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Decontamination of Soil Contaminated with *Bacillus anthracis* Spores

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Decontamination of Soil Contaminated with *Bacillus anthracis* Spores

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

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Executive Summary

The U.S. Environmental Protection Agency (EPA) Office of Research and Development is striving to protect human health and the environment from adverse impacts resulting from acts of terror by investigating the effectiveness and applicability of technologies for homeland security (HS)-related applications. The purpose of this investigation was to determine the efficacy of four chemical decontaminants for inactivating *Bacillus anthracis* (causative agent for anthrax) spores in soil. The decontaminants that were evaluated included two liquid biocides (pH-amended bleach and sodium persulfate) and two fumigants (methyl bromide and metam sodium). The objective of this study was to provide an understanding of the performance (i.e., efficacy) of these decontamination technologies to guide their use and implementation in HS applications for hard-to-decontaminate materials such as soil. In the assessment of options for decontamination following an intentional release of *B. anthracis* spores, it is important to know what operational factors can impact the decontamination efficacy.

This investigation focused on decontamination of two types of soil material: topsoil and Arizona Test Dust (AZTD). These two soil types were selected for testing in an attempt to span the range in expected organic content of soils. Decontamination efficacy tests were conducted with spores of *B. anthracis* or *B. subtilis*, the latter microorganism included to assess its potential as a surrogate for future studies related to *B. anthracis*. Decontamination efficacy was quantified in terms of log reduction (LR), based on the difference in the number of bacterial spores (as colony forming units) recovered from the positive controls (soil samples not exposed to decontaminant) and test samples. Tests were conducted with varying operational parameters (e.g., contact time, number of applications of the decontaminant, decontaminant concentration) to assess the effect of these parameters on decontamination efficacy.

Summary of Results

pH-Amended Bleach

At the most robust treatment with pH-amended bleach (seven-day contact time, eight applications), the decontamination efficacy for topsoil was minimal: less than 0.5 LR for both microorganisms. In contrast, pH-amended bleach was successful in decontaminating AZTD with greater than 7.0 LR obtained for both *B. anthracis* and *B. subtilis* with four applications and a two- hour contact time. For AZTD, efficacy generally decreased with decreasing number of applications and contact time.

Sodium Persulfate

All five tests conducted with sodium persulfate used a contact time of seven days while varying the number of times the decontaminant was applied to the soil samples. The most efficacious treatment, in which sodium persulfate was applied to the samples six times, resulted in complete inactivation (no spores detected) of *B. anthracis* on both soil materials. The next robust treatment (three applications of the decontaminant, all applied within the first two hours) provided greater than a 7 LR for *B. anthracis* on both soils. Efficacy generally decreased with decreasing number of persulfate applications. The decontamination efficacy results for topsoil and AZTD were not significantly different for the majority of tests.

Methyl Bromide

Eight tests were conducted with this fumigant, with concentrations ranging from 100 to 212 milligrams per liter (mg/L). All tests were conducted at 25 °C with a contact time of 36 hours, except for the last test at 24 hours. The relative humidity (RH) levels in the test chamber were measured but uncontrolled, although for a few tests, attempts were made to manipulate the RH by adding or removing moisture from the soil prior to testing. The two most efficacious treatments evaluated (utilizing 212 mg/L MeBr, 36 hour contact time, no drying of soil) resulted in complete inactivation of *B. anthracis* spores on AZTD and greater than 7.0 LR on topsoil. Overall, MeBr was effective (greater than 6 LR achieved) against *B. anthracis* on topsoil and AZTD at 25 °C when using a concentration of at least 180 mg/L and contact time of 36 hours. (One minor exception is the test in which the soil samples were dried beforehand, which resulted in a 5.9 LR on topsoil.) As expected, decontamination efficacy generally decreased with decreasing concentration and contact time. With respect to the effect of soil type, the decontamination efficacies obtained for *B. anthracis* were slightly higher on AZTD compared to topsoil.

Metam Sodium

This decontaminant was significantly more effective on the AZTD compared to the topsoil for the majority of the tests. For all but one of the eight tests with AZTD, *B. anthracis* was completely inactivated, whereas just one test with *B. anthracis*-contaminated topsoil resulted in completely inactivation. Metam sodium was effective (greater than 6 LR) against *B. anthracis* on topsoil in three of the tests conducted.

Operational factors such as doubling the amount of metam sodium applied to the soil materials improved efficacy significantly for both microorganisms on topsoil and for *B. subtilis* on AZTD. (Tests were conducted with either 80 or 160 µL.) Increasing contact time (up to 14 days) generally improved efficacy for the inactivation of *B. anthracis* on topsoil but not significantly. Decontamination efficacy for *B. anthracis* on topsoil increased with increasing soil moisture content, with efficacy greater than 6 LR when the soil moisture was at its highest levels (~46%).

Comparing efficacy results for B. anthracis and B. subtilis

There were no tests in which *B. subtilis* was inactivated to a significantly higher degree than *B. anthracis*, and, for pH-amended bleach, there were no significant differences in decontamination efficacy for the two microorganisms. For the other three decontaminants, the inactivation efficacy for *B. subtilis* was significantly less than the efficacy for *B. anthracis* for the majority of the tests conducted. For MeBr in particular, the differences in efficacy for the two microorganisms were greater than 5-6 LR for more than half of the tests.

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Abbreviations/Acronyms

ATCC	American Type Culture Collection
AZTD	Arizona Test Dust
B. anthracis	*Bacillus anthracis* (Ames strain)
B. subtilis	*Bacillus subtilis* (ATCC 19659)
BBRC	Battelle Biomedical Research Center
BSC	biological safety cabinet
°C	degree(s) Celsius
CBR	Chemical, biological, and radiological
CFU	colony forming unit(s)
CI	confidence interval
cm	centimeter(s)
DNA	deoxyribonucleic acid
EPA	U.S. Environmental Protection Agency
ft	feet
g	gram
HCl	Hydrogen chloride
H_2O_2	hydrogen peroxide
hr	hour(s)
HS	homeland security
HSRP	Homeland Security Research Program
kGy	Kilogray(s)
LAL	Limulus Amebocyte Lysate
L	liter(s)
LED	Light emitting diode
LR	log reduction
M	Molarity
MeBr	methyl bromide
mg	milligram(s)
MITC	methyl isothiocyanate
mL	milliliter(s)
µL	microliter(s)
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
$Na_2S_2O_8$	sodium persulfate
NHSRC	National Homeland Security Research Center
ORD	Office of Research and Development
oz	ounce
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline + 0.1% Triton® X-100
PCR	polymerase chain reaction
ppm	part(s) per million
PVC	polyvinyl chloride
QA	quality assurance
QC	quality control

QMP	Quality management plan
RH	relative humidity
rpm	revolution(s) per minute
SD	standard deviation
SE	standard error
SFW	sterile filtered water (cell-culture grade)
STS	sodium thiosulfate
TSA	technical systems audit(s)

1.0 Introduction

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

As part of the above effort, EPA investigates the effectiveness and applicability of technologies for homeland security (HS)-related applications by developing test plans that are responsive to the needs of the HSRP's EPA Program Office partners, conducting tests, collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and high quality are generated and that the results are defensible. EPA provides high-quality information that is useful to decision makers in purchasing or applying the tested technologies.

The purpose of this investigation was to develop an understanding of the effectiveness of four different chemical biocides or technologies to decontaminate two types of soil materials. Residual biological agent (such as *B. anthracis*) on surfaces following decontamination after an intentional release could present a potential health risk. This report documents the impact of operational factors on the efficacy of two liquid decontaminants (pH-amended bleach, sodium persulfate) and two fumigants (methyl bromide, metam sodium) against spores of *B. anthracis* and *B. subtilis* using one cm deep topsoil and AZTD. *B. subtilis* was included in the tests to assess its potential use as a benign surrogate microorganism (so that future decontamination tests could be conducted without the use *B. anthracis*). Decontamination efficacy was determined based on the log reduction (LR) in viable spores recovered from the inoculated samples, with and without exposure to the decontaminants.

2.0 Technology Descriptions and Test Matrices

2.1 Technology Descriptions

Table 2-1 describes the four decontamination technologies (or biocides) evaluated in this investigation. Information is provided on the manufacturer, product name (where applicable), chemical components and active ingredients. Some of the decontaminants are mixtures that react to produce other chemicals that are responsible for the sporicidal activity. Note that Ultra Clorox® Germicidal Bleach is registered as a disinfectant, but the pH-amended solution is not. Further details on the chemical composition, preparation, and decontamination application procedures are provided in Section 4.

Table 2-1 Decontamination Technology Descriptions

Decontaminant	Product Name and Vendor	Active Ingredients and Sporicidal Chemical	Components	EPA Registration
Methyl Bromide	Methyl Bromide Matheson Tri-Gas Basking Ridge, NJ	Methyl bromide	99.5% methyl bromide gas with 0.5% chloropicrin added as a warning irritant	None
Metam Sodium	Metam concentrate, Buckman Laboratories, Inc. Memphis, TN	Sodium N-methyldithiocarbamate (metam sodium), methyl isothiocyanate (MITC)	42.5% metam sodium; aqueous solution	1448-107
pH-Amended Bleach	Ultra Clorox®Germicidal Bleach, Clorox® Professional Products Co. Oakland, CA	Sodium hypochlorite, hypochlorous acid	Sodium hypochlorite 6.15%, sodium hydroxide <1%; diluted with sterile filtered water (SFW); with 5% acetic acid added to reduce pH to 6.5 - 7.0.	67619-8 (disinfectant)
Sodium Persulfate	Klozur™ FMC Corporation Philadelphia, PA	Sodium persulfate, activated with hydrogen peroxide; sulfate radicals	Sodium persulfate ($Na_2S_2O_8$) >99% purity (used as a 12% (0.5M) aqueous solution, activated with an 8% hydrogen peroxide solution	None

Methyl bromide was selected for testing because it has been demonstrated to be effective against *B. anthracis* on building materials[1], but has not been tested against *B. anthracis* on soils. Furthermore, although MeBr use is being phased out under the Montreal Protocol, MeBr is still currently and widely used via critical use exemptions as a soil and commodity (quarantine) fumigant[2]. Metam sodium was selected for testing because it is the most widely used soil fumigant in the US[3]. If proven to be effective against *B. anthracis*, it would greatly improve preparedness in the event of an outdoor release of *B. anthracis* to have a decontaminant that is widely available and commonly used. Bleach (with its pH lowered) was selected for testing because this decontaminant has been demonstrated to be effective against *B. anthracis* on some materials, is easily made using off-the-shelf chemicals, and is often the decontaminant of choice for remediation officials[4]. Lastly, sodium persulfate was included in this evaluation because this chemical is used to remediate soil contaminated with organic chemicals. In addition, sodium

persulfate was shown to be moderately effective against *B. anthracis* in soil in screening tests[5], so more robust test conditions were planned for this evaluation.

2.2 Test Matrices for Liquid Decontamination

In general, the conditions selected for testing (e.g., contact time, concentration) were based on previous, similar *B. anthracis* efficacy tests (as described above), as well as how the decontaminants are currently used in practice.

The test matrices for the pH-amended bleach and sodium persulfate (activated with hydrogen peroxide (H_2O_2)) liquid sporicide tests are shown in Tables 2-2 and 2-3, respectively. For the bleach tests, topsoil was tested only once under the most robust test condition (Test 1; it was completely ineffective). For the sodium persulfate tests, a contact time of seven days was used for each test. For each test listed below, separate subtests were conducted for each combination of microorganism and soil type.

Table 2-2 pH-Amended Bleach Test Matrix

Test #	Biological Agent	Soil type	Application Frequency (total number of applications)	Contact Time (hr)
1	*B. anthracis* *B. subtilis*	Topsoil AZTD	0.5 mL every 15 minutes for 2 hr (8)	168
2	*B. anthracis* *B. subtilis*	AZTD	0.5 mL every 15 minutes for 2 hr (8)	24
3	*B. anthracis* *B. subtilis*	AZTD	0.5 mL every 30 minutes for 2 hr (4)	2
4	*B. anthracis* *B. subtilis*	AZTD	0.5 mL every 30 minutes for 1 hr (2)	1

Table 2-3 Sodium Persulfate (Klozur™) Test Matrix

Test #	Biological Agent	Soil Type	Application Frequency* (total number of applications)	Contact Time (days)
1	*B. anthracis* *B. subtilis*	Topsoil AZTD	Every 60 minutes (6)	7
2	*B. anthracis* *B. subtilis*	Topsoil AZTD	Every 60 minutes (3)	7
3	*B. anthracis* *B. subtilis*	Topsoil AZTD	Days 0, 2 and 4 (3)	7
4	*B. anthracis* *B. subtilis*	Topsoil AZTD	Time 0 and 1 Hr (2)	7
5	*B. anthracis* *B. subtilis*	Topsoil AZTD	Day 0 (1)	7

* = Each application consisted of 1 mL Klozur™ followed by 1 mL 8% H_2O_2.

2.3 Test Matrices for Fumigant Decontamination

The test matrices for the MeBr and metam sodium fumigation tests are shown in Tables 2-4 and 2-5.

For MeBr, all tests were conducted at 25 °C and with RH measured but not controlled. However, soil moisture did affect the RH level, so soil moisture was adjusted in a few experiments to assess its impact on RH and subsequent decontamination efficacy.

Metam sodium requires some moisture in the soil to produce MITC gas, the chemical responsible for biocidal activity[6]. Two mL of water were therefore added to all soil samples prior to testing, and additional amounts of water were added to the soils as an experimental variable. The two methods used to pre-sterilize the soil (irradiation and autoclaving) also affected soil moisture, so soil sterilization method was also used as a test parameter. Due to the potential for having residual metam sodium in the soil following the contact time, the soil samples were allowed to aerate for varying amounts of time (mimicking how the product is used in the field). Further details on the metam sodium related test procedures may be found in Section 4.4 and Appendix A.

Table 2-4 MeBr Test Matrix

Test #	Biological Agent	Soil type	Target Concentration (mg/L)	Contact Time (hr)
1	*B. anthracis* *B. subtilis*	Topsoil AZTD	212 ± 21	36
2	*B. anthracis* *B. subtilis* †	Topsoil AZTD	212 ± 21	36
3	*B. anthracis* *B. subtilis* ‡	Topsoil AZTD	212 ± 21	36
4	*B. anthracis* *B. subtilis*	Topsoil AZTD	100 ± 10	36
5	*B. anthracis* *B. subtilis*†	Topsoil AZTD	100 ± 10	36
6	*B. anthracis* *B. subtilis*	Topsoil AZTD	180 ± 18	36
7	*B. anthracis* *B. subtilis*	Topsoil AZTD	140 ± 14	36
8	*B. anthracis* *B. subtilis*	Topsoil AZTD	212 ± 21	24

† 2 mL SFW added prior to sample inoculation.
‡ Samples dried prior to sample inoculation.

