 4.7 software included in the BioTek Synergy HTX Multi-Mode microplate reader using the equation:

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

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

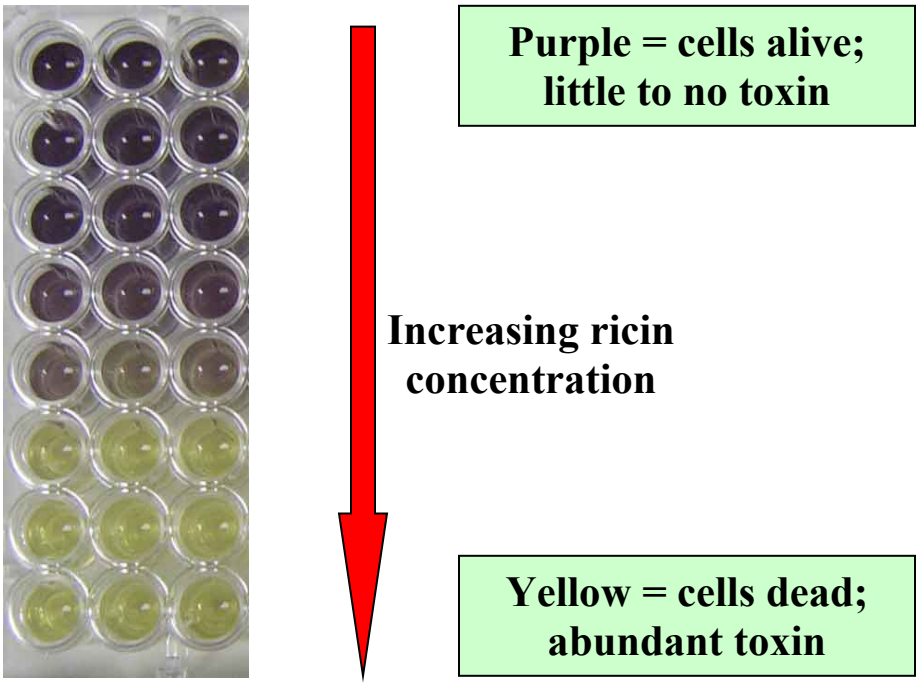

Figure 2-6. Visual Demonstration of MTT Assay on a Microplate

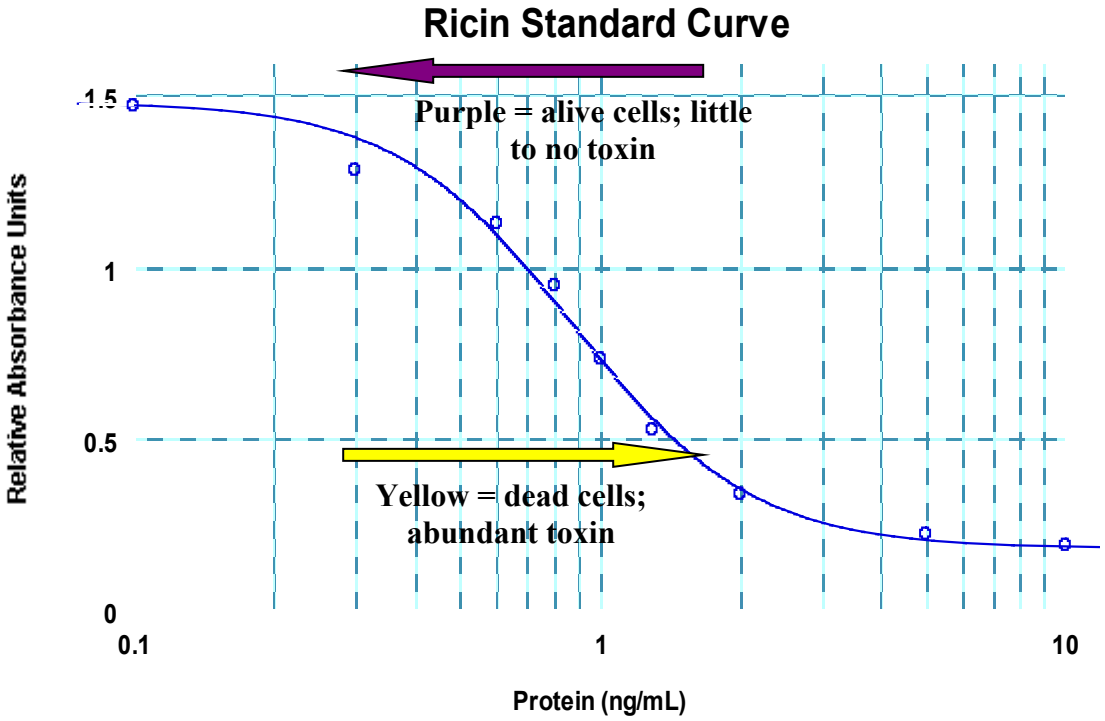

Figure 2-7. Example of Ricin Cytotoxic Profile with Corresponding Absorbance Measured Using a Microplate Reader

Initially and then throughout the study, the inherent cytotoxicity of material coupon extracts from laboratory and procedural blank coupons was assessed during each test, to determine a starting dilution that could mitigate any potential confounding cytotoxic effects observed in the ricin bioassay. To account for this potential for coupon extract-induced cytotoxicity in the ricin bioassay, the dilution factor of coupon extracts exhibiting cytotoxicity of less than 20%, when compared to negative controls (cell culture medium only), was selected as the usable starting dilution for each corresponding test material sample.

2.7 Percent Reduction Calculation

The percent reduction of active ricin was assessed by determining the mass of bioactive toxin extracted from each test coupon subjected to specified H_2O_2 concentrations as compared to the average mass of bioactive toxin extracted from the associated positive control coupons.

Neutralization of ricin in terms of percent reduction for a given test concentration, time point, and material was calculated as the difference between the mean control mass values and the mean test mass values, divided by the mean control mass values, i.e.:

$$\frac{\overline{Massc}_{ij} - \overline{Masst}_{ij}}{\overline{Massc}_{ij}} \times 100\ \% = \left(1 - \frac{\overline{Masst}_{ij}}{\overline{Massc}_{ij}}\right) \times 100\ \%. \quad (2)$$

where Massc$_{ij}$ refers to the j individual mass values obtained from the positive control coupons, Masst$_{ij}$ refers to the j individual mass values obtained from the corresponding test coupons, and the overbar designates a mean value. In this study, there were three positive controls and three corresponding test coupons (*i.e.*, $j = 3$) for each coupon material and each contact time.

