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Fogging of Chlorine-Based Sporicidal Liquids for the Inactivation of Bacillus Anthracis Surrogate Spores

Office of Research and Development
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Disclaimer

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Executive Summary

The U.S. Environmental Protection Agency's (EPA) 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. As part of the HSRP, EPA is investigating the effectiveness and applicability of technologies for homeland security-–related applications. The purpose of this investigation was to determine the sporicidal efficacy of a fogging technology using chlorine-based sporicidal liquids for inactivating bacterial spores (*Bacillus* [*B.*] *atrophaeus*, a surrogate for *B. anthracis*) in an office or indoor environment. The use of fogging technology to disseminate sporicidal solutions via microscopic droplets has the potential to be a less arduous, more economical volumetric decontamination alternative to fumigation.

Twenty-seven pilot-scale tests were conducted overall. Test surfaces, or coupons, were typical indoor and outdoor building materials and included carpet, ceiling tile, concrete, glass, laminate, painted wallboard (PWB) paper, galvanized metal, and wood. Known amounts of *B. atrophaeus* spores were inoculated onto the material coupons, and then the coupons were placed in three locations in a mock office: under a desk, on top of a desk, and above the ceiling tiles (one ceiling tile was removed to allow for fog distribution). The chlorine-based decontamination solutions investigated were pH-adjusted bleach (pAB), diluted bleach (1 in 4 dilution), sodium dichloro-s-triazinetrione (dichlor), and aqueous chlorine dioxide (ClO_2). One or two foggers were used to disseminate the sporicidal solutions throughout the chamber in the form of an aerosol.

Experimental parameters included the sporicidal solution, active ingredient concentration (AIC) of the liquid sporicide, disseminated volume of solution, dwell time, and chamber air exchange. The efficacy of the fogging treatment was characterized in terms of $\log_{10}$ reduction (LR), which was calculated as the difference between the log of the number of bacterial spores (as colony-forming units, or CFU) recovered from the coupons before (positive controls) and log of the number after decontamination. A decontaminant is considered to be an effective sporicide if a 6 LR or greater is achieved based upon appropriate laboratory testing.

Summary of Results

The decontamination efficacy results were variable and depended greatly on the material. The nonporous materials tested were easier to decontaminate, i.e., had generally higher decontamination efficacies, while materials that are porous or comprised of organic chemical constituents proved more difficult to effectively decontaminate. In the majority of the tests, galvanized metal, glass, laminate, and PWB paper were effectively decontaminated (≥ 6 LR). Fogging of the chlorinated decontaminants was moderately effective for concrete, with only one test achieving an average ≥ 6 LR on this material (but several tests in which ≥ 5 LR was achieved). Ceiling tile, carpet, and wood (porous and organic-based materials) were the most difficult materials to decontaminate. There were no tests in which ceiling tile or carpet were effectively decontaminated as defined as ≥ 6 LR.

Statistical analyses of results showed that the disseminated volume of solution proved to have a significant effect on decontamination efficacy. Further, maximizing the fogged solution quantity (up to approximately 336 mL per cubic meter volume to be decontaminated) and the AIC generally produced similar results for all sporicides. More specifically, the average decontamination efficacy for all materials in

the tests at these optimized operating conditions was generally above 5 LR, and was independent of the sporicide fogged.

Analysis of the data showed a significant yet minor average improvement (~ 0.5 LR) in the decontamination efficacy for the coupons placed on the desk location compared to the other locations. Coupons located under the desk and above the ceiling showed the same average decontamination efficacy. Overall, these differences in decontamination results as a function of test chamber location were minor and generally imply the fog was well distributed.

An evaluation of the neutralization requirements for coupon samples containing dichlor residue during the extraction process determined that there were statistically insignificant differences in spore recovery between samples extracted with buffer solution plus neutralizer and those extracted with just the buffer. This was shown to be the case for all materials. Additionally, the recovery of viable bacterial spores inoculated onto coupons already having a dichlor residue was significantly diminished.

This study has demonstrated the potential of using chlorine-based decontaminants applied with a commercially available fogging technology for volumetric decontamination of surfaces typical of indoor environments contaminated by *B. anthracis* surrogate spores. However, this decontamination approach may be better suited for areas that do not contain significant quantities of porous or organic materials such as carpet, ceiling tile, or wood.

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Acronyms and Abbreviations

ADA	Aerosol Deposition Apparatus
AIC	active ingredient concentration
ANOVA	analysis of variance
B.	*Bacillus*
BI	biological indicator
BSC	biological safety cabinet
Cl_2	chlorine
ClO_2	chlorine dioxide
CFU	colony-forming unit(s)
cm	centimeter(s)
COMMANDER	Consequence Management and Decontamination Evaluation Room
DI	deionized
dichlor	sodium dichloro-s-triazinetrione
EPA	U.S. Environmental Protection Agency
FAC	free available chlorine
g	gram(s)
HEPA	high-efficiency particulate air
Hg	mercury
HSRP	Homeland Security Research Program
i.d.	inner diameter
in.	inch(es)
kg	kilogram(s)
L	liter(s)
lb	pound(s)
LR	log reduction
µL	microliter(s)
m	meter(s)
MDI	metered-dose inhaler
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
NCASI	National Council of the Paper Industry for Air and Stream Improvement, Inc.
NHSRC	National Homeland Security Research Center (EPA/ORD)
NIST	National Institute of Standards and Technology
o.d.	outer diameter
pAB	pH-adjusted bleach

PBST	phosphate-buffered saline with Tween® 20
PPE	personal protective equipment
ppm	part(s) per million
PVC	polyvinyl chloride
QA	quality assurance
QAPP	quality assurance project plan
QC	quality control
RH	relative humidity
RPM	revolutions per minute
s	second(s)
SCADA	supervisory control and data acquisition
SD	standard deviation
STS	sodium thiosulfate
TNTC	too numerous to count
TSA	tryptic soy agar
VHP®	vaporized hydrogen peroxide

1 Introduction

The U.S. Environmental Protection Agency's (EPA) 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, the HSRP is working to develop tools and information that will help detect, contain, and decontaminate radiological, chemical, or biological contaminants resulting from an intentional introduction of these agents into buildings, water systems, or the outdoor environment, as well as facilitate the treatment and disposal of materials resulting from remediation activities. As part of this effort, and in response to the needs of the HSRP partners, EPA is investigating the effectiveness and applicability of technologies for homeland security–related applications by developing test plans, conducting tests, collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with quality assurance (QA) protocols to ensure that data of known and high quality are generated.

In 2001, the introduction of a few letters containing *Bacillus (B.) anthracis* (anthrax) spores into the U.S. Postal Service system resulted in contamination of several facilities. Although most of the facilities in which these letters were processed or received were heavily contaminated, they were successfully remediated with approaches such as fumigation with chlorine dioxide (ClO_2) or vaporized hydrogen peroxide (VHP®) (Canter et al., 2005). Large-scale use of sporicidal chemicals to decontaminate large buildings was unprecedented (Rastogi et al., 2010), and the overall cost of remediation activities for the letter attacks was estimated to be approximately $320 million (Schmitt and Zacchia, 2012). It is generally agreed that additional rapid, effective, and economical decontamination methods that can be employed over wide areas (outdoor and indoor) are needed to increase preparedness for such a release.

While previous tests have been conducted by EPA to evaluate the inactivation of *B. anthracis* spores using peracetic acid solutions (Wood et al., 2013), there are few data available in the literature related to decontamination efficacy when fogging chlorine-based solutions. Thus to fill this gap, the study reported here evaluated the effectiveness of different chlorine-based sporicidal liquids, disseminated using a commercially available fogging device, to inactivate bacterial spores in a pilot-scale decontamination chamber. (The efficacy of bleach-based decontaminants in inactivating spores has been evaluated on a number of different materials when applied as a spray (Ryan et al., 2014; U.S. EPA, 2006; U.S. EPA, 2012; U.S. EPA, 2015; Wood et al., 2011), but again, few data are available on efficacy when chorine-based decontaminants are applied as a fog.) Experimental variables included material, location within test chamber, sporicidal solution, quantity of solution, AIC, and air exchange. The chlorine-based sporicidal decontaminants chosen for testing were solutions of diluted bleach (1 in 4 dilution), pH-amended bleach (pAB), aqueous chlorine dioxide, and a concentrated aqueous solution of sodium dichlor-s-trianzinetrione dihydrate (a pool sanitizer chemical commonly referred to as dichlor).

Twenty-five tests were conducted to evaluate decontamination efficacy for the fogging of chlorine-based sporicidal solutions. Two additional tests were conducted to assess the neutralization requirements for coupon samples containing dichlor residue.

2 Materials and Methods

2.1 Biological Organism and Spore Deposition

The test organism for this study was *B. atrophaeus* (American Type Culture Collection 9372) in a dry powder form of spores mixed with silicon dioxide particles. *B. atrophaeus,* formerly known as *B. subtilis var. niger* or *B. globigii*, was used as a surrogate for *B. anthracis* in three decontamination test rounds (including ClO_2 fumigation) of the Bio-response Operational Testing and Evaluation study (U.S. EPA, 2013). The bacterial spores were prepared by the U.S. Army Dugway Proving Ground as reported in Brown et al. (2007a).

The test surfaces (coupons) were loaded with a target dose of 10^7 colony-forming units (CFU) of the dry spore mix using a procedure specifically developed for this purpose. Briefly, each sterilized coupon was aseptically mounted on the top of a cylindrical coupon holder and topped with a metered-dose inhaler (MDI) actuator. The pre-weighed MDI was vortexed and hand agitated to evenly distribute the dry spore mix and then placed inside the actuator, which was activated to disperse the spore mix onto a circular area in the center of the coupon's surface. Refer to Lee *et al.* (2011) for further details.

2.2 Decontamination Chamber and Test Environment

All tests were conducted in the Consequence Management and Decontamination Evaluation Room, or COMMANDER. COMMANDER consists of a stainless steel–lined inner chamber built specifically for decontamination testing, with internal dimensions of approximately 3.4 m wide, 2.5 m deep, and 2.8 m high. At the entrance to the chamber is an airlock compartment, and enclosing the chamber and airlock is an exterior steel shell. When desired, all three components can be kept under cascading slightly negative pressure (with the greatest negative pressure in the inner chamber) by using separate air streams with valve controls on the inlet and outlet of each. Air entering the decontamination chamber passes through a high-efficiency particulate air (HEPA) filter, and exhaust air from the chamber is ducted to an activated carbon bed and HEPA filter prior to release to the facility exhaust system. Fans were used inside the chamber to provide internal mixing during fumigation. The inner chamber inlet and outlet duct fans (blowers) were turned off during fumigation, and the inlet duct valve was closed. Further details and a diagram of COMMANDER can be found elsewhere (Wood et al., 2013).

Temperature, relative humidity (RH), air pressures, and flow rates within the decontamination chamber are controlled and/or their data logged continuously using a supervisory control and data acquisition (SCADA) system. Temperature and RH within the chamber were measured using a temperature and RH transmitter (model HMD40Y, Vaisala Inc., Helsinki, Finland). This instrument was calibrated prior to each test by comparing its RH data with known RH values generated in the sealed headspace above individual saturated solutions of various salt compounds. The RH meters were replaced if calibration criteria could not be met. During fogging events, the RH and temperature within the chamber were monitored but not controlled. Typically, RH measurements neared or exceeded the maximum range of the RH meter during the fogging events.

2.3 Mock Office

The stainless steel surfaces of the decontamination chamber were covered by materials typical of an indoor office setting. The floor was covered with plywood and then industrial carpet tiles (P/N 54594,

Multiplicity carpet tile, Shaw Floors, Sherwin-Williams, Durham, NC). The rear and side walls were framed and faced with 1.27-cm-thick drywall (P/N GB4080-0800, GoldBond, Home Depot, Durham, NC). The drywall was patched with joint compound (P/N 380119048, USG Sheetrock, Lowes, Durham, NC) and joint tape (P/N 382199010, USG Sheetrock, Lowes, NC) according to typical building practices and then primed (P/N 20005, Kilz, Lowes, Durham, NC) and painted (P/N 105001, Behr, Lowes, Durham, NC). At the top of the walls, a drop ceiling was installed and consisted of acoustic ceiling tile panels (P/N SC1135c, Armstrong, Home Depot, Durham, NC) and two plenum grilles to enable conditioning of the interior chamber air using the existing RH and temperature controls. The chamber was furnished with office equipment consisting of a laminated desk, an office chair, a file cabinet, books/catalogs, and an oscillating fan for chamber mixing. Figure 2-1 shows the mock office with furniture and fogger positions.

Figure 2-1. Mock office setup and fogger placement.

2.4 Fogging System and Methods

Decontamination tests were conducted using an ultra-low volume fogger (SANI-TIZER™, Curtis Dyna-fog, Ltd., Westfield, IN), which consisted of a motor/blower assembly, nozzle system, nozzle housing, 1-gallon formulation tank, and metering valve. The sporicide was drawn from the formulation tank through the control valve and into the nozzle system where it was pneumatically sheared into droplets. The droplets were then disseminated throughout the chamber by ambient air passing through the nozzle system.

The fogger was operated by first transferring a measured volume of sporicidal solution into the formulation tank and then weighing the fogger. (The formulation tank could accommodate slightly more than 4 L [1 gallon] of sporicide, so two foggers were used for volumes greater than 4 L.) The fogger was transferred to COMMANDER and placed on the floor in front of the chamber door, facing the back wall with the nozzle positioned at an angle of approximately 70° from horizontal. The metering valve knob was positioned on the low setting to regulate the mean droplet size at 14–20 microns, according to the manufacturer. The fogger was plugged into an unenergized power outlet and the fogger's power switch moved to the on position. COMMANDER was sealed and the target air exchange set by adjusting the chamber's air supply valve. The fogger was activated remotely using the SCADA system by increasing the voltage output of the power outlet from 0% to 100%. Fogging typically began within 1-2 hours after the active ingredient concentration was measured. At the completion of testing, the fogger was removed from the chamber, weighed, and drained of sporicide. The drained sporicide was collected in a graduated cylinder where the volume was measured, and then discarded. The empty fogger was purged with deionized water and reused if the post-test dissemination rate was within 20% of the initial rate at factory condition. If the criteria were not met, the fogger was removed from service.

2.5 Sporicidal Solutions

The type, volume, and concentration of sporicidal solution used for fogging were some of the independent variables for this investigation. The solutions used for this effort are detailed in Table 2-1, and were all tested at laboratory ambient temperature of approximately 22 °C.

Table 2-1. Summary of Sporicides

Sporicidal Solution	Active Ingredient	Vendor
pH-adjusted bleach (pAB)	Sodium hypochlorite, hypochlorous acid	Produced on-site with Clorox® concentrated germicidal bleach (EPA registration 5813-102; Lowes, Durham, NC) and 5% acetic acid
Diluted bleach	Sodium hypochlorite	Produced on-site with Clorox® concentrated germicidal bleach
Stabilized chlorinating granules (dichlor)	Sodium dichloro-s-triazinetrione Hydrated, hypochlorous acid	Pool Solutions, Pool Supply World, P/N PSW-CSC158-5; Brilliance for spas, B & G Builders Pools & Spas, Durham, NC
Aqueous ClO_2	Aqueous chlorine dioxide	P/N G0005, GO2 International, Buena Park, CA

The pAB was prepared as follows: one part Clorox® concentrated germicidal bleach (Clorox Corp., Oakland, CA) was diluted with approximately eight parts of deionized water and one part 5% (v/v) acetic acid (P/N 13025 or equivalent, Fisher Scientific, Pittsburgh, PA;). This brand of bleach is registered with EPA as an antimicrobial pesticide and has a hypochlorite concentration of 8.3%. The pH was adjusted to 6.5–7.0 with additional 5% acetic acid, as needed. For the first two tests, the free available chlorine (FAC) content was adjusted to 6000–6700 ppm with deionized water after preparation. The FAC levels for subsequent tests with pAB had higher FAC levels that were more consistent with the FAC levels of the diluted bleach tests.

Initially, diluted bleach was prepared by mixing Clorox® concentrated germicidal bleach with deionized water to reach the target FAC, and the pH was recorded. Most of the tests thereafter with diluted bleach

used one part bleach and three parts water, to give an FAC level of 20,000 ppm or higher. For tests with lower FAC concentrations, the ratio of bleach to water was varied to meet the target FAC specification.

The dichlor solutions were prepared by dissolving 0.33 lb of the dichlor granules per 1 gallon of deionized water, except for Test 24, in which 0.5 lb of the dichlor product was added per gallon of water. Note that the actual measured FAC level (discussed next) of the dichlor solutions does not correspond directly to the above reported quantities (mass) of the product added per liter of water. This is because the pool chemical used does not result in 100% conversion of FAC, as measured by the technique discussed below.

The aqueous ClO_2 solutions were prepared according to the manufacturer's instructions by first dissolving the active aqueous ClO_2 brand component A (52% sodium chlorite) in 5 L of tap water and then adding component B (97% sodium bisulfate). The solution was gently stirred to promote even mixing, and then required three hours to complete the reactions to fully produce the ClO_2 solution. All ClO_2 solutions were prepared in chemical resistant containers (polyethylene or polypropylene) and used within 1 hour after completion of the required 3-hour reaction hold time. These aqueous solutions were typically at ambient temperature (~ 23 °C) prior to fogging. Safety precautions were taken to protect personnel from liberated chlorine and chlorine dioxide gas.

The FAC concentration of the formulations for pAB, diluted bleach, and dichlor was measured using the HACH® high-range bleach test kit (Method 10100, [model CN-HRDT], HACH, Loveland, CO) which was adapted from ASTM Method D2022-89. A 1 or 5-mL aliquot of the decontaminant solution was mixed with approximately 150 mL of deionized water in a 250 mL glass beaker. The size of aliquot depended on the expected concentration of the FAC. Usually, for solutions with target FAC concentrations less than 10,000 mg/L, a 5 mL aliquot was used and, for those solutions with target FACs greater than 10,000 mg/L, a 1 mL aliquot was used. A potassium iodide powder pillow (HACH®, P/N 20599-96) was added and mixed until completely dissolved. The sample was acidified with an acid reagent powder pillow (HACH®, P/N 1042-99) then iodometrically titrated with sodium thiosulfate (STS) to a colorless end point. The bleach solution aliquot was taken and analyzed immediately after formulation and mixing.

The ClO_2 concentration of the aqueous ClO_2 solutions was also determined using the HACH® high-range bleach test kit, but modified as follows to measure only ClO_2. A 1 mL aliquot of solution was mixed with approximately 150 mL of deionized water in a 250 mL glass beaker. A potassium iodide powder pillow was added and mixed until completely dissolved. A neutral (no acid added) titration was performed iodometrically with sodium thiosulfate (STS) to a colorless end point.

The pH of each solution was measured with an Oakton Acorn® series pH 5 meter (Oakton Instruments, Vernon Hills, IL). This meter was calibrated daily.

Sporicide volumes ranged from 1 L to 8 L, but the volume added to the fogger was not necessarily the volume disseminated. Typically, dissemination limitations caused by equipment efficiency resulted in approximately 50 mL of sporicide remaining in the fogger after testing. However, equipment malfunctions in some tests resulted in more than 50 mL of unused sporicide. Two foggers were deployed for tests requiring more than 4 L of sporicide. The test parameters for each solution are discussed in Section 4.