Table 2-5 Metam Sodium Test Matrix

Test #	Biological Agent†	Soil type	Amount SFW Added Prior to Addition of Metam Sodium (mL)	Quantity of Metam Sodium Applied (µL)	Soil Sterilization Method	Contact Time/ Aeration Time (days)
1	*B. anthracis* *B. subtilis*	Topsoil AZTD	--	80	Gamma Irradiation at 40 kGy	5/0
2	*B. anthracis* *B. subtilis*	Topsoil AZTD	--	160	Gamma Irradiation at 40 kGy	5/0
3	*B. anthracis* *B. subtilis*	Topsoil AZTD	1	160	Gamma Irradiation at 60 kGy	7/7
4	*B. anthracis* *B. subtilis*	Topsoil AZTD	2	160	Gamma Irradiation at 60 kGy	7/7

5	*B. anthracis* or *B. subtilis*	Topsoil AZTD	3	160	Gamma Irradiation at 60 kGy	7/7
6	*B. anthracis* or *B. subtilis*	Topsoil AZTD	1	160	Gamma Irradiation at 60 kGy	14/28
7	*B. anthracis* or *B. subtilis*	Topsoil AZTD	1	160	Autoclave (121 °C; 1 hr)	7/7
8	*B. anthracis* or *B. subtilis*	Topsoil AZTD	1	160	Autoclave (121 °C; 1 hr)	14/28

3.0 Summary of Test Procedures

Test procedures were performed in accordance with a pre-approved Quality Assurance Project Plan (QAPP) (available upon request) and are summarized in this chapter.

3.1 Biological Agent

The *B. anthracis* spores used for this testing were prepared from a qualified stock of the Ames strain at the Battelle Biomedical Research Center (BBRC, West Jefferson, OH). All spore lots were subject to a stringent characterization and qualification process required by Battelle's standard operating procedure for spore production. Specifically, all spore lots were characterized prior to use by observation of colony morphology, direct microscopic observation of spore morphology and size and determination of percent refractivity and percent encapsulation (of the vegetative bacterial colonies). In addition, the number of viable spores was determined by colony count and expressed as colony forming units per milliliter (CFU/mL). Theoretically, once plated onto bacterial growth media, each viable spore germinates and yields one CFU. Variations in the expected colony phenotypes were recorded. Endotoxin concentration of each spore preparation was determined by the Limulus Amebocyte Lysate (LAL) assay to assess whether contamination from gram-negative bacteria occurred during the propagation and purification process of the spores. Genomic deoxyribonucleic acid (DNA) was extracted from the spores and DNA fingerprinting by polymerase chain reaction (PCR) was performed to confirm the genotype. The virulence of the spore lot was measured by challenging guinea pigs intradermally with a dilution series of spore suspensions, and virulence was expressed as the intradermal median lethal dose.

To ensure spores are used in testing (and not vegetative cells), various steps are taken, described as follows. The spore stock is stored in purified water and characterized via visual purity. The stock is viewed under the microscope, viable spores are then counted and any cell debris is noted. The spore preparation must have a minimum 95% purity vs. debris and non-viable spores. The spore prep is also heat shocked prior to removing from our fermenter. In addition, testing was conducted for robustness of the spores via hydrochloric acid (HCl) resistance.

The *B. subtilis* spores (BBRC stock culture; American Type Culture Collection [ATCC] 19659) underwent the same characterization tests as described above for *B. anthracis*, except that the LAL assay, DNA fingerprinting, and virulence testing were excluded. Qualitative PCR was performed using a custom PCR assay to confirm *B. subtilis.* Primers were designed that targeted a conserved region of *B. subtilis* chromosomal DNA because multiple strains of this bacterium exist.

The stock spore suspensions were prepared in SFW at an approximate concentration of 1×10^9 CFU/mL and stored under refrigeration at 2 to 8 degrees Celsius (°C).

3.2 Soil Materials

Information on the soil types used for testing is presented in Table 3-1. Soil samples were placed unpacked in one ounce (oz), 1.5 inch diameter glass jars (Qorpak®, #GLC-01596, Bridgeville, PA) at a depth of one cm for testing. The commercial topsoil used for this evaluation was a proprietary mixture of soil, composted cow manure, sand, and other ingredients (also

proprietary). Topsoil was selected for testing since it represents a difficult soil to treat in terms of its organic content. The AZTD was selected for testing since it represents a soil with minimal organic burden.

Soils used in tests with pH-amended bleach, persulfate, and MeBr were prepared for testing by sterilization via gamma irradiation at ~40 kilogray (kGy; STERIS Isomedix Services, Libertyville, IL). Soils were pre-sterilized to minimize contamination that could interfere with colony counting. However, when testing with metam sodium (the last technology to be tested), endogenous bacteria were observed in the topsoil samples, so additional soil sterilization methods were evaluated and used for the tests with metam sodium. (It is unclear why this contamination occurred, since topsoil samples were all from the same lot.) In addition to gamma irradiation at ~40 kGy, samples were gamma irradiated at ~60 kGy or autoclaved at 121 °C for one hr. (Refer to Appendix A for additional details.) Gamma-irradiated soils were sealed in Lock & Lock containers (Farmers Branch, TX) and autoclaved soils were sealed in sterilization pouches (Cat # 01-812-51, Fisher Scientific, Pittsburgh, PA) to preserve sterility until the samples were ready for use.

Table 3-1 Soil Materials

Material*	Lot, Batch, or ASTM No., or Observation	Manufacturer/ Supplier Name	Pre-sterilized moisture content (%)	Pre-sterilized organic carbon content (%)
Topsoil	Earthgro® Topsoil, Product #: 71140180	The Scotts Company Marysville, OH	34	9.3
Arizona Test Dust	ISO 12103-1, A3 Medium	Powder Technology, Inc. Burnsville, MN	0.23	0.40

* A soil sample consisted of a 1.5 in diameter glass jar filled with uncompacted soil to a height of 1 cm.

Prior to decontamination testing, samples (pre- and post-sterilization) were analyzed in triplicate using ASTM D Method 2974-87 for Moisture, Ash and Organic Matter of Peat and Other Organic Soils[7]. The results of these tests are shown in Table 3-1 and in more detail in Appendix A. Note the topsoil has a much higher moisture and organic content compared to the AZTD. The moisture and organic content did not change significantly after the gamma irradiation of the samples. However, slight changes were observed in autoclaved samples.

Because the moisture content of soils could impact the decontamination efficacy of metam sodium, the moisture content of samples used in the tests with metam sodium was also determined using ASTM Method D 2974-87. Further details and results are found in Appendix A.

3.3 Preparation of Soil Samples

Test and positive control soil samples (in their jars) were placed on a flat surface within a Class II biological safety cabinet (BSC) and inoculated with approximately 1 x 10^8 CFU of viable *B. anthracis* or *B. subtilis* spores per sample. A 100 microliter (µL) aliquot of a stock suspension of approximately 1 x 10^9 CFU/mL was dispensed using a micropipette applied as 10 µL droplets across the soil surface. This approach provided a more uniform distribution of spores across the sample surface than would be obtained through a single drop of the suspension. Further details on the inoculation methods can be found elsewhere[8,9]. After inoculation, the samples were left undisturbed overnight in a Class III BSC to dry under ambient conditions, approximately 22 °C and 40% relative humidity (RH). A heat shock test was conducted to confirm that no

germination of cells occurred (only spores present) while spores were left in soil samples overnight.

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

- five test samples (inoculated with *B. anthracis* or *B. subtilis* spores and exposed to decontaminant)
- five positive controls (inoculated with *B. anthracis* or *B. subtilis* spores but not exposed to decontaminant)
- one laboratory blank (inoculated with sterile water only and not exposed to the decontaminant)
- one procedural blank (inoculated with sterile water only and exposed to the decontaminant)

On the day following spore inoculation, the jars of soil samples intended for decontamination (including blanks) were transferred into a test chamber where the decontamination technology was applied using the apparatus and application conditions specified in Section 4 of this report.

3.4 Sample Extraction and Biological Agent Quantification

At the appropriate decontaminant contact time, spores were extracted from the soil samples by adding 10 mL of sterile phosphate-buffered saline extraction buffer containing 0.1% Triton® X-100 surfactant (PBST; Sigma, St. Louis, MO) and neutralizer (to stop sporicidal activity when liquid decontaminant was used; refer to subsection 4.2.2 and Appendix B) to each sample jar. The jars were capped and agitated on an orbital shaker for 15 minutes at approximately 200 revolutions per minute (rpm) at room temperature. Further details on these methods can be found elsewhere[8,9].

Residual viable spores were quantified using a dilution plating approach. Following extraction, the extract was removed and a series of 10-fold dilutions was prepared in sterile water. An aliquot (0.1 mL) of either the undiluted extract and/or each serial dilution was plated onto tryptic soy agar in triplicate and incubated for 18-24 hours (hr) at 35-37 ºC. Colonies were counted manually and CFU/mL was determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Dilution data representing the greatest number of individually definable colonies were expressed as arithmetic mean ± standard deviation of the numbers of CFU observed.

Laboratory blanks controlled for sterility and procedural blanks controlled for viable spores inadvertently introduced to test samples. The blanks were inoculated with an equivalent amount of 0.1 mL SFW. The target acceptance criterion was that extracts of laboratory or procedural blanks were to contain zero CFU of target organism.

After each decontamination test, the BSC III was cleaned thoroughly (using separate steps involving bleach, ethanol, water, then drying) following procedures established under the BBRC Facility Safety Plan.

3.5 Decontamination Efficacy

The mean percent spore recovery from each soil sample was calculated using results from positive control samples (inoculated, not decontaminated), by means of the following equation:

$$\text{Mean \% Recovery} = [\text{Mean } CFU_{pc}/CFU_{spike}] \times 100 \quad (1)$$

where Mean CFU_{pc} is the mean number of CFU recovered from five replicate positive control samples of a single material, and CFU_{spike} is the number of CFU inoculated onto each of those samples. The value of CFU_{spike} is known from enumeration of the stock spore suspension. Spore recovery was calculated for *B. anthracis* or *B. subtilis* on each soil sample, and the results are included in Section 6.

The performance or efficacy of the decontaminants was assessed by determining the number of viable organisms remaining on each soil test sample after decontamination. Those numbers were compared to the number of viable organisms extracted from the positive control samples.

The number of viable spores of *B. anthracis* or *B. subtilis* in extracts of test and positive control samples was determined to calculate efficacy of the decontaminant. Efficacy is defined as the extent (as $\log_{10}$ reduction) to which viable spores extracted from test samples after decontamination were less numerous than the viable spores extracted from positive control samples. The logarithm of the CFU abundance from each sample extract was determined, and the mean of those logarithm values was then determined for each set of control and associated test samples, respectively. Efficacy of a decontaminant for a test organism/test condition on the i^{th} sample material was calculated as the difference between those mean log values, i.e.:

$$Efficacy = \overline{(\log_{10} CFUc_{ij})} - \overline{(\log_{10} CFUt_{ij})} \quad (2)$$

where $\log_{10} CFUc_{ij}$ refers to the j individual logarithm values obtained from the positive control samples, and $\log_{10} CFUt_{ij}$ refers to the j individual logarithm values obtained from the corresponding test samples, and the overbar designates a mean value. In tests conducted under this plan, there were five positive controls and five corresponding test samples (i.e., $j = 5$) for each soil sample. A decontaminant that achieves a 6 LR or greater is considered effective[10].

In the case where no viable spores were detected in any of the five test sample extracts after decontamination, a CFU abundance of 1 was assigned, resulting in a $\log_{10}$ CFU of zero for that material. When this occurs, the spore population on the soil sample is considered to be completely inactivated within the detection limit of 33 CFU per soil sample. With complete spore inactivation, the decontaminant achieves the maximum efficacy possible or quantifiable. That is, the final efficacy on that material is reported as greater than or equal to ($\geq$) the value calculated by Equation 2. With complete inactivation, the reported LR value is dependent on the positive control recovery, and in most cases, the LR $\geq$ 7.5.

The variances (i.e., the square of the standard deviation) of the $\log_{10} CFUc_{ij}$ and $\log_{10} CFUt_{ij}$ values were also calculated for both the control and test samples (i.e., S^2c_{ij} and S^2t_{ij}), and were used to calculate the pooled standard error (SE) for the efficacy value calculated in Equation 2, as follows:

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

where the number 5 again represents the number *j* of samples in both the control and test data sets. Each efficacy result is reported as an LR value with an associated 95% confidence interval (CI), calculated as follows:

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

The significance of differences in efficacy across different test conditions and spore types was assessed based on the 95% confidence interval of each efficacy result. Differences in efficacy were judged to be significant if the 95% CIs of the two efficacy results did not overlap. Any results based on this formula are hereafter noted as significantly different. Note this comparison is not applicable when the two efficacy results being compared are both reported with LRs as ≥ some value.

3.6 Discoloration of Soils

The physical effect of the decontaminants on the soil materials was also monitored qualitatively during the evaluation. This approach provided a gross visual assessment of whether the decontaminants altered the appearance of the soil, e.g., discoloration. The procedural control (sample that is decontaminated, but has no spores applied) was visually compared to a laboratory blank sample (a sample not exposed to the decontaminant and that has no spores applied).

4.0 Decontamination Procedures

4.1 Liquid Decontaminant Preparation

4.1.1 pH-Amended Ultra Clorox® Germicidal Bleach

The pH-amended bleach consisted of bleach diluted in water with its pH adjusted by addition of acetic acid. Specifically, Ultra Clorox® Germicidal Bleach was used, which contains 6.15% by weight sodium hypochlorite (NaOCl) and <1.0% sodium hydroxide (NaOH) in aqueous solution. This product has a pH between 11 and 12, and a density of 1.08 to 1.11 grams (g)/mL. The pH adjustment to 6.5 – 7.0 is achieved by the addition of 5% acetic acid. The primary active decontaminating agent in this final solution is hypochlorous acid. The recipe for preparation of pH-amended bleach for use as a decontaminant was as follows:

- Prepare 5% acetic acid solution by diluting 50 mL of glacial acetic acid up to 1 L with SFW in a volumetric flask.
- Mix 9.4 parts SFW, 1 part Ultra Clorox® Germicidal Bleach, and 1 part 5% acetic acid. The resulting solution will have a mean total chlorine content (estimated based on dilution) of about 5,400 parts per million (ppm) (or mg/L). The pH is verified before every test to be 6.5 – 6.6.