The variance of the mean percent reduction was estimated through propagation of error using Taylor series approximation. Let S^2c_i be the variance of the three positive control coupons and let S^2t_i be the variance of the three test coupons. Then the estimated standard error (SE) of percent reduction is:

$$\sqrt{\frac{\overline{Masst_i}^2}{\overline{Massc_i}^2}\left(\frac{S^2t_i}{\overline{Masst_i}^2}+\frac{S^2c_i}{\overline{Massc_i}^2}\right)}{3} * 100\%. \quad (3)$$

where the number 3 represents the number j of coupons in the control and test data sets. Each decontamination result is reported as a mass value with an associated 95% confidence interval (CI), calculated as follows:

$$95\ \%\ \text{CI} = \textit{Neutralization (\% Mass Reduction)} \pm (1.96 \times \text{SE}) \quad (4)$$

Significant differences in ricin neutralization efficacy for the different test conditions may be assessed visually in some of the figures presented in Section 4, based on whether the 95% CI values for each percent reduction result overlapped. However, significant effects of test variables were more robustly analyzed using the statistical procedures described below.

2.8 Statistical Analysis

For the purposes of conducting the statistical analysis, i.e., to eliminate skewness of the data, percent reductions were also calculated as the difference between the log-transformed mean control crude ricin mass values and log-transformed test coupon mass values, divided by the log-transformed mean control mass values, as follows:

$$\frac{\log_{10}(\overline{Massc}_{ij}) - \log_{10}(Masst_{ij})}{\log_{10}(\overline{Massc}_{ij})} \times 100\ \% = \left(1 - \frac{\log_{10}(Masst_{ij})}{\log_{10}(\overline{Massc}_{ij})}\right) \times 100\ \%. \quad (5)$$

where Massc$_{ij}$ refers to the j individual mass values obtained from the positive control coupons, Masst$_{ij}$ refers to the j individual mass values obtained from the corresponding test coupons, and the overbar designates a mean value.

