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Evaluation of the Inactivation of Ricin Toxin on Surfaces Using Vapor Phase Hydrogen Peroxide

Office of Research and Development
National Homeland Security Research Center

Disclaimer

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Executive Summary

The U.S. Environmental Protection Agency (EPA), Office of Research and Development (ORD) is striving to protect human health and the environment from adverse impacts resulting from acts of terror by investigating the effectiveness and applicability of technologies for homeland security (HS)-related applications. The purpose of this investigation was to determine the decontamination efficacy of vapor phase hydrogen peroxide (VPHP) in inactivating ricin toxin on indoor materials representative of a mail sorting facility. The objective of this study was to provide an understanding of the performance of VPHP to guide its use and implementation in HS applications. When assessing options for decontamination following intentional release of ricin toxin, it is important to know the extent to which factors such as VPHP concentration measured in parts per million (ppm) and duration of exposure may impact the decontamination efficacy.

This investigation focused on the decontamination of eight types of materials representative of a mail sorting facility: aluminum, industrial carpet, ceramic tile, neoprene rubber, optical plastic, paper, stainless steel, and unpainted concrete. Decontamination efficacy tests were conducted using two different VPHP generators – STERIS 1000ED and a Bioquell Clarus C – against two forms of ricin toxin: a commercially-available purified version and a crude version prepared from castor beans. Using a cell-based assay, decontamination efficacy was quantified in terms of percent (%) reduction in the mass of bioactive ricin recovered from test coupons compared to the positive control (non-decontaminated) coupons. Tests were conducted using cycles developed for both vapor-generating technologies and by varying the time of the third phase (fumigant contact phase) of the decontamination process. These data were utilized to assess the effect of these fumigation parameters on decontamination efficacy.

Summary of Results

The STERIS and Bioquell VPHP generators used in this study have four similar user-defined phases that comprise each decontamination test. The phase parameters developed for this testing were unique to the VPHP test chamber utilized for this study and should not be directly applied to larger spaces. Cycle development is required to obtain optimal conditions for each unique space to be decontaminated. Phase 1 is a chamber conditioning phase in which injection lines and chamber surfaces are warmed, and the chamber atmosphere is dehumidified to a cycle-defined level. Phase 1 set points remained consistent throughout testing for both generators. Phase 2 defined the hydrogen peroxide injection rate, which varied from 2.5 to 3.8 g/min for 20 minutes. Phase 3 consisted of a defined injection rate and delivery time, which varied from 2.5 to 3.8 g/min for 30 minutes to 16 hour (hr) contact times over a total of 10 tests. Phase 4 (aeration phase) was allowed to run until the testing chamber reached a concentration of VPHP that was ≤10 ppm. Table ES-1 shows the phase parameters required to achieve greater than 99 % reduction on selected material types tested except unpainted concrete at all operational parameters. Unpainted concrete was removed from testing due to low recovery from control materials, which may have been a result of the caustic nature of this material, affecting the bioactivity of the ricin.

Table ES-1. Parameters Required to Achieve >99 % Reduction on All Materials*

Technology	Ricin Form/Target Mass	Avg VPHP ppm±SD[b]	Phase 1	Phase 2		Phase 3		Phase 4
			Duration (min)	Injection Rate (g/min)	Duration (min)	Injection Rate (g/min)	Duration (h:min)	Duration (h:min)
Bioquell	Pure/250 µg	279±45.0	15	3.8	20	1.0	8:00	11:41
Bioquell	Crude/250 µg	301±37.8	15	3.8	20	1.0	16:00	6:26
Bioquell*	Pure/500 µg	240±40.3	15	3.8	20	1.0	16:00	7:42
STERIS*	Pure/500 µg	398±44.2	15	2.5	20	2.2	13:40	10:09
STERIS*	Crude/500 µg	398±44.2	15	2.5	20	2.2	13:40	10:09
STERIS†	Crude/500 µg	392±18.5	15	2.5	20	2.2	13:40	4:47

* Limited materials tested were industrial carpet, optical plastic, paper, and stainless steel.

† Limited materials tested were neoprene rubber, aluminum, ceramic tile, and unpainted concrete.

[a] Detailed data from each test number can be referenced in Appendix A.

[b] Concentration of hydrogen peroxide measured in the vapor phase during Phases 2 and 3.

The data generated from this investigation suggest that VPHP reduces the bioactivity of both a commercially-available purified form of ricin toxin, as well as a crude form produced by Battelle from whole castor beans. The purified ricin, as well as the whole castor beans, were purchased from Vector Laboratories (Vector Labs, Burlingame, CA). A Bioquell Clarus C Phase 3 contact time of 8 or 16 h was required to achieve greater than 99 % reduction of pure and crude ricin, respectively, on all materials tested at target inoculation level of 250 micrograms (µg). A Phase 3 contact time of 16 h was required on carpet, plastic, paper and stainless steel with an increased inoculum target of 500 µg. STERIS 1000ED required Phase 3 contact time of 13 h 40 min and a modified injection rate of 2.2 g/m to achieve greater than 99 % reduction of pure and crude ricin toxin at the increased inoculum target of 500 µg.

Testing two VPHP technologies was not the original intent of the study. However, due to repeated unforeseen system failures of the STERIS generator at an injection rate of 1.0 g/min (injection rate failure), it was necessary to utilize a second technology (Bioquell) to complete testing. Mitigation of this failure required the STERIS injection rates to be adjusted from 3.8 and 1.0 g/min to 2.5 and 2.2 g/min, respectively, and the exhaust connections slightly opened to allow for a dilution effect. The dilution resulted in obtaining the targeted 400 ppm at a higher Phase 3 injection rate, allowing the STERIS generator to be used again starting with Test 7.

VPHP appears to be an effective decontaminant against ricin toxin utilizing the STERIS 1000ED at a targeted 400 ppm for 14 h of hydrogen peroxide injection (Phases 2 and 3) as well as with the Bioquell Clarus C, targeting microcondensation for 8 or 16 h. In general, the crude form of ricin was more difficult to inactivate on plastic and carpet (Tests 1-4, 6-8).

Contents

Figures

Tables

List of Appendices

Abbreviations/Acronyms

4-PL	four-parameter logistic
BSC	biological safety cabinet
CI	confidence interval
cm	centimeter(s)
°C	degree(s) Celsius
E-beam	electron beam
EPA	U.S. Environmental Protection Agency
HEPA	high efficiency particulate air
HSRP	Homeland Security Research Program
HVAC	heating, ventilation, and air conditioning
h	hour
HS	homeland security
IV	intravenous injection
kg	kilogram(s)
kGy	kilogray(s)
L	liter(s)
lb	pound
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)
NHSRC	National Homeland Security Research Center
ORD	Office of research and development
PBS	phosphate buffered saline
ppm	part(s) per million
QA	quality assurance
QC	quality control
QMP	Quality Management Plan
RH	relative humidity
rpm	revolution(s) per minute
SD	standard deviation
SE	standard error
SFW	sterile filtered water (cell-culture grade)
T&E	Technology and Evaluation
TSA	technical systems audit
VPHP	vapor phase hydrogen peroxide

1.0 Introduction

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program (HSRP) is helping protect human health and the environment from adverse impacts resulting from the release of chemical, biological, or radiological agents. With an emphasis on decontamination and consequence management, water infrastructure protection, and threat and consequence assessment, HSRP is working to develop tools and information that will help with the cleanup of chemical or biological contaminants introduced into buildings or water systems.

In 2013, several letters containing ricin toxin were sent to various locations, including the White House and the office of the New York City mayor according to the US Attorney's Office in a memo dated June 28, 2013. These contaminated letters had the potential to contaminate the corresponding mail sorting facilities and equipment, creating an exposure risk for those working in the area. Ricin toxin is a highly toxic protein produced within the beans of the *Ricinus communis* plant. 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.[1]

This investigation was conducted as a screening process in which the efficacy of vapor phase hydrogen peroxide (VPHP) was tested against both a pure and a crude form of ricin toxin applied to materials representative of a mail sorting facility (aluminum, industrial carpet, ceramic tile, neoprene rubber, optical plastic, paper, stainless steel, and unpainted concrete) to provide efficacy data assessing the suitability of VPHP as a decontaminant for ricin toxin. Decontamination efficacy was quantified as a percent reduction in the mass of ricin toxin that induced cellular cytotoxicity recovered from test coupons compared to the mass of toxin recovered from positive control coupons. Lastly, these data provide a side-by-side efficacy comparison for the pure form of ricin as compared to a likely real-world crude preparation that could be used to assess the suitability of using the purified toxin for future fumigant decontamination investigations.

2.0 Technology Description and Test Matrices

2.1 Technology Description

Two commercially available VPHP technologies were used for testing, the STERIS VHP 1000ED (Mentor, OH) generator (Figure 2-1) and the Bioquell Clarus C (Horsham, PA) generator (Figure 2-2). These fumigant-generating technologies are advantageous for large-scale room decontamination due to the ease of fumigant delivery, ease of distribution within the targeted space, and the relatively low toxicity of the hydrogen peroxide in comparison to other fumigants such as chlorine dioxide, ethylene oxide or formaldehyde.[2] Cycle development is required for each unique space or chamber to ensure proper concentration delivery as well as equal distribution throughout the space.

Each technology has four common phases for the delivery of the hydrogen peroxide into the target enclosure to be decontaminated. These phases define how quickly and in what concentration the hydrogen peroxide is delivered into the VPHP test chamber as well as how long the concentration is maintained. Phase 1 is a chamber conditioning phase in which injection lines and chamber surfaces are warmed up and the space is dehumidified to a cycle-defined level (e.g., ≤40 % relative humidity [RH]). For this phase, the two technologies differ in that the STERIS generator dehumidifies to a much lower RH during this phase compared to the Bioquell Clarus C. The result of starting at a lower RH is the increased capacity to carry the hydrogen peroxide in the vapor phase upon injection of the STERIS 35 % Vaprox solution (Cat. No. PB006US proprietary hydrogen peroxide) without condensation. The Bioquell generator is designed so that any commercially-available 30 to 35 % hydrogen peroxide (Cat. No. H325-4, Fisher Scientific) can be injected until saturation is achieved and microcondensation forms on all surfaces. Because condensation is the endpoint, the starting RH is less critical. Phase 2 is defined by setting an injection rate of the peroxide solution and time of delivery. The purpose of the higher injection rate during this phase is to allow for a rapid increase in concentration to the desired parts per million (ppm) level (STERIS) or to achieve microcondensation (Bioquell). Phase 3 is defined by further setting an injection rate and delivery time, but in most cases at a reduced injection rate to maintain the ppm or microcondensation achieved in Phase 2. The fourth and final phase is aeration, in which the hydrogen peroxide concentration is reduced catalytically to water and oxygen until low or no measurable hydrogen peroxide remains. A list of tested parameters is shown in Table 2-1.

Figure 2-1. STERIS, Inc. 1000ED

Throughout the entirety of the run, the STERIS 1000ED unit catalytically breaks down the delivered hydrogen peroxide upon returning to the unit. The air is then transferred through a desiccant chamber, dried, and passed through the vaporizer to add additional hydrogen peroxide before injecting back into the VPHP test chamber. At the conclusion of the run, a 3 hour (h) regeneration cycle is required to heat the desiccant material to remove collected moisture and make the desiccant material ready for the next decontamination cycle.

Figure 2-2. Bioquell, Inc. Clarus™ C

In contrast, the Bioquell Clarus C generator uses a dual-loop system in which air is recirculated through the vaporizer to continually add additional hydrogen peroxide into the system. During the aeration phase, the unit switches to a second loop where the hydrogen peroxide is catalytically degraded into water and oxygen and the moisture is removed via a refrigerated coil. The condensate is then pumped into a waste collection bottle.

Testing two VPHP technologies was not the original intent of the study. However, due to repeated unforeseen system failures of the STERIS generator at an injection rate of 1.0 g/min (injection rate failure), it was necessary to utilize a second technology (Bioquell) to complete testing.

2.2 Test Matrix

The test matrix for the VPHP fumigation tests is shown in Table 2-1. Tests 6 and 9 were evaluated utilizing a downselected set of materials. This selection was made to maintain a representative selection of porous and non porous materials while selecting some of the more difficult to decontaminate materials such as carpet and plastic. Test 10 materials were selected to complete the samples that had not been tested at the higher inoculum level.