4.1.2 Sodium Persulfate (Klozur™)

Klozur™ was used as the source of sodium persulfate and is a solid reagent made by FMC Corporation used for *in situ* and *ex situ* chemical oxidation of contaminants in environmental remediation applications (e.g., soil). Klozur™ consists of >99% pure sodium persulfate ($Na_2S_2O_8$) in the form of white odorless crystals. In remediation applications, Klozur™ is injected into contaminated soil or groundwater and activated by mixing in appropriate proportions of up to 8% H_2O_2 by weight, according to instructions published by FMC Corporation[11]. Activation of Klozur™ with H_2O_2 generates sulfate radicals (SO_4•), which are capable of destroying a wide range of organic contaminants while maintaining oxidative ability in a soil (organic) environment. For testing, a 0.5 M solution of sodium persulfate was prepared by dissolving 12 g of Klozur™ in SFW and diluting to 100 mL. This solution was 11.9% persulfate by weight. Commercially prepared 8% H_2O_2 solution was purchased for use in testing.

4.2 Liquid Decontamination Test and Control Chambers and Procedures

4.2.1 Test and Control Chambers

All liquid decontaminant tests were conducted at ambient conditions inside a climate-controlled laboratory. The temperature inside the testing chamber was equilibrated to the ambient laboratory temperature of approximately 20 °C. The temperature and RH were both monitored and recorded with a HOBO® data logger (Onset Computer Corporation, Cape Cod, MA), but no attempt was made to control either. All experiments took place in a Class III BSC.

4.2.2 Neutralization Determination

Sodium thiosulfate (STS) was used to neutralize Klozur™/H_2O_2 and pH-amended bleach decontaminants after the desired contact times were achieved. The optimum concentration of STS in the extraction buffer was determined in trial runs for each liquid decontaminant and application regimen (number of applications of the decontaminant and contact time) that was

tested. In each of those trials, a range of STS concentrations was assessed to determine the STS concentration that most effectively stopped the action of the decontaminant (indicated by the maximum recovery of viable spores in the sample extracts). Further details of the methods and results of the neutralization trials to determine the optimum amount of STS to use are summarized in Appendix B.

4.2.3 pH-Amended Bleach Decontamination Procedure

The number of applications and contact times were selected for testing was based on previous tests with pH-amended bleach on soil materials[5]. Each application consisted of injecting 0.5 mL of pH-amended bleach into each sample (or SFW into the positive control samples) using a laboratory pipette, and mixing the soil and pH- amended bleach solution thoroughly in the sample jar using a glass stirring rod. The solution was re-applied at intervals specified in Table 2-2, mixing between each application with the stirring rod. After the last indicated application, all samples were left at ambient temperature and RH (with the cap removed) in the Class III BSC for the required contact time. At the end of the required time, all samples were extracted as described in Section 3.4.

4.2.4 Sodium Persulfate Decontamination Procedure

For the Klozur™ tests, a 1 mL volume of the 0.5 M persulfate solution was added to each sample jar and mixed with a glass stirring rod. A 1 mL volume of the 8% H_2O_2 activating solution was immediately applied and mixed in the same manner. SFW was applied to the control samples at all application times in the same manner. This process was repeated for multiple applications as shown in Table 2-3. After the last application, all samples were left uncapped in the Class III BSC until the end of the specified contact time (all tests used a seven-day contact time). At the end of the contact time, all samples were dry, and extracted as described in Section 3.4. Equal volumes of the persulfate and H_2O_2 solutions resulted in a H_2O_2/persulfate molar ratio of 5 to 1, a typical ratio recommended for the use of Klozur™ in soil remediation[11]. A contact time of one week was selected, based on information indicating this oxidant can persist in subsurface environments for hours to weeks[12].

4.3 MeBr Fumigation Test and Control Chambers and Procedures

Methyl bromide is a colorless and odorless volatile gas. Chloropicrin was added to the MeBr source gas (0.5% chloropicrin, 99.5% MeBr) as a warning irritant (lacrimator) for the safety of laboratory staff. The gas mixture was used at full strength and injected into the test chamber at the indicated concentrations.

Figure 4-1 shows a schematic drawing of the MeBr test chamber and containment system. The primary test chamber was glass with a 23 L volume (approximately 29 x 29 x 29 cm^3). The chamber was insulated to prevent condensation on the inside chamber walls. The glass chamber was needed for MeBr as the gas exhibits high penetration through many materials. The high toxicity and penetrability of MeBr also required a secondary containment chamber for protection of laboratory personnel. A Class III BSC (SG603, Baker, Sanford, ME) provided secondary containment. Temperature was controlled using a heated water bath and the RH was uncontrolled in the MeBr test chamber.

The MeBr concentration in the test chamber was measured continuously during the contact period using a Fumiscope™ Version 5.0 (Key Chemical and Equipment Company, Clearwater, FL). MeBr was added to the chamber, as necessary, to maintain the specified concentration within ±10%. The Fumiscope meter was calibrated by the manufacturer for MeBr, displaying the concentration on a digital light-emitting diode (LED) display in ounces of MeBr per 1000 cubic feet (ft^3). One oz per 1000 ft^3 is approximately 257 ppm at 25 °C and is approximately 1 mg/L (independent of temperature). The Fumiscope meter included an air pump that pulled a gas sample from the test chamber through the thermal conductivity meter at a controlled rate and exhausted the gas back into the test chamber. Moisture was removed from the gas sample before it was measured in the Fumiscope to eliminate interference from water. At the end of a given trial, the test chamber was flushed with ambient air to <250 ppm and opened.

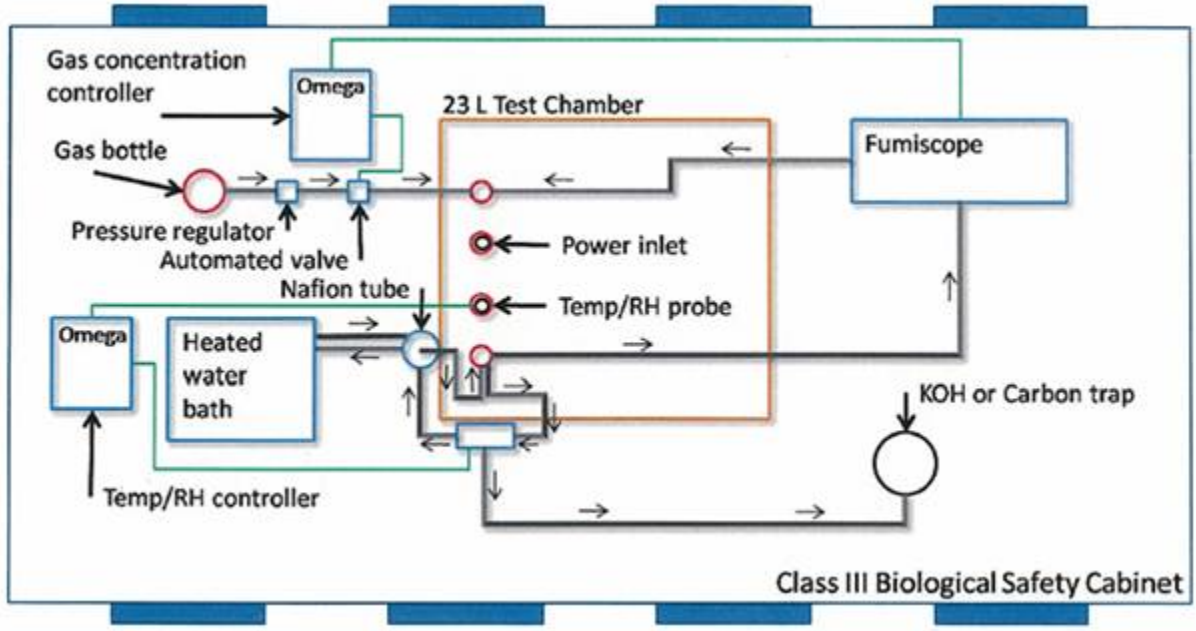


Figure 4-1 Schematic of MeBr Decontamination Test Chamber.

A 9 L Lock & Lock® airtight container served as the positive control chamber. After the addition of control and laboratory blank samples, this chamber was kept at ambient laboratory temperature with no attempts made to control temperature. The RH in the chamber was also left uncontrolled to mimic the RH in the test chamber as closely as possible. However, both temperature and RH were measured with an iTHP-2 temperature and humidity probe (Omega Engineering, Stamford, CT).

As in previous studies with MeBr[1], multiple samples of each soil type were inoculated with the biological agent and placed on a wire rack inside the test chamber. Blank (i.e., uninoculated) and positive control (i.e., inoculated but not decontaminated) samples were also prepared for each soil material and were utilized with data from the test samples (inoculated and decontaminated) to determine decontamination efficacy.

The eight MeBr tests were conducted at concentrations ranging from 100 to 212 mg/L, as shown in Table 2-4. All tests were conducted using a 36 hr contact time, except the last test that was conducted for 24 hr. All tests were conducted at 25 °C and with RH measured but not controlled. The initial RH levels in the chamber just prior to injection of MeBr were at ambient

levels and ranged from approximately 35-65% RH. The RH levels in the chamber increased over time, presumably due to the release of moisture from the soil samples into the chamber air. Two of the tests (Tests 2 and 5) were conducted with soils in which 2 mL SFW was added, to assess the impact on RH levels and decontamination efficacy. One test (Test 3) was conducted with the soil materials dried prior to testing, also to assess impact on RH level (presumably would be relatively lower) and decontamination efficacy.

4.4 Metam Sodium Fumigation Test and Control Chambers and Procedures

Metam sodium is a clear or yellow to yellow-green liquid with a slight sulfide odor; the source we used was an aqueous solution of metam sodium, 42.5% by weight[13].

Upon exposure to the environment, metam sodium decomposes to MITC (the primary biologically active ingredient) and eventually decomposes to hydrogen sulfide and other degradation products containing hydrogen, sulfur, and nitrogen. The conversion rate of metam sodium to MITC depends on soil moisture, pH, soil type, temperature, organic content, and other factors. For optimum performance, it is recommended that the soil be free of clods and soil moisture be between 50-80% of field capacity. Metam sodium can be applied with tillers, sprinklers, or other means of distribution to mix into the soil. Once the metam sodium is added to the soil, it is common practice to place a tarp or cap over the soil to prevent the loss of MITC. The time between application of the metam sodium and planting depends on whether a tarp is used and can vary between 2-4 weeks[6]. After removal of the tarp, the soil may need to be aerated prior to planting[14].

All metam sodium tests were completed under ambient laboratory conditions with no attempt made to control the temperature or RH. Each sample jar served as its own primary container. Immediately prior to inoculation of spores to the soil sample, 2 mL of SFW was added to each test, control, and blank sample and mixed with a glass stirring rod. Once mixed, all test and control samples were inoculated with biological agent and allowed to dry overnight as described in Section 3.3. Because of the potential importance of soil moisture in the formation of MITC, following the overnight drying period, additional amounts of water were added to the soil samples (varied from 0-3 mL of SFW; refer to Table 2-5), and the mixture was stirred.

During the course of testing with metam sodium, endogenous bacteria (morphologically distinct from our target organisms) were observed in the topsoil samples. This observation of endogenous bacteria occurred in the soils that were treated with gamma irradiation at ~40 kGy and also in subsequent soil samples irradiated at ~60 kGy or autoclaved at 121 °C for one hr. Because the sterilization method could impact soil moisture, the moisture content of samples used in the tests with metam sodium was determined for each test. Refer to Appendix A for additional details.

Following the addition of any water, the 42.5 % metam sodium solution was applied with a pipette using small droplets (i.e., 10 µL/droplet). Decontamination tests were conducted using either 80 µL (Test 1) or 160 µL (Tests 2-8) metam sodium applied to each soil sample. Based on the surface area of the sample jars used, these amounts correspond to approximately 75 gallons per acre or 150 gallons per acre, respectively. After application of the metam sodium, the sample jars were capped. These airtight containers allowed the liquid metam sodium to off-gas to MITC, and keep the gas in close contact with each soil sample (mimicking a tarped application in agriculture). Following the conclusion of the contact time, samples were either extracted and plated (refer to Section 3.4) immediately, or the caps were removed, samples stirred, and then the

samples were allowed to aerate with their lids off for a specified amount of time prior to extraction.

Starting with the fourth metam sodium test, we assessed the presence of MITC (potentially indicating the presence of residual metam sodium in the soil) after the contact time to determine the need for aeration of soil samples. Air samples were drawn from the headspace of one *B. anthracis* topsoil test sample and one *B. anthracis* AZTD test sample using specialized MITC detection tubes (Cat # 800-03485, SKC Gulf Coast Inc., Houston, TX; detection limit of 0.1 ppm). These air samples were taken immediately following the removal of the sample caps and stirring of each sample. Air samples were then taken again at the end of the aeration period. This procedure was utilized to ensure that no MITC remained in the headspace of the samples at the end of the contact time and aeration period. These tests did show the presence of MITC after each contact time (indicating the potential presence of residual metam sodium), but no MITC was detected after the same samples were aerated.

5.0 Quality Assurance/Quality Control

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

5.1 Equipment Calibration

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

5.2 QC Results

Quality control efforts conducted during decontaminant testing included positive control samples (inoculated, not decontaminated), procedural blanks (not inoculated, decontaminated), laboratory blank (not inoculated, not decontaminated), and inoculation control samples (analysis of the stock spore suspension).

All positive control results were within the target recovery range of 1 to 150% of the inoculated spores, and all procedural and laboratory blanks met the criterion of no observed CFU for both organisms.

Inoculation control samples were taken from the spore suspension on the day of testing and serially diluted, nutrient plated, and counted to establish the spore density used to inoculate the samples. The spore density levels met the QA target criterion of 1×10^9 CFU/mL (±1 log) for all tests.

5.3 Audits

5.3.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during these experiments. Table 5-1 summarizes the performance evaluation audits that were performed.

No performance evaluation audits were performed to confirm the concentration of *B. anthracis* or *B. subtilis* spores. Unlike chemical analytes, commercially available quantitative standards do not exist for these organisms. The control samples and blanks support the spore measurements.

Table 5-1 Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume of liquid from micropipettes	Gravimetric evaluation	± 10%	± 0.57%
Time	Compared to independent clock	± 2 sec/hr	0 sec/hr
Temperature	Compared to independent calibrated thermometer	± 2 °C	± 0.36 °C
Relative Humidity	Compare to independent calibrated hygrometer	± 10%	± 2%
Fumiscope™ thermal conductivity meter	Instrument was certified as calibrated at the time of use	± 10%	0%
Balance	Compared to independent calibrated weight sets	± 0.5g	± 0.03g

5.3.2 Technical Systems Audit

Observations and findings from the technical systems audit (TSA) were documented and submitted to the laboratory staff lead. TSAs were conducted on December 6 and December 13, 2011, to ensure that the tests were being conducted in accordance with the test/QA plan and Quality Management Plan (QMP). As part of the audit, test procedures were compared to those specified in the test/QA plan and data acquisition and handling procedures were reviewed. None of the findings of the TSA required corrective action.