For data analysis, log transformation of recoveries makes data distribution closer to normal distribution (better for modeling assumptions), plus it allows the models to better determine patterns in the dependent variable. In Formula 5, each individual test sample percent log reduction was calculated, allowing better modeling power with more data points (three points per condition vs one average). Since the individual control recoveries don't necessarily correspond to

a specific test sample, calculating percent reduction using one arbitrary control sample of the three tested may not accurately reflect deactivation activity, so average of the three is used.

Regression models were fitted to percent reduction ratios calculated using Formula 5 for each material. Models included main effects for exposure time and LCHPV concentration, as well as polynomial interactions for exposure time. The following model structure was fitted to the percent reduction of the log-transformed ratio response, separately for each material type tested:

$$y_{ijkn} = \beta_{0k} + \beta_{1k} \times Concentration_{ik} + \beta_{2k} \times Time_{jk} + \beta_{3k} \times Time_{jk}^2 + \beta_{4k} \times Time_{jk}^3$$

where y_{ijkn} is the observed log-transformed value of the n^{th} replicate for concentration n_i, time $_j$, and material $_k$. The parameter β_{0k} is the model intercept constant and parameters β_{1k}- β_{4k} are coefficient constants for each model effect, each being unique to the model fitted to material $_k$ data. A graphical representation of the model for each material is presented in Figure 2-8, displaying the fit of log-transformed percent reduction values calculated by the models (curves) to the true data points obtained during testing (points), by LCHPV concentration.

The regression models prepared were used to estimate the percent reduction of the log-transformed active ricin recoveries, with 95 percent confidence for each material and combination of time and LCHPV concentration. For the purposes of this study, the effects of test variables were reported as significant if the p-values were less than or equal to 0.05. All statistical analysis was performed using R[19, 20].