2.6 Measurement of ClO_2 Gas Levels

Gas samples using a modified version of Method 4500-ClO_2 E (Standard Methods Online, 2005) were taken to monitor the ClO_2 gas concentration inside the mock office, when fogging aqueous ClO_2 solutions. This method is an amperometric titration suitable for aqueous ClO_2 concentrations between 0.1 and 100 mg/L. This method does not address gas-phase sampling. The full method is quite complex in that a multi-titration scheme is used to differentiate several chlorine-containing analytes. A modification of this method to incorporate gas-phase sampling requires the use of a buffered potassium iodide bubbler sample collection and restricting the official method to a single acidic titration versus a two-step neutral and acidic titration. The neutral titration analyzes the combined chlorine, chlorine dioxide, and chlorite as a single value. It can only be applied where chlorite and chlorate are not present. Since the modified standard method described below is applied to gas-phase samples, the presumption of the absence of chlorite and chlorate is valid.

The modified method was performed as follows:

- A series of four impingers were assembled: Impingers 1 and 2 contained 20 mL of potassium iodide (KI) phosphate buffer solution (KIPB) with a pH of 7.2. The solution was prepared using 25 g of KI in 500 mL of phosphate buffer). Impinger 3 was empty, and impinger 4 contained silica desiccant.
- ClO_2 gas was impinged from the chamber into the KIPB solution in the impingers in series at a flow rate 0.5 L/min for a time necessary for the KIPB to turn from clear to a solid yellow color.
- The 20 mL of KIPB solution from each impinger were combined into a 200-mL volumetric flask. The impingers were rinsed thoroughly with deionized water and the rinse was collected in a 250-mL beaker.
- 1 mL of 6 N HCl was added to the solution.
- The solution was placed in the dark for 5 min.
- The solution was titrated with 0.01 N STS, and the volume of STS used in the titration was recorded.

Conversion from titrant volume to ClO_2 concentration was based on the modified 4500 E method and calculated as follows:

$$ClO_2\ (mg/L)\ = \frac{T_A\ x\ 13490\ x\ N}{0.025\ (fraction\ of\ gas\ titrated)}$$

where

T_A = volume of STS (mL)

N = Normality of STS

This method removed many of the possible interferences listed in Method 4500-ClO_2 E. The initial presence of KI in excess prevents iodate formation, which can occur in the absence of KI and leads to a negative bias. The presence of the pH 7 buffer during impinging prevents oxidation of iodide by oxygen,

which occurs in strongly acidic solutions. Other interferences are unlikely to be a problem in this application as manganese, copper, and nitrate are unlikely in a gaseous sample.

The second impinger filled with KIPB solution was added in series to reduce the likelihood of breakthrough. The second impinger was not analyzed independently, but was combined with the first impinger for analysis.

2.7 Extractive Sampling Method for Cl_2 Gas Measurement

The Cl_2 extractive sampling method was used to monitor mock office Cl_2 levels in the gas phase when fogging pAB, diluted bleach, and dichlor solutions. Extractive samples were collected continuously (one after another) while the foggers were active. The method was developed by the National Council of the Paper Industry for Air and Stream Improvement, Inc. (NCASI) for monitoring Cl_2 and ClO_2 in bleach plants (NCASI, 1997). This method is suitable for Cl_2 concentrations above 1 mg/L for a 10-L sample. Briefly, an air sample is extracted from the mock office and passed through impingers containing potassium iodide phosphate buffer solution. The NCASI method involved an assessment of iodine formed at neutral and acidic pH for quantitative assessment of Cl_2. The method was modified, however, to a single, acidic titration. As mentioned previously, omission of the neutral titration can only be applied where chlorite and chlorate are not present. Since ClO_2 was not present in the air sample, the presumption of the absence of chlorite and chlorate is, again, quite valid.

Cl_2 extractive sampling was performed as follows:

- A series of four impingers were assembled: Impingers 1 and 2 contained 20 mL of potassium iodide (KI) phosphate buffer solution (KIPB) with a pH of 7.2. The solution was prepared using 25 g of KI in 500 mL of phosphate buffer). Impinger 3 was empty, and impinger 4 contained silica desiccant.
- Cl_2 gas was impinged from the chamber into the KIPB solution in the impingers in series at a flow rate 0.5 L/min for a time necessary for the KIPB to turn from clear to a solid yellow color.
- The 20 mL of KIPB solution from each impinger were combined into a 200-mL volumetric flask. The impingers were rinsed thoroughly with deionized water and the rinse was collected in the 250-mL beaker.
- 10 mL of 10% sulfuric acid was added to the solution.
- The solution was titrated with 0.01 N STS, and the volume of STS used in the titration was recorded.

Conversion from titrant volume to Cl_2 concentration was calculated as follows:

$$Cl_2(ppm) = \frac{Cl_2\ Moles\ x\ 24.04 \frac{L}{mole} x\ 10^6}{S_c\ x\ t_s}$$

where

S_c =corrected sampling flow rate

T_s = time sampled, min

Possible interferences for this method include sulfur dioxide and hydrogen peroxide, neither of which were present during testing. Results of an accuracy assessment performed for this sampling method are discussed in Section 3.3.

2.8 Coupon Materials and Biological Indicators

All eight materials used for this study were fabricated so that they could be mounted onto circular aluminum scanning electron microscopy stubs (18 mm in diameter). Oak wood coupons were 8-mm-thick plugs (part SPO750, Woodworks Ltd, Haltom City, TX). The borosilicate glass coupons were 3.3-mm-thick plugs (Prism Research Glass, Inc., Research Triangle Park, NC). The 18-mm discs were cut from 26-gauge galvanized metal (East Coast Metal, Durham, NC), carpet (Multiplicity 54594, Shaw Industries Group, Dalton, GA), laminate flooring (Pergo Estate Oak Laminate Flooring, Home Depot SKU 257063 – no longer available), and ceiling tile (Armstrong, P/N 949, Lowes SKU 40684). Concrete coupons were cast from sand and cement mix (P/N 110360, Quikrete, Lowes, Durham, NC). The front facing of drywall (P/N GB00090800, GoldBond, Lowes SKU 34137) was primed (Kilz, 2-gallon, P/N 20005, Home Depot SKU 317390) and painted (Behr Premium Plus flat white latex, P/N 105001, Home Depot SKU 923827). The paper was then removed from the gypsum before the 18-mm discs were cut from the paper. All materials were then mounted to 18-mm aluminum stubs (P/N 16119, Ted Pella, Inc., Redding, CA) using double-sided adhesive tape (P/N 16073-2, Ted Pella, Inc., Redding, CA) and placed in holder trays. To prevent contamination and bias of results due to non-target organisms, all coupons and stubs were sterilized with ethylene oxide using an EOGas AN333 sterilization system (Andersen Products, Haw River, NC).

Biological indicators (BI) were also used to assess the effectiveness of fogging in inactivating bacterial spores. The BIs comprised nominally 10^6 *B. atrophaeus* spores inoculated onto stainless steel discs and wrapped in Tyvek envelopes. The BIs, obtained from Mesa Labs (model 1-6100ST, Lakewood, CO), were placed in triplicate in each of the three locations inside the mock office and in the enclosure (the unexposed location just outside the exposure chamber). The BIs were collected upon completion of each test and analyzed according to manufacturer instructions to determine whether any of the BIs exhibited growth of bacteria (survival of any spores).

2.9 Test Sequence

Each fogging event consisted of three phases: 1) Active fogging, characterized as the segment of the test during which the fogger(s) were powered on. During active fogging, chamber conditions such as temperature, RH, and sporicide concentration were monitored. 2) The dwell phase began when the fogger(s) were turned off. Monitoring of control chamber conditions continued during the dwell phase; however, the wet chemistry samples were collected for a relatively short time during the beginning of the phase. The dwell phase continued overnight. 3) The aeration phase began the next morning when the inner chamber valves were opened and the exhaust duct blower was turned on. Chamber aeration continued until the concentration of sporicide was safe for reentry.

The following general test sequence was used for all 27 mock office tests:

- Material coupons were prepared for testing as follows: Coupons designated as positive controls (not exposed to the fog) and test coupons were inoculated within 24 hours of testing, with the exception of Tests 14 and 15. Tests 14 and 15 coupons were inoculated 72 hours prior to exposure and stored in a refrigerator at 4 °C. Procedural blanks, field blanks, and negative control coupons (not inoculated with spores) were stored in the same manner as the inoculated coupons.

- Test coupons and BIs were placed in COMMANDER in three predetermined locations: 1) on the floor underneath the office desk, 2) on top of the office desk, and 3) in the ceiling on the ceiling tile (one ceiling tile was removed from the tile framing to allow for fog to reach above the remaining tiles). Procedural blank coupons were placed on the floor underneath the office desk. Positive and negative control coupons remained outside the chamber and were not exposed to fogging conditions. BIs were also placed in the unexposed enclosure area just outside the exposure chamber.

- The chamber was sealed and fogging initiated (including operation of fan). Chamber conditions during each test are detailed in Section 4.1. The fogger flow (dissemination) rate varied somewhat, but averaged about 175 mL per minute when using two foggers. Thus for an eight-liter decontaminant solution, this would require approximately 45 minutes to disseminate.

- Dwell was typically overnight, although there were a few tests with a dwell of only 2 hours, to assess the effect of this operating parameter.

- After the dwell, the chamber was aerated for the time required to achieve a safe level of decontaminant concentration prior to reentry. The aeration duration was typically only about five minutes following fogging of bleach solutions, and was approximately an hour for the fogging of dichlor solutions.

- Upon completion of the aeration phase, the chamber was entered and the test and procedural blank coupons collected. In some instances, it was necessary to enter using supplied air respirators to collect samples in a timely manner.

- Positive control, field blank, and material blank coupons were collected in empty sterile sample tubes.

- The coupons were transferred to the National Homeland Security Research Center (NHSRC) biocontaminant laboratory (biolab) for storage in a refrigerator over the weekend until subsequent analysis.

2.10 Bacterial Spore Sampling and Analysis

Numerous microbiological samples and assays were used to characterize bacterial spore presence or absence in the mock office for each experiment (116 total for each test). Coupons were collected and spores were extracted, serial plated, filter plated (if needed) and enumerated as CFU as described in Wood et al. (2016). Samples or assays were either quantitative (providing a numerical result) or qualitative (indicating either presence or absence of bacterial growth). Laboratory blanks of items such as growth media and sampling materials were also employed in each experiment to check for aseptic conditions. A summary of the number and type of samples/assays for each experiment is shown in Table 2-2. Each sample or assay is further described in the narrative below.

Table 2-2. Summary of Samples Used for each Test

Sample or Assay Type	No. Procedural Blanks	No. Positive Controls	No. Exposed Samples
Biological indicators	0	3	9
Material coupons	8	24	72
Total	8	27	81

CFU counts per coupon were calculated by multiplying the number of counted colonies by the dilution factor and by the volume of the sample extract. Efficacy is defined as the extent (by log reduction, or LR) to which the agent extracted from the material surface after the treatment with the decontamination procedure is reduced below that extracted from a similar material coupon before decontamination. Efficacy was calculated for each material (j) (eight materials were used for each fog test (i)) as follows:

$$LR_{ijk} = \frac{\sum_{c=1}^{n} (\log_{10} C_{ijc})}{n_{ijc}} - \log_{10}\left(N_{ijk} \right) \qquad (2\text{-}1)$$

where

C_{ijc} is the number of viable organisms recovered from control coupons for the *i*th test and *j*th material,

n_{ijc} is the number of control coupons for the *i*th test and *j*th test material (n = 3 positive controls were used for each material, each test), and

N_{ijk} is number of viable organisms recovered on the *k*th replicate test coupon for the *i*th test and *j*th test material (9 replicates were used for each material, each test; 3 replicates were placed in each of the 3 locations within the test chamber).

If no viable spores were detected, then the detection limit of the sample was used for N_{ijk} and the efficacy reported as greater than or equal to the value calculated by Eqn. 2-1. The detection limit of a sample depends on the analysis method and so might vary. The detection limit of a spread-plate is 1 CFU, but half of this value was used in calculating the detection limit of the sample. For instance, the detection limit of a 0.1-mL plating of a 20-mL sample suspension is 100 CFU (0.5 CFU / 0.1 mL * 20 mL), but if all 20 mL of the sample is filter plated, the detection limit would be 0.5 CFU.

The standard deviation (SD) of the LR values for a particular material and test was calculated with MS Excel as the standard deviation of the sample (STDEV function).

2.11 Dichlor Residue Evaluations

2.11.1 Spore Recovery Evaluations

During the process of collecting samples exposed to dichlor fog, residue was observed on the coupon surfaces and in COMMANDER itself. Figure 2-2 shows the residue formed on the surfaces of the material coupons and the desk after exposure to dichlor fog.

Figure 2-2. Coupons coated with residue after exposure to dichlor fog.

In response, tests were conducted to determine the impact, if any, of dichlor residue on the recovery of spores subsequently inoculated onto coupons. During one test with dichlor solution, six sterile stainless steel coupons (14 in. x 14 in.) were placed in various locations throughout the chamber (the majority were located on the floor in sterile trays for handling) in addition to the 18-mm material coupons. Figure 2-3 shows the placement of these coupons during exposure. The fog test was performed as described in Section 2.9, but the stainless steel coupons remained in the chamber for 72 hours after treatment to allow them to dry. (The chamber was entered as usual the day following treatment to retrieve the 18-mm coupons and BIs, but the stainless steel coupons were left behind). After the 72-hour drying time, the stainless steel coupons were removed from the chamber and inoculated with spores via an MDI as follows: three positive control coupons (inoculated onto coupons not unexposed to dichlor treatment), five test coupons (inoculated onto coupons that were exposed to dichlor treatment), one procedural blank coupon (uninoculated and exposed to dichlor treatment), and one laboratory blank coupon (uninoculated and unexposed to dichlor treatment). After inoculation, the spores were allowed 48 hours of contact with the coupon surfaces. After the contact time, coupons were sampled using a sponge wipe method and relinquished for extraction, serial plating, and enumeration.

Figure 2-3. 14 in. x 14 in. stainless steel coupons.

2.11.2 Neutralization Evaluations

Two additional, separate experiments were conducted to assess whether neutralization was needed to stop any potential lingering sporicidal activity of dichlor residue when processing samples.

The purpose of the first neutralization test was to identify the need, if any, to neutralize the exposed sample coupons during the extraction process. During the coupon extraction process, dichlor residue may dissolve into the extraction solution with the extracted spores. There was some concern that potentially elevated FAC levels in the extraction solution could result in residual inactivation of viable spores, or assay conditions that prevent the germination and outgrowth of viable spores.

Four material types were selected for this experiment to represent differing levels of material porosity: carpet and concrete (porous) and galvanized metal and laminate (nonporous). Positive control and test coupons were inoculated as before. Two sets of each material were placed in triplicate in each of the three sample locations inside the mock office and positive controls were placed in a location immediately outside COMMANDER. The sample collection procedure remained consistent with that of previous fogging tests. But now samples were extracted using two procedures. The extraction procedure used for the first set of coupons remained unchanged from that of other tests with the use of 10 mL of phosphate-buffered saline with Tween® 20 (PBST) as the extraction solution. The extraction procedure used for the second set of coupons differed with the addition of an STS neutralizing solution to PBST in stoichiometric equivalent quantities.

The purpose of the second neutralization test was to identify the need to neutralize the exposed sample coupons during the 72-hour period over the weekend when coupons were stored until they could be extracted. As outlined in Section 2.9, at the completion of a fog treatment, exposed coupons were collected in empty sterile sample tubes and then refrigerated for approximately 72 hours prior to the extraction process. Thus for this second neutralization test, three material types were selected for this test to represent varying levels of material porosity: carpet, laminate, and concrete. Positive control and test coupons were inoculated as in the initial neutralization test. Three sets of each inoculated material was placed in triplicate at each of the three sample locations inside the mock office, and positive controls (in triplicate) were placed in a location immediately outside the chamber. The sample collection/storage procedure was modified from fogging tests for two of the three sets of samples. The procedure used to collect and store the first set of samples was consistent with that used for previous fogging tests. The samples were aseptically transferred into empty sterile sampling tubes. To determine the effect of simply diluting any dichlor residue, the coupons from the second set were transferred into sampling tubes preloaded with 10 mL of PBST. Finally, to assess neutralization requirements during the 72-hour hold time, coupons from the third set were transferred into sampling tubes preloaded with PBST and STS neutralizer.

2.12 Decontamination Efficacy Characterization

Spore loading (i.e., positive control spore levels) was quantified by taking the logarithm_{10} of the CFU count for each material coupon and then calculating the mean and standard deviation of the log values (the mean of a series of log values is equivalent to the log of the geometric mean for the same series) for each set of triplicates (positive controls were inoculated in sets of three per material). Post-decontamination results are presented in terms of spore recovery as well and were calculated in the same manner as the positive control results. Results are also presented in terms of decontamination efficacy, which was quantified as LR. The LR was calculated as the mean of the log values for each positive control average CFU count minus the mean of the log values for each test sample average CFU count. Occasionally results were reported by noting whether the average LR for a particular coupon or surface test was ≥ 6.0, since a decontaminant that achieves ≥ 6 LR is considered effective as a sporicidal decontaminant based upon appropriate laboratory testing (U.S. EPA, 2010). We note, however, that while a decontamination efficacy ≥ 6 LR may be considered “effective” when reporting test results, in an actual *B. anthracis* release event, the goal for decontamination would be to minimize the number of recoverable viable spores, regardless of LR. Hence, we also report results in terms of the number of samples in which spores were not detected.

When no spores were detected for a sample, this result implied the highest decontamination efficacy quantifiable and achievable, and the LR was reported as ≥ the positive control recovery minus the recovery from the test sample (calculated based on imputing a 0.5 CFU value on the filter plate and adjusting for the filter plate volume)

2.13 Statistical Analyses

Decontamination efficacies associated with study parameters were compared using one-way analysis of variance (ANOVA) in MS Excel. The p-value from two-sided (non-directional) tests were used to test the hypotheses ($\alpha = 0.05$). Note, all of the tests contained the same distribution of materials and locations within the room therefore it was assumed that a material or location by volume fogged, air exchange, dwell, or AIC interaction did not occur. This could be evaluated further and may influence the significance testing reported here.

3 Quality Assurance / Quality Control

Quality assurance/quality control (QC) procedures were performed in accordance with the NHSRC Quality Management Plan and the Quality Assurance Project Plan (QAPP-J15-011.0 and J15-011.A1). The QA/QC procedures and results are summarized below.

3.1 Sampling, Monitoring, and Equipment Calibration

Approved operating procedures were used for the maintenance and calibration of all laboratory equipment. All equipment was verified as being certified calibrated or having the calibration validated by EPA's metrology laboratory at the time of use. Standard laboratory equipment such as balances, pH meters, biological safety cabinets (BSC), and incubators were routinely monitored for proper performance. Calibration of instruments was done at the frequency shown in Tables 3-1 and 3-2. Any deficiencies were noted. Any deficient instrument was adjusted to meet calibration tolerances and recalibrated within 24 hours. If tolerances were not met after recalibration, additional corrective action was taken, including recalibration or/and replacement of the equipment.

Table 3-1. Sampling and Monitoring Equipment Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
Meter box	Volume of gas is compared to National Institute of Standards and Technology (NIST)–traceable dry gas meter annually	± 2%
Flow meter	Calibration using a flow hood and a Shortridge manometer	± 5%
RH and temperature sensor	Compare RH to the head space of three calibration salt solutions in an enclosed space once a week; thermistor (for temperature) part of RH sensor and calibrated by manufacturer	± 5%
Stopwatch	Compare against NIST Official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java once every 30 days	± 1 min/30 days

Table 3-2. Analysis Equipment Calibration Frequency

Equipment	Calibration Frequency	Calibration Method	Responsible Party	Acceptance Criteria
Pipettes	Annually	Gravimetric	Carter Calibrations, Manassas, VA	± 1% target value
Incubator Thermometers	Annually	Compared to NIST-traceable thermometer	Metrology Laboratory	± 0.2 °C
Scale	Before each use	Compared to Class S weights	Laboratory staff	± 0.01% target

3.2 Acceptance Criteria for Critical Measurements

QA/QC checks associated with this project were established in the QAPP. A summary of these checks is provided in Table 3-3.