5.3.3 Data Quality Audit

At least 10% of the data acquired during the evaluation were audited. The data was traced from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

5.4 Test/Quality Assurance Plan Deviations

5.4.1 pH-Amended Ultra Clorox® Germicidal Bleach Test Matrix

Table 5 of the test/QA plan shows that four tests would be completed using pH-amended Ultra Clorox® Germicidal Bleach using both topsoil and AZTD. After showing minimal efficacy (0.36 LR against *B. anthracis* and 0.10 with *B. subtilis*) under the most robust test conditions planned, there appeared to be no need to investigate the efficacy of pH-amended bleach for topsoil further. The remaining tests were conducted using AZTD only.

5.4.2 Extraction buffer

For the seven-day test using pH-amended Ultra Clorox® Germicidal Bleach, the neutralization extraction buffer (1.5% sodium thiosulfate [STS]) was made with phosphate-buffered saline (PBS) instead of the required PBST. This buffer was used to extract all test samples (both topsoil and AZTD) and associated blanks. Minimal effect on results was expected, as exemplified in the positive control recovery results.

5.5 QA/QC Reporting

Each assessment and audit was documented in accordance with the test/QA plan and QMP. For these tests, findings were noted (none significant) in the TSA or data quality audit, but no follow-up corrective action was necessary. The findings for the TSA were minor with one item noted (see Section 5.4.2, above). The findings for the data quality audit were mostly minor data transcription errors requiring some recalculation of efficacy results, but none were gross errors in recording.

5.6 Data Review

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

6.0 Results and Performance Summary for Liquid Biocides

6.1 pH-Amended Bleach Results

The quantitative decontamination efficacy results (in terms of LR) for pH-amended bleach against spores of *B. anthracis* and *B. subtilis* are presented in detail in Tables 6-1 and 6-2 and summarized in Figure 6-1.

For the seven-day contact time (Test 1), the decontamination efficacy on topsoil was minimal (less than 0.5 LR for both organisms), and because of this lack of efficacy, additional tests under the less robust conditions were discontinued for topsoil. The poor efficacy is most likely due to the organic content of the topsoil; refer to Table 3-1.

At this same decontamination treatment for AZTD, both microorganisms were completely inactivated. In general, the pH-amended bleach was more successful in decontaminating the AZTD, with greater than 7 LR obtained for both *B. anthracis* and *B. subtilis* with only a 2 hr contact time (Test 3). There were no significant differences in efficacy between the two microorganisms tested.

6.2 Sodium Persulfate Results

The quantitative efficacy results for Klozur^{TM} are presented in detail in Tables 6-3 through 6-6 and summarized graphically in Figure 6-2. All tests were conducted with a contact time of seven days, with the number of applications of the sodium persulfate/H_2O_2 decontaminant ranging from 1 to 6. The most robust treatment, the six application regimen (Test 1), resulted in complete inactivation of *B. anthracis* on both soil materials. The next robust treatment (three applications of the decontaminant, all applied within the first 2 hours; Test 2) provided greater than a 7 LR for *B. anthracis* on both soils. Efficacy generally decreased with decreasing number of persulfate applications; none of the other sodium persulfate application conditions resulted in greater than a 6 LR.

When comparing the results for the topsoil and AZTD, the decontamination effectiveness of the sodium persulfate against *B. anthracis* was not significantly different for the two soil types for the majority of tests. Against *B. subtilis*, however, the sodium persulfate technology was generally more effective on the topsoil than the AZTD. (The highest LR obtained for *B. subtilis* on AZTD was 1.1.) These results generally indicate that the organic content of the topsoil did not diminish efficacy, which is consistent with its persistent oxidative ability and commercial use as a soil remediation technology. When comparing results for the two microorganisms, *B. subtilis* was significantly more difficult to inactivate than *B. anthracis* in all but one of the tests.

Two tests were conducted to assess whether the frequency of the application of sodium persulfate affected decontamination efficacy. In these two tests, we used a contact time of seven days and three applications of the sodium persulfate, but in one test it was applied once every hour (Test 2), and in the other, it was applied every 48 hours (Test 3). When applied every hour, the efficacy was significantly greater than when it was applied every 48 hours (on Days 0, 2 and 4) against *B. anthracis*: 7.07 vs. 5.53 LR on topsoil and 7.38 vs. 5.24 LR on AZTD. However, with respect to *B. subtilis*, the frequency of the application resulted in no significant difference in efficacy.

Table 6-1 Inactivation of *Bacillus anthracis* Spores on Soil using pH-Amended Ultra Clorox® Germicidal Bleach[a]

Contact Time (Number of Applications) – Material Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (8) Topsoil[a] - #1				
Positive Controls[b]	9.87×10^7	7.54 ± 0.04	35.60 ± 3.48	[g]
Test Samples[c]	9.87×10^7	7.19 ± 0.06	15.74 ± 2.29	0.36 ± 0.07
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (8) – AZTD - #1				
Positive Controls	9.87×10^7	7.88 ± 0.05	77.08 ± 9.72	-
Test Samples	9.87×10^7	0	0	≥7.88 ± 0.05
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (8) - AZTD - #2				
Positive Controls	9.43×10^7	7.87 ± 0.03	79.30 ± 5.07	-
Test Samples	9.43×10^7	0	0	≥7.87 ± 0.02
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Two Hours (4) – AZTD - #3				
Positive Controls	1.05×10^8	7.91 ± 0.04	77.35 ± 6.99	-
Test Samples	1.05×10^8	0	0	≥7.91 ± 0.03
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
One Hour (2) – AZTD - #4				
Positive Controls	1.04×10^8	7.82 ± 0.06	63.75 ± 9.00	-
Test Samples	1.04×10^8	2.66 ± 1.79	0.0066 ± 0.0088	5.16 ± 1.57
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.

Table 6-2 Inactivation of *Bacillus subtilis* Spores on Soil using pH-Amended Ultra Clorox® Germicidal Bleach[a]

Contact Time (Number of Applications) – Material Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (8) – Topsoil[a] - #1				
Positive Controls[b]	1.25×10^8	7.41 ± 0.15	21.58 ± 6.96	[g]
Test Samples[c]	1.25×10^8	7.31 ± 0.03	16.29 ± 1.02	0.10 ± 0.14
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (8) – AZTD - #1				
Positive Controls	1.25×10^8	7.81 ± 0.16	54.77 ± 17.15	-
Test Samples	1.25×10^8	0	0	≥7.81 ± 0.14
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (8) - AZTD - #2				
Positive Controls	1.19×10^8	7.93 ± 0.02	71.14 ± 3.40	-
Test Samples	1.19×10^8	0	0	≥7.93 ± 0.02
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Two Hours (4) – AZTD - #3				
Positive Controls	1.14×10^8	7.95 ± 0.03	77.98 ± 5.33	-
Test Samples	1.14×10^8	0.74 ± 1.66	0.00090 ± 0.0021	7.21 ± 1.46
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
One Hour (2) – AZTD - #4				
Positive Controls	8.80×10^7	7.69 ± 0.05	56.40 ± 6.60	-
Test Samples	8.80×10^7	4.42 ± 1.67	0.23 ± 0.36	3.28 ± 1.47
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE). Differences in efficacy may be significant if the 95% CIs of the two efficacy results do not overlap; however, this comparison is not applicable when the two efficacy results being compared are both reported with log reductions as ≥ some value.
[g] "-" Not Applicable.

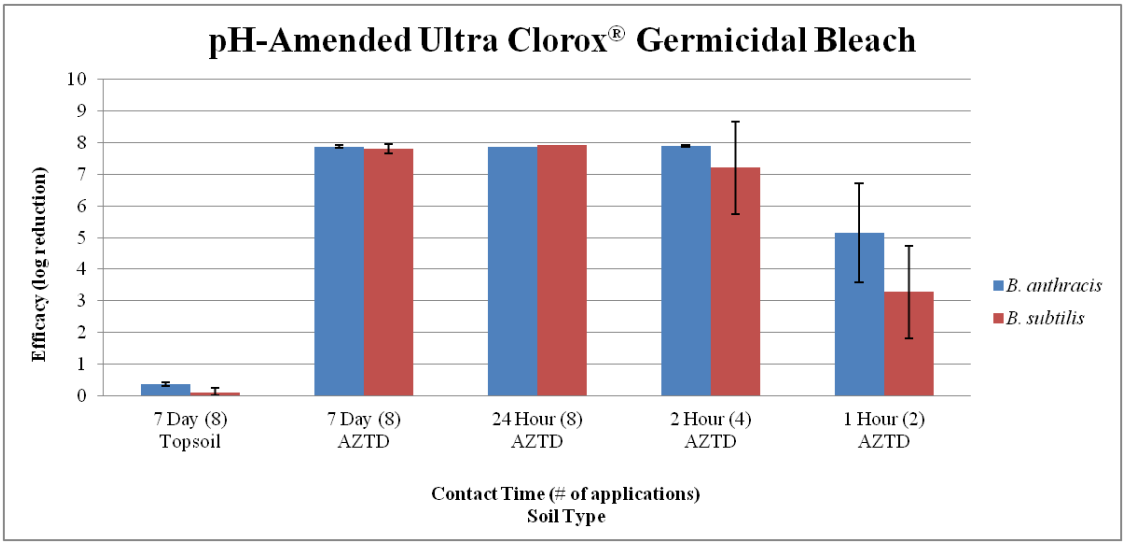


Figure 6-1 Summary of Decontamination Efficacies (with 95% confidence intervals) for pH-Amended Bleach Testing on Topsoil and AZTD

Table 6-3 Inactivation of *Bacillus anthracis* Spores on Topsoil with Klozur™, [a]

Contact Time (Number of Applications) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (6)[†] - #1				
Positive Controls[b]	8.97×10^7	7.82 ± 0.04	74.35 ± 7.50	-[g]
Test Samples[c]	8.97×10^7	0	0	≥7.82 ± 0.04
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (3)[†] - #2				
Positive Controls	8.97×10^7	7.72 ± 0.04	58.51 ± 5.65	-
Test Samples	8.97×10^7	0.65 ± 1.46	0.00041 ± 0.00090	7.07 ± 1.28
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (3)[‡] - #3				
Positive Controls	1.12×10^8	7.62 ± 0.09	37.98 ± 8.74	-
Test Samples	1.12×10^8	2.09 ± 1.40	0.00093 ± 0.0014	5.53 ± 1.23
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (2)[†] - #4				
Positive Controls	1.23×10^8	7.83 ± 0.08	55.45 ± 9.91	-
Test Samples	1.23×10^8	4.03 ± 1.13	0.00089 ± 0.0017	3.80 ± 1.00
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (1) - #5				
Positive Controls	8.83×10^7	7.68 ± 0.08	54.90 ± 10.81	-
Test Samples	8.83×10^7	6.75 ± 0.31	0.076 ± 0.042	0.93 ± 0.28
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
[†] The decontaminant was applied every 60 minutes until the total number of applications was reached.
[‡] The decontaminant was applied on days 0, 2 and 4.

Table 6-4 Inactivation of *Bacillus anthracis* Spores on AZTD with Klozur™, [a]

Contact Time (Number of Applications) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (6)[†] - #1				
Positive Controls[b]	8.97×10^7	7.87 ± 0.10	83.94 ± 19.52	-[g]
Test Samples[c]	8.97×10^7	0	0	≥7.87 ± 0.09
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (3)[†] - #2				
Positive Controls	8.97×10^7	7.69 ± 0.04	55.21 ± 5.04	-
Test Samples	8.97×10^7	0.31 ± 0.70	0.0000091 ± 0.000018	7.38 ± 0.61
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (3)[‡] - #3				
Positive Controls	1.12×10^8	7.79 ± 0.10	56.46 ± 12.98	-
Test Samples	1.12×10^8	2.55 ± 0.59	0.00054 ± 0.00051	5.24 ± 0.53
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (2)[†] - #4				
Positive Controls	1.23×10^8	7.48 ± 0.31	30.32 ± 25.09	-
Test Samples	1.23×10^8	3.31 ± 1.33	0.025 ± 0.050	4.17 ± 1.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (1) - #5				
Positive Controls	8.83×10^7	7.95 ± 0.13	104.24 ± 32.81	-
Test Samples	8.83×10^7	3.58 ± 0.26	0.0049 ± 0.0026	4.37 ± 0.26
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
[†] The decontaminant was applied every 60 minutes until the total number of applications was reached.
[‡] The decontaminant was applied on days 0, 2 and 4.