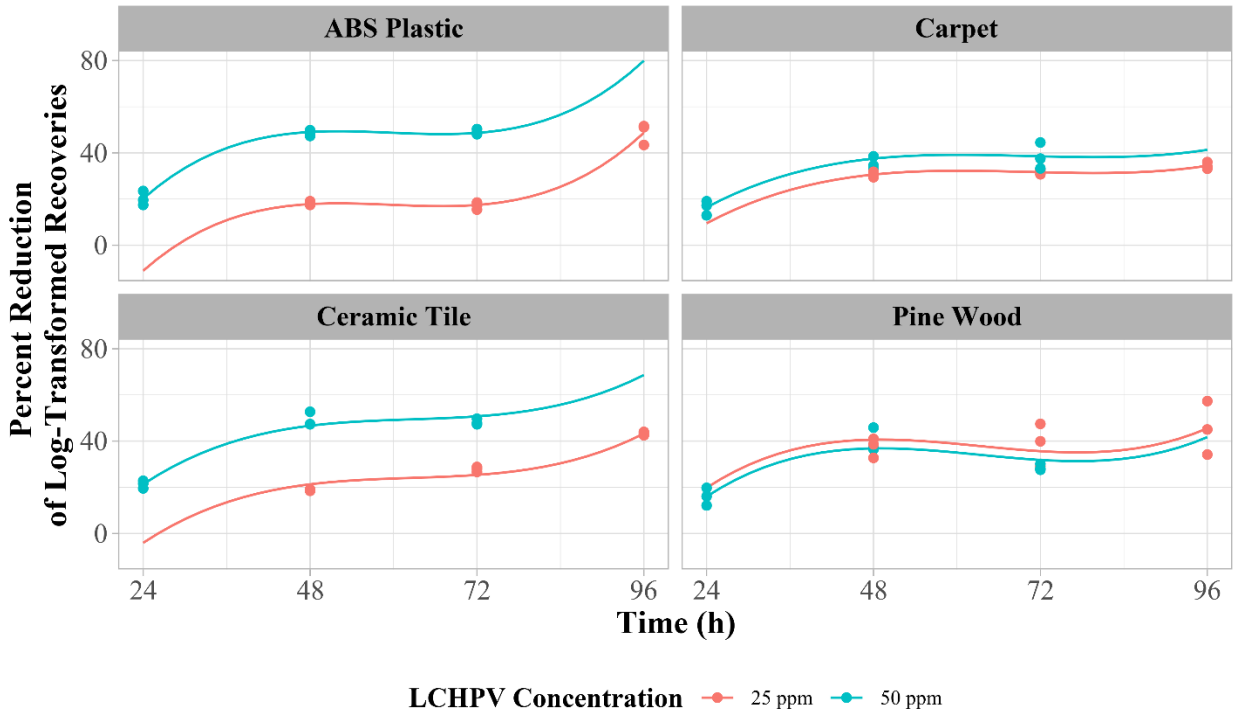

Figure 2-8. Percent Reduction of Log-Transformed Recoveries: Regression-Calculated Values (lines) and Test Sample Values (Points) by Time and Hydrogen Peroxide Concentration.

2.9 Surface Damage

The physical effect of the LCHPV on the materials was qualitatively monitored during the evaluation. This approach provided a gross visual assessment of whether the LCHPV changed the appearance of the test materials. The procedural blank (coupon that is exposed to environmental conditions, but has no toxin applied) was visually compared to a laboratory blank coupon (a coupon exposed to the LCHPV conditions and having no toxin applied). No obvious visible damage, which might include structural damage, surface degradation, discoloration, or other aesthetic impacts was observed at either of the targeted H_2O_2 concentrations (25 and 50 ppm).

3.0 Quality Assurance/Quality Control

Quality assurance (QA)/quality control (QC) procedures were performed in accordance with the Scientific, Technology, Research, Engineering, and Modeling Support (STREAMS IV) Program Quality Management Plan (QMP), Version 2 and the associated task order Quality Assurance Project Plan (QAPP). The QA/QC procedures and results are summarized below.

3.1 Equipment Calibration

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

3.2 QC Results

QC efforts conducted during testing included positive control samples (inoculated, held for duration of specified timepoint, then recovered), procedural blanks (not inoculated, decontaminated), laboratory blanks (not inoculated, not decontaminated), and inoculation control samples (analysis of the stock ricin suspension).

Positive control samples were run during each test to determine the loss of cytotoxicity over the specified contact period. The amount of ricin recovered from these positive controls was sufficient to determine percent reduction due to the cytotoxicity assay standard range of 0.1 to 10 ng.

All procedural and laboratory blanks met the acceptance criteria using dilution to mitigate inherent material specific cytotoxicity, as previously discussed. Inoculation control samples were taken from the purified and crude stock toxin suspension each day of testing and assayed against the 4-PL (parameter logistic) standard curve. Control samples were assessed for outliers using a Grubbs test. One outlier sample was identified (carpet, Test 1) and removed for statistical modeling analysis. The outlier sample is included in all other data reporting.

3.3.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during these experiments. Table 3-1 summarizes the performance evaluation audits that were performed.

Table 3-1. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume of liquid from micropipettes	Gravimetric evaluation	± 10%	± 0.8% to 2.5%
Time	Compared to independent clock	± 2 seconds/hour	0 seconds/hour
Temperature	Compared to independent calibrated thermometer	± 2 °C	0.10 to 0.22°C
Relative Humidity	Compare to independent calibrated hygrometer	± 10%	1.51 to 2.25%

3.3.2 Technical Systems Audit

Observations and findings from a technical systems audit (TSA) were documented and submitted to the laboratory technical lead for response. The TSA was conducted on January 24, 2023, to ensure that tests were being conducted in accordance with the appropriate QAPP and QMP. As part of the audit, test procedures were compared to those specified in the QAPP, and data acquisition and handling procedures were reviewed. The result of this TSA was no adverse findings noted.

3.3.3 Deviations

One deviation occurred during this study. This deviation was prepared to address the failure of the Bioquell commercial LCHPV generator described in Section 2.5. This failure occurred during Test 3, when a liquid flow error caused the generator to turn off in the early morning hours of January 13, 2023. This caused the test chamber to fall below the target of 25 ppm for a duration of 433 minutes. The concentration within the test chamber fell as low as 8.5 ppm. The impact of this deviation was considered minimal since corrective actions were taken in consultation with the TOCOR prior to the conclusion of the test, to add additional time (196 minutes at the correct concentration). This added time corrected for the anticipated concentration x time (CT) value with the observed CT during the event.