Table 3-3. Summary of QA/QC Checks

Matrix	Measurement	QA/QC Check	Frequency	Acceptance Criteria	Corrective Action
Negative test coupon samples (field blank)	CFU/sample	Field blank	One per material type per test	0 CFU (no detection)	Revise handling procedures; investigate sources of contamination; reject results of the same order of magnitude
Biolab materials	CFU/sample	Biocontaminant material blanks of PBST, dilution tubes, and plating beads (check that plating materials are not contaminated)	3 per each material used per test	0 CFU (no detection)	Investigate sterilization procedure; investigate sources of contamination
Positive test samples	CFU	Positive controls (inoculated w/ spores, but not subject to any treatment)	3 per material per test	5×10^6 to 5×10^7 CFU	Revise deposition or sampling protocol if mandated by WACOR
Test coupon samples	CFU	Agreement of triplicate plates of single coupon at each dilution	Each sample	Each CFU count must be within 50% of the other two replicates	Replate or filter samples
Chamber air	RH	2-point calibration	Once per test	±5%	Replace Vaisala sensor
Chamber air	Temperature	5-point calibration	Annually	±1 °C accuracy	Replace thermocouple
Chamber air	Cl_2 concentration	2-point calibration	Annually	Factory calibration with ACD Cal 2000 chlorine generator, ±5%	Replace sensor
Chamber air	ClO_2 concentration	2-point calibration	Prior to each use	NIST-traceable transmission band-pass optical filters	Replace sensor
Sporicidal solutions	pH, effective concentration of hydrogen ions in solution	Oakton Acorn meter	1 per use	> 6.5 and < 7.0 for fresh pAB	Reject solution; replace reagents and prepare a new solution
Sporicidal solutions containing bleach or dichlor	Concentration of FAC in fresh pAB, diluted bleach, and, dichlor solutions	HACH test kit, model CN-HRDT	Once upon production	±10% of target concentration	Reject solution; replace reagents and prepare a new solution
Sporicidal solutions containing ClO_2	Concentration of ClO_2	HACH test kit, model CN-HRDT	Once upon production	±10% of target concentration	Reject solution; replace reagents and prepare a new solution
Exposure/dwell	Time	NIST-traceable timer, comparison with official NIST U.S. time	Once per 1 second	± 0.5 second	Synchronize timer with official NIST U.S. time

3.3 Data Quality

Temperature and RH measurement devices were maintained within the calibration tolerances listed in Table 3-1. The extractive sampling method was validated against a known concentration of certified Cl_2 gas (Airgas Specialty Gases, Durham, NC). During extractive sampling, Cl_2 concentrations were sampled

using impingers, and the impinger liquid was analyzed using wet chemistry techniques. An accuracy assessment was performed by comparing the wet chemistry measurement to the certified Cl_2 gas concentration (target of 53.21 ppm ± 2%). Average wet chemistry values were shown to be within 5% (0.51 SD) of the certified gas. Figure 3-1 shows the results of the assessment.

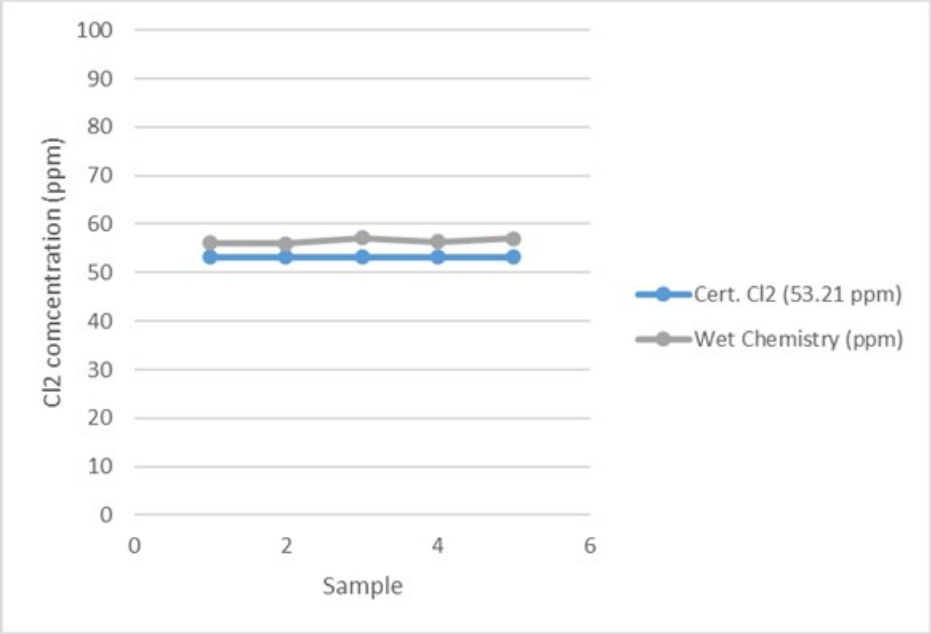


Figure 3-1. Cl_2 measurement accuracy assessment

Sporicide solution pH levels were measured using a high-accuracy (± 0.01 pH) waterproof pH meter. Three-point calibrations using certified buffer solutions were performed on the pH measurement device prior to each use. The device was maintained within ± 0.02 pH of each point prior to use.

Figure 3-2 shows the number of field blank samples that returned CFU counts (11 out of approximately 200 field blanks overall for the study) for the target organism for the overall study. Field blank samples were handled the same as test samples except that they were not inoculated with spores. For each test, the field blank coupons (one coupon per material) were placed in separate stages from the test coupons, inside COMMANDER underneath the desk.

Although the intention was to minimize the presence of contamination, the levels detected on field blanks were considered minor (in the rare occurrence the target organism was found on a field blank, it was typically less than five CFU on a filter plate) and were not expected to impact study results. Spores present on the field blanks could indicate cross-contamination during sampling or sample collection, confounding post-fumigation results of the same order of magnitude.

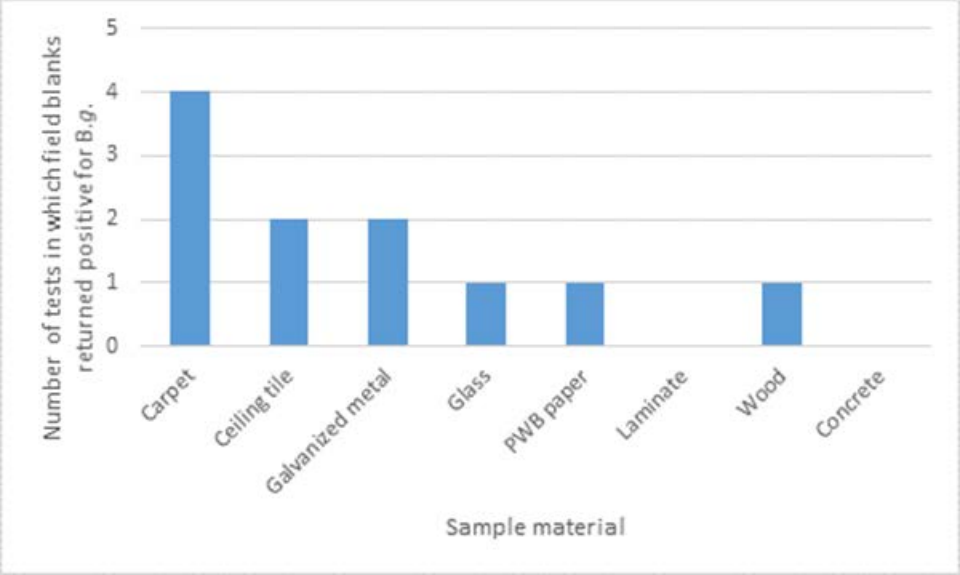


Figure 3-2. Field blank test sample results for tested materials

4 Results and Discussion

4.1 Test Matrix Summary and Fumigation Conditions

A summary of the conditions for each fogging test is shown in Table 4-1. (Note there were a total of 28 tests conducted in the study, although Test 5 is not included in the table since it was used for method development. In addition, the last two tests were conducted to investigate issues related to dichlor residue, which are further discussed below.) These values include the actual level of the active ingredient (either FAC or ClO_2) in the sporicidal solution, the actual volume of liquid sporicide disseminated, the mass of AIC disseminated (concentration of AIC times volume disseminated), dwell time, RH, and temperature. All fogging tests were conducted at ambient temperature and RH. Additionally, chamber chlorine gas and ClO_2 gas concentrations were monitored in the air but not controlled. Except as noted in the table, tests were performed in a closed system; four of the last five decontamination tests were conducted with controlled room air exchange to test its effect on decontamination efficacy.

Initially in the study, tests were conducted with solution volumes less than the fogger capacity of 1 gallon. However, as testing progressed and with the intent to improve decontamination efficacy, two foggers were used in some tests, which allowed fogging up to 2 gallons of liquid sporicide. After conducting tests with 2-gallon fogging, it was decided that 2 gallons would be the maximum amount to fog due to substantial wetting and dripping of sporicide from the ceiling.

The actual volumes of solution disseminated via the fogger(s) were, in general, within 12% of the target volume and, in most cases, within 5%. Tests 6, 24, and 25 were exceptions in that the volumes disseminated were 21%, 87%, and 39% less than the target volumes, respectively. In these cases, equipment malfunctions occurred during fogging, reducing the flow of solution through the fogger.

Seven tests were performed with pAB solution. Initially, the target FAC was 6000 mg/L to 6800 mg/L (Tests 1 and 2). During subsequent tests, a new procedure for preparing pAB was adopted that allowed for higher FAC concentration targets. The FAC for these tests ranged from 7,840 mg/L to 18,701 mg/L.

Eight tests were performed with diluted bleach solution. FAC levels ranged from 15,920 mg/L to 24,201 mg/L.

Seven decontamination tests were performed with dichlor solution. FAC levels of the dichlor solutions ranged from 20,601 mg/L to 21,901 mg/L with the exception of Test 24. The dichlor solution formulation was modified for Test 24, from 0.33 lb of stabilized chlorine granules per 1 gallon of DI water to 0.5 lb/gallon, resulting in an increased FAC level of 32,502 mg/L. Two additional tests (Tests 27 and 28) were conducted with dichlor to investigate issues related to the residue affecting CFU counts, as discussed in section 2.11.

Three tests were performed with the ClO_2 solution. ClO_2 levels in the aqueous phase ranged from 4,763 mg/L to 5,907 mg/L.

The pH levels for dichlor and pAB were in the range of 6-7; diluted bleach pH levels ranged from around 11-12; and aqueous ClO_2 pH levels were rather acidic (~ pH of 2).

Table 4-1. Summary of Fogging Conditions

Test Number	Sporicidal Solution	Actual Sporicidal Solution Volume Disseminated (mL)	AIC in Aqueous Solution (mg/L)	Mass of AIC disseminated (grams)	pH	Mean AIC in Chamber Air (ppm)	Max AIC in Chamber Air (ppm)	Dwell Time (h)	Mean RH (%)	Max RH (%)	Mean T (°C)	Air Exchange (fraction of chamber volume replaced each hour)
1	pAB	887	6440	5.71	6.8	3	5	20	68	71	28	0
2	pAB	2921	6480	18.93	7.0	7	12	24	67	78	26	0
3	Diluted bleach	2891	17401	50.31	11.4	8	17	23	77	82	28	0
4	pAB	3941	7840	30.90	6.8	7	16	20	64	90	27	0
6	Diluted bleach	4840	16721	80.93	NA	10	18	20	42	35	28	0
7	Dichlor	5873	20601	120.99	NA	4	8	37	76	90	22	0
8	Diluted bleach	5300	15920	84.38	12.01	27	40	2	64	90	26	0
9*	pAB	5891	15701	92.49	7.20	35	40	0	69	87	26	0
10	ClO_2	1910	5906	11.28	2.04	59	72	19	72	73	25	0
11	ClO_2	3960	4763	18.86	2.24	73	97	19	96	98	24	0
12	pAB	5817	18301	106.46	6.67	89	125	19	97	98	28	0
13	Dichlor	7165	20701	148.32	7.82	12	22	17	91	100	28	0
14*	Diluted bleach	7642	19001	145.21	11.14	34	93	19	100	100	26	0
15	ClO_2	7738	5907	45.71	1.66	36	155	19	90	100	26	0
16	pAB	7229	17401	125.79	6.24	131	219	19	80	88	27	0
17*	Diluted bleach	7776	24201	188.19	11.15	48	103	16	87	102	29	0
18	Dichlor	7915	21301	168.60	6.61	20	32	16	81	86	30	0
19**	Diluted Bleach	7766	23701	184.06	11.12	46	106	19	90	32	27	0
20	pAB	7860	18701	146.99	6.28	52	224	20	68	95	26	0
21***	Diluted bleach	7780	23001	178.95	11.31	9	78	18	73	27	24	0
22	Dichlor	7778	21901	170.35	6.52	11	26	21	69	79	25	0.75
23	Dichlor	7396	20701	153.10	NA	14	22	18	84	85	25	0
24*	Dichlor	3141	32502	102.09	6.74	8	15	17	56	73	27	0.75
25	Dichlor	5406	20801	112.45	6.57	9	16	19	65	92	27	0.75

Test Number	Sporicidal Solution	Actual Sporicidal Solution Volume Disseminated (mL)	AIC in Aqueous Solution (mg/L)	Mass of AIC disseminated (grams)	pH	Mean AIC in Chamber Air (ppm)	Max AIC in Chamber Air (ppm)	Dwell Time (h)	Mean RH (%)	Max RH (%)	Mean T (°C)	Air Exchange (fraction of chamber volume replaced each hour)
26	Diluted bleach	7674	22201	170.37	11.13	16	27	20	66	76	29	0.75
27	Dichlor	7948	22501	178.84	6.74	17.2	21.6	18	97.8	101.1	26.2	0
28	Dichlor	7897	23104	182.45	6.77	11.4	18	19	82.1	84.0	27.6	0

*Insufficient air circulation due to mixing fan malfunction. **Replaced mock office ceiling tiles prior to experiment. ***Fogger nozzles inadvertently positioned toward back wall instead of ceiling.
The active ingredient for liquids were measured as FAC for tests performed with diluted bleach, dichlor, and pAB. ClO_2 was the active ingredient measured for tests performed with aqueous ClO_2.
The AIC as measured in air was Cl_2 gas when fogging diluted bleach, dichlor, and pAB; and ClO_2 gas when fogging aqueous ClO_2
Test 5 not included in table since this test was used for method development, in which peracetic acid (a non-chlorine based decontaminant) was fogged.

4.2 Results

This section presents results for the overall effectiveness of each chlorine-based sporicide (in terms of LR) in inactivating *B. atrophaeus* spores on contaminated material surfaces. Effectiveness was determined by comparing the viable spore recoveries of fogged material coupons to their unexposed positive control counterparts. A 7 log spore challenge (inoculation of test and positive control materials with ~5 x 10^7 spores) was used across all tests and materials. This study utilized the generally accepted criterion of 6 LR to consider an approach effective. Recovery of no viable spores following treatment was considered highly effective.

4.2.1 Spore Recovery from Positive Controls

Spore recoveries of positive control samples for each material for the entire study using the bacterial spore sampling and analysis method detailed in Section 2.10 are shown in Figure 4-1. On average, 2.83E+07 (± 9.38E+06) CFU were recovered from coupon materials. The extractive sampling method successfully recovered the required 1 x 10^6 CFU (minimum amount required to demonstrate 6 LR) consistently from each material throughout the test series.

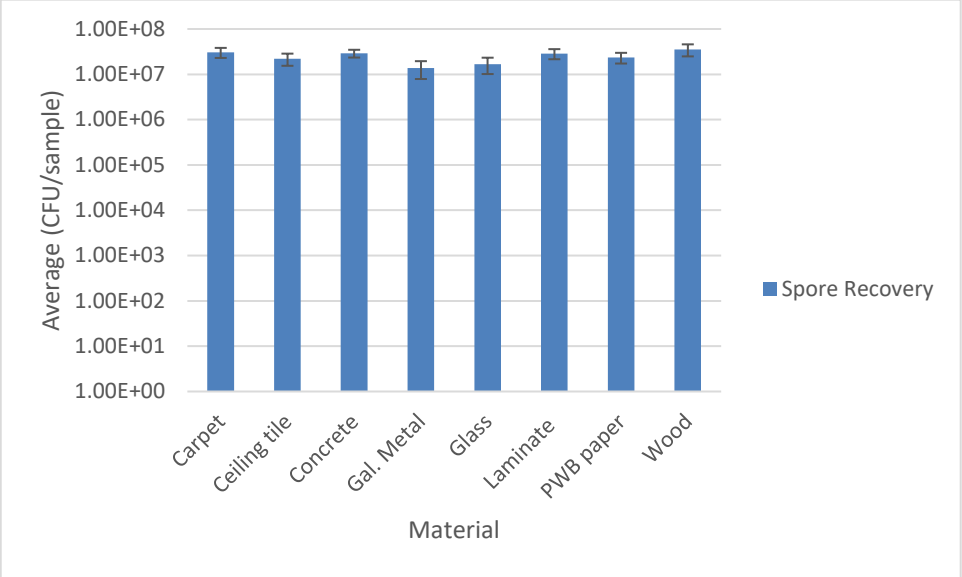


Figure 4-1. Average CFU recovery (± SD) from positive controls for each material

4.2.2 Efficacy of Individual Fogging Tests

Figure 4-2 shows the average efficacy results for each of the 25 chlorine-based fog decontamination tests, organized by sporicide solution. The three tests providing the greatest average LR values in the study were Test 19 (diluted bleach; 5.86 ± 0.80 LR), Test 15 (aqueous ClO_2; 5.80 ± 1.08 LR), and Test 18 (dichlor; 5.76 ± 0.52 LR). It can be observed from Figure 4-2 that the lower numbered tests, which tended to have less solution fogged and/or lower AIC values (refer to Table 4-1), generally resulted in lower LR

values. As the test program proceeded, we endeavored to improve efficacy for a particular sporicide by increasing AIC or liquid volume fogged. Thus maximizing the volume of solution fogged (maximum of approximately 8 L) and the AIC (maximum of approximately 22,000 mg/L for pAB, diluted bleach, and dichlor solution; tests performed with aqueous ClO_2 had maximum ClO_2 concentrations of approximately 5,900 mg/L) produced similar LR results for all sporicides. That is, average decontamination efficacies associated with fogging relatively greater amounts of liquid and using higher AIC levels were generally ≥ 5 LR for all four of the chlorinated sporicidal liquids. Note that because these LR values are averaged across all materials, this resulted in standard deviation values greater than 2 LR for some tests; statistical analyses comparing efficacy results and effects of test variables are further discussed below.

For additional details, refer to Appendix A, Table A-1, for the average LR values for each material for each test. Refer to Appendix B for efficacy results graphically displayed for each test, indicating average LR for each material at each location within the test chamber.