Table 2-1 VPHP Test Matrix

Test Number	Materials	Ricin mass µg	VPHP Technology	Test Chamber	Decontamination Parameters					
					Phase 1	Phase 2		Phase 3		Phase 4
					Duration min	Rate g/m	Duration min	Rate g/m	Duration h	
1	Stainless Rubber Plastic Aluminum Carpet Ceramic Concrete Paper	250	STERIS	774 liter (L) Class III	15	3.8	20	1.0	0.5	≤ 10 ppm
2					15	3.8	20	1.0	4	
3			Bioquell		15	3.8	20	1.0	4	
4					15	3.8	20	1.0	8	
5					15	3.8	20	1.0	16	
6*	Stainless Plastic Carpet Paper	500			15	3.8	20	1.0	16	
7	Stainless Rubber Plastic Aluminum Carpet Ceramic Concrete Paper	250	STERIS	774L Class III with HVAC	15	2.5	20	2.2	8	
8					15	2.5	20	2.2	13.7	
9*	Stainless Plastic Carpet Paper	500			15	2.5	20	2.2	13.7	
10*	Rubber Aluminum Ceramic Concrete	500			15	2.5	20	2.2	13.7	

*Only four materials tested to allow for increased inoculum.

3.0 Test Procedures

This section provides an overview of the procedures used for the bench-scale evaluation of VPHP to inactivate both pure and crude forms of ricin toxin on eight different materials. Testing was performed in accordance with a peer reviewed and EPA approved Test/Quality Assurance (QA) Plan.

3.1 Ricin Toxins

Testing was conducted with a commercially available form of ricin toxin (Cat. No. L-1090: *Ricin communis* agglutinin II, 5 mg per milliliter [mg/mL] protein concentration, Vector Laboratories, Burlingame, CA), which was stored at 2 to 8 degrees Celsius (°C) and used as received. In addition, a crude preparation of the toxin was extracted from whole castor beans obtained from Vector Laboratories (Vector Laboratories, Inc., Burlingame, CA). The crude version of ricin toxin was prepared in house according to Battelle methods derived from the scientific literature. [(4)] Briefly, the whole castor beans were de-husked and homogenized into a slurry, precipitated from the solution, dialyzed, and rinsed in sterile phosphate buffered saline (PBS). The final crude ricin toxin was prepared in sterile PBS at an approximate concentration of 5 mg/mL, and stored at 2 to 8 °C.

3.2 Test Materials

The test materials used for decontamination testing included aluminum, industrial carpet, ceramic tile, neoprene rubber, optical plastic, paper, stainless steel, and unpainted concrete. Information on these materials is presented in Table 3-1, and a picture of each is presented in Figure 3-1. Material coupons were cut to uniform length and width (Table 3-1) from larger pieces of stock material. Materials were prepared for testing by either sterilization via electron beam (E-beam) irradiation at ~200 kilogray (kGy; E-beam Services Inc., Lebanon, OH) or autoclaving at 121 °C for 15 minutes (min). E-beam-irradiated material coupons were sealed in 6 mil (thousandth of an inch) Uline Poly Tubing (Cat. No. S-2940, Uline, Chicago, IL), and autoclaved coupons were sealed in sterilization pouches (Cat. No. 01-812-50, Fisher, Pittsburgh, PA) to preserve sterility until the coupons were ready for use. Sterilization was intended to minimize contamination by microorganisms that might interfere with the cell-based assay used to assess ricin bioactivity.

Table 3-1. Test Materials

Material	Lot, Batch, or ASTM No., or Observation	Manufacturer/ Supplier Name Location	Approximate Coupon Size, Width x Length x Thickness	Material Preparation
Stainless Steel	Grade 304, gauge 12	Adept Products, West Jefferson, OH	1.9 centimeters (cm) x 7.5 cm x 0.2 cm	Autoclave
Neoprene Rubber	Nonmarking Neoprene Rubber Part # 8837K214	McMaster Carr Aurora, OH	1.9 cm x 7.5 cm x 0.3 cm	E-Beam
Optical Grade Plastic	Optically Clear Cast Acrylic Sheet McMaster Item #8560K263	McMaster Carr Aurora, OH	1.9 cm x 7.5 cm x 0.3 cm	E-Beam
Aluminum	Grade 2024	Adept Products, West Jefferson, OH	1.9 cm x 7.5 cm x 0.2 cm	Autoclave
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
Ceramic Tile	Style Selections White Matte Ceramic Floor Tile Item #: 437485	Lowes, Hilliard, OH	1.9 cm x 7.5 cm x 0.8 cm	Autoclave
Unpainted Concrete	Cut Cinder Block	Lowes, Hilliard, OH	1.9 cm × 7.5 cm x 0.7 cm	Autoclave
Paper	Boise Aspen Laser Paper 24 pounds (lb) Part #BPL-2411-RC	Office Max, Hilliard, OH	1.9 cm x 7.5 cm x 0.3 cm	E-Beam

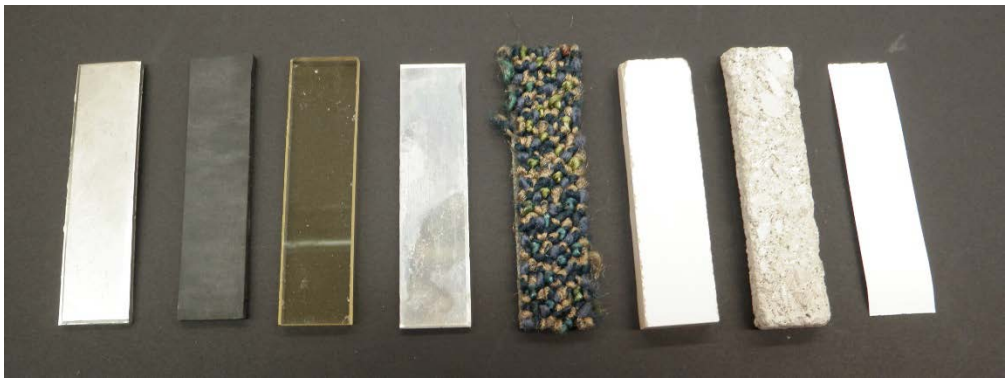

Figure 3-1. Coupon Types from Left to Right: Aluminum, Neoprene Rubber, Optically Clear Acrylic, Stainless Steel, Industrial Carpet, Ceramic Tile, Unpainted Concrete, and Paper

3.3 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 value of approximately 250 or 500 µg of either the purified or crude ricin toxin. Actual delivered mass of toxin per material was determined by cell-based bioassay. A 50 or 100 µL aliquot of a stock suspension of approximately 5 mg/mL was dispensed using a micropipette and applied as a single or double streak across the coupon surface (see Figure 3-2). This approach provided decreased drying times (~1.5 h) and a more uniform

distribution of toxin across the coupon surface than would be obtained through a single drop of the suspension. After inoculation, the coupons were transferred to a Class III BSC and left undisturbed to dry for approximately 1 h (or until visually dry) under ambient conditions, ~22 °C and 40 % RH.

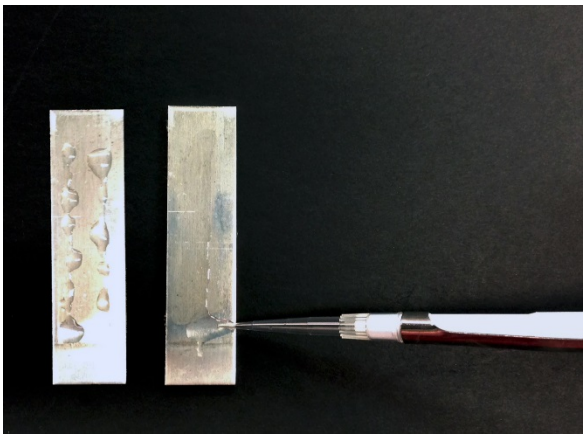

Figure 3-2. Liquid Inoculation of Coupon Using a Micropipette

The number and type of replicate coupons used for each combination of material, decontaminant, concentration, and environmental condition included were:

- Three test coupons (inoculated with ricin toxin and exposed to VPHP)
- Three dry time controls (inoculated with ricin toxin and extracted after 1 h drying time, conducted for test one only)
- Three positive controls (inoculated with ricin toxin but not exposed to VPHP, stored at ambient conditions)
- One laboratory blank (not inoculated and not exposed to VPHP)
- One procedural blank (not inoculated and exposed to VPHP).

Approximately 1 h post inoculation (or until materials were visually dry), coupons intended for decontamination (including blanks) were transferred into the test chamber and exposed to the VPHP fumigant using the apparatus and application conditions specified in Section 3.4. Control coupons were added to the control chamber as described in Section 3.4.

3.4 Fumigation Description and Procedures

Figure 3-3 shows a schematic of the VPHP test chamber and vapor generating system. Vapor phase hydrogen peroxide decontamination testing was conducted within a test chamber comprised of a 774 L Class III BSC (The Baker Company, Sanford, ME) that was hard-ducted to the facility heating, ventilation, and air conditioning (HVAC) filtered exhaust system. The VPHP test chamber was modified with sensors capable of monitoring temperature, RH and VPHP concentration. A low-speed fan was placed inside the test chamber to ensure a homogeneous distribution of VPHP throughout. The STERIS and Bioquell generators were utilized based on availability and performance throughout testing. The VPHP generators were connected to the test chamber, and the

hydrogen peroxide delivered via supply and return hoses. In addition to each generator having internal high efficiency particulate air (HEPA) filters, external in-line HEPA filters were used to maintain containment and eliminate any potential contamination of the two technologies.

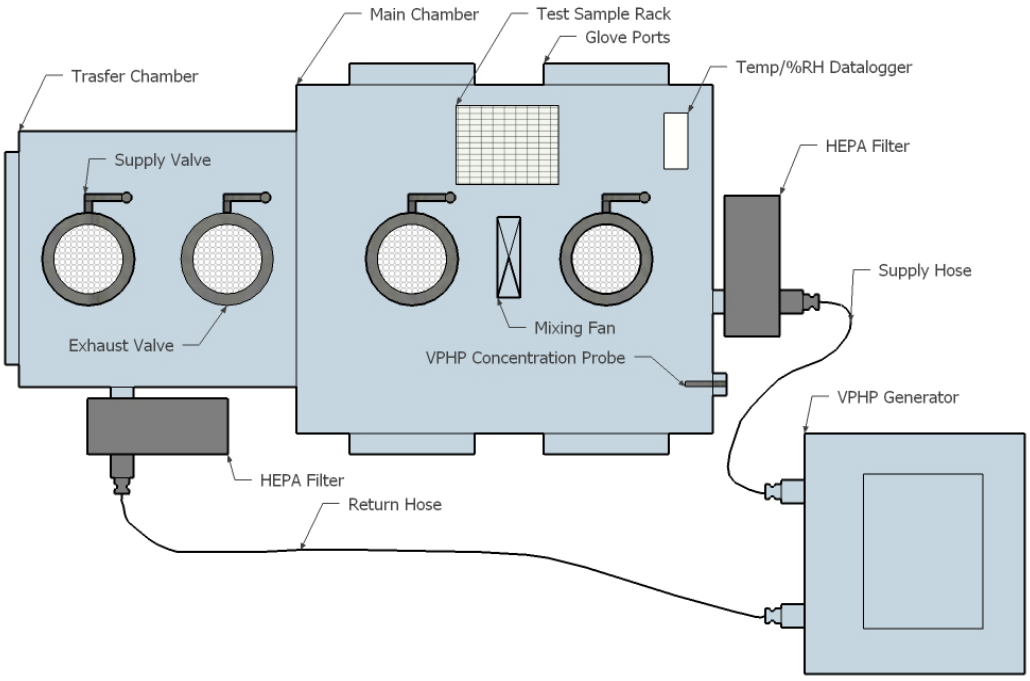

Figure 3-3. Aerial Schematic of VPHP Test Chamber and Attached Fumigant Generator

Temperature and RH were monitored and recorded every minute within the VPHP test chamber using a U14 HOBO data logger (Cat. No. U12-12, Onset Corp., Bourne, MA), the hydrogen peroxide concentrations were monitored using an ATI B-12 wet gas transmitter (Cat. No. B12-34-8-2000-1, Analytical Technology Inc., Collegeville, PA), and the data were recorded by a UX120 HOBO data logger (Cat. No. UX120-006M, Onset Corp., Bourne, MA). During each test, inoculated test samples were placed inside the VPHP test chamber and the chamber was sealed. The test samples were allowed to dry for approximately 1 h (or until visually dry). Once dry, the controls were removed by placing samples into a 9 L Lock & Lock® airtight control chamber (Cat. No. HPL838 Lock & Lock, Farmers Branch, TX) and removed from the VPHP test chamber and placed into a Class II BSC for the remainder of the test. Once the control samples were moved, the predetermined decontamination cycle was performed. As previously stated, each technology has four similar phases in common but differs in regards to the endpoint either being vapor phase or microcondensation mode of decontamination.

Figure 3-4 shows a representative graph of both a STERIS 1000ED and Bioquell Clarus C decontamination run.