Table 6-5 Inactivation of *Bacillus subtilis* Spores on Topsoil with Klozur™, [a]

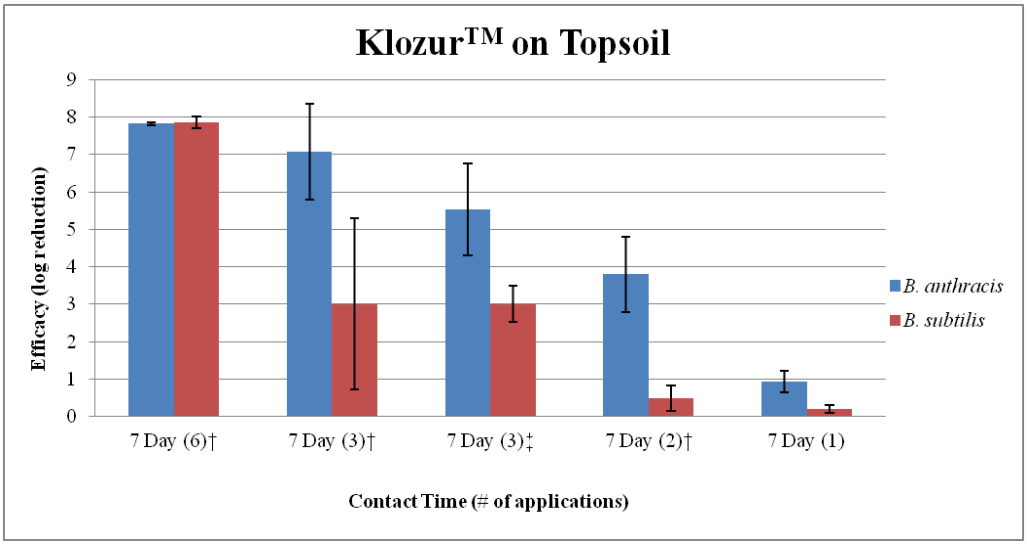
Contact Time (Number of Applications) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (6)[†] - #1				
Positive Controls[b]	1.12×10^8	7.86 ± 0.18	68.71 ± 25.25	-[g]
Test Samples[c]	1.12×10^8	0	0	≥7.86 ± 0.16
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (3)[†] - #2				
Positive Controls	9.30×10^7	7.58 ± 0.05	41.57 ± 5.19	-
Test Samples	9.30×10^7	4.57 ± 2.61	0.71 ± 0.71	3.01 ± 2.28
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (3)[‡] - #3				
Positive Controls	1.31×10^8	7.72 ± 0.09	40.41 ± 8.02	-
Test Samples	1.31×10^8	4.71 ± 0.53	0.064 ± 0.057	3.01 ± 0.48
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (2)[†] - #4				
Positive Controls	1.18×10^8	7.68 ± 0.40	57.02 ± 50.50	-
Test Samples	1.18×10^8	7.20 ± 0.05	13.37 ± 1.59	0.49 ± 0.35
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (1) - #5				
Positive Controls	9.53×10^7	7.74 ± 0.08	58.91 ± 10.70	-
Test Samples	9.53×10^7	7.54 ± 0.09	37.00 ± 8.84	0.20 ± 0.11
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

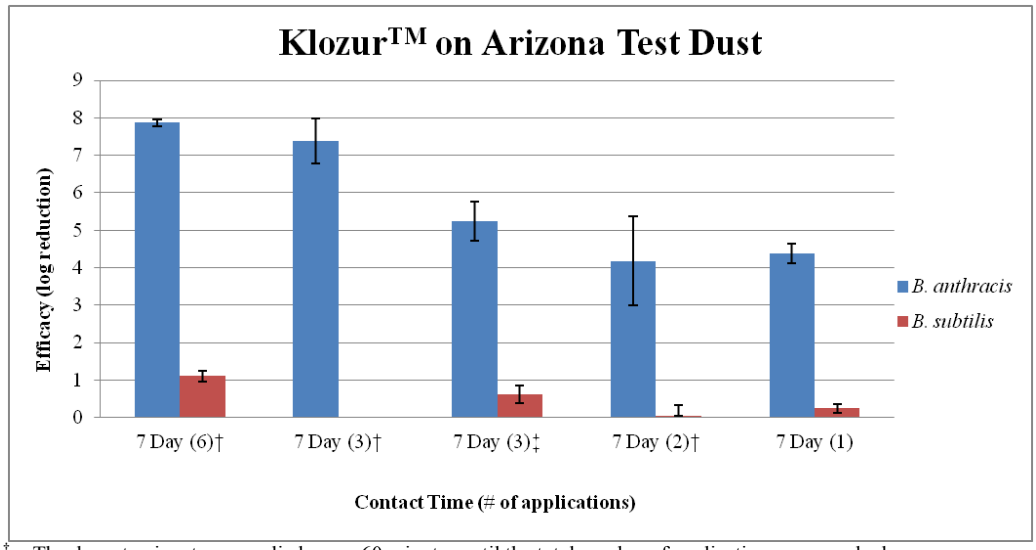
[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
[†] The decontaminant was applied every 60 minutes until the total number of applications was reached.
[‡] The decontaminant was applied on days 0, 2 and 4.

Table 6-6 Inactivation of *Bacillus subtilis* Spores on AZTD with Klozur™[a]

Contact Time (Number of Applications) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
Seven Days (6)[†] - #1				
Positive Controls[b]	1.12×10^8	8.01 ± 0.11	94.99 ± 25.64	-[g]
Test Samples[c]	1.12×10^8	6.91 ± 0.13	7.53 ± 2.07	1.10 ± 0.15
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Seven Days (3)[‡] - #2				
Positive Controls	9.30×10^7	7.64 ± 0.09	47.89 ± 9.94	-
Test Samples	9.30×10^7	7.69 ± 0.05	52.71 ± 5.42	0.00 ± 0.00
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (3)[‡] - #3				
Positive Controls	1.31×10^8	8.00 ± 0.03	75.86 ± 5.57	-
Test Samples	1.31×10^8	7.37 ± 0.27	20.88 ± 12.67	0.62 ± 0.24
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (2)[†] - #4				
Positive Controls	1.18×10^8	7.89 ± 0.31	76.26 ± 33.36	-
Test Samples	1.18×10^8	7.84 ± 0.04	59.15 ± 5.40	0.05 ± 0.28
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (1) - #5				
Positive Controls	9.53×10^7	7.96 ± 0.04	95.55 ± 9.06	-
Test Samples	9.53×10^7	7.72 ± 0.12	56.69 ± 14.75	0.24 ± 0.11
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = sample inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated .
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
[†] The decontaminant was applied every 60 minutes until the total number of applications was reached.
[‡] The decontaminant was applied on days 0, 2 and 4.





† = The decontaminant was applied every 60 minutes until the total number of applications was reached.
‡ =The decontaminant was applied at the start of the test and on day 2 and 4.

Figure 6-2 Summary of Decontamination Efficacies (with 95% confidence intervals) for Klozur™ Liquid Testing on Topsoil and AZTD

7.0 Results and Performance Summary for Fumigant Biocides

7.1 MeBr Results

The decontamination efficacy of MeBr against *B. anthracis* and *B. subtilis* was evaluated at target concentrations ranging from 100 to 212 mg/L at a target temperature of 25 °C, using a contact time of 36 hr for all tests except for one test performed at 24 hr. The RH levels in the test chamber were uncontrolled, but for a few tests, attempts were made to manipulate RH by adding or removing moisture from the soil prior to testing. The actual fumigation conditions for each test, including RH, are shown in Table 7-1. The detailed decontamination efficacy results are shown in Tables 7-2 through 7-5 and summarized graphically in Figure 7-1.

Table 7-1 Actual Fumigation Conditions for Tests with MeBr

Test #	Actual Mean MeBr Concentration ± SD (mg/L)	Soil Moisture Condition	Actual mean temperature ± SD (° C)	Actual Mean RH ± SD	Contact Time (hr)
1	213 ± 0.74	No change	25.3 ± 0.41	79.3 ± 3.53	36
2	213 ± 0.73	2 mL SFW added	25.2 ± 0.16	82.8 ± 2.16	36
3	213 ± 0.68	Samples dried	25.4 ± 0.53	54.5 ± 2.26	36
4	102 ± 1.36	No change	25.5 ± 0.62	76.9 ± 3.96	36
5	101 ± 0.51	2 mL SFW added	25.3 ± 0.53	85.1 ± 1.91	36
6	181 ± 0.68	No change	25.2 ± 0.22	82.0 ± 2.38	36
7	141 ± 0.53	No change	25.2 ± 0.18	82.9 ± 2.08	36
8	213 ± 0.81	No change	25.2 ± 0.30	76.2 ± 4.01	24

The two most robust treatment conditions in terms of concentration, contact time, and soil moisture (212 mg/L MeBr, 36 hour, no drying of soil; Tests 1 and 2) resulted in complete inactivation of *B. anthracis* spores on AZTD, and > 7.0 LR on topsoil. Overall, MeBr was effective (greater than 6 LR achieved) against *B. anthracis* on topsoil and AZTD at 25 °C when using a concentration of at least 180 mg/L and contact time of 36 hours. One minor exception is the test in which the soil samples were dried beforehand (Test 3), which resulted in a comparatively lower RH (55%), and a lower decontamination efficacy (LR of 5.9) on topsoil. The two tests in which water was added to the soil beforehand (Tests 2 and 5) resulted in slightly higher RH levels (compared to tests under the same conditions without water added), but the added water had no significant effect or resulted only in slightly decreased decontamination efficacy for *B. anthracis*. As expected, decontamination efficacy generally decreased with decreasing concentration and contact time.

With respect to the effect of soil type, the decontamination efficacies obtained for *B. anthracis* were generally slightly higher on AZTD compared to topsoil, although half of the test results for the two soil types were not significantly different. *B. subtilis* was significantly more difficult to inactivate compared to *B. anthracis* for all tests conducted. The highest LR value obtained for *B. subtilis* was 2.1, with the majority of the LR values for *B. subtilis* below 1.0. The scope of this study did not allow for us to examine the mechanisms to explain why *B. subtilis* is significantly

more resistant to MeBr than *B. anthracis*. However, this result is consistent with a previous study.[1]

Table 7-2 Inactivation of *Bacillus anthracis* Spores on Topsoil with MeBr[a]

Contact Time (Actual Concentration [mean mg/L ± SD]) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
36 Hours (213 ± 0.74) #1				
Positive Controls[b]	8.97×10^7	7.77 ± 0.037	66.38 ± 5.47	-[g]
Test Samples[c]	8.97×10^7	0.36 ± 0.82	0.000016 ± 0.000033	7.41 ± 0.72
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
36 Hours (213 ± 0.73)* #2				
Positive Controls	9.97×10^7	7.92 ± 0.065	83.61 ± 12.71	-
Test Samples	9.97×10^7	0.51 ± 1.15	0.000074 ± 0.00016	7.40 ± 1.01
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (213 ± 0.68)† #3				
Positive Controls	7.57×10^7	7.65 ± 0.080	60.21 ± 11.30	-
Test Samples	7.57×10^7	1.78 ± 1.75	0.0028 ± 0.0058	5.87 ± 1.54
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (102 ± 1.36) #4				
Positive Controls	8.73×10^7	7.72 ± 0.080	61.49 ± 11.27	-
Test Samples	8.73×10^7	6.28 ± 0.037	2.18 ± 0.19	1.45 ± 0.08
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (101 ± 0.51)* #5				
Positive Controls	8.40×10^7	7.81 ± 0.11	79.43 ± 21.13	-
Test Samples	8.40×10^7	6.81 ± 0.075	7.84 ± 1.29	1.00 ± 0.12
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (181 ± 0.68) #6				
Positive Controls	8.83×10^7	7.66 ± 0.036	51.55 ± 4.31	-
Test Samples	8.83×10^7	0.97 ± 0.90	0.000031 ± 0.000031	6.68 ± 0.79
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (141 ± 0.53) #7				
Positive Controls	1.43×10^8	8.05 ± 0.020	78.18 ± 3.58	-
Test Samples	1.43×10^8	4.59 ± 0.13	0.028 ± 0.0075	3.46 ± 0.11
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (213 ± 0.81) #8				
Positive Controls	1.25×10^8	7.79 ± 0.045	49.01 ± 4.82	-
Test Samples	1.25×10^8	4.54 ± 0.38	0.037 ± 0.033	3.25 ± 0.34
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated .
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
* 2 mL SFW added to samples prior to inoculation.
† = Samples dried in oven prior to inoculation.

Table 7-3 Inactivation of *Bacillus anthracis* Spores on AZTD with MeBr[a]

Contact Time (Actual Concentration [mean mg/L ± SD]) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
36 Hours (213 ± 0.74) #1				
Positive Controls[b]	8.97×10^7	7.74 ± 0.80	62.81 ± 12.48	-[g]
Test Samples[c]	8.97×10^7	0	0	≥7.74 ± 0.07
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
36 Hours (213 ± 0.73)* #2				
Positive Controls	9.97×10^7	7.90 ± 0.062	80.52 ± 11.32	-
Test Samples	9.97×10^7	0	0	≥7.90 ± 0.05
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (213 ± 0.68)† #3				
Positive Controls	7.57×10^7	7.77 ± 0.050	77.75 ± 9.08	-
Test Samples	7.57×10^7	0.30 ± 0.68	0.000010 ± 0.000019	7.46 ± 0.60
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (102 ± 1.36) #4				
Positive Controls	8.73×10^7	7.75 ± 0.056	64.83 ± 8.69	-
Test Samples	8.73×10^7	5.41 ± 0.44	0.38 ± 0.20	2.34 ± 0.39
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (101 ± 0.51)* #5				
Positive Controls	8.40×10^7	7.72 ± 0.061	63.33 ± 8.79	-
Test Samples	8.40×10^7	6.32 ± 0.28	2.89 ± 1.49	1.40 ± 0.25
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (181 ± 0.68) #6				
Positive Controls	8.83×10^7	7.75 ± 0.052	63.87 ± 7.55	-
Test Samples	8.83×10^7	1.49 ± 1.47	0.00056 ± 0.0011	6.26 ± 1.29
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (141 ± 0.53) #7				
Positive Controls	1.43×10^8	8.13 ± 0.031	94.69 ± 6.79	-
Test Samples	1.43×10^8	1.94 ± 1.78	0.00074 ± 0.00069	6.19 ± 1.56
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (213 ± 0.81) #8				
Positive Controls	1.25×10^8	7.87 ± 0.084	60.85 ± 11.51	-
Test Samples	1.25×10^8	0.70 ± 0.98	0.000022 ± 0.000034	7.17 ± 0.86
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
* 2 mL SFW added to samples prior to inoculation.
† Samples dried in oven prior to inoculation.