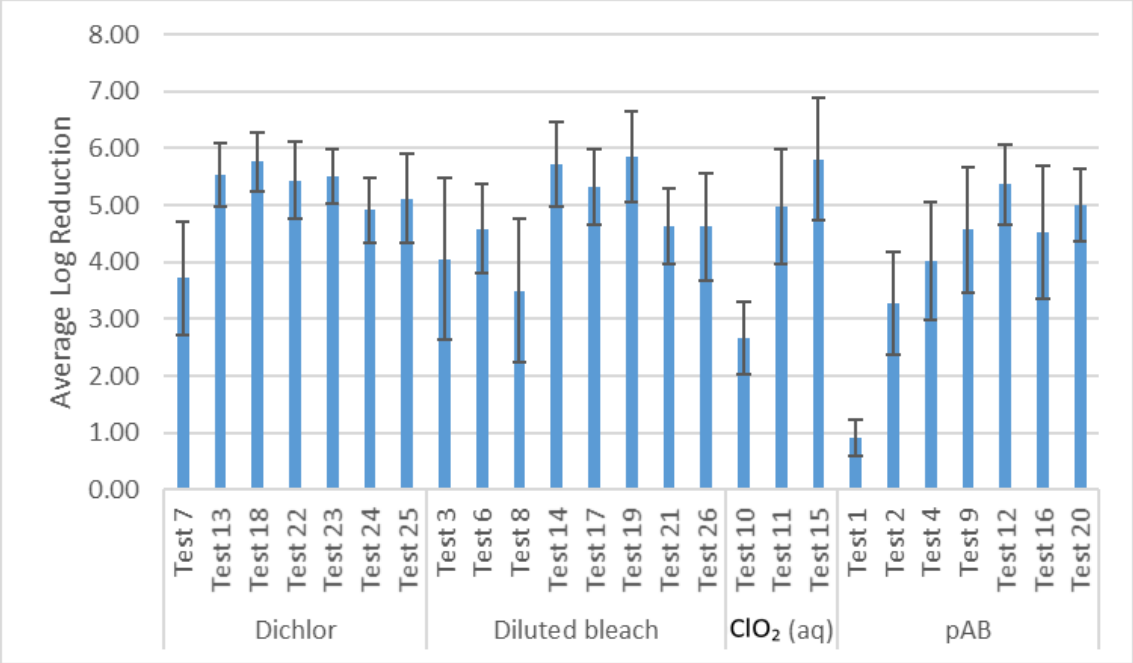


Figure 4-2. Efficacy results (Avg. LR) grouped by sporicide (±SD)

4.2.3 Decontamination Results by Material

Figure 4-3 shows the average LR of viable spores for each material for all tests performed in this study (analysis includes combined effects of sample location, sporicide, and test conditions). On average, non-porous materials such as galvanized metal, glass, laminate, and PWB paper were easier to decontaminate, with overall average efficacies of 6.08 ± 1.89, 6.54 ± 1.63, 6.08 ± 1.86, and 5.78 ± 1.86, respectively. Conversely, porous materials proved more difficult to effectively decontaminate via the fogging of chlorinated decontaminants, with average LR values for carpet, ceiling tile, wood, and concrete being 2.69 ± 1.88, 1.89 ± 0.76, 3.49 ± 1.83, and 4.31 ± 1.82, respectively. Of the materials tested, ceiling

tile was the most difficult to decontaminate; the highest LR achieved in the study for ceiling tile was 3.24 ± 0.20 (Test 19). Glass proved to be the least difficult to decontaminate overall with results showing > 6 LR for 21 of the 25 treatments tested. Notably the three most difficult materials to decontaminate (carpet, ceiling tile, and wood) are also comprised of organic based constituents. These general results related to the effect of materials on decontamination efficacy when fogging chlorine-based decontaminants are similar to results when pAB was applied as a spray or when materials were immersed. See for example, US EPA (2006); Wood et al. (2011a); Wood et al. (2011b); and Calfee et al. (2011).

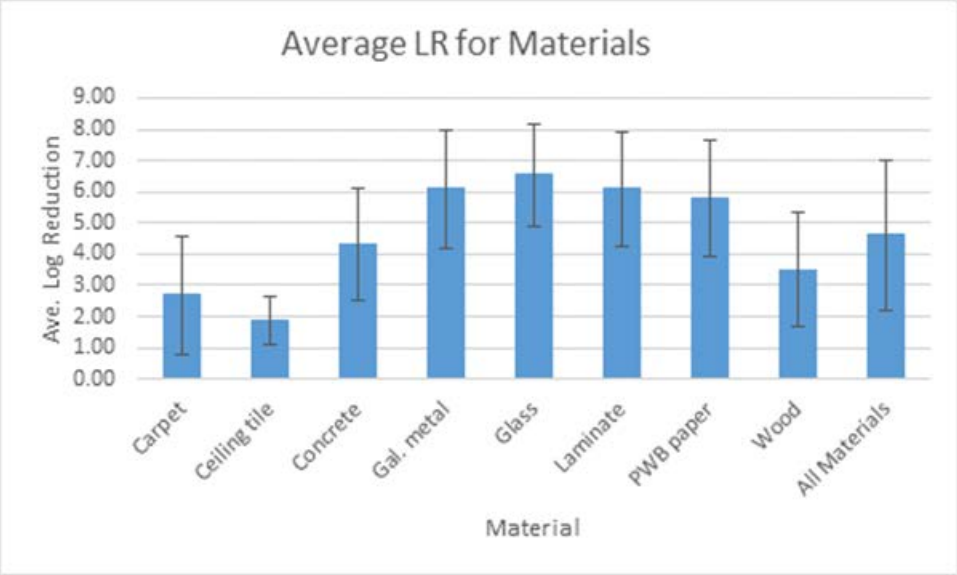


Figure 4-3. Average LR for materials (±SD)

Figure 4-4 displays efficacy results, but organized by material and sporicide. The average LR by material was generally similar for each sporicide, although there were a few exceptions (e.g., the aqueous ClO_2 solution much less effective on the galvanized metal compared to the other decontaminants). All four chlorinated decontaminants were generally ineffective on carpet, concrete, wood, and ceiling tile.

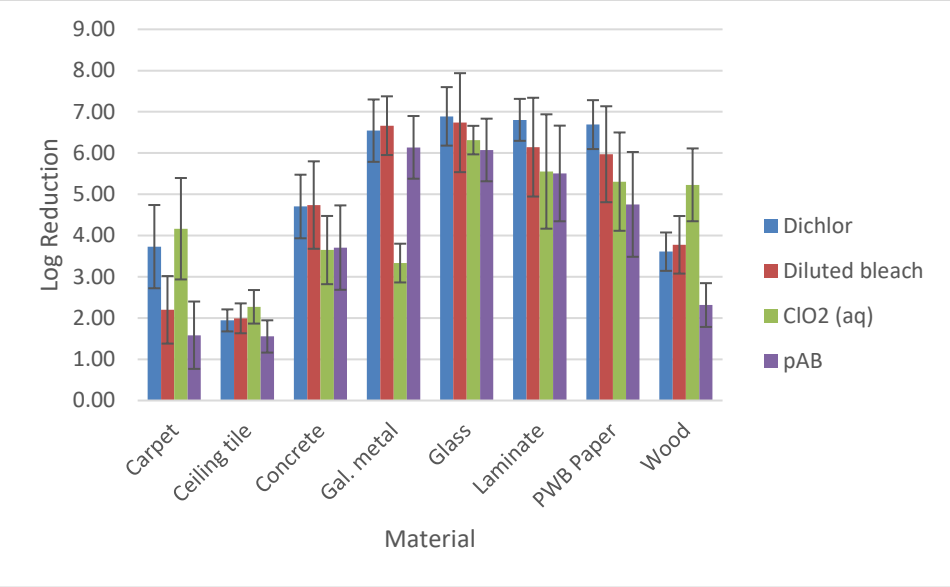


Figure 4-4. Average efficacy for sporicidal solutions with respect to material

4.2.4 Decontamination Results for Each Location

The average LR (all tests, sporicides, and materials) for each mock office location and the average LR for all locations combined are presented in Figure 4-5. Material coupons located on the desk show the highest average LR, at 4.94 ± 2.17. Coupons located under the desk (average LR 4.47 ± 2.30) and above the ceiling (LR 4.42 ± 1.45) show effectively the same decontamination efficacy. An ANOVA showed no significant difference between the average LR of coupons located under the desk and that of coupons in other locations (p-value = 0.15). Findings were similar for coupons located above the ceiling (p-value = 0.28). Data analysis showed a significant difference (albeit small, i.e., ~ 0.5 LR difference) in the decontamination efficacy of the desk location compared to the other locations (p-value = 0.012). Overall, these minor differences in efficacy results as a function of test chamber location generally imply the fog was fairly well distributed.

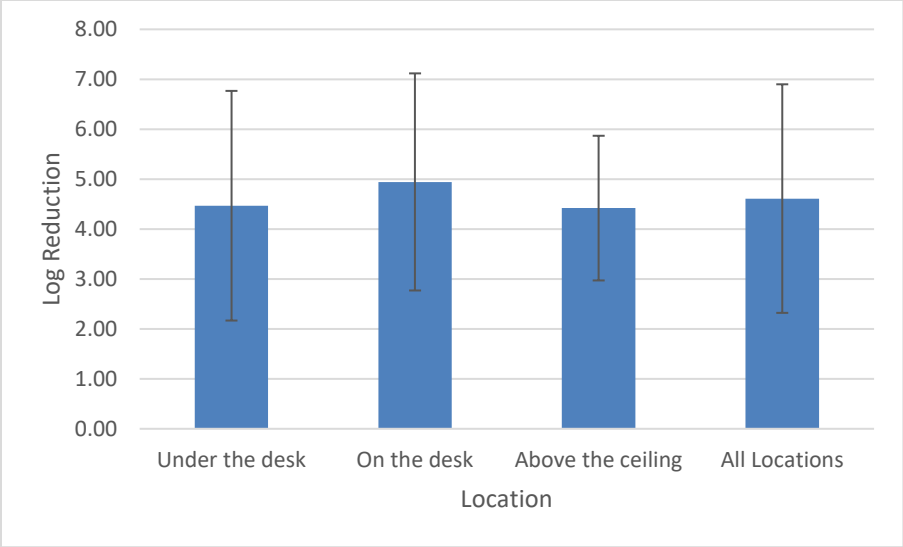


Figure 4-5. Average LR for mock office locations (±SD)

4.3 Summary of Efficacious Test Conditions and Impact of Test Variables

Although no one test proved efficacious for all materials, the majority (24 of 25) of the decontamination tests returned > 6 LR for one or more materials. Table 4-2 summarizes the tests in which each material was effectively decontaminated (achieved an average of > 6 LR) and a summary of the associated treatment conditions (effective ranges for volume disseminated, AIC in solution, and dwell time). Refer to Table 4-1 for a detailed summary of conditions for individual tests. There were no tests in which carpet or ceiling tile was effectively decontaminated, and just one test (Test 19) in which concrete was effectively decontaminated, and so these materials are excluded from the table.

Efficacious decontamination conditions for galvanized metal were achieved with pAB volumes as low as 2,921 mL at 6,480 mg/L FAC. Similarly, effective decontamination with diluted bleach on galvanized metal was achieved using 2,891 mL at 17,400 mg/L FAC. While smaller volumes of dichlor were sufficient for effective decontamination on galvanized metal, all tests were performed with relatively high FAC concentrations (at least 20,000 mg/L). Maximizing pAB and diluted bleach volume and FAC concentration was not required to achieve > 6 LR for galvanized metal. Low volumes of dichlor at higher FAC concentration proved efficacious, but information is not available for treatments at lower FAC concentrations.

Similar to galvanized metal, efficacious fogging conditions were achieved for glass at lower pAB volumes and FAC concentrations (2,921 mL pAB at 6,480 mg/L FAC). Also, fogging with low volumes of diluted bleach and FAC concentrations (as low as 4,840 mL and 16,720 FAC) were effective with zero air exchanges. Volumes of dichlor as low as 7,165 mL with FAC concentrations of 20,701 were proven effective for glass with zero air exchanges. Aqueous ClO_2 solution also proved effective for glass material at low volumes (3,960 mL).

Small volumes of pAB at lower FAC concentrations (as low as 3,941 mL and 7840 mg/L FAC) were effective for decontaminating laminate. Low volumes of diluted bleach were effective with increased levels

of FAC (2,891 mL and 17,401 mg/L FAC). Aqueous ClO_2 solution was effective at low volumes and ClO_2 concentrations (3,960 mL and 4,763 mg/L). Larger volumes of dichlor at higher concentrations (7,165 mL and 20,701 mg/L) were required for full decontamination of laminate coupons.

Painted wallboard paper required smaller volumes of pAB at higher FAC concentrations for effective decontamination (5,817 mL required 18,301 mg/L FAC). Relatively high volumes and FAC concentrations (at least 7,642 mL and 19,001 mg/L FAC) of diluted bleach were required for effective decontamination. Aqueous ClO_2 solution proved effective at low volumes (3,960 mL). Similar to diluted bleach, high volumes and FAC concentrations (at least 7,165 mL and 20,701 mg/L FAC) were required for successful decontamination of painted wallboard paper with dichlor.

Wood material was successfully decontaminated with diluted bleach and aqueous ClO_2. A relatively large volume of diluted bleach at high FAC levels was required for effective decontamination. Aqueous ClO_2 proved efficacious with relatively low volumes (3,960 mL).

In the case of nonporous materials, dichlor fogging proved effective at low volumes and 0.75 air exchanges, but exceedingly high concentrations were required (3,141 mL and 32,502 mg/L FAC). It was suspected that the high FAC levels of dichlor resulted in failure of the fogging equipment (one of two foggers malfunctioned during testing). Further testing is required to assess equipment compatibility with concentrated sporicide solutions.

Figure 4-6 is a distillation of the above information, and shows the minimum mass of AIC disseminated (concentration of AIC X volume fogged) that resulted in effective decontamination, as a function of each material and sporicide.

Table 4-2. Summary of Efficacious (≥ 6 LR) Decontamination Conditions per Material

Coupon Material	Efficacious Test Conditions and Test Identification (ID) Numbers			
	pAB	Diluted bleach	Aqueous ClO_2	Dichlor
Galvanized metal	Volume disseminated: 2,921–7,860 mL AIC: 6,480–18,701 mg/L Dwell: 0–20 hours Tests: 2, 4, 9, 12, 16, 20	Volume disseminated: 2,891–7,674 mL AIC: 16,721–24,201 mg/L Dwell: 16–23 hours Tests: 3, 6, 14, 17, 19, 21, 26	–	Volume disseminated: 3,141–7,915 mL AIC: 20,701–32,502 mg/L Dwell: 16–21 hours Tests: 13, 18, 22–25
Glass	Volume disseminated: 2,921–7,860 mL AIC: 6,480–18,701 mg/L Dwell: 0–20 hours Tests: 2, 4, 9, 12, 16, 20	Volume disseminated: 4,840–7,674 mL AIC: 16,721–24,201 mg/L Dwell: 16–20 hours Tests: 6, 14, 17, 19, 21, 26	Volume disseminated: 3,960–7,738 mL AIC: 4,763–5,907 mg/L Dwell: 19 hours Tests: 11, 15	Volume disseminated: 3,141–7,915 mL AIC: 20,701–32,502 mg/L Dwell: 16–21 hours Tests: 13, 18, 22–25
Laminate	Volume disseminated: 3,941–7,860 mL AIC: 7,840–18,701 mg/L Dwell: 19–21 hours Tests: 4, 12, 16, 20	Volume disseminated: 2,891–7,766 mL AIC: 17,401–24,201 mg/L Dwell: 16–23 hours Tests: 3, 14, 17, 19	Volume disseminated: 3,960–7,738 mL AIC: 4,763–5,907 mg/L Dwell: 19 hours Tests: 11, 15	Volume disseminated: 3,141–7,915 mL AIC: 20,701–32,502 mg/L Dwell: 16–21 hours Tests: 13, 18, 22–25
PWB paper	Volume disseminated: 5,817–7,229 mL AIC: 17,401–18,301 mg/L Dwell: 19 hours Tests: 12, 16	Volume disseminated: 7,642–7,766 mL AIC: 19,001–24,201 mg/L Dwell: 16–19 hours Tests: 14, 17, 19	Volume disseminated: 3,960–7,738 mL AIC: 4,763–5,907 mg/L Dwell: 19 hours Tests: 11, 15	Volume disseminated: 3,141–7,915 mL AIC: 20,701–32,502 mg/L Dwell: 16–21 hours Tests: 13, 18, 22–25
Wood	–	Volume disseminated: 7,766 mL AIC: 23,701 mg/L Dwell: 19 hours Tests: 19	Volume disseminated: 3, 960–7,738 mL AIC: 4,763–5,907 mg/L Dwell: 19 hours Tests: 11, 15	–

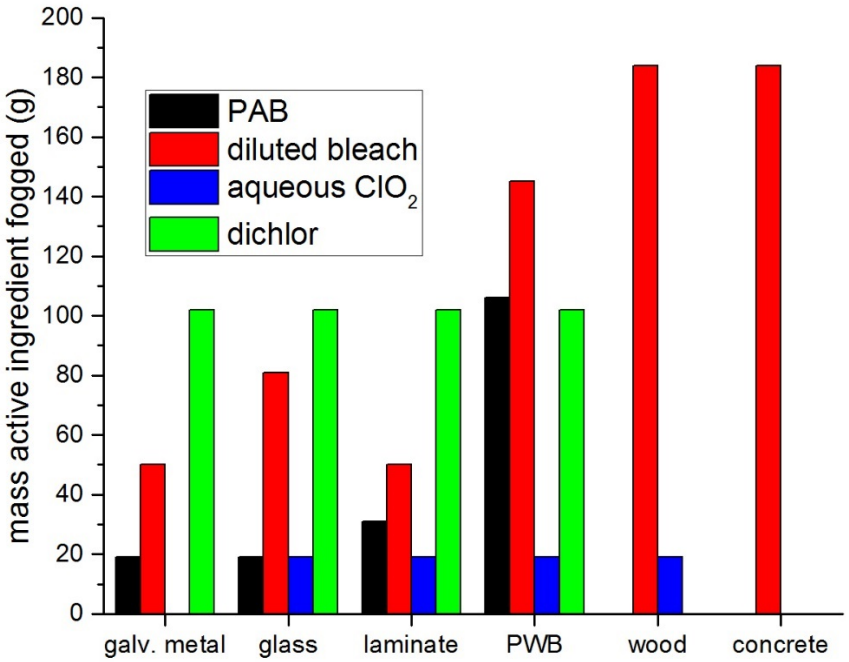


Figure 4-6. Minimum mass of active ingredient needed to achieve effective decontamination

4.3.1 *Effect of Disseminated Volume on Fogging Efficacy*

The effect of liquid volume fogged on decontamination efficacy was examined by comparing tests subjected to similar conditions (solution type, AIC, dwell time, pH, RH and T) but with dissimilar volumes of solution (mL) fogged. Tests paired for comparative analysis include Tests 7 and 13, 1 and 2, and 10 and 15. Table 4-1 summarizes the test conditions for each set of tests evaluated (AIC, volume of solution disseminated, and dwell time) as well as the average LR and statistical analysis findings (ANOVA).

Tests 7 and 13 used dichlor solution; Test 7 conditions included an FAC concentration of 20,601 mg/L, a disseminated volume of 5,873 mL, and 17 hours of dwell. Test 13 conditions included an FAC concentration of 20,701 mg/L, a disseminated volume of 7,165 mL, and 17 hours of dwell. Statistical analysis of these data indicated that varying the volume of diluted bleach solution disseminated has a statistically significant effect on decontamination efficacy at the prescribed test conditions (0.00122 p-value).

Tests 1 and 2 were performed with pAB solution. Test 1 conditions included an FAC concentration of 6,440 mg/L, a disseminated volume of 6,480 mL, and 20 hours of dwell. Test 2 conditions included an FAC concentration of 6,480 mg/L, a disseminated volume of 2,921 mL, and 24 hours of dwell. Statistical analysis of these data indicated that varying the volume of pAB solution disseminated has a statistically significant effect on decontamination efficacy at the prescribed test conditions (1.22E-05 p-value).