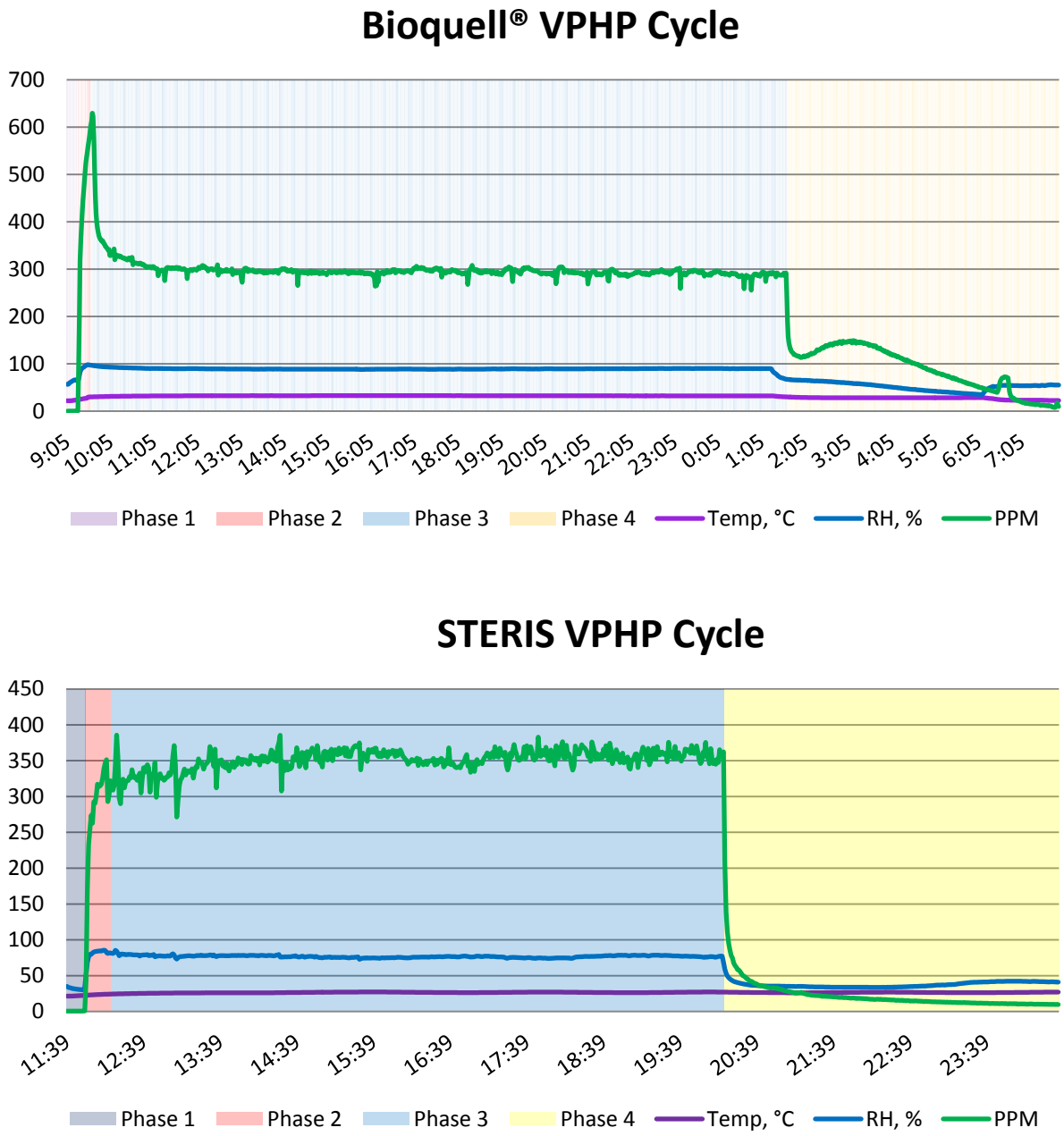

Figure 3-4. Representative Graph of Bioquell and STERIS Decontamination Cycles

The VPHP test chamber heating, ventilation and air conditioning (HVAC) exhaust was utilized during aeration to speed up the process (Phase 4). The test chamber was allowed to aerate until the VPHP levels in the chamber reached ≤10 ppm. At this time, the samples were removed and processed as described in Section 3.5.

The control samples were held inside a 9 L Lock & Lock® airtight container at ambient laboratory conditions for the duration of the experiment. The temperature and RH were not controlled within this control chamber. The temperature and RH of the control chamber were measured and data logged using a HOBO® data logger model U12 (Cat. No U12-11, Onset Corp., Bourne, MA).

As in previous studies,[2,5] multiple coupons of each material were inoculated with the ricin toxin and placed on a wire rack inside the VPHP test chamber. Blank (i.e., not inoculated) and positive control (i.e., inoculated but not decontaminated) samples were also prepared for each material and were utilized with data from the test samples (inoculated and decontaminated) to determine decontamination efficacy.

Ten VPHP decontamination tests were conducted at predetermined cycles as shown in Table 2-1. Phase 1 was consistent throughout testing for both technologies at 15 min. Contact times and injection rates for Bioquell Phase 2 were 3.8 g/min and 20 minutes. STERIS Phase 2 parameters ranged from 3.8 to 2.5 g/min at 20 min. The Bioquell Phase 3 injection rate was 1.0 g/min while the contact time ranged from 4 to 6 h. The STERIS Phase 3 injection rate varied from 1.0 to 2.2 g/min and contact times ranged from 30 min to 13.7 h. Phase 4 parameters were the same for all tests at ≤ 10 ppm VPHP. The change in injection rates for the STERIS generator was due to three failed attempts at Test 3 (data not reported) in which the lower published injection rate limit for the STERIS unit of 1.0 g/min prompted injection rate failure alarms. When in alarm mode, the STERIS generator automatically aborted the cycle and initiated aeration mode (Phase 4). The Bioquell generator was utilized for Tests 3 to 6 while the STERIS generator was used for Tests 1, 2, and 7 to 10.

3.5 Coupon Extraction and Ricin Toxin Quantification

After decontamination, test coupons, positive controls, and blanks were individually placed in 50 mL polypropylene conical tubes containing 10 mL of sterile PBS for 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. Residual active toxin in the test and control coupon extracts was determined using the bioassay approach described below.

The mechanism of action by which ricin toxin exerts its toxic effect is through inhibition of protein synthesis within cells. Such inhibition of protein production leads to cell death. Therefore, an *in vitro* cytotoxicity assay was used to evaluate the level of bioactive ricin toxin extracted from both decontaminated and 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.[4] Cytotoxicity is reported as mass of bioactive toxin as determined using a reference standard prepared from a purified form of ricin toxin.

To conduct this assay, Vero cells (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 various coupon extracts (test, positive controls and blank controls) by adding 100 µL of extract or dilution to each well. Following 48 to 72 h exposure to 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 SPECTRAmax PLUS^{384} microplate reader (Molecular Devices, Sunnyvale, CA), is directly proportional to the number of living cells and inversely proportional to the cytotoxic potential of ricin toxin (Figures 3-5 and 3-6). For all dilutions and sample transfers into the individual wells of a 96-well plate, a micropipette was used in which the pipette tip was replaced between wells to ensure that cross contamination did not occur.

To determine the concentration of ricin toxin from each test sample, a ricin toxin standard (purified, Vector Laboratories, Inc., Burlingame, CA) was prepared from the commercial purchased stock solution and assayed in parallel on each test plate. The ricin toxin stock solution (purified) was used to prepare a seven-point standard curve of absorbance versus calculated mass of 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 nanograms (ng)/mL, and a four-parameter logistic (4-PL) curve was generated by the SoftMax Pro Version 4.7 software included in the SPECTRAmax microplate (Molecular Devices, Sunnyvale, CA) reader using the equation:

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

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

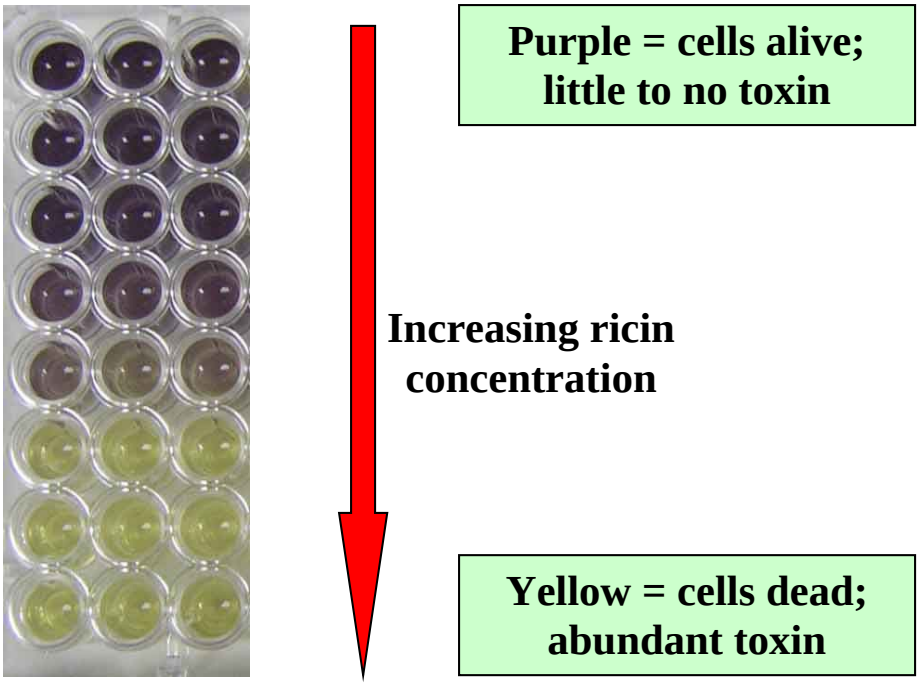

Figure 3-5. Visual Demonstration of MTT Assay on a Microplate

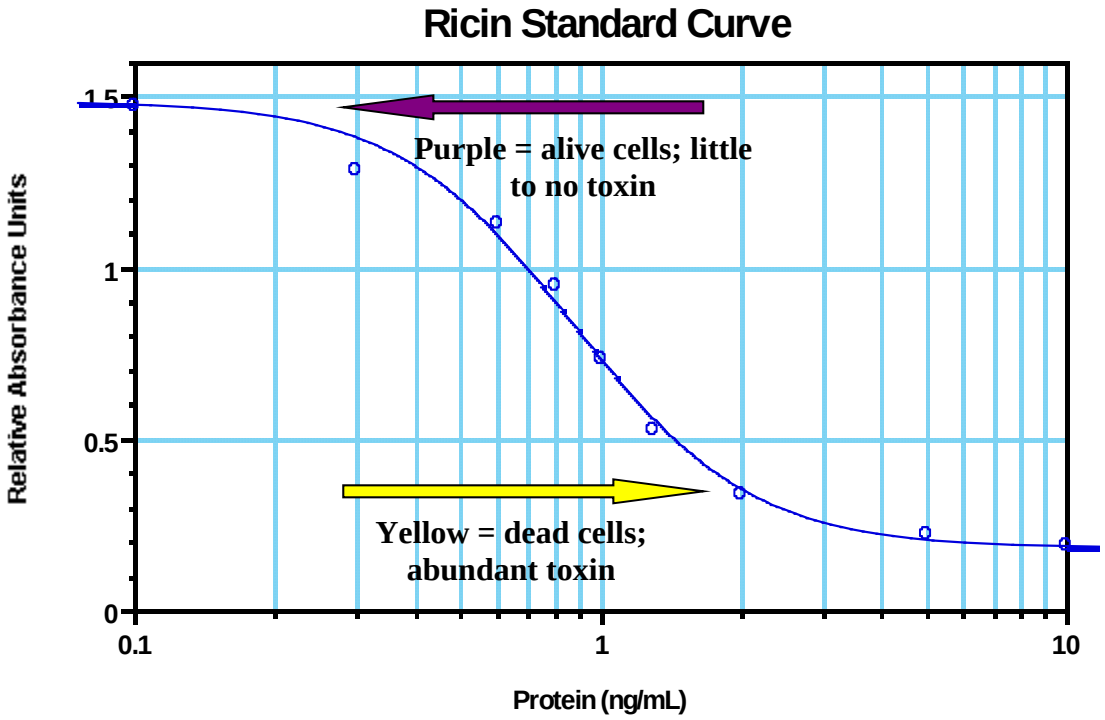

Figure 3-6. Example of Ricin Cytotoxic Profile with Corresponding Absorbance Measured Using a Microplate Reader

Throughout the study, the inherent cytotoxicity of coupon extracts from laboratory and procedural blank coupons was assessed to determine a starting dilution that would 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) were selected as the starting dilution for all test samples. The dilution scheme effectively baselined the cytotoxicity of the test coupons (see Table 3-2).

Table 3-2. Average Dilution Factors per Coupon Material

Material	Dilution Factors Required to "Zero Out" Coupon Cytotoxicity
Stainless Steel	1:35
Neoprene Rubber	1:23
Optical Grade Plastic	1:6
Aluminum	1:10
Carpet	1:166
Ceramic Tile	1:27
Unpainted Concrete	1:58
Paper	1:34

3.6 Decontamination Efficacy

The performance, or efficacy, of VPHP was assessed by determining the mass of bioactive toxin extracted from each test coupon after decontamination compared to the average mass of bioactive toxin extracted from the positive control coupons.