Table 7-4 Inactivation of *Bacillus subtilis* Spores on Topsoil with MeBr[a]

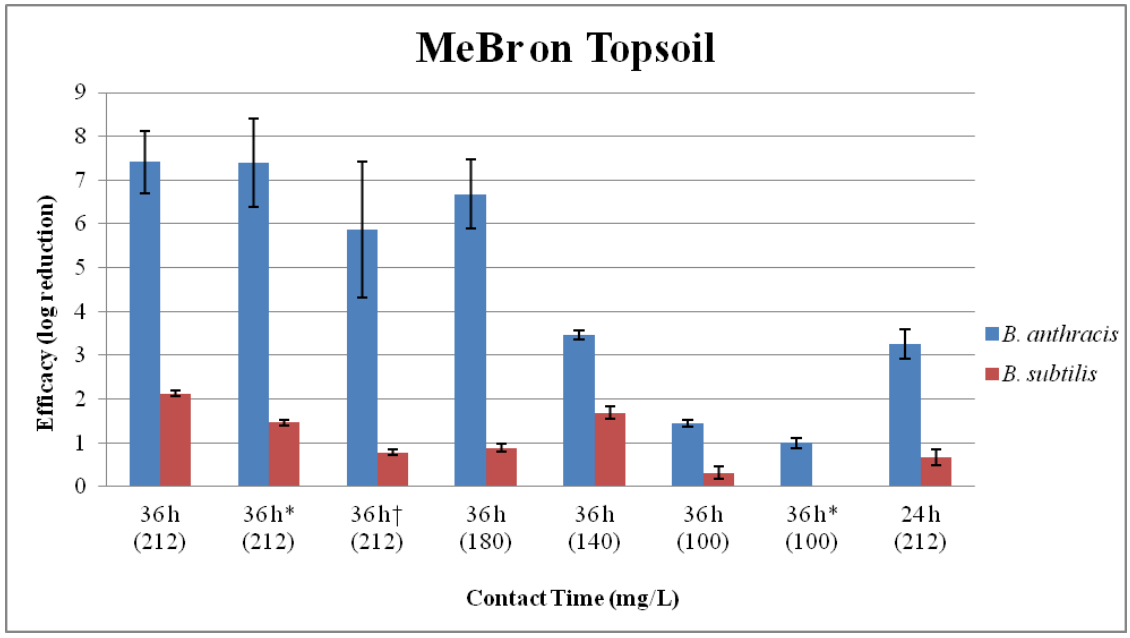
Contact Time (Actual Concentration [mean mg/L ± SD]) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
36 Hours (213 ± 0.74) #1				
Positive Controls[b]	6.10×10^7	7.75 ± 0.047	92.59 ± 9.37	-[g]
Test Samples[c]	6.10×10^7	5.62 ± 0.045	0.69 ± 0.07	2.13 ± 0.06
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
36 Hours (213 ± 0.73)* #2				
Positive Controls	1.36×10^8	8.00 ± 0.051	73.59 ± 8.68	-
Test Samples	1.36×10^8	6.54 ± 0.063	2.55 ± 0.37	1.46 ± 0.07
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (213 ± 0.68)† #3				
Positive Controls	1.03×10^8	7.52 ± 0.024	32.37 ± 1.78	-
Test Samples	1.03×10^8	6.74 ± 0.061	5.35 ± 0.68	0.78 ± 0.06
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (102 ± 1.36) #4				
Positive Controls	9.23×10^7	7.77 ± 0.035	64.53 ± 5.15	-
Test Samples	9.23×10^7	7.45 ± 0.17	32.46 ± 9.93	0.32 ± 0.15
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (101 ± 0.51)* #5				
Positive Controls	8.50×10^7	7.77 ± 0.014	69.32 ± 2.15	-
Test Samples	8.50×10^7	7.79 ± 0.025	71.93 ± 4.17	0.00 ± 0.00
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (181 ± 0.68) #6				
Positive Controls	9.53×10^7	7.66 ± 0.083	48.69 ± 9.30	-
Test Samples	9.53×10^7	6.78 ± 0.061	6.30 ± 0.91	0.88 ± 0.09
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (141 ± 0.53) #7				
Positive Controls	1.42×10^8	8.11 ± 0.076	91.83 ± 15.87	-
Test Samples	1.42×10^8	6.42 ± 0.15	1.91 ± 0.61	1.69 ± 0.15
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (213 ± 0.81) #8				
Positive Controls	1.36×10^8	8.03 ± 0.0066	79.26 ± 1.21	-
Test Samples	1.36×10^8	7.36 ± 0.22	18.33 ± 6.76	0.67 ± 0.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

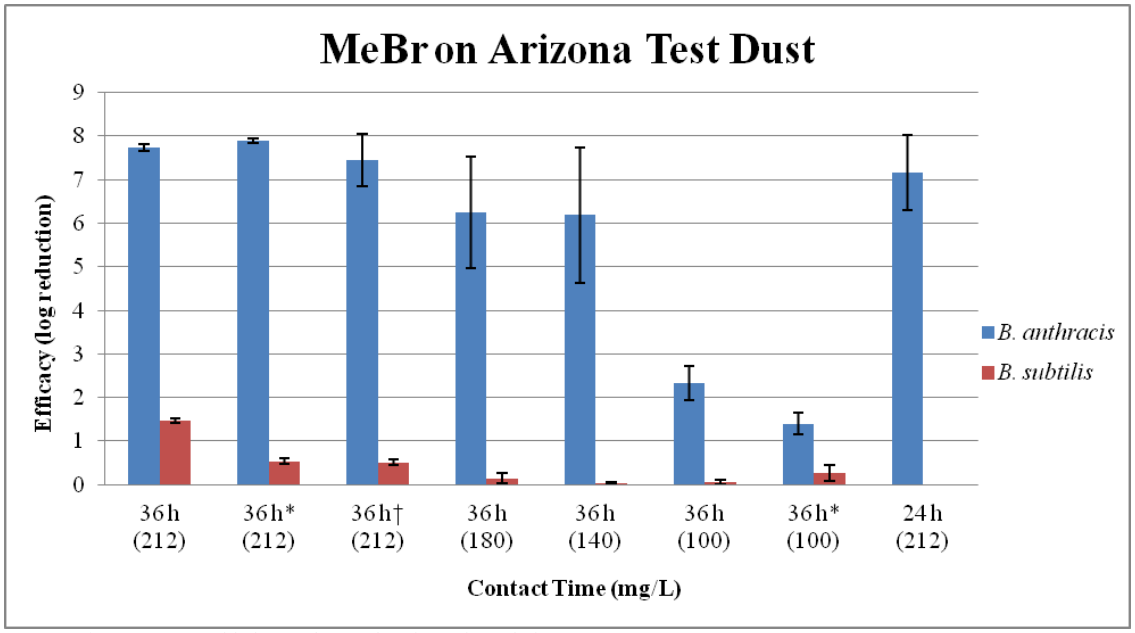
[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
* 2 mL SFW added to samples prior to inoculation.
† Samples dried in oven prior to inoculation.

Table 7-5 Inactivation of *Bacillus subtilis* Spores on AZTD with MeBr[a]

Contact Time (Actual Concentration [mean mg/L ± SD]) Test #	Inoculum (CFU)	Mean of Logs of Observed CFU	Mean % Recovery	Decontamination Efficacy ± CI[f]
36 Hours (213 ± 0.74) #1				
Positive Controls[b]	6.10×10^7	7.77 ± 0.038	96.82 ± 8.23	-[g]
Test Samples[c]	6.10×10^7	6.30 ± 0.059	3.32 ± 0.45	1.47 ± 0.06
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
36 Hours (213 ± 0.73)* #2				
Positive Controls	1.36×10^8	8.03 ± 0.031	79.01 ± 5.67	-
Test Samples	1.36×10^8	7.49 ± 0.061	23.03 ± 3.47	0.54 ± 0.06
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (213 ± 0.68)† #3				
Positive Controls	1.03×10^8	7.58 ± 0.071	37.36 ± 5.94	-
Test Samples	1.03×10^8	7.07 ± 0.047	11.57 ± 1.24	0.51 ± 0.07
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (102 ± 1.36) #4				
Positive Controls	9.23×10^7	7.78 ± 0.063	65.35 ± 8.88	-
Test Samples	9.23×10^7	7.72 ± 0.067	56.86 ± 8.52	0.06 ± 0.08
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (101 ± 0.51)* #5				
Positive Controls	8.50×10^7	7.77 ± 0.012	68.61 ± 1.97	-
Test Samples	8.50×10^7	7.50 ± 0.22	40.59 ± 19.15	0.27 ± 0.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (181 ± 0.68) #6				
Positive Controls	9.53×10^7	7.77 ± 0.12	64.16 ± 14.69	-
Test Samples	9.53×10^7	7.64 ± 0.052	45.67 ± 5.37	0.14 ± 0.12
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
36 Hours (141 ± 0.53) #7				
Positive Controls	1.42×10^8	8.04 ± 0.041	76.86 ± 7.25	-
Test Samples	1.42×10^8	8.00 ± 0.026	71.27 ± 4.38	0.03 ± 0.04
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
24 Hours (213 ± 0.81) #8				
Positive Controls	1.36×10^8	7.92 ± 0.090	62.16 ± 13.14	-
Test Samples	1.36×10^8	7.97 ± 0.024	68.09 ± 3.77	0.00 ± 0.00
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
* 2 mL SFW added to samples prior to inoculation.
† Samples dried in oven prior to inoculation.





* 2 mL SFW added to each sample prior to inoculation.
† samples dried in oven prior to inoculation.

Figure 7-1. Summary of Decontamination Efficacies for MeBr Fumigant Testing on Topsoil and AZTD

7.2 Metam Sodium Results

The detailed decontamination efficacy results for metam sodium against *B. anthracis* and *B. subtilis* on topsoil and AZTD are summarized in Tables 7-6 through 7-9 and summarized graphically in Figure 7-2.

In terms of the number of test conditions in which the soil samples were completely inactivated, the metam sodium was significantly more effective against both microorganisms on the AZTD compared to the topsoil for the majority of the tests. For example, in all but one of the eight tests on AZTD, *B. anthracis* was completely inactivated, whereas there was just one test (Test 6) on topsoil in which *B. anthracis* was completely inactivated.

In all the tests on topsoil, *B. subtilis* was significantly more difficult to inactivate compared to *B. anthracis*. On AZTD, *B. subtilis* was significantly more difficult to inactivate compared to *B. anthracis* in half the tests.

The effect of doubling the amount of metam sodium applied to the soil materials can be seen in reviewing results for Tests 1 and 2. Efficacy improved significantly for both microorganisms on topsoil and for *B. subtilis* on AZTD.

Increasing contact time and aeration time generally improved efficacy for the inactivation of *B. anthracis* on topsoil but not significantly. This effect can be seen by comparing the results between Test 2 and 3 (contact time/aeration time increased from 5/0 to 7/7 days); Tests 5 and 6 (contact time/aeration time increased from 7/7 to 14/28 days using 60 kGy irradiated soils); and Tests 7 and 8 (contact time/aeration time increased from 7/7 to 14/28 days for autoclaved soils). Similar improvements in efficacy were seen with *B. subtilis* on AZTD.

The moisture content of the soil samples was affected by the amount of water added prior to decontamination testing, the soil sterilization method, and the overnight dry time; refer to Appendix A for further details. The effect of moisture content on decontamination efficacy of the metam sodium is readily apparent in the results for *B. anthracis* on topsoil (refer to Figure 7-3). In Figure 7-3, results are aggregated by contact time/aeration time (results for Tests 1 and 2 are excluded because different amounts of metam sodium were used), which shows that efficacy increases with increasing levels of moisture. For Tests 3, 5, and 6, in which efficacy was greater than 6.0, the soil moisture was notably at its highest levels. The effect of moisture on efficacy was not readily apparent for the results with *B. subtilis* on topsoil (all results less than a 1.13 LR) or for both microbes on AZTD (all results greater than 7 LR for *B. anthracis*). For AZTD, the lack of apparent effect of moisture may be because the moisture content for Tests 3-8 was generally uniform at approximately 18%, with one exception for Test 5, which had a moisture content at 27%.

Table 7-6 Inactivation of *Bacillus anthracis* Spores on Topsoil with Metam Sodium[a]

Contact Time (Aeration Time) Test #	Inoculum (CFU)	Mean Logs Observed (CFU ± SD)	Mean % Recovery (±SD)	Decontamination Efficacy ± CI [f]
Five Days (0 Days) - #1				
Positive Controls[b]	1.37×10^8	7.94 ± 0.064	64.82 ± 9.68	-[g]
Test Samples[c]	1.37×10^8	7.11 ± 0.23	10.69 ± 6.58	0.83 ± 0.21
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Five Days (0 Days) - #2				
Positive Controls	1.11×10^8	8.00 ± 0.060	90.63 ± 12.64	-
Test Samples	1.11×10^8	3.41 ± 3.11	0.27 ± 0.26	4.59 ± 2.73
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) † - #3				
Positive Controls	1.03×10^8	7.83 ± 0.52	66.08 ± 8.04	-
Test Samples	1.03×10^8	1.82 ± 2.50	0.014 ± 0.020	6.01 ± 2.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) ‡ - #4				
Positive Controls	1.04×10^8	7.74 ± 0.062	52.75 ± 7.56	-
Test Samples	1.04×10^8	3.96 ± 0.48	0.014 ± 0.013	3.77 ± 0.42
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) § - #5				
Positive Controls	9.97×10^7	7.89 ± 0.082	79.78 ± 14.46	-
Test Samples	9.97×10^7	1.37 ± 2.01	0.0054 ± 0.12	6.52 ± 1.76
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days) † - #6				
Positive Controls	9.67×10^7	7.84 ± 0.046	71.09 ± 7.58	-
Test Samples	9.67×10^7	0	0	≥7.84 ± 0.04
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) † - #7				
Positive Controls	1.11×10^8	8.04 ± 0.0099	98.56 ± 2.26	-
Test Samples	1.11×10^8	6.15 ± 0.22	1.39 ± 0.61	1.89 ± 0.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days) † - #8				
Positive Controls	1.00×10^8	7.94 ± 0.042	87.18 ± 8.42	-
Test Samples	1.00×10^8	4.96 ± 0.40	0.12 ± 0.071	2.98 ± 0.35
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated .
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
***** 2 mL SFW added to all samples prior to inoculation.
† 1 mL SFW added prior to addition of metam sodium.
‡ 2 mL SFW added prior to addition of metam sodium.
§ 3 mL SFW added prior to addition of metam sodium.

Table 7-7 Inactivation of *Bacillus anthracis* Spores on AZTD with Metam Sodium[a]

Contact Time (Aeration Time) Test #	Inoculum (CFU)	Mean Logs Observed (CFU ± SD)	Mean % Recovery (±SD)	Decontamination Efficacy ± CI [f]
Five Days (0 Days) - #1				
Positive Controls[b]	1.37×10^8	7.84 ± 0.054	51.18 ± 6.26	-[g]
Test Samples[c]	1.37×10^8	0.63 ± 1.40	0.00020 ± 0.00045	7.22 ± 1.23
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Five Days (0 Days) - #2				
Positive Controls	1.11×10^8	7.77 ± 0.10	54.47 ± 14.03	-
Test Samples	1.11×10^8	0	0	≥7.77 ± 0.09
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[†] - #3				
Positive Controls	1.03×10^8	7.81 ± 0.040	63.36 ± 5.83	-
Test Samples	1.03×10^8	0	0	≥7.81 ± 0.03
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[‡] - #4				
Positive Controls	1.04×10^8	7.68 ± 0.061	46.65 ± 6.47	-
Test Samples	1.04×10^8	0	0	≥7.68 ± 0.054
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[§] - #5				
Positive Controls	9.97×10^7	7.91 ± 0.053	82.57 ± 10.22	-
Test Samples	9.97×10^7	0	0	≥7.91 ± 0.47
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days)[†] - #6				
Positive Controls	9.67×10^7	7.79 ± 0.025	64.14 ± 3.55	-
Test Samples	9.67×10^7	0	0	≥7.79 ± 0.02
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[†] - #7				
Positive Controls	1.11×10^8	7.81 ± 0.045	58.49 ± 5.96	-
Test Samples	1.11×10^8	0	0	≥7.81 ± 0.04
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days)[†] - #8				
Positive Controls	1.00×10^8	7.74 ± 0.062	55.86 ± 7.92	-
Test Samples	1.00×10^8	0	0	≥7.74 ± 0.05
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
* 2 mL SFW added to all samples prior to inoculation.
[†] 1 mL SFW added prior to addition of metam sodium.
[‡] 2 mL SFW added prior to addition of metam sodium.
[§] 3 mL SFW added prior to addition of metam sodium.