Tests 10 and 15 used aqueous ClO_2 solution. Test 10 conditions included a ClO_2 concentration of 5,906 mg/L, a disseminated volume of 5,907 mL, and 19 hours of dwell. Test 15 conditions included a $ClO_{2\,(aq)}$ concentration of 5,907 mg/L, a disseminated volume of 1,910 mL, and 19 hours of dwell. Statistical analysis of these data indicated that varying the volume of aqueous ClO_2 solution disseminated has a statistically significant effect on decontamination efficacy at the prescribed test conditions (1.03E-07 p-value).

The disseminated volume of solution proved to have a significant effect on efficacy for each of the three comparisons evaluated, i.e., increasing the volume fogged increased the efficacy. This effect seemingly persisted regardless of the AIC (relatively high or low).

Table 4-3. Decontamination Efficacy Comparison of Similar Tests with Different Volumes of Disseminated Solution

Test ID	Sporicidal Solution	AIC in Solution (mg/L)	Volume of Solution Disseminated (mL)	Dwell Time (h)	Avg. LR	p-Value (α = 0.05)
7	Dichlor	20601	**5873**	17	3.71 ± 1.58	0.00122
13		20701	**7165**	17	5.53 ± 1.90	
1	pAB	6440	**887**	20	0.90 ± 0.32	1.22E-05
2		6480	**2921**	24	3.28 ± 0.91	
10	Aqueous ClO_2	5906	**1910**	19	2.66 ± 0.52	1.03E-07
15		5907	**7738**	19	5.80 ± 1.13	

4.3.2 Effect of Air Exchange on Decontamination Efficacy

Two air exchange rates were used for this study: 0 and 0.75 air exchanges per hour. The majority of fog tests used no air exchange, while there were four tests that used an air exchange of 0.75. Statistical analyses were performed to assess the impact of air exchange on decontamination efficacy using paired tests with similar conditions, but with and without air exchange. Sets compared included Tests 22 and 23, 7 and 25, and 26 and 21. Table 4-3 summarizes test conditions (the AIC for the sporicidal solution, the volume of solution disseminated, and the total air exchanges [fraction of chamber volume replaced each hour]) as well as the comparative analysis (ANOVA) findings. The results show lower average efficacy in all three comparisons where air exchange was used, however the effect was significant only for the comparison between Tests 7 and 25 and therefore the lower values for air exchange in the other two comparisons were due to natural variation.

Table 4-4. Efficacy Comparison of Similar Tests with Different Rates of Air Exchange

Test ID	Sporicidal solution	AIC in Solution (mg/L)	Volume of Solution Disseminated (mL)	Air Exchange (fraction of chamber volume replaced per hour)	Avg. LR (± SD)	p-Value (α = 0.05)
22	Dichlor	21901	7778	0.75	5.44 ± 0.67	0.89
23		20701	7396	0	5.52 ± 0.48	
7	Dichlor	20601	5873	0.75	3.72 ± 0.99	0.027
25		20801	5406	0	5.12 ± 0.78	
26	Diluted bleach	22201	7674	0.75	4.55 ± 0.93	0.91
21		23001	7780	0	4.63 ± 0.65	

4.3.3 Effect of Dwell on Decontamination Efficacy

For this study, the dwell period (the post fogging time period during which the chamber environment was allowed to remain undisturbed prior to starting aeration) was typically overnight. However, two tests were performed with low dwell times (0 and 2 hours) to assess the impact of dwell on decontamination efficacy. Comparative analysis was performed using two sets of paired tests with similar conditions but different dwell times. Test sets included Tests 8 and 6 and 9 and 16. Table 4-4 summarizes test conditions (AIC for the sporicidal solution, volume of solution disseminated, and dwell time) as well as the average LR and statistical analysis findings (ANOVA).

Table 4-5. Efficacy Comparison of Similar Tests with Different Dwell Times

Test ID	Sporicidal Solution	AIC in Solution (mg/L)	Volume Disseminated (mL)	Dwell Time (h)	Average LR (± SD)	p-Value (α = 0.05)
8	Diluted Bleach	15920	5300	2	3.5 ± 1.25	0.059
6		16721	4840	20	4.59 ± 0.79	
9	pAB	15701	5891	0	4.56 ±1.11	0.96
16		17401	7229	19	4.53 ± 1.17	

Tests 8 and 6 using diluted bleach were paired for comparison. Test 8 conditions included 2 hours of dwell, an FAC concentration of 15,920 mg/L, and a disseminated volume of 5,300 mL. Test 6 conditions

included 20 hours of dwell, an FAC concentration of 16,721 mg/L, and a disseminated volume of 4,840. Analysis of these data indicated dwell time did not have a statistically significant effect on decontamination efficacy at the time prescribed test conditions (0.059 p-value).

Tests 9 and 16 using pAB were paired for comparison. Test 9 conditions included 0 hours of dwell, an FAC concentration of 15,701 mg/L, and a disseminated volume of 5,891 mL. Test 16 conditions included 19 hours of dwell, an FAC concentration of 17,401 mg/L, and a disseminated volume of 7,229 mL. Statistical analysis indicated dwell time has no statistically significant effect on decontamination efficacy at the prescribed test conditions (0.96 p-value).

4.3.4 Effect of Active Ingredient Concentration on Decontamination Efficacy

The effect of AIC on decontamination efficacy was assessed by comparing paired tests that had similar test conditions but different AICs. Test sets examined were Test 14 and 17 and Tests 2 and 4. Table 4-5 summarizes the tests conditions (AIC for the sporicidal solution, volume of solution disseminated, and dwell time) as well as the average LR and statistical analysis findings (ANOVA).

Table 4-6. Efficacy Comparison for Tests with Varying AICs

Test ID	Sporicidal Solution	AIC in Solution (mg/L)	Volume Disseminated (mL)	Dwell Time (h)	Average LR (± SD)	p-Value (α = 0.05)
14	Diluted bleach	19001	7642	19	5.73 ± 0.74	0.52
17		24201	7776	16	5.32 ± 0.66	
2	pAB	6480	2921	24	3.28 ±0.91	0.29
4		7840	3941	21	4.03 ± 1.03	

While statistical analysis for both diluted bleach and pAB tests did not indicate any significant effects of FAC levels on decontamination efficacy, this is most likely because the difference in AIC was not large for the relationship to be apparent.

4.4 Biological Indicator Results

Although previous tests (Rastogi et al., 2010) have shown that spore populations on BIs are typically much easier to inactivate than spores associated with coupons from building materials or actual environmental surfaces, BIs were included in fogging tests to demonstrate the general concept and utility of fogging technology.

Figure 4-7 presents the BI results for tests that included BIs, in terms of the percent of BIs inactivated (typically nine were used in each test). All positive control BIs (those not exposed to fogging conditions) from every experiment tested positive for growth. In experiments with air exchange (Tests 22, 24, and 25), a portion of the BIs showed positive growth. In two instances (Tests 10 and 24), all BIs tested positive. Other tests that had less than a 100% inactivation rate of BIs (Tests 2, 4, and 7) used low disseminated volumes of sporicide. In general, relatively low dissemination volumes and the presence of air exchange appear to challenge BI decontamination efficacy (similar to *B. atrophaeus* spore inactivation results discussed above) to a higher extent than other parameters tested in this study.

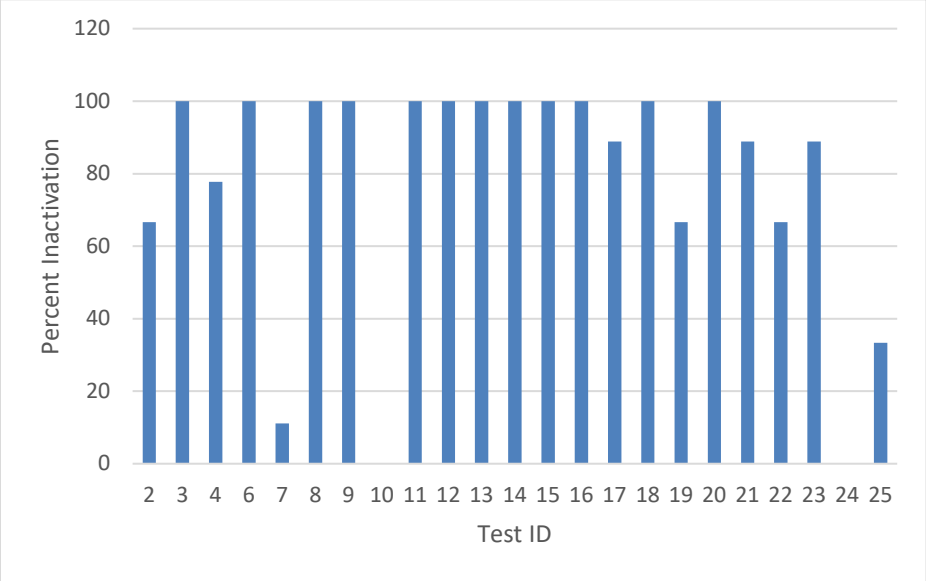


Figure 4-7. Percent of BIs inactivated for each test with BIs (n = 9)

Figure 4-8 shows the percentage of inactivated BIs as a function of location. BIs located on the desk showed the highest inactivation percentage of 84.1%. BIs under the desk were inactivated at a rate of 81.9%, and BIs located above the ceiling had the lowest inactivation percentage of 69.6%.

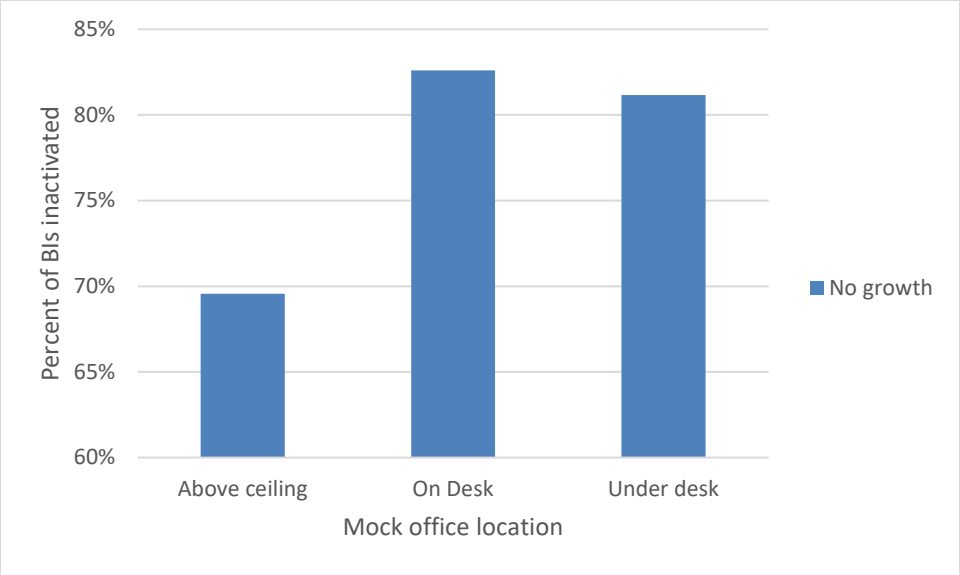


Figure 4-8. BI inactivation percentages in each mock office location (n = 69, all tests combined)

4.5 Impact of Dichlor Residue on Spore Recovery

During the process of collecting samples exposed to dichlor fog, residue was observed on the coupon surfaces and in COMMANDER itself. Subsequent tests confirmed that a dichloroisocyanurate salt was present in the residue. Because of this residue, a test was conducted to determine the impact of the dichlor residue on the recovery of spores subsequently inoculated onto coupons.

The results of this test are shown in Figure 4-9, and show a considerable effect of the residue, i.e., a 7 log difference between viable spores recovered from surfaces containing dichlor residue (4.67E+01) and those recovered from clean surfaces (5.83E+07). In response to these findings, additional neutralization tests were performed to identify potential biases in data gathered from fogging tests with dichlor solution.

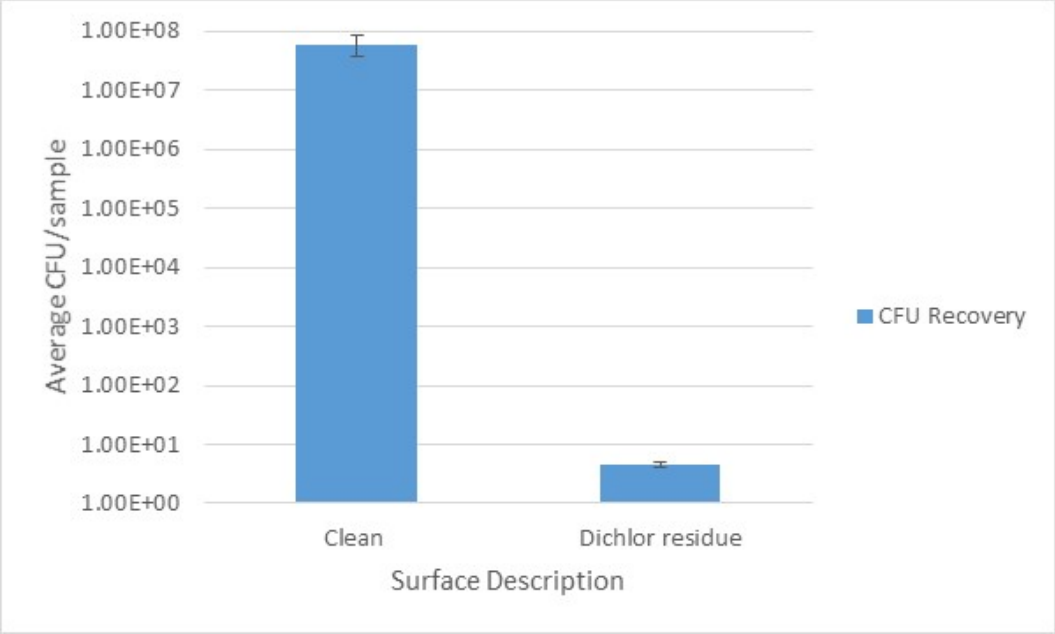


Figure 4-9. Average CFU recoveries from stainless steel clean surfaces vs. surfaces loaded with dichlor fog residue (±SD)

4.5.1 Extractive Sample Neutralization Scoping Test

Test 27 was performed to determine the impact of dichlor residue on the recovery of viable spores during the coupon extraction process. There was some thought that during the extraction process, any dichlor residue remaining on the coupon surface would dissolve and potentially inactivate any viable spores in the extraction solution (Section 2.11).

The results for Test 27 are shown in Figure 4-10 and summarized Table 4-7. An ANOVA of the two extraction procedures was performed for each material. The analysis showed p-values greater than 0.05 for all materials, indicating no statistically significant difference between the two coupon extraction procedures (the normal extraction process using only PBST, or extraction using PBST plus STS to neutralize any remaining dichlor). With respect to porous materials (carpet and concrete), the addition of STS to samples located on the desk appeared to increase spore recovery by approximately 2 logs. With the exception of the concrete coupons located in the ceiling, the addition of STS had no significant effect on the recovery of spores from porous material located on the floor and above the ceiling. The addition of the STS neutralizer to laminate samples collected from the desk appeared to reduce average LR (with large variances) compared to the other materials that showed either a moderate increase or no change. We acknowledge that these results showing no overall significant effect with the use of STS may be difficult to interpret because we did not start with a known titer (CFU depended on efficacy of dichlor fog), leading to the possibility of too much variability to detect these effects. That said, the variability of recovered CFU (see error bars in Figure 4-10) in this experiment was relatively low.

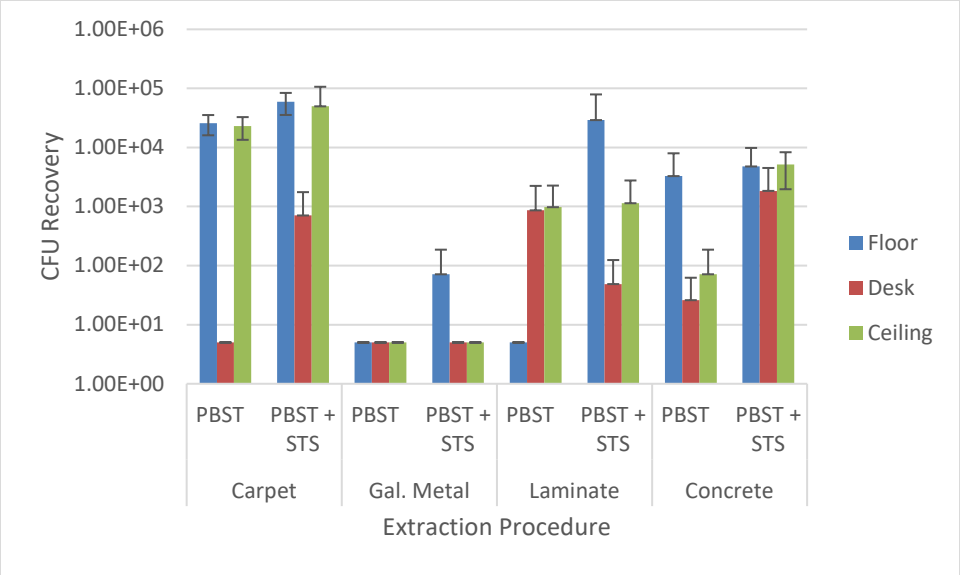


Figure 4-10. Effect of neutralizer during extraction on average CFU recovery (± SD)

Table 4-7. Average CFU Recoveries for Extraction Procedures

Material	Extraction Solution	Floor	p-Value	Desk	p-Value	Ceiling	p-Value
Carpet	PBST	2.57E+04 ± 9.66E+03	0.087	5.00E+00 ± 0.00E+00	0.31	2.31E+04 ± 9.60E+03	0.47
	PBST + STS	5.97E+04 ± 2.42E+04		7.06E+02 ± 1.05E+03		4.97E+04 ± 5.69E+04	
Galvanized Metal	PBST	5.00E+00 ± 0.00E+00	0.37	5.00E+00 ± 0.00E+00	n/a	5.00E+00 ± 0.00E+00	n/a
	PBST + STS	7.13E+01 ± 1.15E+02		5.00E+00 ± 0.00E+00		5.00E+00 ± 0.00E+00	
Laminate	PBST	5.00E+00 ± 0.00E+00	0.37	8.62E+02 ± 1.38E+03	0.36	9.76E+02 ± 1.29E+03	0.90
	PBST + STS	2.91E+04 ± 5.00E+04		4.87E+01 ± 7.56E+01		1.14E+03 ± 1.63E+03	
Concrete	PBST	3.28E+03 ± 4.69E+03	0.73	2.60E+01 ± 3.64E+01	0.30	7.13E+01 ± 1.15E+02	0.051
	PBST + STS	4.77E+03 ± 5.05E+03		1.84E+03 ± 2.66E+03		5.14E+03 ± 3.17E+03	

4.5.2 Coupon Storage Neutralization Test

The purpose of this second neutralization test (Test 28) was to identify the need to neutralize the coupons (after fogging of dichlor) during the 72-hour period over the weekend when coupons were stored in a refrigerator until they could be processed and extracted the following week. Figure 4-11 shows the results for this test (Test 28) and Table 4-8 provides a summary. An ANOVA of the three sample hold procedures (dry, in 10 mL PBST, or in 10 mL PBST+STS) for samples with dichlor residue was performed for each of the three materials studied. For laminate and concrete, the analysis resulted in p-values greater than 0.05 for each material, indicating no statistically significant difference between the three coupon hold procedures. For these materials, neither storing samples in 10 mL of PBST nor adding neutralizer to the PBST appeared to improve average spore recoveries significantly compared to storing the samples dry. However, in the case of carpet, an ANOVA comparison of the three storage procedures showed a

statistical difference within the set, i.e., spore recoveries for carpet samples appeared to increase significantly for samples stored in PBST with neutralizer (ANOVA p-value = 3.05E-09).