Efficacy (% reduction) of a decontaminant for a test toxin/test condition on the i^{th} coupon material was calculated as the difference between the mean control mass values and the individual test mass values, i.e.:

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

where $Mass_{ci}$ refers to the *j* individual mass values obtained from the positive control coupons, $Mass_{ti}$ refers to the *j* individual mass values obtained from the corresponding test coupons, and the overbar designates a mean value. In tests conducted under this plan, there were three positive controls and three corresponding test coupons (*i.e.*, $j = 3$) for each coupon.

In samples where no bioactive toxin was observed in any of the three test coupon extracts after decontamination, an adjusted limit of detection (LOD) value for that material was assigned. The adjusted LOD was defined as mass of ricin toxin that corresponded to the lowest dilution factor

in the standard curve. The assigning of adjusted LOD values for the test samples occurred when the decontaminant was highly effective, and diluted sample values were below the linear range of the 4-PL standard curve. In such cases, the final efficacy (adjusted LOD) for that material was calculated by multiplying the lowest value assayed in the standard curve by the lowest dilution factor for each test material based on coupon extract cytotoxicity limits (determined as described in Section 3.5). The resultant ricin mass values were reported as greater than or equal to (≥) the adjusted LOD.

The variance of the mean percent reduction can be estimated using the 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 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 both the control and test data sets. Each efficacy result is reported as a mass value with an associated 95 % confidence interval (CI), calculated as follows:

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

Significant differences in efficacy for the different test conditions and toxin types were assessed using the 95 % CI of each efficacy result. Differences in efficacy were judged to be significant if the 95 % CIs of the two efficacy results did not overlap. Any results based on this formula are hereafter noted as significantly different. Note this comparison is not applicable when the two efficacy results being compared are both reported with MASS as ≥ LOD.

3.7 Surface Damage

The physical effect of VPHP on the materials was qualitatively monitored during the evaluation. This approach provided a gross visual assessment of whether the decontaminant altered the appearance of the test materials. The procedural blank (coupon that is decontaminated, but has no toxin applied) was visually compared to a laboratory blank coupon (a coupon not exposed to the decontaminant and having no toxin applied). Obvious visible damage might include structural damage, surface degradation, discoloration, or other aesthetic impacts.

4.0 Quality Assurance/Quality Control

QA/quality control (QC) procedures were performed in accordance with the Technology Testing and Evaluation (T&E) Program Quality Management Plan (QMP) and the test/QA plan. The QA/QC procedures and results are summarized below.

4.1 Equipment Calibration

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

4.2 QC Results

QC efforts conducted during decontaminant testing included dry time control samples (inoculated, dried for ~1.5 h, and not decontaminated), procedural blanks (not inoculated, decontaminated), laboratory blanks (not inoculated, not decontaminated), and inoculation control samples (analysis of the stock toxin suspension).

Dry time control samples were run once during Test 1 to determine the loss of cytotoxicity over the ~1.5 h drying period. Percent recoveries ranged from 1.02 to 1.77 %. The amount of ricin recovered from these controls was sufficient to determine % reduction due to the cytotoxicity assay standard range of 0.1 to 10 ng. Outlier tests were not performed as this test was conducted only once.

All procedural and laboratory blanks met the acceptance criterion by the use of 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 standard curve. Using a Grubbs outlier test, the inoculation control samples were assessed and no outliers were found for target inoculum levels of 250 µg. The increased inoculum target of 500 µg was not assessable via this test as six replicates are required and only three tests were conducted with 500 µg inoculum.

4.3 Audits

4.3.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during these experiments. Table 4-1 summarizes the performance evaluation audits that were performed.

Table 4-1. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume of liquid from micropipettes	Gravimetric evaluation	± 10 %	± 0.00 % to 7.63 %
Time	Compared to independent clock	± 2 seconds/hour	0 seconds/hour
Temperature	Compared to independent calibrated thermometer	± 2 °C	± 0 to 0.3 °C
Relative Humidity	Compare to independent calibrated hygrometer	± 10 %	± 1 %

4.3.2 Technical Systems Audit

Observations and findings from technical systems audits (TSAs) were documented and submitted to the laboratory technical lead for response. TSAs were conducted on May 29, June 10, and June 20, 2014 to ensure that tests were being conducted in accordance with the appropriate test/QA plan and QMP. As part of the audit, test procedures were compared to those specified in the test/QA plan and data acquisition and handling procedures were reviewed. One deviation was noted during the TSA.

4.3.3 Deviations

A deviation was prepared to address the finding in which no sterile filtered water (SFW) was inoculated onto the blank coupons. In practice, this inoculation of coupons with diluent only had been eliminated from previous work assignments, and its inclusion was an oversight in test/QA plan preparation. An additional deviation from the test/QA plan included a change to the Phase 4 stopping point from ≤1 ppm to ≤10 ppm. This change had little effect on overall testing and enabled sample processing during normal business hours.

4.3.4 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 data transcription errors that were corrected.

4.4 QA/QC Reporting

Each assessment and audit were documented in accordance with the test/QA plan and QMP. For these tests, findings were noted (none significant) in the data quality audit, and no followup corrective action was necessary. The findings were mostly minor data transcription errors requiring some recalculation of efficacy results, but none were gross errors in recording.

4.5 Data Review

Records and data generated in the evaluation received a QC/technical review before they were utilized in calculating or evaluating results and prior to incorporation in this report.

5.0 Summary of Results and Discussion

The decontamination efficacy of two VPHP generators was evaluated against purified and crude ricin toxin inoculated onto porous and nonporous material coupons. For the ten tests in this evaluation, the decontamination cycles varied Phase 2 injection rates from 2.5 to 3.8 g/min for 20 min and varied Phase 3 injection rates from 2.5 to 3.8 g/min from 30 min to 16 h contact times. The STERIS VPHP cycle achieved a lower RH throughout the decontamination cycle, which enabled the hydrogen peroxide to remain in the vapor phase for the entire decontamination cycle. Decontamination runs using the Bioquell unit exhibited a much higher RH, which resulted in the formation of micro condensation. This deposition of hydrogen peroxide on all surfaces within the VPHP test chamber reduced the level of hydrogen peroxide measured in the vapor phase. Thus, the actual hydrogen peroxide concentrations deposited onto the surface were assumed to be higher than in the vapor phase, but were not measured. During the Bioquell aeration phase, a second increase of VPHP was observed as a result of the condensed hydrogen peroxide evaporating off the surfaces VPHP test chamber and returning to the vapor phase. In a larger chamber or room setting, size, complexity of floor plan, and material compatibility must be considered and chamber specific cycle parameters must be developed.

Testing two VPHP technologies was not the original intent of the study. However, due to repeated unforeseen system failures of the STERIS generator at the lowest published injection rate of 1.0 g/min (injection rate failure), it was necessary to utilize a second technology (Bioquell) to complete testing. Mitigation of this failure required the STERIS injection rates to be adjusted from 3.8 and 1.0 g/min in a sealed VPHP test chamber, to 2.5 and 2.2 g/min, respectively, and the exhaust connections slightly opened to allow for a dilution effect. The dilution resulted in obtaining the targeted 400 ppm at a higher Phase 3 injection rate, allowing the STERIS generator to be used again starting with Test 7.

VPHP appears to be an effective decontaminant against pure and crude forms of ricin toxin utilizing the STERIS 1000ED at a targeted 400 ppm for 14 h of hydrogen peroxide injection (Phases 2 and 3, Tests 9-10) as well as with the Bioquell Clarus C, targeting microcondensation for 8 or 16 h (Tests 4-6). In some cases the test temperature/RH was notably higher than the control chamber. Additional testing is needed to study the effect of increased temperature and RH on the inactivation of ricin toxin in the absence of VPHP. Additional testing is also needed to confirm these data at higher inoculum levels, as only three tests were completed using a targeted 500 µg inoculation quantity. Further testing is also needed to confirm the data presented here as well as to test additional surface materials and combinations of cycle parameters including lower concentrations of VPHP and longer contact times to potentially address application challenges when needed in larger area applications.

5.1 Operational Parameters

The temperature, RH, and VPHP concentrations during each test were controlled by each respective generator technology, as described in Section 3.0. These VPHP generating technologies were set to the target injection rates and contact times and initiated upon test sample readiness. Readings were

taken once every minute for the duration of each test. The actual operational parameters for each test are shown in Table 5-1 and reported as the average value ± standard deviation (SD).

Table 5-1. Actual Fumigation Conditions for VPHP Tests

Test Number	Technology	VPHP Concentration (ppm)		Temperature (°C)[a]		RH (%)[a]		Phase 4 Time (h:min)
		Target	Actual*	Fumigation Actual*	Control Actual*	Fumigation Actual*	Control Actual*	
1	STERIS	400	480±175	25.1±3.75	20.3±0.50	26.6±19.2	30.7±1.61	17:41
2	STERIS	400	414±108	27.2±2.97	22.3±0.23	28.3±23.3	57.0±1.18	16:00
3	Bioquell	NA[†]	310±58.9	29.8±1.83	22.0±0.24	45.7±30.7	59.7±0.36	13:22
4	Bioquell	NA[†]	279±45.0	30.4±2.67	21.7±0.13	82.2±9.87	60.5±0.41	11:41
5	Bioquell	NA[†]	301±37.8	30.7±2.87	20.8±0.28	78.6±17.5	58.5±0.37	6:26
6	Bioquell	NA[†]	240±40.3	29.8±3.51	21.6±0.69	70.3±24.1	53.9±1.54	7:42
7	STERIS	400	349±23.9	25.7±1.89	22.3±0.54	56.1±17.6	57.9±1.08	10:47
8	STERIS	400	387±21.7	25.5±1.78	22.4±0.53	62.6±9.01	53.5±1.17	9:09
9	STERIS	400	398±44.2	25.4±1.20	22.5±0.47	65.8±16.4	53.2±1.36	10:09
10	STERIS	400	392±18.5	25.1±1.68	21.8±0.41	70.3±13.6	56.6±1.01	4:47

* Data reported as average ± SD.

[†] Bioquell technology targets micro condensation in lieu of ppm.

[a] No defined temperatures or RH were targeted.

Table 5-2 shows the operational parameters required to achieve >99 % reduction on all material types tested (aluminum, industrial carpet, ceramic tile, neoprene rubber, optical plastic, paper, and stainless steel) except unpainted concrete (Tests 4-6, 9-10). Data for unpainted concrete were not included in the summarized results due to little to no recoverable ricin toxin from positive control samples. Although not evaluated, the caustic nature of this material may have affected the bioactivity of the ricin. Actual operational parameters as measured were well within acceptable ranges and are detailed above. The detailed decontamination efficacy results are provided in Appendix A. As seen in Table 5-2, a Phase 3 contact time of 8 or 16 h was required to achieve >99 % reduction of pure and crude ricin with the Bioquell Clarus C VPHP generator using an inoculum of ~250 µg. A Phase 3 contact time of 16 h was required to achieve >99 % reduction when the amount of inoculum was increased to ~500 µg, but this contact time was only tested on industrial carpet, optical plastic, paper, and stainless steel. These test materials were down selected to represent porous and non-porous surfaces. STERIS 1000ED required a 13 h 40 min Phase 3 time with a target of 400 ppm to achieve >99 % reduction on materials for the crude and pure form of ricin at an inoculum of ~500 µg.

Table 5-2. Parameters Required to Achieve >99 % Reduction on All Materials

Technology	Ricin Form/Target Mass µg	Avg VPHP ppm±SD[b,c]	Phase 1	Phase 2		Phase 3		Phase 4
			Duration (min)	Injection Rate (g/m)	Duration (min)	Injection Rate (g/m)	Duration (h:min)	Duration (h:min)
Bioquell	Pure/250	279±45.0	15	3.8	20	1.0	8:00	11:41
Bioquell	Crude/250	301±37.8	15	3.8	20	1.0	16:00	6:26
Bioquell*	Pure/500	240±40.3	15	3.8	20	1.0	16:00	7:42
STERIS*	Pure/500	398±44.2	15	2.5	20	2.2	13:40	10:09
STERIS*	Crude/500	398±44.2	15	2.5	20	2.2	13:40	10:09
STERIS†	Crude/500	392±18.5	15	2.5	20	2.2	13:40	4:47

* Materials tested were limited to industrial carpet, optical plastic, paper, and stainless steel.

† Materials tested were limited to neoprene rubber, aluminum, ceramic tile, and unpainted concrete.

[a] Detailed data from each test number can be referenced in Appendix A.

[b] Concentration of hydrogen peroxide measured in the vapor phase during Phases 2 and 3.