Table 7-8 Inactivation of *Bacillus subtilis* Spores on Topsoil with Metam Sodium[a]

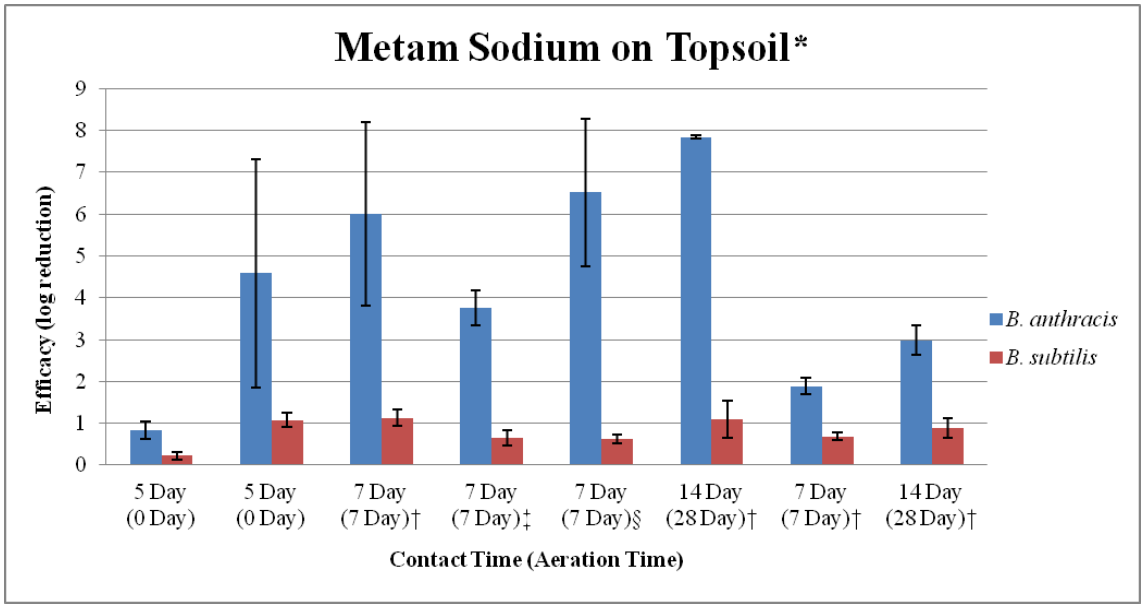
Contact Time (Aeration Time) Test #	Inoculum (CFU)	Mean Logs Observed (CFU ± SD)	Mean % Recovery (±SD)	Decontamination Efficacy ± CI [f]
Five Days (0 Days) - #1				
Positive Controls[b]	1.07×10^8	7.99 ± 0.010	91.53 ± 2.05	-[g]
Test Samples[c]	1.07×10^8	7.77 ± 0.094	56.45 ± 11.96	0.22 ± 0.08
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Five Days (0 Days) - #2				
Positive Controls	1.35×10^8	8.06 ± 0.010	84.15 ± 2.00	-
Test Samples	1.35×10^8	6.97 ± 0.19	7.53 ± 3.51	1.08 ± 0.17
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) † - #3				
Positive Controls	1.04×10^8	7.99 ± 0.055	95.52 ± 11.83	-
Test Samples	1.04×10^8	6.86 ± 0.21	7.76 ± 3.74	1.13 ± 0.19
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) ‡ - #4				
Positive Controls	9.73×10^7	7.72 ± 0.048	54.12 ± 6.10	-
Test Samples	9.73×10^7	7.06 ± 0.19	12.72 ± 5.32	0.66 ± 0.18
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) § - #5				
Positive Controls	9.07×10^7	7.81 ± 0.058	71.51 ± 9.24	-
Test Samples	9.07×10^7	7.18 ± 0.11	16.89 ± 3.61	0.63 ± 0.11
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days) † - #6				
Positive Controls	8.97×10^7	7.85 ± 0.072	80.13 ± 14.57	-
Test Samples	8.97×10^7	6.75 ± 0.51	8.94 ± 5.63	1.10 ± 0.45
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days) † - #7				
Positive Controls	1.13×10^8	7.87 ± 0.035	65.86 ± 5.21	-
Test Samples	1.13×10^8	7.19 ± 0.10	14.09 ± 2.90	0.68 ± 0.09
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days) † - #8				
Positive Controls	1.06×10^8	7.82 ± 0.070	63.15 ± 10.07	-
Test Samples	1.06×10^8	6.94 ± 0.27	9.23 ± 4.52	0.89 ± 0.24
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

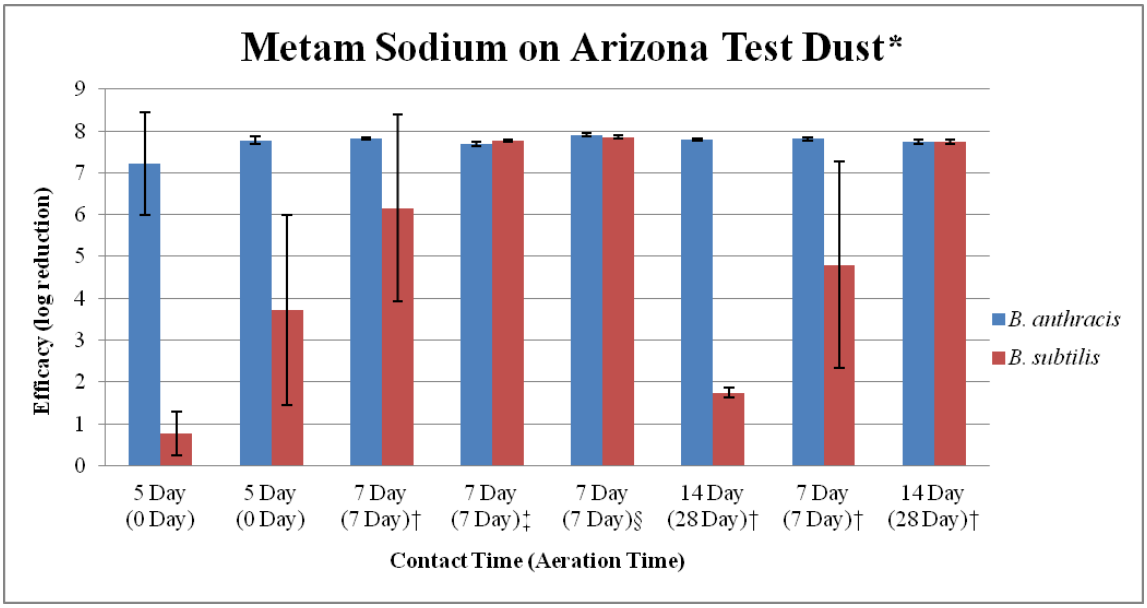
[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
***** 2 mL SFW added to all samples prior to inoculation.
† 1 mL SFW added prior to addition of metam sodium.
‡ 2 mL SFW added prior to addition of metam sodium.
§ 3 mL SFW added prior to addition of metam sodium.

Table 7-9 Inactivation of *Bacillus subtilis* Spores on AZTD with Metam Sodium[a]

Contact Time (Aeration Time) Test #	Inoculum (CFU)	Mean Logs Observed (CFU ± SD)	Mean % Recovery (±SD)	Decontamination Efficacy ± CI [f]
Five Days (0 Days) - #1				
Positive Controls[b]	1.07×10^8	7.83 ± 0.46	83.14 ± 42.82	-[g]
Test Samples[c]	1.07×10^8	7.06 ± 0.37	13.99 ± 11.29	0.77 ± 0.52
Laboratory Blank[d]	0	0	-	-
Procedural Blank[e]	0	0	-	-
Five Days (0 Days) - #2				
Positive Controls	1.35×10^8	7.87 ± 0.049	55.72 ± 6.17	-
Test Samples	1.35×10^8	4.15 ± 2.59	0.56 ± 0.76	3.72 ± 2.28
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[†] - #3				
Positive Controls	1.04×10^8	8.02 ± 0.030	101.87 ± 7.14	-
Test Samples	1.04×10^8	1.86 ± 2.56	0.019 ± 0.027	6.16 ± 2.24
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[‡] - #4				
Positive Controls	9.73×10^7	7.76 ± 0.033	58.64 ± 4.33	-
Test Samples	9.73×10^7	0	0	≥7.76 ± 0.029
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[§] - #5				
Positive Controls	9.07×10^7	7.85 ± 0.045	78.88 ± 8.00	-
Test Samples	9.07×10^7	0	0	≥7.85 ± 0.039
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days)[†] - #6				
Positive Controls	8.97×10^7	7.80 ± 0.044	70.14 ± 7.45	-
Test Samples	8.97×10^7	6.06 ± 0.13	1.32 ± 0.41	1.74 ± 0.12
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
Seven Days (7 Days)[†] - #7				
Positive Controls	1.13×10^8	7.78 ± 0.048	53.27 ± 5.79	-
Test Samples	1.13×10^8	2.97 ± 2.82	0.27 ± 0.59	4.80 ± 2.47
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-
14 Days (28 Days)[†] - #8				
Positive Controls	1.06×10^8	7.75 ± 0.054	53.34 ± 6.61	-
Test Samples	1.06×10^8	0	0	≥7.75 ± 0.05
Laboratory Blank	0	0	-	-
Procedural Blank	0	0	-	-

[a] Data are expressed as the mean (± SD) of the logs of the number of spores (CFU) observed on five individual samples, the mean percent recovery on those five samples, and decontamination efficacy (log reduction).
[b] Positive Controls = samples inoculated, not decontaminated.
[c] Test Samples = samples inoculated, decontaminated.
[d] Laboratory Blank = samples not inoculated, not decontaminated.
[e] Procedural Blank = samples not inoculated, decontaminated.
[f] CI = confidence interval (± 1.96 × SE).
[g] "-" Not Applicable.
***** 2 mL SFW added to all samples prior to inoculation.
[†] 1 mL SFW added prior to addition of metam sodium.
[‡] 2 mL SFW added prior to addition of metam sodium.
[§] 3 mL SFW added prior to addition of metam sodium.





* 2 mL SFW added to all samples prior to inoculation.
† 1 mL SFW added prior to addition of metam sodium.
‡ 2 mL SFW added prior to addition of metam sodium.
§ 3 mL SFW added prior to addition of metam sodium.
Test results are presented in numerical order

Figure 7-2. Summary of Decontamination Efficacies for Metam Sodium Fumigant Testing on Topsoil and AZTD

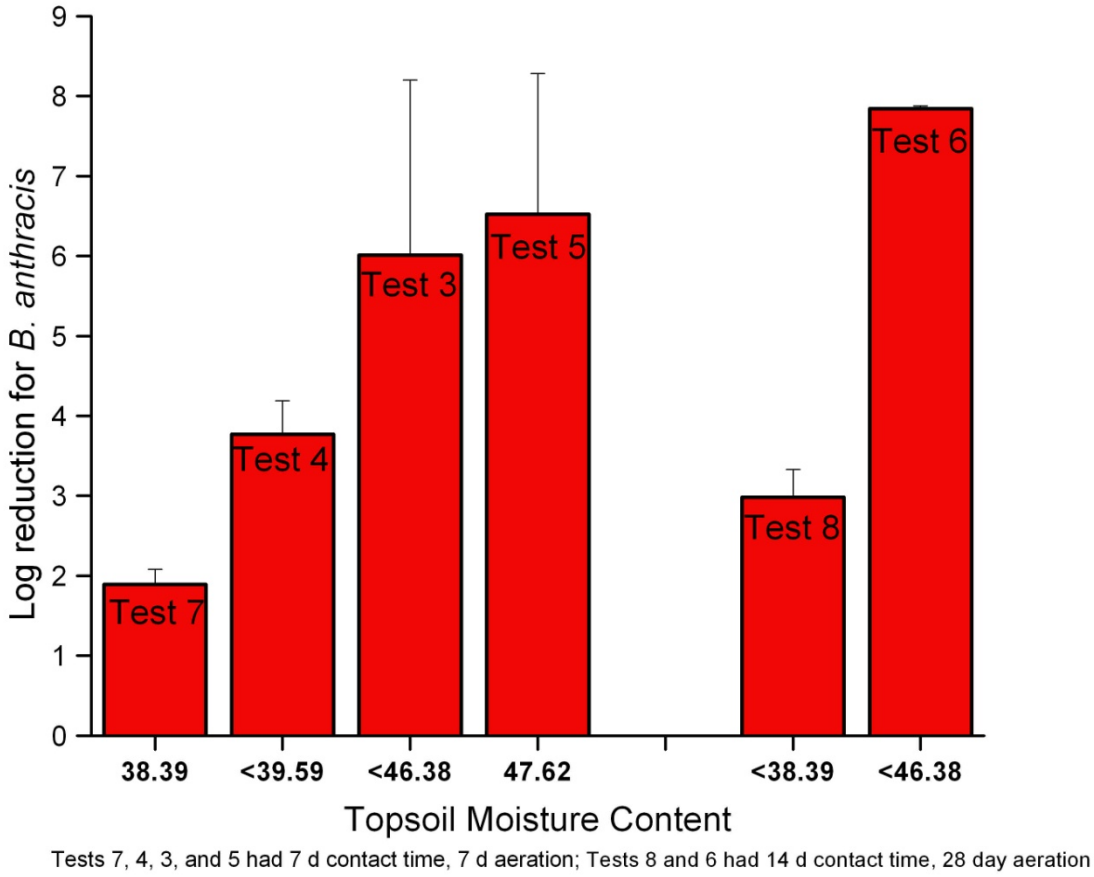


Figure 7-3. Effect of Topsoil Moisture Content on Decontamination Efficacy for *B. anthracis*

7.3 Discoloration of Soils

At the end of each decontamination test, the procedural blanks were compared visually to the laboratory blanks, and test samples were compared visually to positive controls, to assess any impact (i.e., discoloration) the decontaminants may have had on each material type. Based on the visual appearance of the decontaminated samples, there were no apparent changes in the color of the two soil types after being exposed to MeBr, metam sodium, pH-amended bleach, or sodium persulfate.