3.3.4 Data Quality Audit

At least 10% of the 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 verified. Only minor issues were noted with the data, mostly manual data transcription errors that were corrected.

3.3 QA/QC Reporting

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

3.4 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 this report.

4.0 Summary of Results and Discussion

The neutralization of crude ricin toxin inoculated onto porous and nonporous material coupons was evaluated using two concentrations of LCHPV over elapsed times. For the four tests in this evaluation, the environmental conditions ranged from 22-23 °C and 37-69% RH for durations of 24 to 96 hours. Tests 1 and 4 examined four different material types at 50 ppm, while Tests 2 and 3 examined these same materials at 25 ppm.

4.1 Test Environmental Conditions

The environmental conditions for each test are shown in Table 4-1 and reported as the average value ± standard deviation (SD) for the positive controls and test coupons. Control and test temperatures were typically within 1 °C.

Table 4-1. Environmental and Decontamination Conditions for Each Test

Test	Control Temperature °C	Test Temperature °C	Control %RH	Test %RH	LCHPV Concentration (ppm)	Contact Time (Hours)
1	23.08 ± 0.52	22.05 ± 0.20	36.90 ± 0.24	49.22 ± 1.71	50.56 ± 0.60	48
2	23.42 ± 0.61	22.26 ± 0.32	69.83 ± 0.44	51.03 ± 3.19	25.37 ± 0.46	48
3	22.78 ± 0.76	22.01 ± 0.25	37.92 ± 0.25	49.57 ± 5.27	24.45 ± 3.22	99.23*
	22.72 ± 0.67	22.02 ± 0.27	41.83 ± 0.60	51.10 ± 5.12	24.34 ± 3.41	75.23*
4	23.22 ± 0.36	22.35 ± 0.22	39.73 ± 0.20	51.20 ± 3.27	50.26 ± 0.38	72
	23.29 ± 0.34	22.30 ± 0.19	39.59 ± 0.29	50.3 ± 1.94	50.23 ± 0.32	24

* Time added (196 min) to correct for low concentration deviation per section 3.3.3

4.2 Recovery of Active Ricin from Positive Controls

The average percent recoveries of active ricin from the positive control test coupons are shown in Figure 4-1. These are the study-wide averages of the percent active ricin recovered one hour after the coupons were inoculated compared to the calculated (concentration x volume) quantity of crude active ricin applied to each test material. Average positive control recoveries by material ranged from 113 to 213%, with lowest recovery of ricin from pine wood and highest average recovery from carpet coupons. This high percent recovery could have been associated with error in crude ricin titer values (explained in section 2.4) but is also similar to previous testing showing higher recovery of 127% from materials like industrial carpet [(2)]. Negative control samples ensured that the measured cytotoxicity values were not impacted by extracted chemicals (cytotoxicity) from the test materials. Therefore, the resulting higher recovery as measured from the control coupons resulted from high control coupon recovery, low initial titer value, or more likely a combination of both variables.