5.2 Efficacy Comparison of Ricin Forms

Results comparing the average percent reduction ± SD for the pure and crude ricin are shown in Figure 5-1 and Table 5-3. These results are averages for all tests conducted using both VPHP technologies, different inoculum amounts, and various testing conditions. In general, the results in Table 5-3 show that little difference exists when comparing crude to pure ricin with percent reduction ranging from 73.8 to 98.1 % (excluding concrete) for crude ricin, and 90.1 to 97.9 % (excluding concrete) for pure ricin. When compared to the crude ricin, the pure ricin on neoprene rubber, optical plastic, industrial carpet, and paper exhibited an average difference in efficacy ranging from -7.44 to -16.3 percent. In contrast, on stainless steel, aluminium, and ceramic tile, the crude ricin was less resistant to VPHP than pure ricin with average differences ranging from 0.10 to 1.03 percent. A positive result indicates that the crude ricin was inactivated to a higher degree (less resistant) than pure ricin.

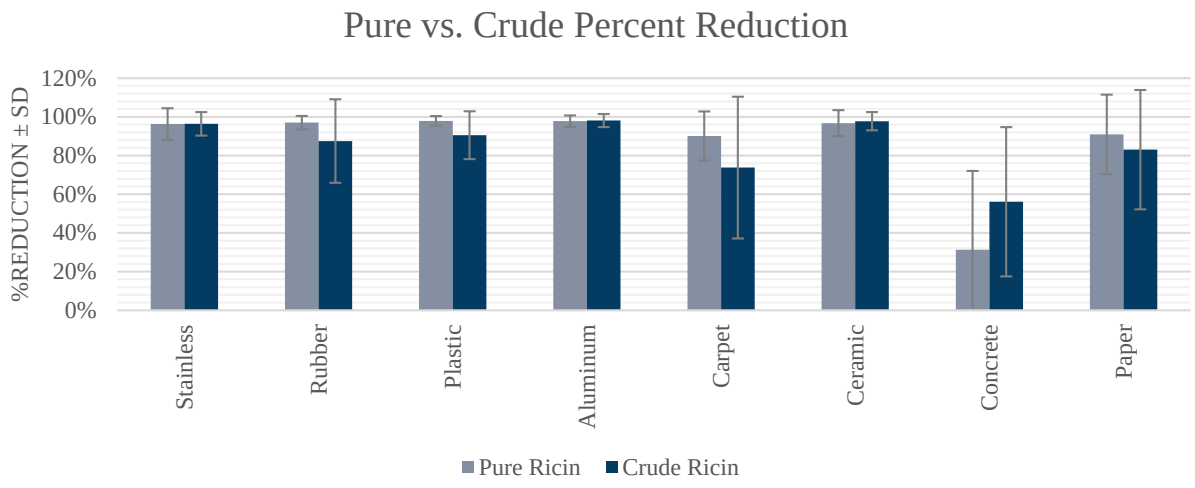

Figure 5-1. Summary of Average Percent Reduction between Pure Ricin and Crude Ricin per Material Type ± Standard Deviation

Table 5-3. Summary of Average Percent Reduction between Pure Ricin and Crude Ricin per Material ± 95% Confidence Interval Type

Material Type	Average Percent Reduction[†]		Difference (%)[*]
	Pure Ricin (%)	Crude Ricin (%)	
Stainless Steel	96.29 ± 8.19	96.40 ± 6.05	+0.10
Neoprene Rubber	97.02 ± 3.50	87.49 ± 21.6	-9.53
Optical Plastic	97.92 ± 2.49	90.48 ± 12.4	-7.44
Aluminum	97.77 ± 2.99	98.10 ± 3.40	+0.33
Industrial Carpet	90.13 ± 12.7	73.79 ± 36.6	-16.34
Ceramic Tile	96.71 ± 6.75	97.75 ± 4.73	+1.03
Unpainted Concrete	31.26 ± 40.7	56.08 ± 38.6	+24.82
Paper	90.92 ± 20.6	83.03 ± 30.9	-7.88

[*]Results shown as difference in average efficacy (percent reduction) ± standard deviation. A positive result indicates that the crude ricin was inactivated to a higher degree than pure ricin.

[†]Averaged performed across all 10 tests.

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5.3 Effects of STERIS VPHP efficacy for Pure and Crude Ricin

Results comparing the average percent reduction for the pure and crude ricin tested using the STERIS 1000ED are shown in Figure 5-2. These results are averages including all tests performed using the STERIS generator, different inoculum amounts and various testing conditions. Although some significant differences between crude and pure ricin are shown in Figures 5-3 to 5-5 for individual tests, the averages in Figure 5-2 show there is little to no difference in decontamination efficacy when comparing the crude and pure forms of ricin when decontaminated with the STERIS 1000ED.

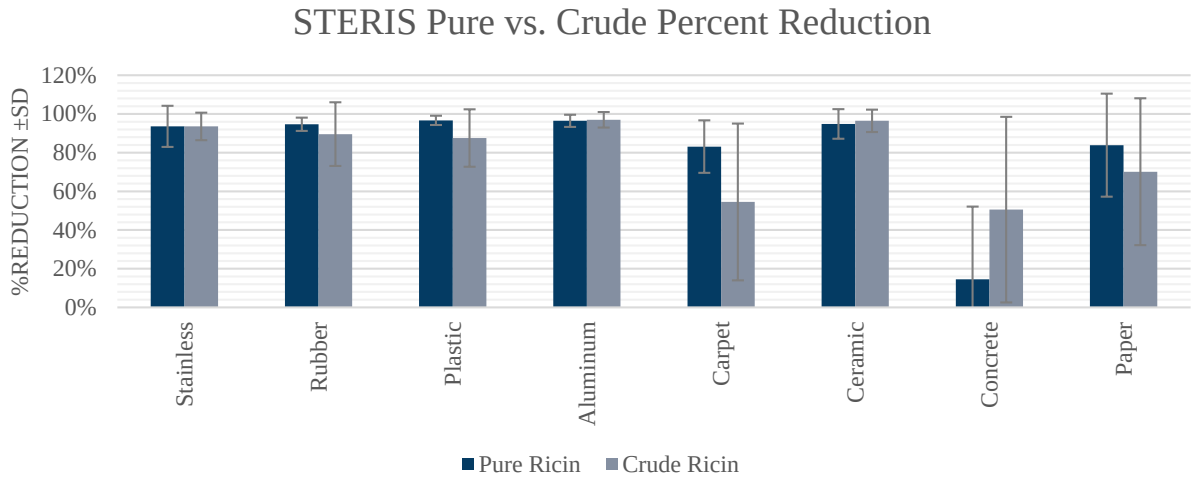

Figure 5-2. Summary of Average Percent Reduction for STERIS 1000ED VPHP Generator between Pure Ricin and Crude Ricin per Material Type ± Standard Deviation

The percent reduction results by material, for each test, are shown in Figures 5-3 through 5-5. Differences in efficacy between the two ricin forms on a material are significant if the 95 % CIs of the two efficacy results do not overlap. The STERIS 1000ED, when testing at suboptimal conditions with crude ricin, was more difficult to inactivate on plastic, carpet, and paper shown in Figure 5-3

(Test 1 and 2). When Phase 2 contact times were increased for Tests 8 and 9 to 13 h 40 min, and the inoculum was increased to target of 500 µg (excluding concrete), a >99 percent reduction was achieved on all materials inoculated with crude ricin and all materials inoculated with pure ricin except industrial carpet (98.7 percent reduction). Detailed values for the decontamination efficacy results are provided in Appendix A.

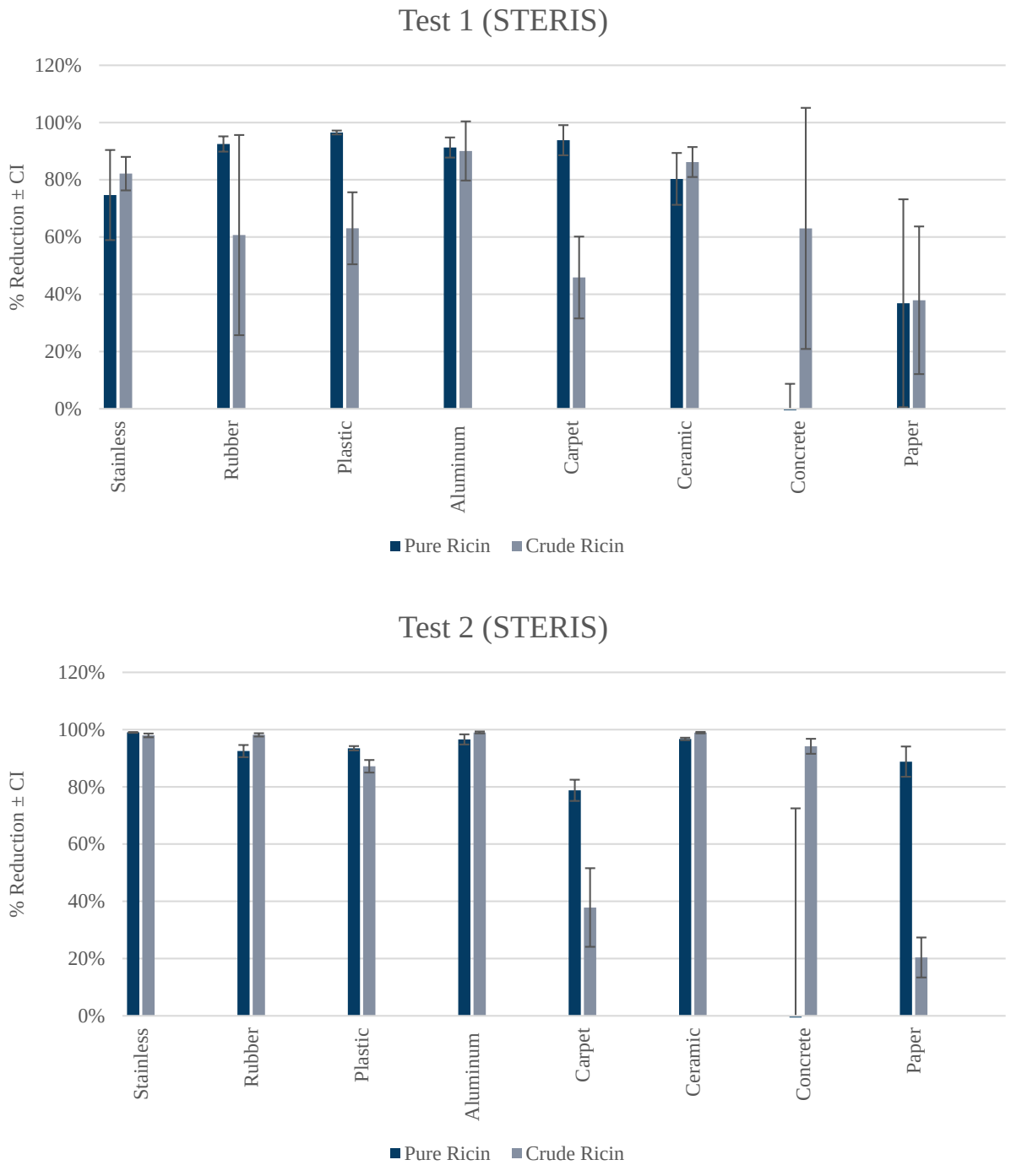

Figure 5-3. Summary of VPHP Efficacy (Tests 1 and 2) Results, by Material, Comparing Pure and Crude Ricin ± 95% Confidence Interval