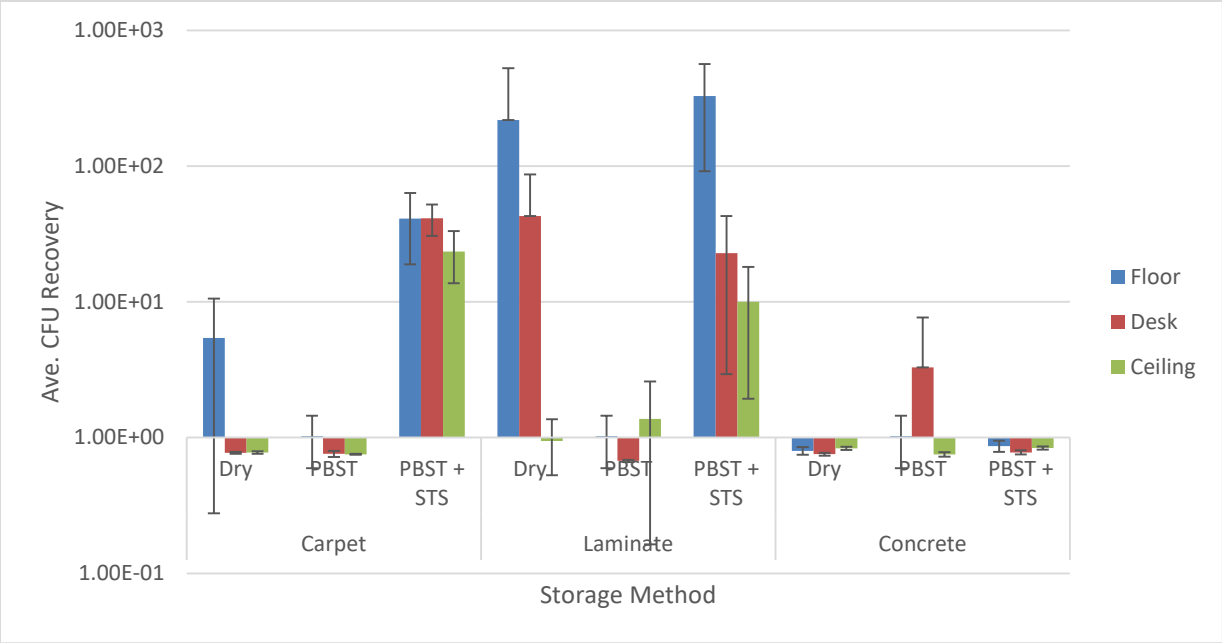


Figure 4-11. Effect of neutralizer during coupon storage (± SD)

Table 4-8. Average CFU Recoveries for Alternative Storage Procedures

Material	Storage Procedure	CFU Recovery (± SD)			p-Value
		Floor	Desk	Ceiling	
Carpet	Dry	5.42E+00 ± 5.14E+00	7.73E-01 ±1.37E-02	7.77E-01 ± 1.83E-02	3.29E-08
	PBST	1.02E+00 ± 4.27E-01	7.59E-01 ± 4.06E-02	7.54E-01 ± 6.53E-03	
	PBST + STS	4.10E+01 ± 2.22E+01	4.13E+01 ± 1.07E+01	2.35E+01 ± 9.74E+00	
Laminate	Dry	2.19E+02 ± 3.07E+02	4.28E+01 ± 4.41E+01	9.46E-01 ± 4.18E-01	0.26
	PBST	1.02E+00 ± 4.27E-01	6.73E-01 ± 1.37E-02	1.38E+00 ± 1.21E+00	
	PBST + STS	3.29E+02 ± 2.37E+02	2.28E+01 ± 1.99E+01	1.00E+01 ± 8.08E+00	
Concrete	Dry	8.00E-01 ± 5.09E-02	7.54E-01 ± 1.72E-02	8.34E-01 ± 2.45E-02	0.35
	PBST	1.02E+00 ± 4.27E-01	3.30E+00 ± 4.36E+00	7.51E-01 ± 2.86E-02	
	PBST + STS	8.67E-01 ± 8.29E-02	7.78E-01 ± 2.74E-02	8.38E-01 ± 2.16E-02	

The aforementioned results are caveated by the fact that Test 28 coupons were inoculated using an MDI manufactured by a different vendor (Research International) from what was used in all the previous tests. The use of the different MDI may have led to notably lower overall CFU recoveries from the Test 28 control set samples (the set of coupons stored dry; the same storage conditions at Tests 1–26) compared to Test 27 control set samples (the set of samples extracted without addition of neutralizer; same

extraction conditions as Tests 1–26), despite having comparable fog test conditions (see Table 4-1). This is because investigation revealed that when subjected to heat shock, samples inoculated with the newer Research International MDI yielded approximately 50% less CFU than the samples not subjected to heat shock. These results suggest a higher population of vegetative cells were loaded onto Test 28 coupons compared to Test 27. Thus it is possible that results for Test 28 were not representative of previous tests because of the different spore mixture and should be cautiously considered in reference to this study. However, any additional vegetative cells on the coupons in Test 28 would have most likely been inactivated during the fogging, yielding only spores on the coupons that were extracted. And thus the comparison of coupon storage methods (dry, PBST, or PBST + STS) would still be valid.

5 Summary and Conclusions

In this investigation, a series of 27 tests were conducted to assess the efficacy of fogging chlorine-based sporicidal solutions for the decontamination of common materials found in indoor and outdoor environments contaminated with a *B. anthracis* spore surrogate. While previous studies have shown that chlorine-based sporicides applied as a spray are effective in inactivating bacterial spores on a number of materials, commercially available fogging technologies have not been evaluated for their use with chlorine-based sporicides.

The following summarizes some of the more important findings from the study:

- Maximizing the fogged solution volume (approximately 8 L total; or in terms of volume to be decontaminated, 336 mL/m^3) and the AIC (maximum of approximately 22,000 mg/L free available chlorine for pAB, diluted bleach, and dichlor solution; and approximately 5000 mg/L aqueous ClO_2) generally produced similar results for all sporicides. That is, decontamination efficacy averaged for all materials in these tests at these more optimal conditions was generally above 5 LR, independent of the sporicide fogged.
- While no individual test achieved an average decontamination efficacy of > 6 LR for all materials, fogging methods were proven most effective (typically achieved > 6 LR) for the nonporous materials: galvanized metal, glass, painted wallboard paper, and laminate. Fogging of the chlorinated decontaminants was moderately effective for concrete. Ceiling tile, wood, and carpet (porous and organic-based materials) were the most difficult materials to decontaminate. These general trends in efficacy by material using chlorine-based sporicides are consistent with the literature.
- Relatively high volumes of sporicidal solution at high concentrations appeared to cancel any significant effects of increased air exchange rates on decontamination efficacy.
- Increasing the disseminated volume of solution proved to significantly increase decontamination efficacy. This effect persisted regardless of the solution's AIC (high or low).
- Data analysis showed a significant yet minor average improvement (~ 0.5 LR) in the decontamination efficacy of the coupons placed on the desk location compared to the other locations (under the desk and above the ceiling tiles). Coupons located under the desk and above the ceiling showed the same average decontamination efficacy. Overall, these minor differences in efficacy results as a function of test chamber location generally imply the sporicidal fog was fairly well distributed.
- In an experiment to evaluate the neutralization requirements for coupon samples containing dichlor residue during the extraction process, it was determined that there were statistically insignificant differences between samples extracted with PBST plus neutralizer and those extracted with just PBST. This was shown to be the case for all materials. This result is caveated by the fact that only one test was conducted, and that further research to investigate more fully this issue is warranted.
- The recovery of spores inoculated onto coupons already having a dichlor residue was significantly diminished.

This study has demonstrated the potential of using chlorine-based decontaminants applied with a commercially available fogging technology for the decontamination of surfaces typical of indoor

environments contaminated by *Bacillus* spores. However, this decontamination approach may be better suited for areas that do not contain significant quantities of porous or organic materials such as carpet, ceiling tile, or wood.

6 References

Brown, G.S., Betty, R.G., Brockmann, J.E., Lucero, D.A., Souza, C.A., Walsh, K.A., Boucher, R.M., Tezak, M., Wilson, M.C., and Rudolph, T. (2007a) Evaluation of a wipe surface sample method for collection of Bacillus spores from nonporous surfaces. *Appl Environ Microbiol* 73(3):706–710.

Calfee, M. W., Choi, Y., Rogers, J., Kelly, T., Willenberg, Z., & Riggs, K. (2011). Lab-scale assessment to support remediation of outdoor surfaces contaminated with Bacillus anthracis spores. *Journal of Bioterrorism and Biodefense*, *2*(3).

Canter, D.A., Gunning, D., Rodgers, P., O'Connor, L., Traunero, C., Kempter, C.J. (2005) Remediation of Bacillus anthracis contamination in the U.S. Department of Justice mail facility. *Biosecur Bioterror* 3: 119–127.

Lee, S.D., Ryan, S.P. and Snyder, E.G. (2011) Development of an aerosol surface inoculation method for Bacillus spores. Appl Environ Microbiol 77, 1638–1645.

National Council of the Paper Industry for Air and Stream Improvement, Inc. (NCASI). (1997) Methods Manual, *Determination of Chlorine and Chlorine Dioxide in Pulp Mill Bleach Plant Vents*. Research Triangle Park, N.C.

Rastogi, V.K., Ryan, S.P., Wallace, L., Smith, L.S., Shah, S.S., Martin, G.B. (2010) Systematic evaluation of the efficacy of chlorine dioxide in decontamination of building interior surfaces contaminated with anthrax spores*. Appl Environ Microbiol* 76: 3343–3351.

Ryan, S.P., Lee, S.D., Calfee, M.W., Wood, J.P., McDonald, S., Clayton, M., Griffin-Gatchalian, N. (2014) Effect of inoculation method on the determination of decontamination efficacy against Bacillus spores. *World J Microbiol Biotechnol,* DOI 10.1007/s11274-014-1684-2.

Schmitt, K., N.A. Zacchia, N.A. (2012) Total decontamination cost of the anthrax letter attacks. *Biosecur Bioterror* 10:98–107.

Standard Methods Online (2005). Standard Methods 21st Edition, Standard Methods for the Examination of Water and Wastewater. http://standardmethods.org/ (last accessed November 28, 2016).

U.S. EPA (2010) Determining the Efficacy of Liquids and Fumigants in Systematic Decontamination Studies for *Bacillus anthracis* Using Multiple Test Methods, EPA/600/R-10/088. Washington, DC.

U.S. EPA (2013). Bio-response Operational Testing and Evaluation (BOTE) Project - Phase 1: Decontamination Assessment, EPA/600/R-13/168. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA (2012). Assessment of Liquid and Physical Decontamination Methods for Environmental Surfaces Contaminated with Bacterial Spores: Evaluation of Spray Method Parameters and Impact of Surface Grime, EPA/600/R/12/591. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA (2015) Surface Decontamination Methodologies for a Wide-Area *B. Anthracis* Incident, EPA/600/S-15/172, 2015. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA. (2006) Evaluation of Spray-Applied Sporicidal Decontamination Technologies, EPA/600/R-06/146. U.S. Environmental Protection Agency, Washington, DC.

Wood, J.P., Calfee, M.W., Clayton, M., Griffin-Gatchalian, N., Touati, A., Ryan, S., Mickelsen, L., Smith, L., Rastogi, V. (2016) A simple decontamination approach using hydrogen peroxide vapor for *Bacillus anthracis* spore inactivation. *J Appl Microbiol.* Accepted Author Manuscript. doi:10.1111/jam.13284.

Wood, J.P., Calfee, M.W., Clayton, M., Griffin-Gatchalian. N., Touati, A., Egler, K. (2013) Evaluation of peracetic acid fogging for the inactivation of Bacillus spores. *J Hazard Mater* 250–251:61–67.

Wood, J.P., Calfee, M.W., Clayton, M., Griffin-Gatchalian, N., and Touati A. Optimizing acidified bleach solutions to improve sporicidal efficacy on building materials. Article first published online: 27 OCT 2011 | DOI: 10.1111/j.1472-765X.2011.03162.x

Wood, J.P., Choi, Y., and Rogers, J.V. Efficacy of liquid spray decontaminants for inactivation of *Bacillus anthracis* spores on building and outdoor materials. *J. Appl Microbiol***. 2011,** 110, 1262–1273**.**

Appendix A:
Detailed Decontamination Results

Table A-1. Detailed LR Summary

Test ID	Carpet		Ceiling Tile		Concrete		Galvanized Metal		Glass		Laminate		PWB Paper		Wood	
	Avg LR	SD	Avg LR	SD	Avg LR	SD	Avg LR	SD	Avg LR	SD	Avg LR	SD	Avg LR	SD	Avg LR	SD
1	0.33	0.12	0.44	0.10	0.59	0.11	1.58	0.46	1.26	0.58	1.45	0.30	1.11	0.31	0.45	0.22
2	0.84	0.18	1.04	0.14	1.94	0.21	**6.70**	0.94	**6.30**	1.20	4.50	1.49	3.46	1.27	1.50	0.57
3	0.49	0.19	1.11	0.23	4.16	1.67	**6.84**	1.21	5.90	2.45	**6.00**	1.70	5.53	1.62	2.42	0.65
4	0.89	0.21	1.57	0.30	3.55	1.07	**6.88**	1.59	**6.76**	1.43	**6.31**	1.22	4.51	1.07	1.73	0.15
6	1.92	0.21	1.86	0.26	4.77	0.87	**7.28**	0.73	**7.47**	0.01	5.24	0.47	5.60	1.78	2.54	0.41
7	2.25	0.72	1.55	0.32	2.93	0.48	4.59	1.38	5.48	1.87	4.51	0.82	5.78	0.70	2.64	0.66
8	1.36	0.19	1.37	0.11	3.57	1.34	4.78	1.09	5.45	2.17	4.62	1.95	4.52	0.96	2.30	0.42
9	2.09	2.04	1.81	0.20	4.90	0.84	**7.15**	0.37	**7.37**	0.14	5.58	1.21	5.57	1.78	2.05	0.38
10	1.80	0.63	1.21	0.12	2.09	0.34	1.98	0.62	5.18	0.50	3.21	1.20	3.01	0.57	2.78	0.52
11	4.74	1.67	2.40	0.40	3.95	0.74	2.27	0.36	**7.13**	0.29	**6.56**	1.42	**6.50**	1.20	**6.25**	0.89
12	2.78	0.46	2.43	0.24	5.74	1.08	**7.19**	0.01	**7.16**	0.09	**7.10**	0.57	**6.70**	1.12	3.81	0.98
13	3.57	1.15	2.20	0.14	5.33	0.98	**6.88**	0.05	**6.97**	0.06	**7.19**	0.30	**7.29**	0.03	4.84	0.35
14	3.18	1.59	2.23	0.33	5.96	0.52	**7.40**	0.06	**7.17**	0.13	**7.51**	0.53	**7.12**	0.80	5.25	0.72
15	5.96	1.15	3.20	0.57	4.91	1.18	5.75	0.37	**6.63**	0.17	**6.88**	1.52	**6.41**	1.58	**6.66**	1.13
16	1.49	0.30	1.37	0.29	3.47	1.81	**7.12**	0.02	**6.87**	0.44	**6.50**	1.95	**6.39**	1.77	3.00	0.51
17	2.01	0.85	2.09	0.31	5.66	0.95	**7.20**	0.05	**7.26**	0.14	**7.33**	0.96	**7.09**	0.38	3.90	0.83
18	4.64	1.19	2.48	0.20	5.35	0.48	**6.99**	0.18	**6.96**	0.01	**7.33**	0.44	**7.50**	0.01	4.83	0.52
19	3.93	0.79	3.24	0.70	**6.23**	0.54	**6.75**	0.05	**6.83**	0.10	**6.86**	0.62	**6.62**	1.28	**6.41**	1.30
20	2.67	0.34	2.21	0.88	5.77	0.99	**6.35**	0.54	**6.81**	0.10	**7.09**	0.02	5.54	0.95	3.64	0.37
21	2.57	0.55	2.04	0.32	4.46	1.13	5.81	0.92	**6.60**	0.81	5.63	0.23	5.79	0.19	4.12	0.35
22	5.06	1.36	1.82	0.28	5.35	0.97	**6.86**	0.04	**6.91**	0.01	**7.35**	0.03	**6.64**	0.80	3.50	0.22
23	3.56	0.42	2.15	0.43	5.46	0.87	**7.02**	0.22	**7.50**	0.01	**7.35**	0.62	**6.99**	0.02	4.09	0.52
24	3.91	0.94	1.89	0.21	4.97	0.66	**6.63**	0.37	**7.21**	0.00	**6.91**	0.35	**6.03**	0.94	1.75	0.22
25	3.12	0.98	1.50	0.16	3.55	0.76	**6.84**	1.37	**7.20**	0.07	**7.00**	0.59	**6.60**	0.67	N/A	N/A
26	2.12	1.04	2.00	0.35	3.11	0.95	**7.26**	0.06	**7.22**	0.36	5.97	1.71	5.49	1.30	3.25	0.34

Note: Test 5 not included in table since this test was used for method development, in which peracetic acid (a non-chlorine based decontaminant) was fogged. Bold numbers indicate complete inactivation of spore population and no spores were detected.