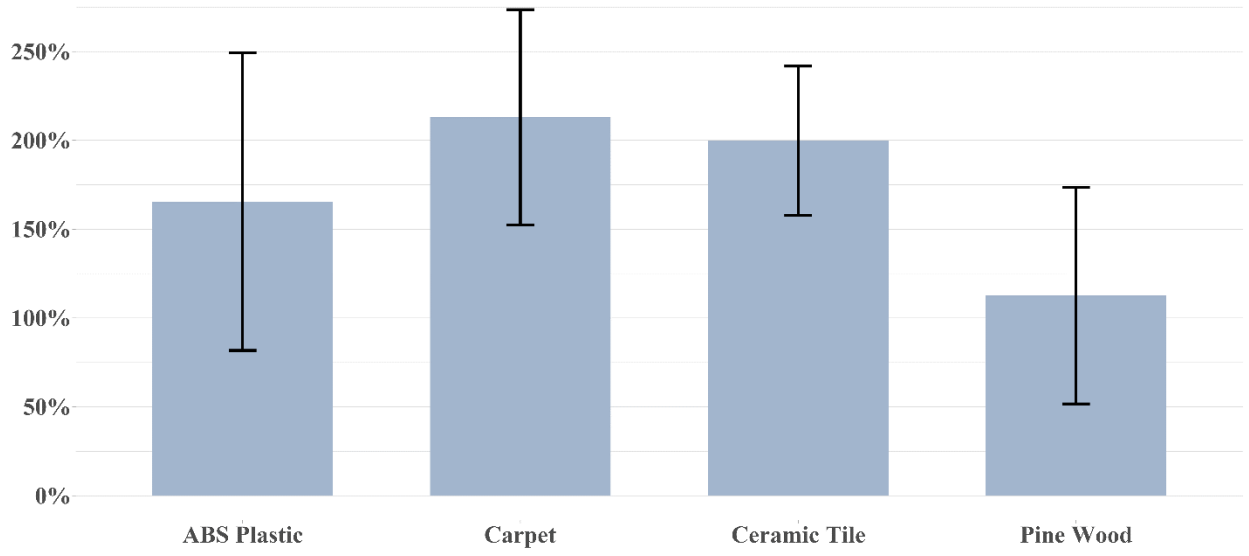

Figure 4-1. Summary of Average Percent Recovery from Positive Controls by Material Type, ± Standard Deviation

4.3 Decontamination Results

The percent reduction results for crude active ricin, by material, LCHPV concentration, and contact time, are summarized in Figure 4-2. Greater than 90% reduction was achieved using 25 and 50 ppm for all materials at exposure times of 96 and 48 hours, respectively. The highest reduction of crude active ricin obtained in the study was 98%, which occurred for the plastic and tile materials at 50 ppm and 72 h contact time. The lowest reduction of crude active ricin occurred with the 25 ppm concentration and 48 h contact time for the ABS plastic.

Reductions generally improved with an increase in contact time, except for ABS plastic at 25 ppm and pine wood at 50 ppm LCHPV exposure. From the statistical analysis, contact time had a significant effect on efficacy, and LCHPV had a statistically significant effect for all materials except pine wood ($p<0.05$). The detailed statistical results of these statistical analyses are presented in Table 4-2.