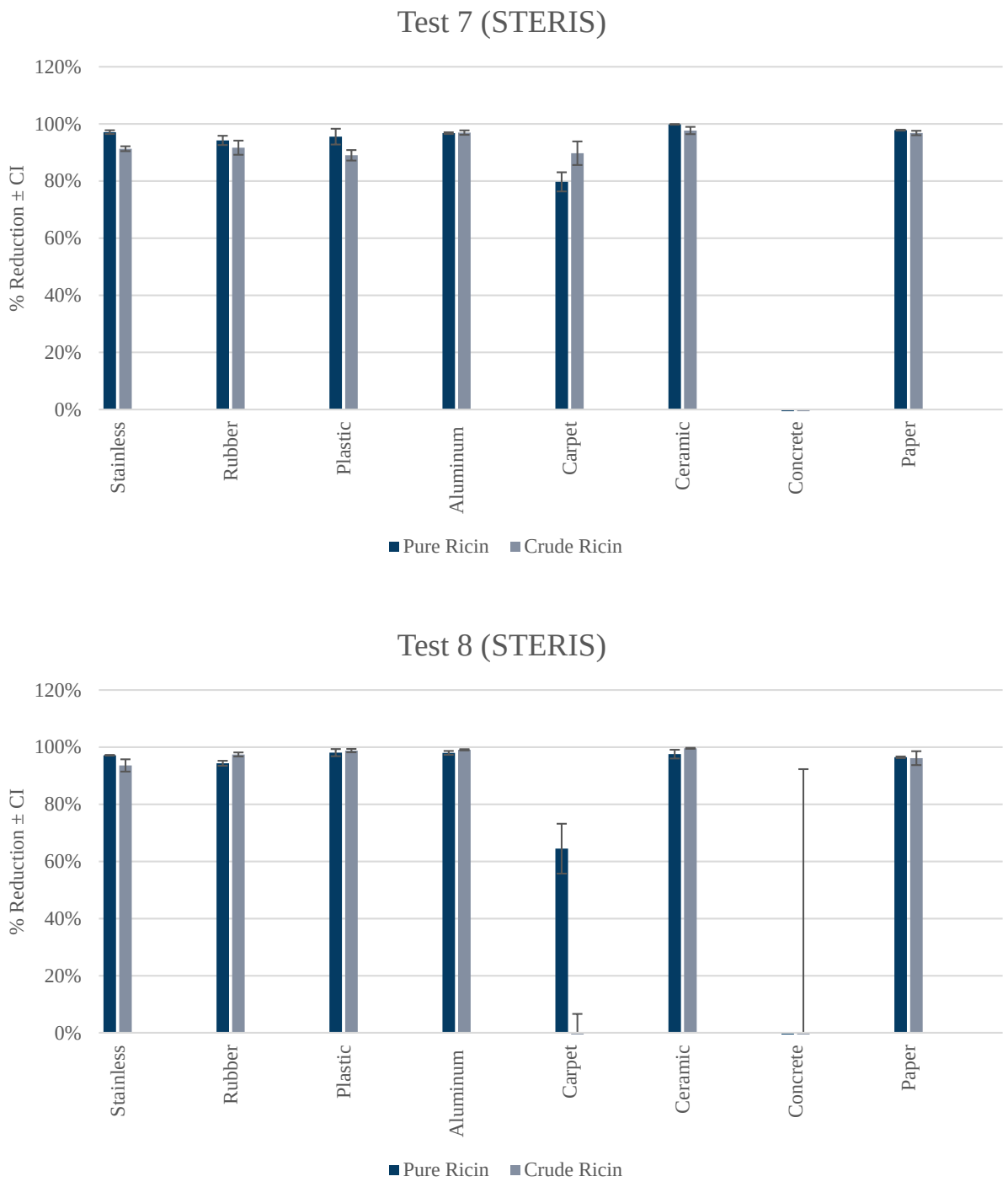

Figure 5-4. Summary of VPHP Efficacy (Tests 7 and 8) Results, by Material, Comparing Pure and Crude Ricin ± 95% Confidence Interval

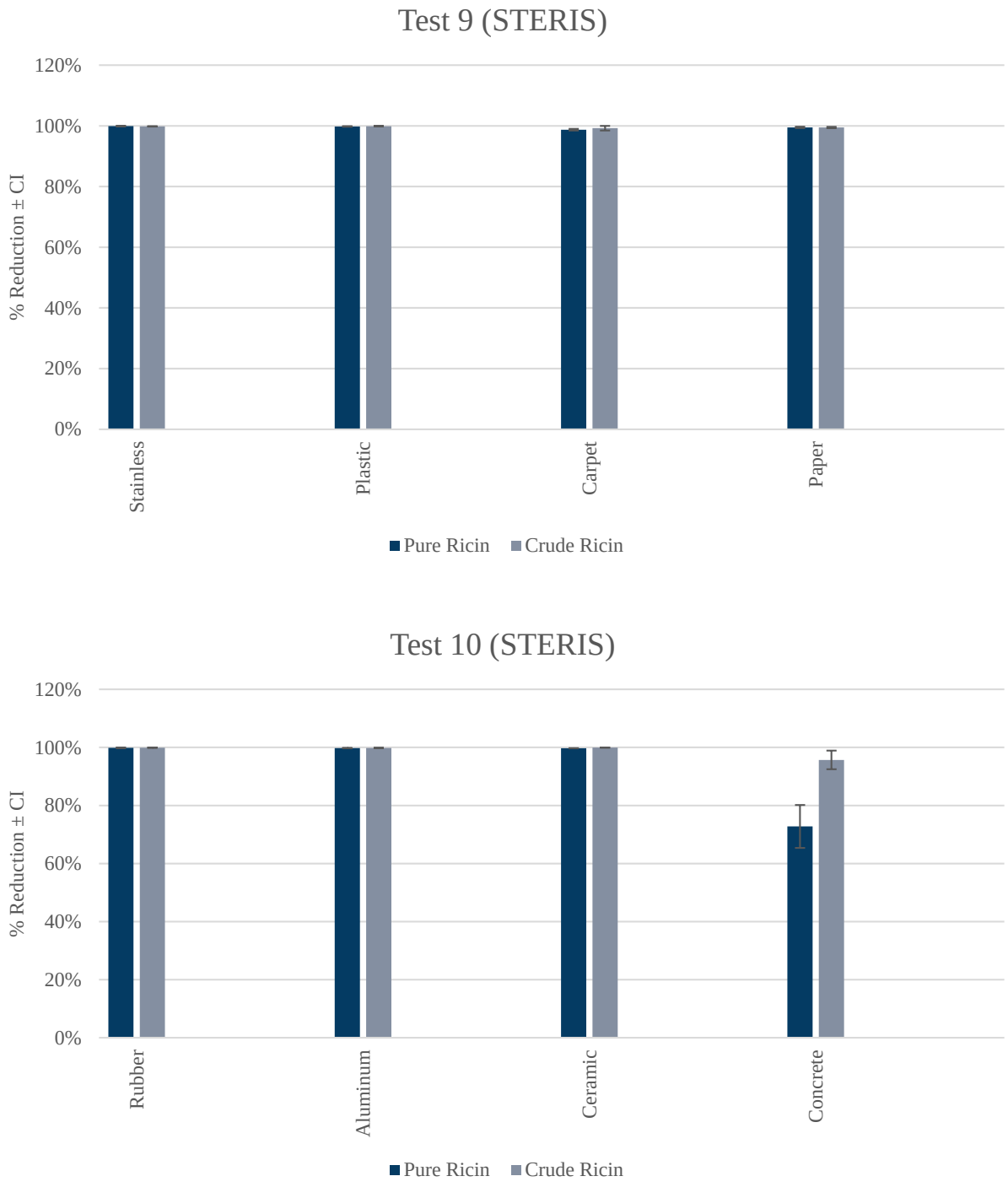

Figure 5-5. Summary of VPHP Efficacy (Tests 9 and 10) Results, by Material, Comparing Pure and Crude Ricin ± 95% Confidence Interval

5.4 Effects of Bioquell VPHP Efficacy for Pure and Crude Ricin

Results comparing the average percent reduction for the pure and crude ricin tested against the Bioquell Clarus C are shown in Figure 5-6. These results are averages including all tests performed using the Bioquell generator, different inoculum amounts and various testing conditions. Although some significant differences between crude and pure ricin are shown in Figures 5-7 to 5-8, the averages in Figure 5-6 show there is little to no difference in decontamination efficacy when comparing the crude and pure forms of ricin when decontaminated with the Bioquell Clarus C.

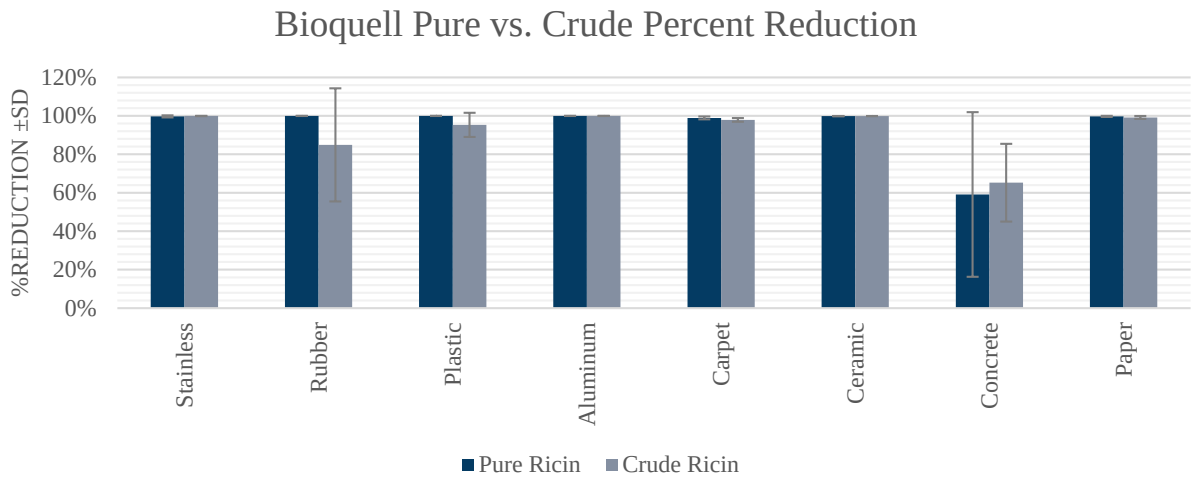

Figure 5-6. Summary of Average Percent Reduction for Bioquell Clarus C VPHP Generator between Pure Ricin and Crude Ricin per Material Type ± Standard Deviation

The percent reduction results by material, for each test, are shown in Figures 5-7 and 5-8. Differences in efficacy between two ricin forms on a material are significant if the 95 % CIs of the two efficacy results do not overlap. When testing the Bioquell Clarus C, crude ricin was more difficult to inactivate on rubber and plastic with a Phase 2 contact time of 4 hours (Test 3; Figure 5-7). When Phase 2 contact times were increased to 8 h (Test 4; Figure 5-7), >99 percent inactivation of pure ricin was achieved on all materials (except concrete) and all materials for crude ricin except paper that exhibited a 98.1 percent reduction. Test 5 increased Phase 2 duration to 16 hours and (excluding concrete) resulted in >99 percent reduction of crude ricin on all materials, while having a slightly reduced efficacy for pure ricin on stainless and carpet (98.9 and 98.8, respectively). When the ricin inoculum was increased to ~500 µg (Test 6; Figure 5-8), >99 percent reduction was achieved for pure ricin on the limited number of materials tested (stainless steel, rubber, carpet, and paper) and crude ricin for all materials except carpet that achieved 97.1 percent reduction. Detailed values for the decontamination efficacy results are provided in Appendix A.

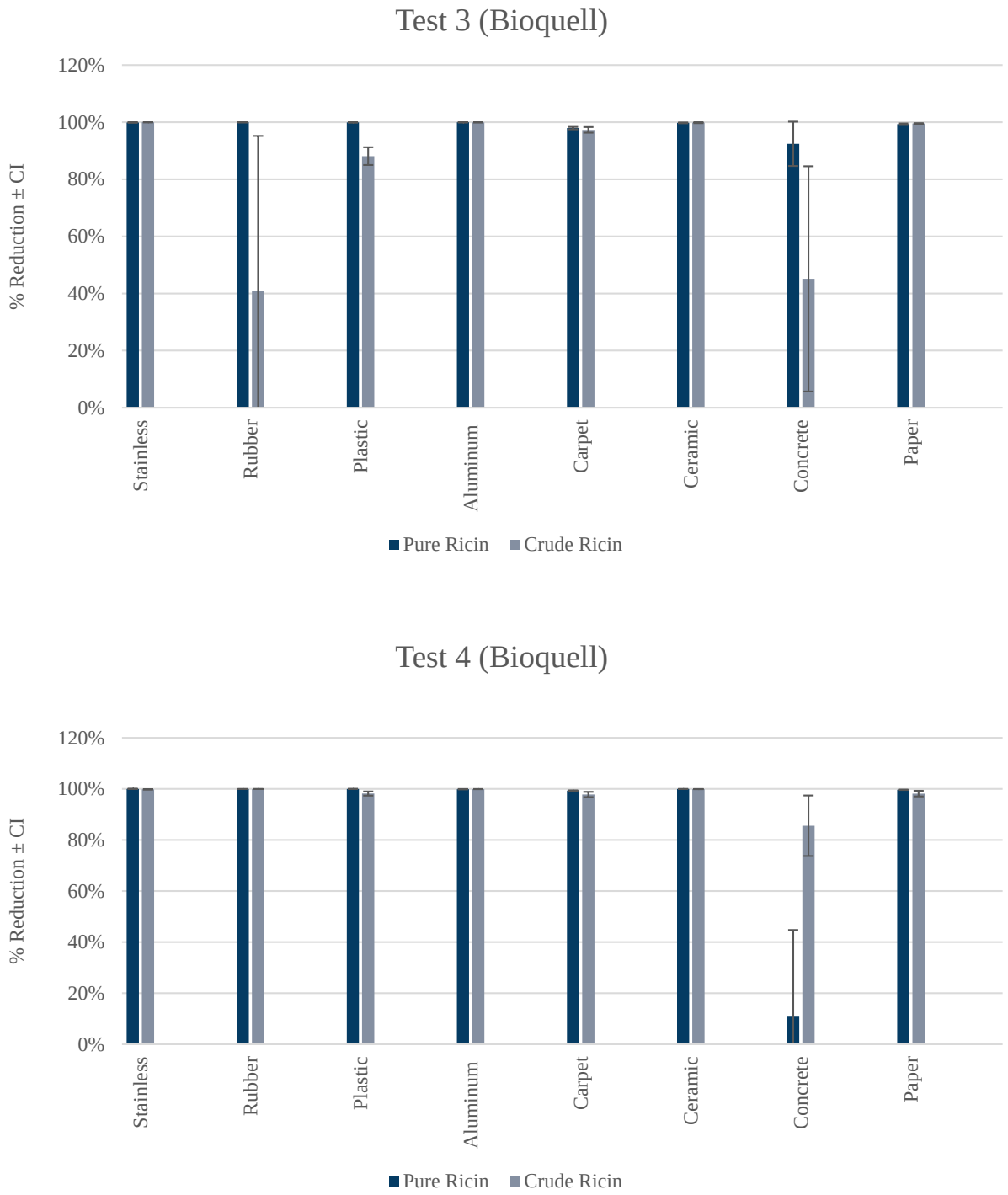

Figure 5-7 Summary of VPHP Efficacy (Tests 3 and 4) Results, by Material, Comparing Pure and Crude Ricin ± 95% Confidence Interval