Appendix B:
Efficacy Charts for Individual Tests

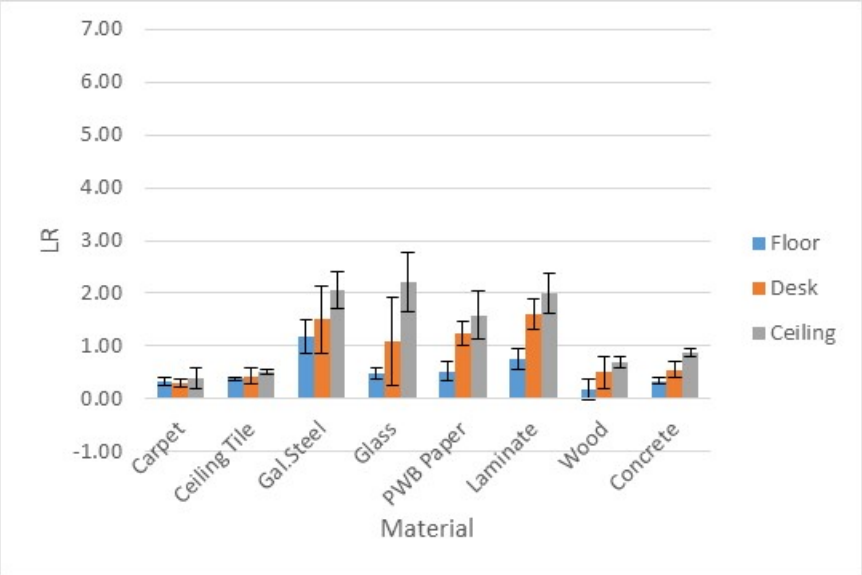


Figure B-1. Test 1 LR Summary (±SD)

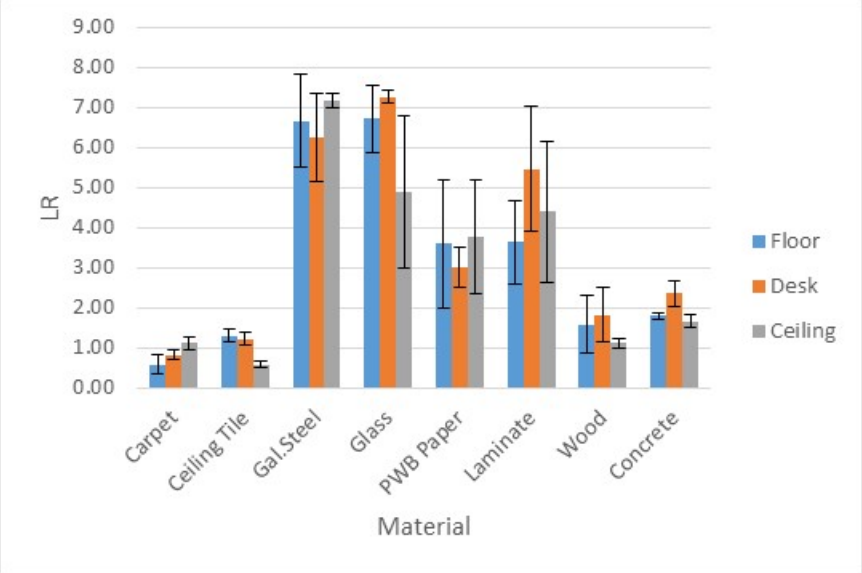


Figure B-2. Test 2 LR Summary (±SD)

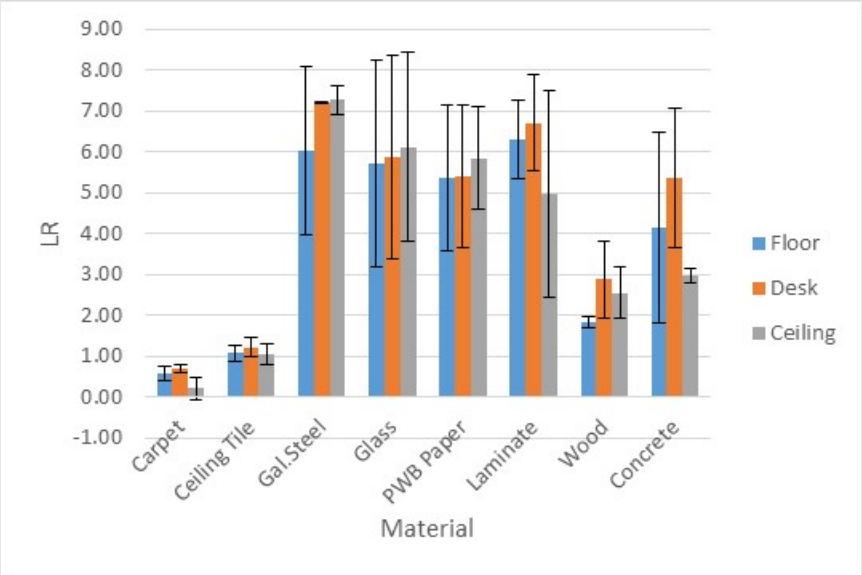


Figure B-3. Test 3 LR Summary (±SD)

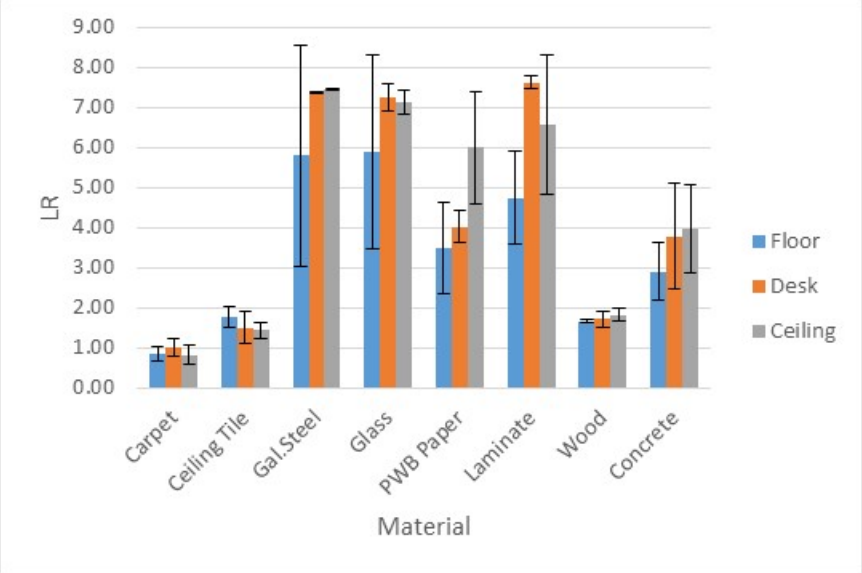


Figure B-4. Test 4 LR Summary (±SD)

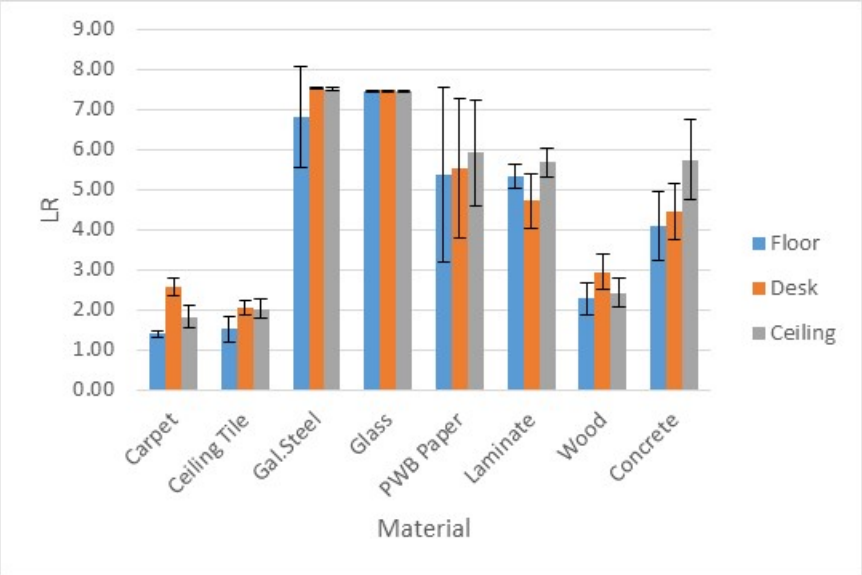


Figure B-5. Test 6 LR Summary (±SD)

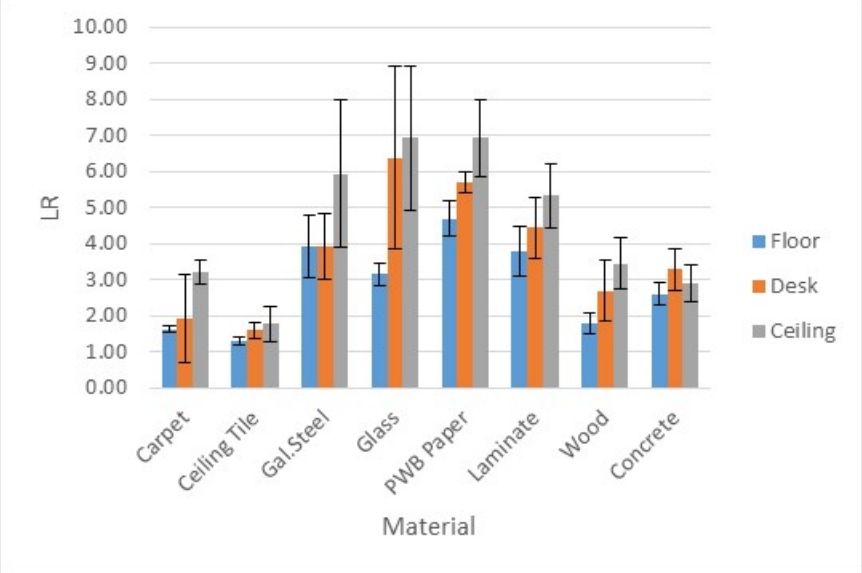


Figure B-6. Test 7 LR Summary (±SD)

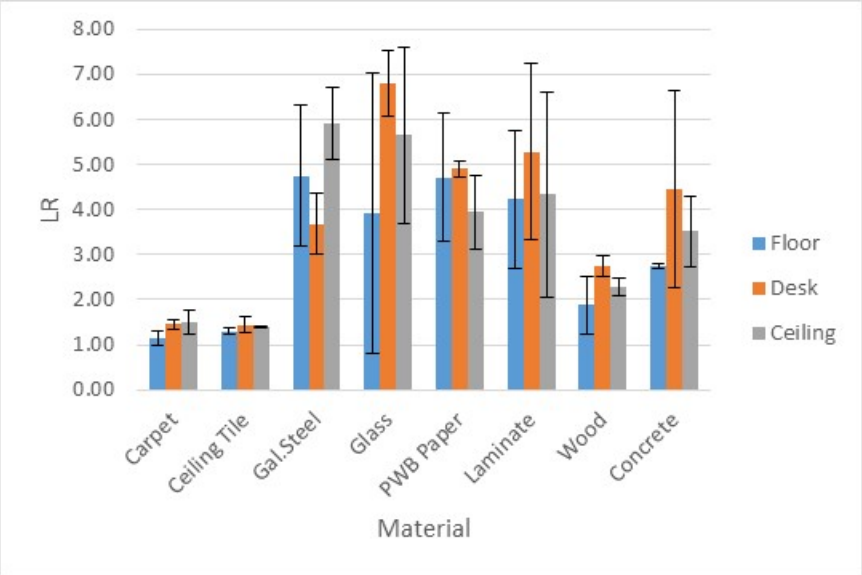


Figure B-7. Test 8 LR Summary (±SD)

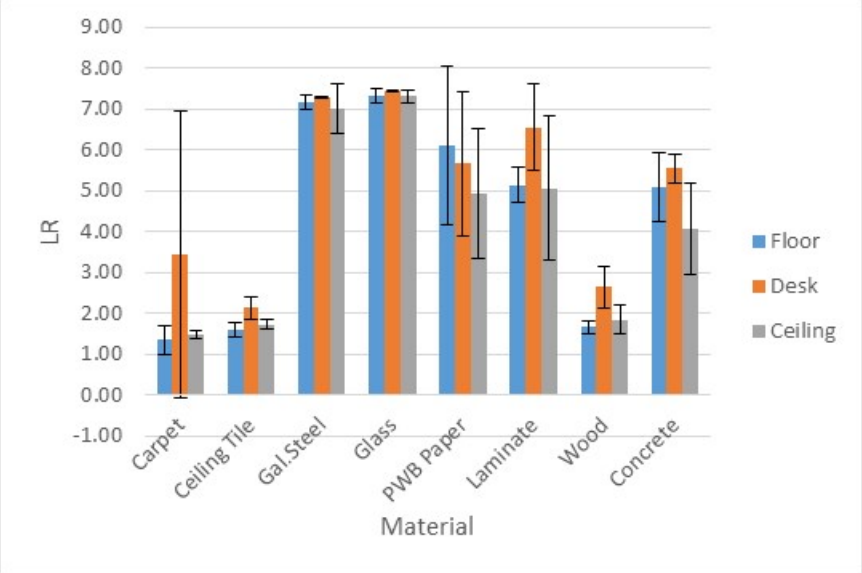


Figure B-8. Test 9 LR Summary (±SD)

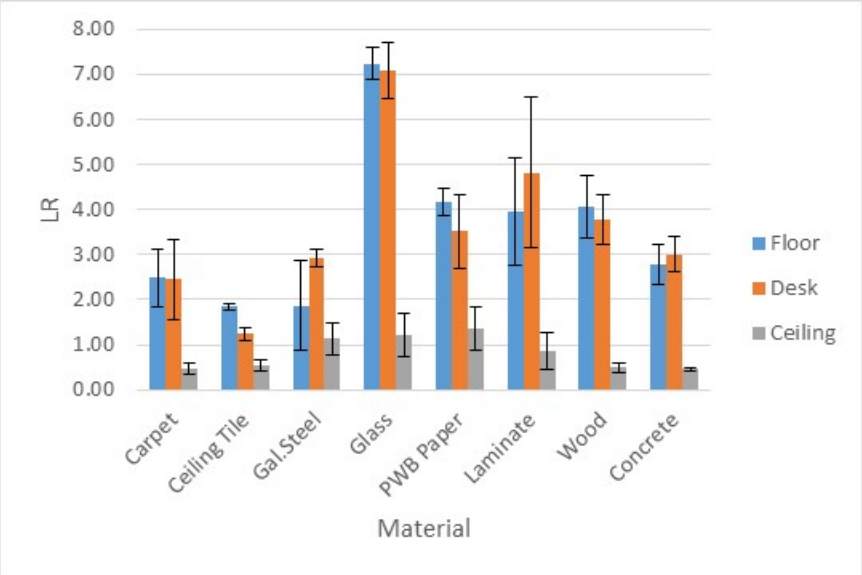


Figure B-9. Test 10 LR Summary (±SD)

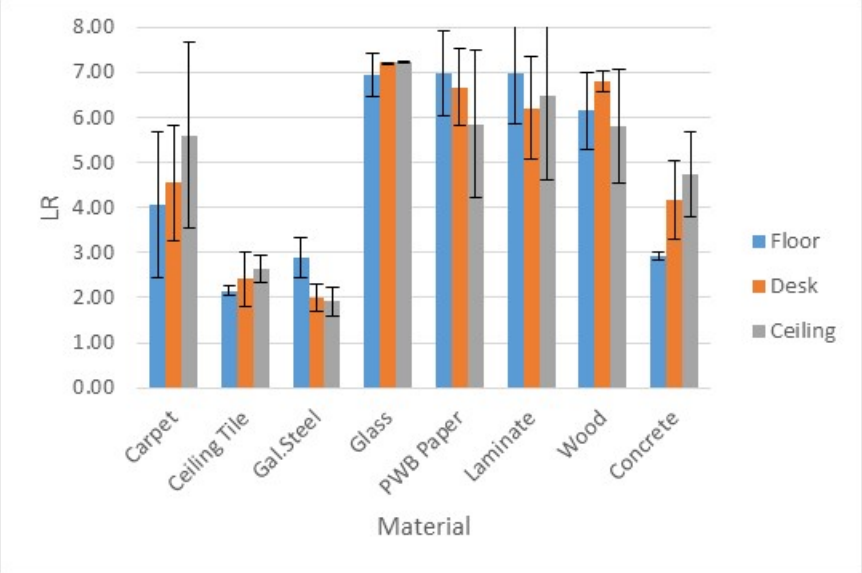


Figure B-10. Test 11 LR Summary (±SD)

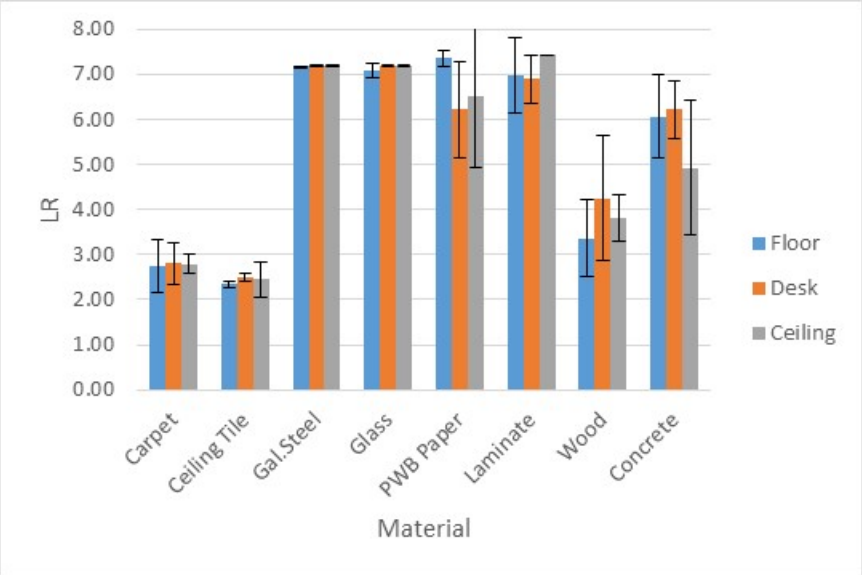


Figure B-11. Test 12 LR Summary (±SD)

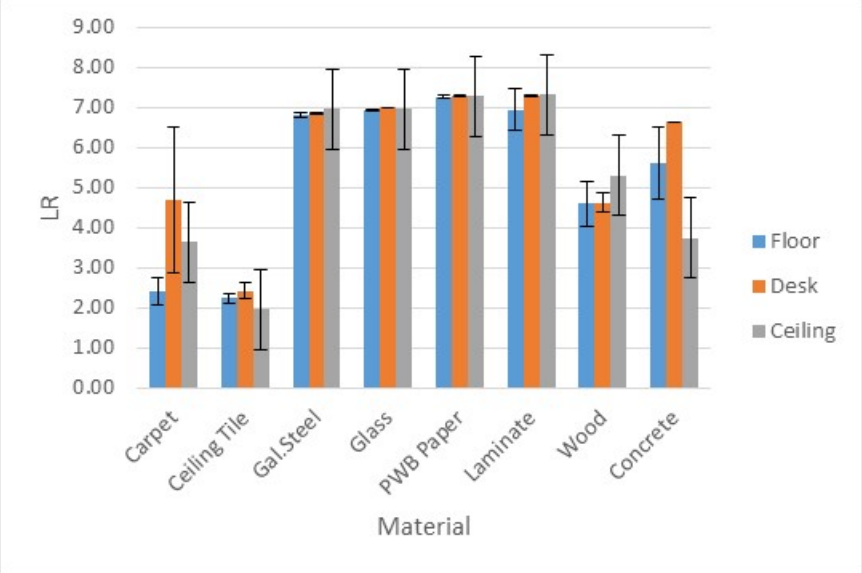


Figure B-12. Test 13 LR Summary (±SD)

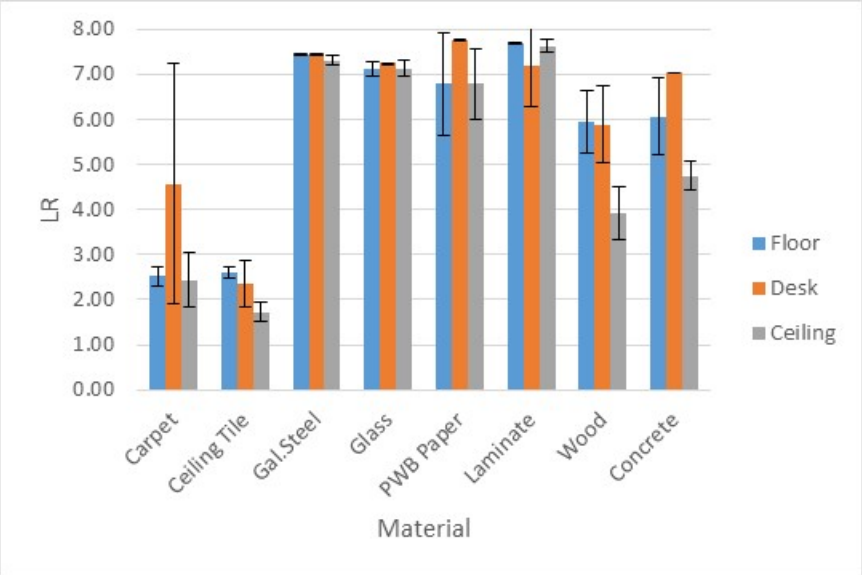


Figure B-13. Test 14 LR Summary (±SD)