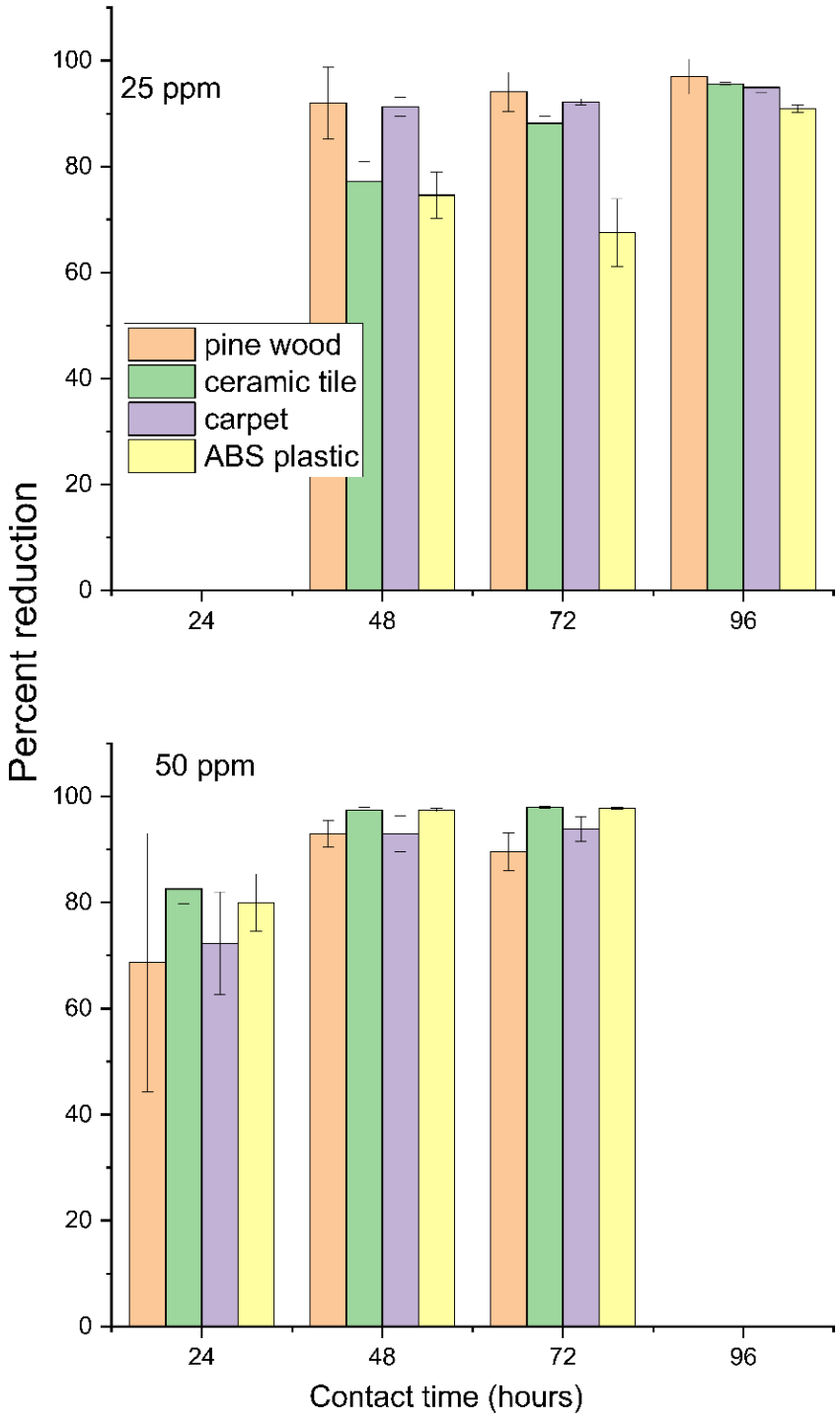

Figure 4-2. Summary of Percent Reduction of Ricin Cytotoxicity (Tests 1-4) Results, by LCHPV Concentration, Time, and Material

An additional regression model was fitted to the entire study dataset (all materials combined) and included coupon material type as a main effect. With ABS plastic as the reference material, material type was not found to be statistically significant, producing p-values of 0.41, 0.66, and 0.77 for carpet, ceramic tile, and pine wood, respectively. Material type was also not statistically significant for the other material comparisons, i.e., resulting in p-values of 0.21 (ceramic tile) and 0.27 (pine wood) with carpet as the reference level, and a p-value of 0.89 for pine wood with ceramic tile as reference level. Concentration, time, $time^2$, and $time^3$ remained as significant effects at the 0.05 level of confidence each producing p-values of <0.0001.

Table 4-2. Details of Statistical Analysis

Material	Model			Adjusted R^2	Root Mean Square Error
	Effect	Coefficient	p-value		
ABS Plastic	Intercept	-161.33	< 0.0001	0.977	2.04
	Concentration	1.25	< 0.0001		
	Time	7.69	< 0.0001		
	$Time^2$	-0.13	< 0.0001		
	$Time^3$	0.00073	< 0.0001		
Carpet	Intercept	-60.53	0.0004	0.870	2.43
	Concentration	0.28	0.001		
	Time	3.81	0.0001		
	$Time^2$	-0.055	0.0007		
	$Time^3$	0.00026	0.002		
Ceramic Tile	Intercept	-111.23	< 0.0001	0.953	2.40
	Concentration	1.01	< 0.0001		
	Time	5.07	< 0.0001		
	$Time^2$	-0.079	< 0.0001		
	$Time^3$	0.00042	< 0.0001		
Pine Wood	Intercept	-63.93	0.067	0.600	6.15
	Concentration	-0.15	0.381		
	Time	5.60	0.007		
	$Time^2$	-0.093	0.011		
	$Time^3$	0.00049	0.015		

4.4 Summary

The data generated from this study demonstrated the efficacy of LCHPV for the neutralization of crude ricin for several common interior materials. Greater than 90% reduction in ricin cytotoxicity was achieved using both 25 and 50 ppm for all materials at exposure times of 96 and 48 hours, respectively. The highest reduction of crude active ricin obtained in the study was 98%, which occurred for the plastic and tile materials at 50 ppm and 72-h contact time. Decontamination procedures using LCHPV neutralization of ricin toxin provide sufficient efficacy for situations where higher concentrations of hydrogen peroxide vapor may not be readily achievable in the field or would likely cause physical damage to contaminated material. LCHPV may also prove useful as part of decontamination procedures for sensitive items or materials.