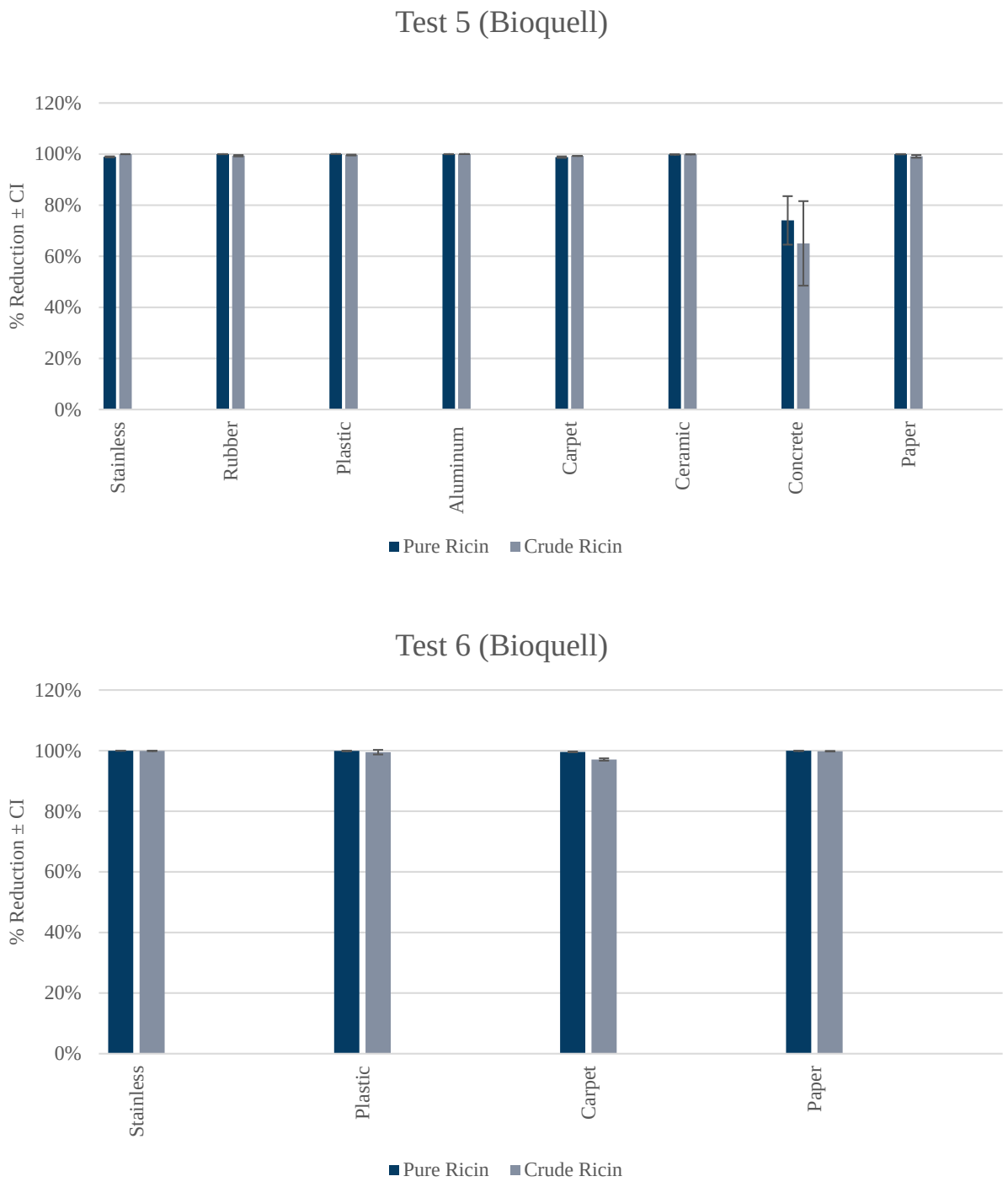

Figure 5-8. Summary of VPHP Efficacy (Tests 5 and 6) Results, by Material, Comparing Pure and Crude Ricin ± 95% Confidence Interval

5.5 Surface Damage to Materials

At the end of each decontamination test, the procedural blanks were visually compared to the laboratory blanks, and test coupons were visually compared to positive controls to assess any impact VPHP may have had on each material type. Based on the visual appearance of the decontaminated coupons, there were no apparent changes in the color, reflectivity, or roughness of the eight material surfaces after exposure to VPHP.

5.6 Summary and Conclusion

The data generated from this project demonstrate that VPHP reduces the bioactivity of both a commercially-available purified form of ricin toxin, as well as a crude form produced from castor beans. The Bioquell Clarus C generator with a contact time of 8 or 16 h demonstrated a greater than 99 % reduction of pure and crude ricin, respectively, on all materials tested at target inoculation level of 250 micrograms (µg). A contact time of 16 h was required for carpet, plastic, paper and stainless steel with an increased inoculum target of 500 µg. The STERIS 1000ED required a contact time of 13 h 40 min and a modified injection rate of 2.2 g/m to achieve greater than 99 % reduction of pure and crude ricin toxin at the increased inoculum target of 500 µg.

VPHP appears to be an effective decontaminant against ricin toxin utilizing the STERIS 1000ED at a targeted 400 ppm for 14 h of hydrogen peroxide injection. Similarly the Bioquell Clarus C required a time of 8 or 16 h depending on the material. In general, the crude form of ricin was more difficult to inactivate on plastic and carpet.

Impact of Study

One of the primary goals of this project was to demonstrate the effectiveness of VPHP for inactivating ricin on surfaces in a mail sorting machine. This work identified the operational conditions necessary to inactivate ricin on a variety of surfaces, including those that are found in a mail sorting machine. VPHP is compatible with most materials and will not damage high value items such as mail sorting machines. VPHP is a viable option for the decontamination of mail sorting machines that may have come into contact with the ricin toxin.

6.0 References

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Appendix A
Detailed Test Results

Efficacy Results

The detailed decontamination efficacy results for VPHP against pure and crude ricin toxin on eight material types (glass, ceiling tile, carpet, painted wallboard paper, bare pine wood and unpainted concrete) are shown in Tables A-1 through A-3. Data highlighted green indicates ≥99% reduction.

Table A-1. Inactivation of Pure Ricin Toxin Using VPHP[a]

Test Number	Test Parameters			Material	Inoculum (µg/coupon)	Mean Recovered Ricin ± SD (µg/coupon)		%Reduction ± CI
	Technology	Phase 2	Phase 3			Positive Control	Test Coupon	
1	STERIS	3.8 g/min 20 min	1.0 g/min 30 min	Stainless Steel	194.3	49.953 ± 24.671	12.648 ± 3.041	74.68 ± 15.74
				Neoprene Rubber		44.137 ± 11.554	3.307 ± 0.563	92.51 ± 2.65
				Optical Plastic		82.850 ± 2.048	2.893 ± 0.507	96.51 ± 0.70
				Aluminum		153.060 ±8.769	13.342 ±4.672	91.28 ± 3.50
				Industrial Carpet		87.611 ± 45.841	5.402 ± 2.947	93.83 ± 5.27
				Ceramic Tile		55.444 ±17.159	10.929 ± 2.872	80.29 ± 9.06
				Unpainted Concrete		2.339 ± 0.243	2.542 ± 0.245	0.00 ± 17.44[f]
				Paper		24.954 ± 11.307	15.758 ± 3.625	36.85 ± 36.31
2	STERIS	3.8 g/min 20 min	1.0 g/min 4 hr	Stainless Steel	152.5	85.883 ± 2.925	0.849 ± 0.204	99.01 ± 0.27
				Neoprene Rubber		60.891 ± 21.002	4.571 ± 1.567	92.49 ± 4.13
				Optical Plastic		65.033 ± 10.721	4.247 ± 0.504	93.47 ± 1.50
				Aluminum		61.795 ± 44.901	2.129 ± 1.088	96.56 ± 3.46
				Industrial Carpet		119.899 ± 19.131	25.438 ± 6.536	78.78 ± 7.26
				Ceramic Tile		189.311 ± 19.775	6.191 ± 1.257	96.73 ± 0.84
				Unpainted Concrete		0.408 ± 0.172	0.600 ± 0.806	0.00 ± 234.28[f]
				Paper		75.628 ± 60.134	8.471 ± 1.545	88.80 ± 10.34
3	Bioquell	3.8 g/min 20 min	1.0 g/min 4 hr	Stainless Steel	166.2	37.328 ± 12.339	0.037 ± 0.001	99.90 ± 0.04
				Neoprene Rubber		77.281 ± 2.541	0.033 ± 0.035	99.96 ± 0.05
				Optical Plastic		76.303 ± 28.462	0.054 ± 0.007	99.93 ± 0.03
				Aluminum		87.220 ± 15.103	0.033 ± 0.005	99.96 ± 0.01
				Industrial Carpet		153.209 ± 2.844	3.161 ± 0.558	97.94 ± 0.41
				Ceramic Tile		27.027 ± 14.392	0.059 ± 0.014	99.78 ± 0.14
				Unpainted Concrete		0.646 ± 0.585	≤0.049 ± 0.000[e]	92.43 ± 7.76
				Paper		80.740 ± 8.564	0.568 ± 0.187	99.30 ± 0.28
4	Bioquell	3.8 g/min 20 min	1.0 g/min 8 hr	Stainless Steel	283.0	123.007 ± 17.719	0.021 ± 0.004	99.98 ± 0.005
				Neoprene Rubber		128.447 ± 33.110	0.076 ± 0.008	99.94 ± 0.02
				Optical Plastic		119.953 ± 30.673	0.037 ± 0.003	99.97 ± 0.01
				Aluminum		50.979 ± 22.141	0.070 ± 0.007	99.86 ± 0.07
				Industrial Carpet		122.050 ± 3.790	0.824 ± 0.014	99.32 ± 0.03
				Ceramic Tile		63.487 ± 20.364	0.040 ± 0.003	99.94 ± 0.02
				Unpainted Concrete		0.219 ± 0.074	≤0.195 ± 0.000[e]	10.83 ± 33.92
				Paper		82.313 ± 6.913	0.285 ± 0.055	99.65 ± 0.08

[a] Data are expressed as the mean (± SD) of the mass of toxin observed on three individual samples, and decontamination efficacy (percent reduction ± CI).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Coupons = samples inoculated, decontaminated.
[d] CI = confidence interval (± 1.96 × standard error [SE]).
[e] Data calculated based on LOD.
[f] Negative value reported as "0".