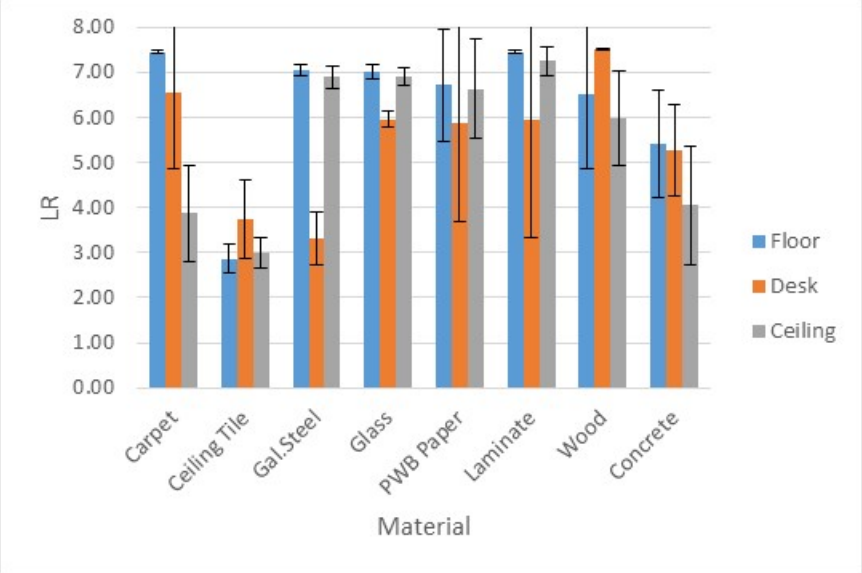


Figure B-14. Test 15 LR Summary (±SD)

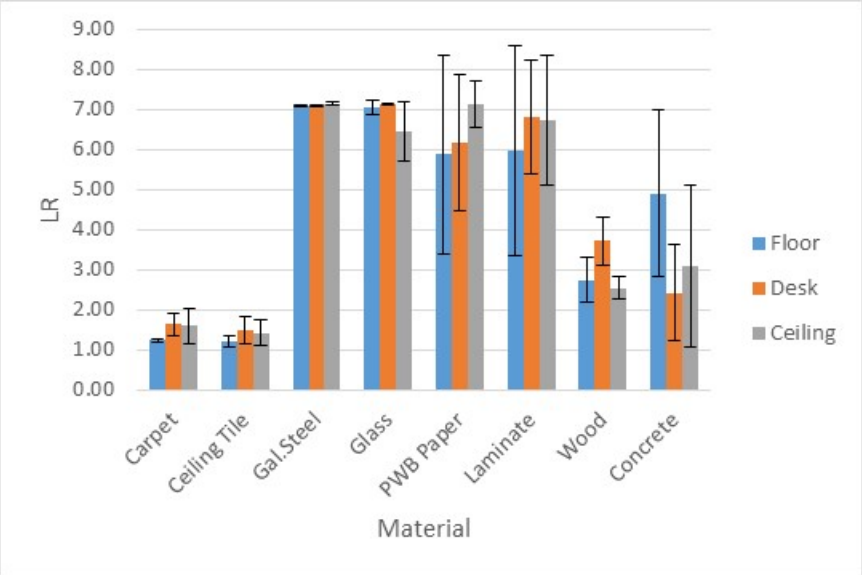


Figure B-15. Test 16 LR Summary (±SD)

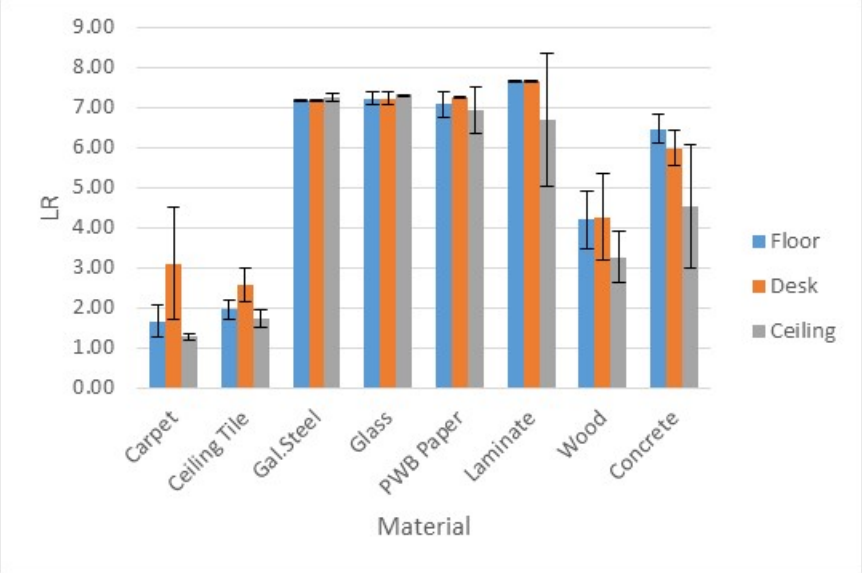


Figure B-16. Test 17 LR Summary (±SD)

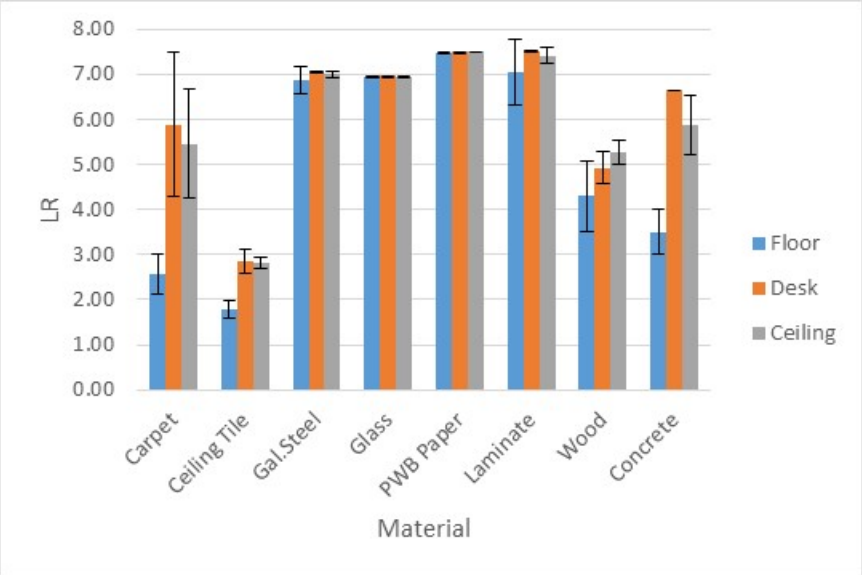


Figure B-17. Test 18 LR Summary (±SD)

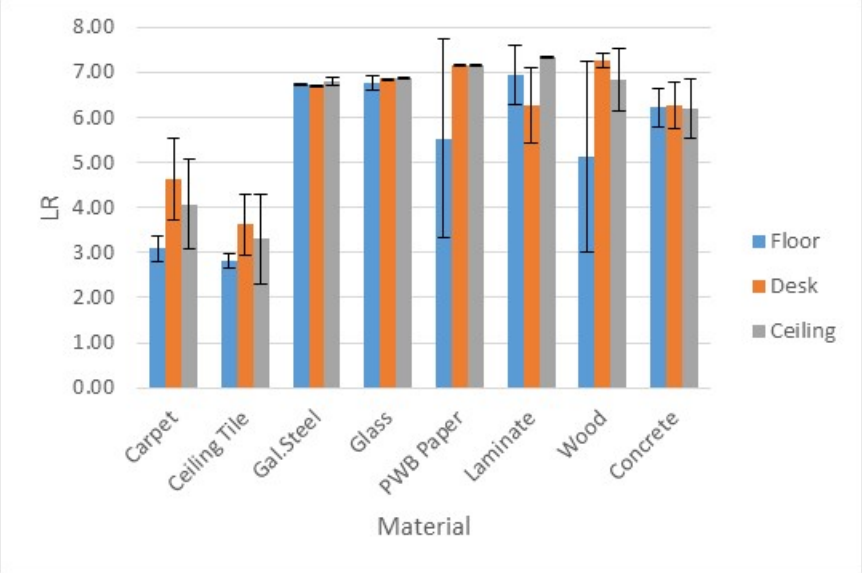
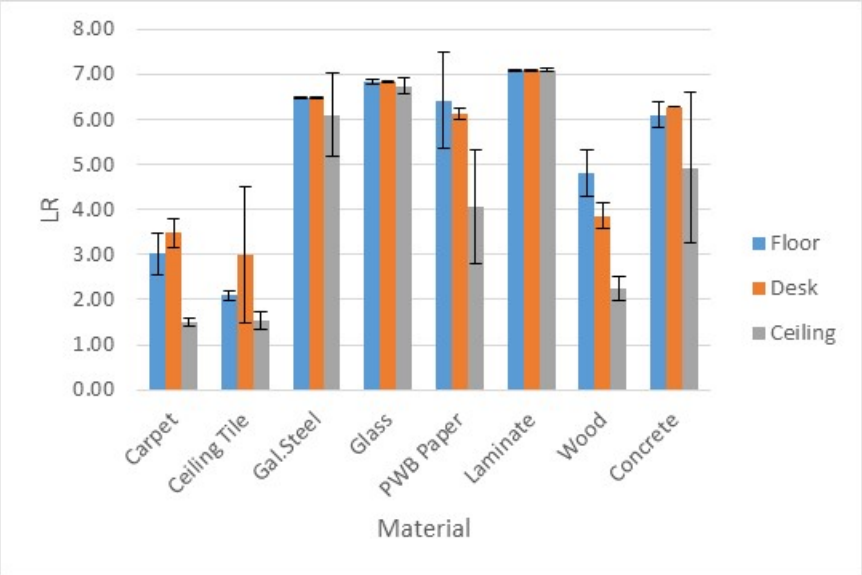


Figure B-18. Test 19 LR Summary (±SD)



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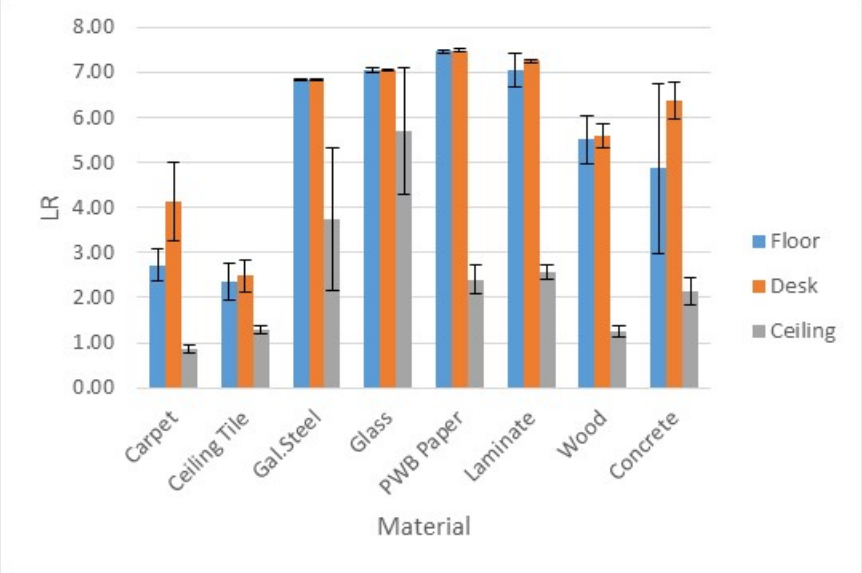

Figure B-20. Test 21 LR Summary (±SD)

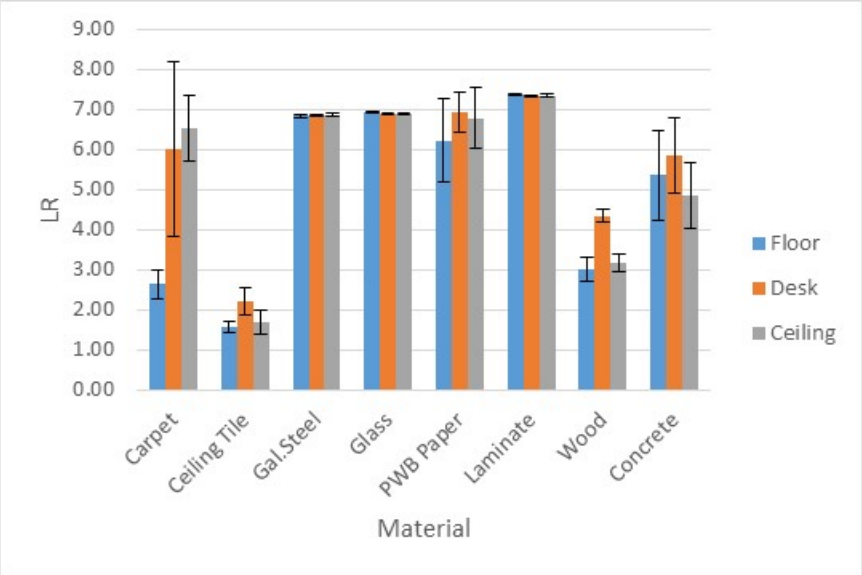

Figure B-21. Test 22 LR Summary (±SD)

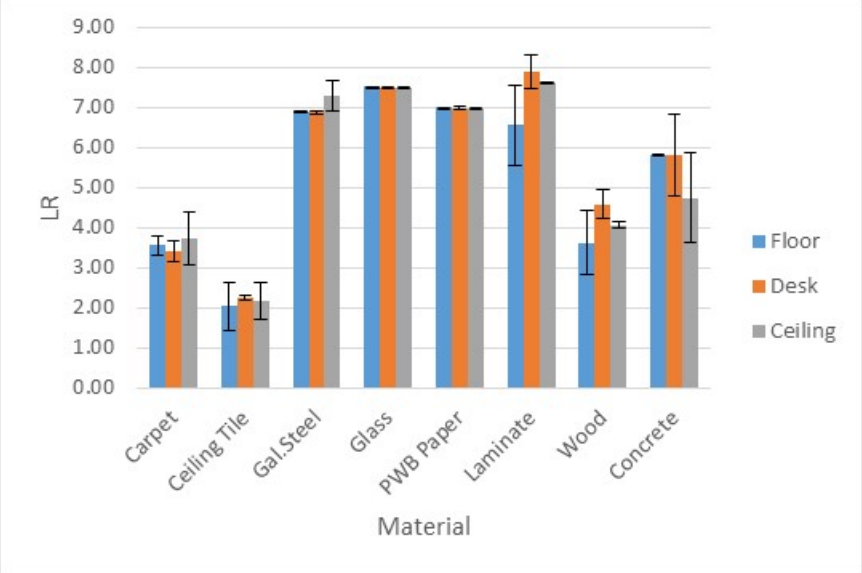

Figure B-22. Test 23 LR Summary (±SD)

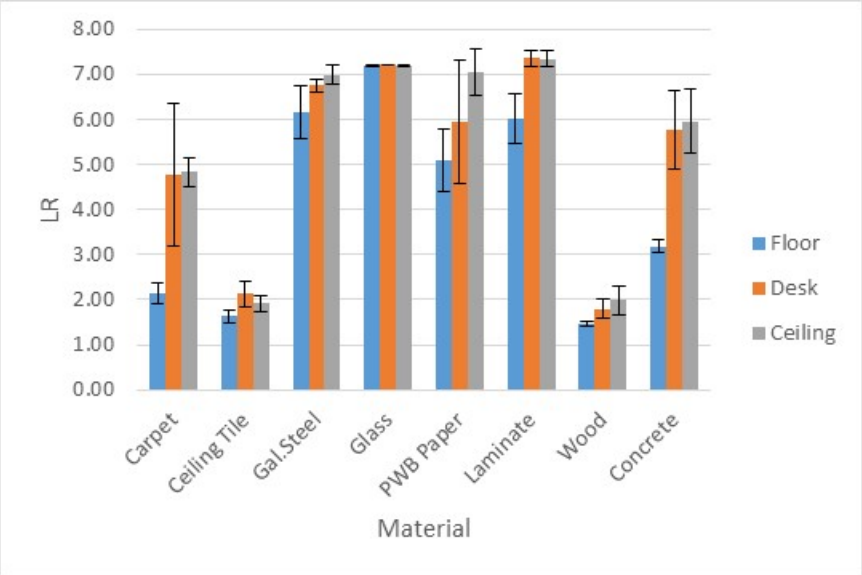

Figure B-23. Test 24 LR Summary (±SD)

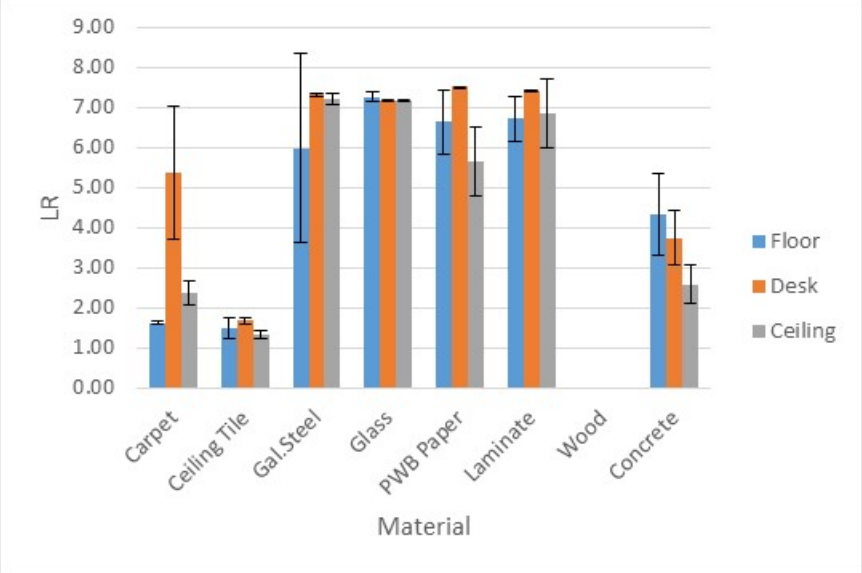

Figure B-24. Test 25 LR Summary (±SD)

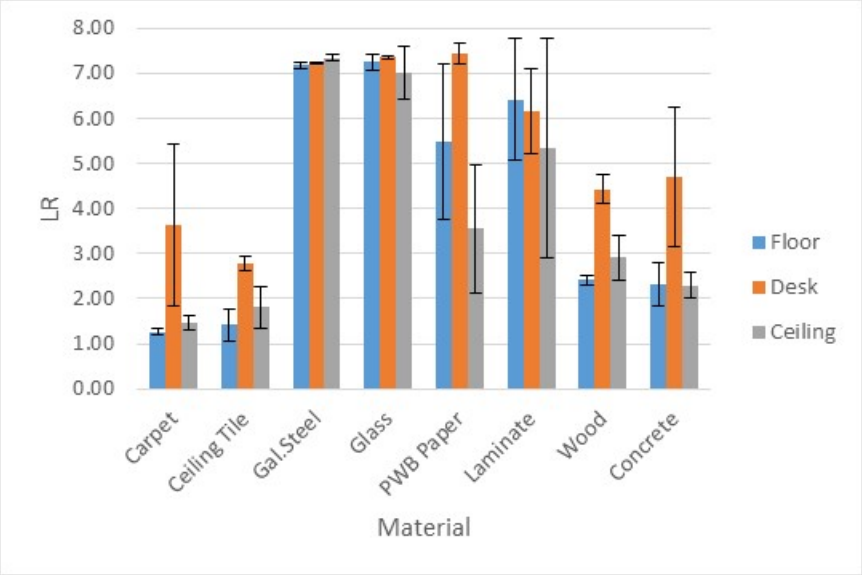

Figure B-25. Test 26 LR Summary (±SD)

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