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Appendix A
Detailed Test Results

Decontamination Results

The detailed ricin neutralization results for varied LCHPV conditions against crude ricin toxin preparations on four material types (bare pine wood, carpet, ABS plastic, and ceramic tile) are shown in Table A-1.

Table A-1. Neutralization of Crude Ricin Toxin[a]

Test Number	Test Parameters				Material	Target Inoculum (µg/coupon)	Mean Recovered Ricin ± SD (µg/coupon)		%Reduction[e] ± CI[d]
	H_2O_2 (ppm)	Temp °C±SD	%RH ±SD	Time (H)			Positive Control[b]	Test Coupon[c]	
1	50.56 ± 0.60	22.05 ± 0.20	49.22 ± 1.71	48	Pine Wood	250	824.2 ± 217.9	58.0 ± 17.7	93.0 ± 2.49
					Industrial Carpet		1912.0 ± 995.018	133.1 ± 25.9	93.0 ± 3.39
					ABS Plastic		2012.6 ± 202.2	49.9 ± 4.8	97.5 ± 0.30
					Ceramic Tile		1965.4 ± 13.1	48.3 ± 10.5	97.5 ± 0.47
2	25.37 ± 0.46	22.26 ± 0.32	51.03 ± 3.19	48	Pine Wood	250	917.4 ± 848.4	73.5 ± 22.1	92.0 ± 6.83
					Industrial Carpet		2977.3 ± 629.1	260.0 ± 24.4	91.3 ± 1.76
					ABS Plastic		1845.9 ± 350.8	469.1 ± 28.3	74.6 ± 4.44
					Ceramic Tile		2517.9 ± 463.8	575.4 ± 14.9	77.2 ± 3.73
3	24.34 ± 3.41	22.02 ± 0.27	51.10 ± 5.12	72	Pine Wood	250	2254.4 ± 219.1	133.0 ± 93.5	94.1 ± 3.67
					Industrial Carpet		3170.9 ± 149.123	246.6 ± 19.2	92.2 ± 0.62
					ABS Plastic		755.8 ± 151.2	245.5 ± 25.3	67.5 ± 6.41
					Ceramic Tile		2165.3 ± 192.6	256.5 ± 21.7	88.2 ± 1.27
	24.45 ± 3.22	22.01 ± 0.25	49.57 ± 5.27	96	Pine Wood	250	1480.3 ± 493.9	66.7 ± 50.8	97.0 ± 3.28
					Industrial Carpet		2306.0 ± 200.0	161.3 ± 17.8	94.9 ± 0.86
					ABS Plastic		3439.1 ± 161.0	68.5 ± 27.5	90.9 ± 0.71
					Ceramic Tile		3076.0 ± 311.9	95.4 ± 5.5	95.6 ± 0.32
4	50.23 ± 0.32	22.30 ± 0.19	50.3 ± 1.94	24	Pine Wood	250	1672.0 ± 1050.6	524.3 ± 147.5	68.7 ± 24.4
					Industrial Carpet		2875.4 ±497.5	795.8 ± 205.4	72.3 ± 9.73
					ABS Plastic		3190.3 ± 82.9	639.3 ± 151.6	80.0 ± 5.41
					Ceramic Tile		3776.5 ± 87.7	657.1 ± 91.3	82.6 ± 2.77
	50.26 ± 0.38	22.35 ± 0.22	51.20 ± 3.27	72	Pine Wood	250	1365.5 ± 319.5	173.5 ± 16.5	89.6 ± 3.63
					Industrial Carpet		3913.6 ± 526.4	174.8 ± 75.6	93.9 ± 2.29
					ABS Plastic		4290.2 ± 206.3	71.2 ± 6.7	97.8 ± 0.20
					Ceramic Tile		4129.3 ± 281.5	74.6 ± 7.7	98.0 ± 0.25

[a] Data are expressed as the mean (± standard deviation (SD)) of the mass of toxin recovered on three replicate individual samples, and neutralization (percent reduction ± CI).

[b] Positive Controls = samples inoculated, not decontaminated (recovered after prescribed contact time).

[c] Test Coupons = samples inoculated, decontaminated.

[d] CI = confidence interval (± 1.96 × standard error [SE]).

[e] % Reduction calculated as (mean ricin recovered positive controls – mean ricin recovered test coupons)/ mean ricin recovered positive controls

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