Table A-1. Inactivation of Pure Ricin Toxin Using VPHP[a] *(Continued)*

Test Number	Test Parameters			Material	Inoculum (µg/coupon)	Mean Recovered Ricin ± SD (µg/coupon)		%Reduction ± CI
	Technology	Phase 2	Phase 3			Positive Control	Test Coupon	
5	Bioquell	3.8 g/min 20 min	1.0 g/min 16 h	Stainless Steel	322.0	153.615 ± 19.043	1.693 ± 0.090	98.90 ± 0.17
				Neoprene Rubber		133.705 ± 5.486	0.058 ± 0.015	99.96 ± 0.01
				Optical Plastic		172.618 ± 2.414	0.023 ± 0.004	99.99 ± 0.002
				Aluminum		143.040 ± 70.931	0.045 ± 0.005	99.97 ± 0.02
				Industrial Carpet		193.877 ± 36.959	2.339 ± 0.136	98.79 ± 0.27
				Ceramic Tile		73.417 ± 3.610	0.122 ± 0.021	99.83 ± 0.03
				Unpainted Concrete		1.064 ± 0.297	0.276 ± 0.045	74.03 ± 9.51
				Paper		86.826 ± 15.870	0.062 ± 0.018	99.93 ± 0.03
6	Bioquell	3.8 g/min 20 min	1.0 g/min 16 h	Stainless Steel	553.7	301.292 ± 37.582	0.049 ± 0.002	99.98 ± 0.002
				Neoprene Rubber		317.757 ± 26.133	0.291 ± 0.048	99.91 ± 0.019
				Industrial Carpet		341.690 ± 15.587	1.437 ± 0.115	99.58 ± 0.044
				Paper		189.273 ± 24.339	0.064 ± 0.004	99.97 ± 0.005
7	STERIS	2.5 g/min 20 min	2.2 g/min 8 h	Stainless Steel	275.3	140.724 ± 27.997	4.067 ± 0.125	97.11 ± 0.66
				Neoprene Rubber		114.479 ± 27.393	6.606 ± 0.422	94.23 ± 1.62
				Optical Plastic		68.567 ± 36.244	3.044 ± 0.449	95.56 ± 2.76
				Aluminum		130.036 ± 8.993	4.184 ± 0.201	96.78 ± 0.31
				Industrial Carpet		327.663 ± 45.399	66.432 ± 3.060	79.73 ± 3.35
				Ceramic Tile		152.556 ± 22.895	0.259 ± 0.144	99.83 ± 0.11
				Unpainted Concrete		2.130 ± 0.513	17.708 ± 1.133	0 ± 235[f]
				Paper		99.913 ± 4.617	2.203 ± 0.114	97.79 ± 0.17
8	STERIS	2.5 g/min 20 min	2.2 g/min 13.7 h	Stainless Steel	150.6	126.976 ± 2.102	3.625 ± 0.113	97.14 ± 0.11
				Neoprene Rubber		122.773 ± 16.098	6.924 ± 0.248	94.36 ± 0.87
				Optical Plastic		148.387 ± 22.372	2.807 ± 1.595	98.11 ± 1.26
				Aluminum		165.282 ± 13.294	3.297 ± 0.947	98.01 ± 0.67
				Industrial Carpet		187.116 ± 19.903	66.448 ± 12.573	64.49 ± 8.72
				Ceramic Tile		128.016 ± 72.227	3.082 ± 0.084	97.59 ± 1.54
				Unpainted Concrete		0.360 ± 0.158	1.282 ± 0.092	0 ± 179[f]
				Paper		83.039 ± 4.516	2.928 ± 0.141	96.47 ± 0.29
9	STERIS	2.5 g/min 20 min	2.2 g/min 13.7 h	Stainless Steel	583.9	324.982 ± 16.358	0.231 ± 0.042	99.93 ± 0.015
				Optical Plastic		257.414 ± 94.242	0.522 ± 0.080	99.80 ± 0.091
				Industrial Carpet		329.371 ± 53.248	4.170 ± 0.386	98.73 ± 0.267
				Paper		153.750 ± 8.282	0.781 ± 0.278	99.49 ± 0.207
10	STERIS	2.5 g/m 20 min	2.2 g/m 13.7 h	Neoprene Rubber	671.5	229.901 ± 76.867	0.348 ± 0.114	99.85 ± 0.080
				Aluminum		254.447 ± 29.183	0.573 ± 0.024	99.77 ± 0.031
				Ceramic Tile		216.001 ± 8.277	0.612 ± 0.058	99.72 ± 0.033
				Unpainted Concrete		0.403 ± 0.083	0.109 ± 0.014	72.77 ± 7.397

[a] Data are expressed as the mean (± SD) of the mass of toxin observed on three individual samples, and decontamination efficacy (percent reduction ± CI).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Coupons = samples inoculated, decontaminated.
[d] CI = confidence interval (± 1.96 × SE).
[e] Data calculated based on LOD.
[f] Negative value reported as "0".

Table A-2. Inactivation of Crude Ricin Toxin Using VPHP[a]

Test Number	Test Parameters			Material	Inoculum (µg/coupon)	Mean Recovered Ricin ± SD (µg/coupon)		%Reduction ± CI
	Technology	Phase 2	Phase 3			Positive Control	Test Coupon	
1	STERIS	3.8 g/min 20 min	1.0 g/min 30 min	Stainless Steel	261.4	95.143 ± 3.458	16.980 ± 4.885	82.15 ± 5.86
				Neoprene Rubber		82.833 ± 27.268	32.559 ± 23.246	60.69 ± 34.97
				Optical Plastic		84.434 ± 23.271	31.181 ± 3.777	63.07 ± 12.58
				Aluminum		123.479 ± 15.888	12.263 ± 11.178	90.07 ± 10.35
				Industrial Carpet		196.871 ± 36.817	106.577 ± 14.901	45.86 ± 14.30
				Ceramic Tile		119.181 ± 26.517	16.447 ± 4.118	86.20 ± 5.23
				Unpainted Concrete		1.840 ± 1.835	0.680 ± 0.088	63.01 ± 42.11
				Paper		74.785 ± 22.401	46.437 ± 9.863	37.91 ± 25.80
2	STERIS	3.8 g/min 20 min	1.0 g/min 4 h	Stainless Steel	370.0	156.477 ± 18.253	3.185 ± 1.773	97.96 ± 1.31
				Neoprene Rubber		117.420 ± 40.827	2.157 ± 0.850	98.16 ± 1.09
				Optical Plastic		140.412 ± 40.177	18.004 ± 1.483	87.18 ± 4.32
				Aluminum		55.849 ± 21.703	0.561 ± 0.250	98.99 ± 0.67
				Industrial Carpet		205.290 ± 41.525	127.600 ± 41.400	37.84 ± 26.89
				Ceramic Tile		130.942 ± 22.749	1.420 ± 0.546	98.92 ± 0.52
				Unpainted Concrete		2.123 ± 1.171	0.124 ± 0.068	94.18 ± 5.15
				Paper		26.318 ± 2.535	20.953 ± 2.470	20.39 ± 13.72
3	Bioquell	3.8 g/min 20 min	1.0 g/min 4 h	Stainless Steel	217.0	152.013 ± 16.460	0.036 ± 0.046	99.98 ± 0.03
				Neoprene Rubber		44.526 ± 36.043	26.367 ± 1.685	40.78 ± 54.41
				Optical Plastic		123.558 ± 14.486	14.705 ± 2.943	88.10 ± 3.12
				Aluminum		159.760 ± 16.519	0.087 ± 0.085	99.95 ± 0.06
				Industrial Carpet		179.894 ± 41.481	4.845 ± 1.132	97.31 ± 1.00
				Ceramic Tile		140.215 ± 37.887	0.222 ± 0.177	99.84 ± 0.15
				Unpainted Concrete		0.353 ± 0.005	0.194 ± 0.123	45.13 ± 39.44
				Paper		140.594 ± 8.712	0.651 ± 0.155	99.54 ± 0.13
4	Bioquell	3.8 g/min 20 min	1.0 g/min 8 h	Stainless Steel	332.4	154.701 ± 52.434	0.285 ± 0.055	99.82 ± 0.08
				Neoprene Rubber		165.291 ± 17.753	0.109 ± 0.019	99.93 ± 0.02
				Optical Plastic		192.846 ± 13.827	3.513 ± 1.393	98.18 ± 0.83
				Aluminum		140.746 ± 56.104	0.099 ± 0.003	99.93 ± 0.03
				Industrial Carpet		177.280 ± 28.875	3.894 ± 1.491	97.80 ± 1.03
				Ceramic Tile		44.841 ± 3.013	0.042 ± 0.006	99.91 ± 0.02
				Unpainted Concrete		1.351 ± 0.980	≤0.195 ± 0.000[e]	85.57 ± 11.85
				Paper		25.883 ± 2.586	0.482 ± 0.252	98.14 ± 1.12

[a] Data are expressed as the mean (± SD) of the mass of toxin observed on three individual samples, and decontamination efficacy (percent reduction ± CI).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Coupons = samples inoculated, decontaminated.
[d] CI = confidence interval (± 1.96 × SE).
[e] Data calculated based on LOD.
[f] Negative value reported as "0".

Table A-2. Inactivation of Crude Ricin Toxin Using VPHP[a] *(Continued)*

Test Number	Test Parameters			Material	Inoculum (µg/coupon)	Mean Recovered Ricin ± SD (µg/coupon)		%Reduction ± CI
	Technology	Phase 1	Phase 2			Positive Control	Test Coupon	
5	Bioquell	3.8 g/min 20 min	1.0 g/min 16 h	Stainless Steel	264.3	122.233 ± 50.002	0.073 ± 0.007	99.94 ± 0.03
				Neoprene Rubber		39.673 ± 11.048	0.256 ± 0.062	99.35 ± .027
				Optical Plastic		52.944 ± 21.963	0.205 ± 0.009	99.61 ± 0.18
				Aluminum		211.581 ± 11.293	0.073 ± 0.007	99.97 ± 0.004
				Industrial Carpet		103.768 ± 3.337	≤0.780 ± 0.000[e]	99.25 ± 0.03
				Ceramic Tile		96.938 ± 18.479	0.098 ± 0.009	99.90 ± 0.02
				Unpainted Concrete		2.191 ± 0.893	0.766 ± 0.070	65.04 ± 16.53
				Paper		17.525 ± 7.143	0.160 ± 0.051	99.09 ± 0.53
6	Bioquell	3.8 g/min 20 min	1.0 g/min 16 h	Stainless Steel	528.7	138.390 ± 34.273	0.076 ± 0.009	99.94 ± 0.017
				Neoprene Rubber		191.177 ± 14.056	0.985 ± 1.278	99.48 ± 0.758
				Industrial Carpet		59.211 ± 5.842	1.734 ± 0.102	97.07 ± 0.380
				Paper		36.479 ± 11.937	0.067 ± 0.017	99.82 ± 0.087
7	STERIS	2.5 g/min 20 min	2.2 g/min 8 h	Stainless Steel	294.5	37.559 ± 2.940	3.269 ± 0.124	91.31 ± 0.86
				Neoprene Rubber		73.161 ± 19.039	6.088 ± 0.229	91.68 ± 2.48
				Optical Plastic		26.776 ± 2.143	2.942 ± 0.365	89.01 ± 1.83
				Aluminum		36.323 ± 5.978	1.099 ± 0.172	96.98 ± 0.78
				Industrial Carpet		95.741 ± 27.082	9.839 ± 2.111	89.72 ± 4.13
				Ceramic Tile		45.574 ± 11.803	1.063 ± 0.433	97.67 ± 1.27
				Unpainted Concrete		1.836 ± 0.680	29.461 ± 2.936	0 ± 696[f]
				Paper		117.354 ± 22.002	3.741 ± 0.384	96.81 ± 0.77
8	STERIS	2.5 g/min 20 min	2.2 g/min 13.7 h	Stainless Steel	124.1	7.557 ± 2.106	0.481 ± 0.050	93.63 ± 2.15
				Neoprene Rubber		8.526 ± 1.833	0.217 ± 0.023	97.46 ± 0.69
				Optical Plastic		12.913 ± 3.972	0.152 ± 0.049	98.82 ± 0.59
				Aluminum		17.367 ± 1.296	0.155 ± 0.030	99.11 ± 0.21
				Industrial Carpet		4.195 ± 0.360	4.652 ± 0.513	0 ± 17.5[f]
				Ceramic Tile		30.955 ± 10.446	0.124 ± 0.017	99.60 ± 0.17
				Unpainted Concrete		0.159 ± 0.047	0.282 ± 0.224	0 ± 170[f]
				Paper		17.966 ± 9.894	0.689 ± 0.066	96.16 ± 2.43
9	STERIS	2.5 g/min 20 min	2.2 g/min 13.7 h	Stainless Steel	579.8	324.122 ± 63.312	0.533 ± 0.104	99.84 ± 0.051
				Optical Plastic		341.860 ± 76.185	0.363 ± 0.099	99.89 ± 0.042
				Industrial Carpet		291.982 ± 257.958	2.194 ± 0.253	99.25 ± 0.758
				Paper		283.505 ± 84.941	1.546 ± 0.353	99.45 ± 0.233
10	STERIS	2.5 g/m 20 min	2.2 g/m 13.7 h	Neoprene Rubber	457.7	396.616 ± 19.176	0.382 ± 0.016	99.90 ± 0.007
				Aluminum		412.201 ± 19.875	0.827 ± 0.223	99.80 ± 0.062
				Ceramic Tile		457.101 ± 35.529	0.318 ± 0.116	99.93 ± 0.029
				Unpainted Concrete		4.016 ± 2.444	0.173 ± 0.043	95.69 ± 3.207

[a] Data are expressed as the mean (± SD) of the mass of toxin observed on three individual samples, and decontamination efficacy (percent reduction ± CI).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Coupons = samples inoculated, decontaminated.
[d] CI = confidence interval (± 1.96 × SE).
[e] Data calculated based on LOD.
[f] Negative value reported as "0".

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