8.0 Summary of Results

8.1 Decontamination Efficacy

The principle goal of this study was to find the necessary decontamination treatment conditions (e.g., concentration of active ingredient, contact time, number of applications, etc.) to effectively decontaminate (≥ 6 LR) topsoil and AZTD using four different biocidal chemistries. The four decontaminants tested were pH-amended bleach, sodium persulfate, methyl bromide, and metam sodium. With the exception of pH-amended bleach in topsoil, greater than 6 LR against *B. anthracis* was obtained with all four decontaminants for both soil types.

Table 8-1 shows the minimum conditions required to obtain at least a 6 LR for each combination of decontamination technology, soil type, and microorganism. More stringent conditions, such as higher concentration, more applications, or longer contact time typically resulted in higher efficacy, and in some cases, complete inactivation. Conversely, there were a few tests in which the most stringent treatment evaluated resulted in a LR less than 6 (indicated in Table 8-1 as "Not found"). Examples of this included the use of pH-amended bleach on topsoil against both biological agents, and methyl bromide against *B. subtilis* (on both soil types).

Table 8-1 Minimum Treatment Required for Effective Decontamination

Decontaminant	Soil type	Microor-ganism	Minimum Treatment for ≥ 6LR
pH-amended bleach	TS	*B.a.*	Not found
pH-amended bleach	TS	*B.s.*	Not found
pH-amended bleach	AZTD	*B.a.*	2 hour contact time, 4 applications
pH-amended bleach	AZTD	*B.s.*	2 hour contact time, 4 applications
Sodium persulfate	TS	*B.a.*	3 applications every 60 minutes
Sodium persulfate	TS	*B.s.*	6 applications every 60 minutes
Sodium persulfate	AZTD	*B.a.*	3 applications every 60 minutes
Sodium persulfate	AZTD	*B.s.*	Not found
Methyl bromide	TS	*B.a.*	180 mg/L MeBr, 24 hour contact time
Methyl bromide	TS	*B.s.*	Not found
Methyl bromide	AZTD	*B.a.*	140 mg/L MeBr, 24 hour contact time
Methyl bromide	AZTD	*B.s.*	Not found
Metam sodium	TS	*B.a.*	160 µL, 7 day contact time, 7 day aeration time, 1 mL water added to soil
Metam sodium	TS	*B.s.*	Not found
Metam sodium	AZTD	*B.a.*	80 µL, 5 day contact time, no aeration period, no moisture added to soil
Metam sodium	AZTD	*B.s.*	160 µL, 7 day contact time, 7 day aeration time, 1 mL water added to soil

One bleach application consisted of 0.5 mL acidified beach, with a mean FAC level of approximately 5,400 ppm and pH 6.5.
One sodium persulfate application consisted of 1 mL 0.5 M sodium persulfate followed by 1 mL 8% H_2O_2. All tests conducted using a 7-day contact time.
All MeBr tests were conducted at 25 °C and RH uncontrolled (all but one test had RH > 75%).
B.a. = B. anthracis; B. s. = B. subtilis.

8.2 Effect of Soil type

The effect of soil type on decontamination efficacy depended on the chemical decontaminant and to some extent, the microorganism. For example, the decontamination efficacy results for pH-amended bleach and metam sodium were significantly higher on AZTD (compared to topsoil) for nearly every test. For the sodium persulfate, however, the decontamination efficacy results were very similar for the two soil types when testing against *B. anthracis*. But interestingly, in the majority of the persulfate tests against *B. subtilis*, higher efficacy was achieved on topsoil. For MeBr, the decontamination efficacies obtained for *B. anthracis* were generally slightly higher on AZTD compared to topsoil, although half of the test results for the two soil types were not significantly different.

8.3 Comparing efficacy for *B. anthracis* and *B. subtilis*

There were no tests in which *B. subtilis* was inactivated to a significantly higher degree than *B. anthracis*, and, for pH-amended bleach, there were no significant differences in decontamination efficacy for the two microorganisms. For the other three decontaminants, the efficacy for the inactivation of *B. subtilis* was significantly less than the efficacy for *B. anthracis* for the majority of the tests conducted. For MeBr in particular, the differences in efficacy for the two microorganisms were greater than 5-6 LR for more than half of the tests.

9.0 References

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Appendix A

Moisture and Organic Content of Soil Samples

Initial Soil Analysis

Soil samples (pre- and post-sterilization via gamma irradiation at ~40 kGy or autoclaving at 121 °C for one hour) were sent to an independent laboratory for analysis of moisture, organic content and other characteristics. The samples were analyzed in triplicate using ASTM D 2974-87, Moisture, Ash and Organic Matter of Peat and Other Organic Soils[7]. (Moisture and organic contents are determined based on loss of sample mass at a given temperature.) The results are shown in Table A-1. The moisture and organic content did not change significantly after the gamma irradiation of the samples. However, autoclaving of the samples did have more of an effect on the soil characteristics. Samples of each soil type were confirmed to be sterile following gamma irradiation and autoclaving by dilution plating samples on tryptic soy agar.

Table A-1 Soil Sample Analysis‡

Soil Type	Pre-Sterilization	Post-Gamma Irradiation†	Post-Autoclave*	Pre-Sterilization	Post-Gamma Irradiation†	Post-Autoclave*
	Water Content (%)			Fraction Organic Carbon (%, 440 °C)		
Topsoil	33.6	32.5	25.4	9.27	9.21	7.29
AZTD	0.233	0.238	0.582	0.399	0.385	0.264
	pH			Recalcitrant Organic Carbon (%, 750 °C)		
Topsoil	6.91	7.23	7.11	1.35	1.39	2.26
AZTD	8.58	8.69	9.09	1.20	1.22	1.15

‡ Data provided by CTL Engineering, Columbus, Ohio.
† Data post-gamma irradiation at ~40 kGy.
* Data post-autoclave at 121 °C for one hour.

Moisture Analysis Related to Metam Sodium Tests

Due to issues with incomplete sterilization of topsoil samples during the metam sodium tests (indicated by the presence of endogenous bacteria), different methods (higher dose of gamma radiation, or autoclaving) were tested to mitigate the presence of the non-target bacteria. Because the sterilization method could affect moisture content, the moisture of the soils was assessed for each sterilization and metam sodium application method used.

Soil moisture tests were completed following method ASTM D 2974-87. Samples were weighed, dried in an oven for ≥16 hours at 105 ± 5 °C, weighed again and the moisture content calculated as:

$$\text{Moisture Content, \%} = [(A\text{-}B) \times 100]/A$$

where:
A = mass of the as-received specimen, g
B = mass of the oven-dried specimen, g

The results of the moisture assessments for the metam sodium decontamination testing are presented in Table A-2. As testing proceeded, we determined that soil moisture wasn't just a function of sterilization method (e.g., autoclaved soils were generally lower in moisture compared to irradiated soils) or the amount of water added to the soil, but that overnight drying time seemed to affect soil moisture results as well. For example, the topsoil used for Test 4, in which 2 mL water was added, had lower moisture content than the soil for Test 3, which only

had 1 mL water added. This discrepancy is most likely due to the longer overnight dry time for Test 4. Overnight dry times are therefore listed in Table A-2. Further, actual overnight dry times prior to the application of metam sodium didn't always coincide with the overnight dry times for conducting the soil moisture test. The soil moisture levels for the decontaminated soil samples were estimated to be greater than or less than the soil samples that underwent moisture tests depending on how the overnight dry times compared for the decontaminated soil samples vs. the soil samples which were tested for moisture.

Table A-2 Soil Moisture Assessments Taken During Metam Sodium Tests

Test #	Volume of Metam Sodium Applied, Contact Time (Days)/ Aeration Time (Days)	Soil Sterilization Method	Soil Type	Amount SFW Added Prior to Addition of Metam Sodium (mL)‡	Estimated Moisture Content (%)† of Soil Undergoing Decontamination	Overnight Time Prior to Decon Test (hr:min)	Actual Moisture of Tested Soil (%)*	Overnight Time for Moisture-Tested Soil (hr:min)
1	80 µL, 5/0	Gamma Irradiation @ 40 kGy	Topsoil	0	≥ 43.56	18:35	43.56	20:04
			AZTD		≥ 15.64		15.64	
2	160 µL, 5/0	Gamma Irradiation @ 40 kGy	Topsoil	0	≤ 43.56	21:30	43.56	20:04
			AZTD		≤ 15.64		15.64	
3	160 µL, 7/7	Gamma Irradiation @ 60 kGy	Topsoil	1	≤ 46.38	22:26	46.38	20:42
			AZTD		≤ 18.04		18.04	
4	160 µL, 7/7	Gamma Irradiation @ 60 kGy	Topsoil	2	≤ 39.59	26:21	39.59	24:17
			AZTD		≤ 18.31		18.31	
5	160 µL, 7/7	Gamma Irradiation @ 60 kGy	Topsoil	3	47.62	19:58	47.62	19:58
			AZTD		27.06		27.06	
6	160 µL, 14/28	Gamma Irradiation @ 60 kGy	Topsoil	1	≤ 46.38	22:54	46.38	20:42
			AZTD		≤ 18.04		18.04	
7	160 µL, 7/7	Autoclave (121 °C; 1 hour)	Topsoil	1	38.39	20:43	38.39	20:42
			AZTD		18.25		18.25	
8	160 µL, 14/28	Autoclave (121 °C; 1 hour)	Topsoil	1	≤38.39	24:04	38.39	20:42
			AZTD		≤18.25		18.25	

† Estimated moisture based on the moisture assessment in comparison to the overnight dry times.
* Actual moisture measured during moisture assessment.
‡ All soil samples had 2 mL SFW added the night before decontamination tests and prior to sample inoculation with spores

Appendix B

Neutralization Tests for Liquid Decontaminants

Neutralization Methodology

Neutralization for the pH-amended bleach and sodium persulfate was achieved with STS. The concentration of STS tested in the neutralization panels was 1.0, 1.5, and 2.0 and 5.0% in the extraction solution. These STS concentrations were selected based on historical data.

The following evaluations were made in each neutralization panel:

(1) Decontamination effectiveness (add spores to decontaminant solution; determine CFU without neutralization). No spores should be recovered.
(2) Assess effectiveness of extraction buffer only (PBST without any STS) to neutralize or dilute sufficiently active ingredient of decontaminant.
(3) Positive control recovery (add spores to extraction buffer without neutralizer, and without decontaminant; determine CFU).
(4) Assess neutralizer effectiveness at terminating decontamination (add spores to neutralized decontamination solution; determine CFU with neutralization).

Based on these results, a specific concentration of neutralizer in the extraction buffer was selected to be used during the liquid decontamination tests.

To specifically assess the neutralizer effectiveness at terminating sporicidal activity (Item 4 above), each decontaminant was applied in the same manner as required for testing and allowed to stay in contact with the soil for the appropriate contact time. At the end of the contact time, the extraction buffer containing the tested level of STS was added and the soil was extracted on an orbital shaker for 15 minutes at 200 rpm at room temperature. A 100 µL aliquot of spores was then added to each sample, with each sample slowly mixed by hand ten times and dilution plated. This level was compared to the positive control recovery.

The results of the neutralization panels are shown in Tables B-1 through B-9. From these trials, the following STS concentrations were determined to be sufficient for neutralization in both soil types (except where indicated) in the following tests:

- 1.5% STS with pH-amended Ultra Clorox® for a seven-day contact time with eight applications over two hours (Test 1).
- 1.5% STS with pH-amended Ultra Clorox® for a 24 hour contact time with eight applications over two hours (Test 2).
- 2.0% STS with pH-amended Ultra Clorox® for a 120 minute contact time with four applications over two hours (Test 3).
- 2.0% STS with pH-amended Ultra Clorox® for a 60 minute contact time with two applications over one hour (Test 4).
- 1.0% STS with Klozur™ for a seven-day contact time with six applications over five hours. (Test 1)

- 2.0% STS with Klozur™ for a seven-day contact time with three applications over two hours. (Test 2)

- 1.0% STS with Klozur™ for a seven-day contact time with three applications over four days. (Test 3)

- 1.0% STS (AZTD) and 2.0% STS (topsoil) Klozur™ for a seven-day contact time with one application at time 0. (Test 5)

- 1.0% STS Klozur™ for a seven-day contact time with two applications over one hour. (Test 4)

Table B-1 Neutralization Testing with *Bacillus subtilis* spores with pH-Amended Bleach, Seven-Day Contact Time, Eight Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
pH-amended bleach + spores	1.08×10^8	0	0
pH-amended bleach + PBS + Triton® X-100 + spores	1.08×10^8	6.87×10^7	65.0
PBS + Triton® X-100 + spores (Control)	1.08×10^8	1.06×10^8	-
pH-amended bleach + PBS + Triton® X-100 + 1.0% STS + spores	1.08×10^8	9.96×10^7	94.2
pH-amended bleach + PBS + Triton® X-100 + 1.5% STS + spores	1.08×10^8	1.03×10^8	97.7
pH-amended bleach + PBS + Triton® X-100 + 2.0% STS + spores	1.08×10^8	1.11×10^8	105
AZTD			
pH-amended bleach + spores	1.08×10^8	0	0
pH-amended bleach + PBS + Triton® X-100 + spores	1.08×10^8	4.85×10^7	58.7
PBS + Triton® X-100 + spores (Control)	1.08×10^8	8.25×10^8	-
pH-amended bleach + PBS + Triton® X-100 + 1.0% STS + spores	1.08×10^8	7.23×10^7	87.6
pH-amended bleach + PBS + Triton® X-100 + 1.5% STS + spores	1.08×10^8	8.05×10^8	97.6
pH-amended bleach + PBS + Triton® X-100 + 2.0% STS + spores	1.08×10^8	9.18×10^7	111

Table B-2 Neutralization Testing with *Bacillus subtilis* Spores with pH-Amended Bleach, 24 Hour Contact Time, 8 Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
AZTD			
pH-amended bleach + spores	1.16×10^8	0	0
pH-amended bleach + PBS + Triton® X-100 + Spores	1.16×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.16×10^8	9.15×10^7	-
pH-amended bleach + PBS + Triton® X-100 + 1.0% STS + spores	1.16×10^8	8.25×10^7	90.2
pH-amended bleach + PBS + Triton® X-100 + 1.5% STS + spores	1.16×10^8	8.73×10^7	95.5
pH-amended bleach + PBS + Triton® X-100 + 2.0% STS + spores	1.16×10^8	8.23×10^7	90.0

Table B-3 Neutralization Testing with *Bacillus subtilis* Spores with pH-Amended Bleach, 120 Minute Contact Time, Four Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
AZTD			
pH-amended bleach + spores	1.24×10^8	0	0
pH-amended bleach + PBS + Triton® X-100 + spores	1.24×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.24×10^8	1.06×10^8	-
pH-amended bleach + PBS + Triton® X-100 + 1.0% STS + spores	1.24×10^8	9.77×10^7	92.0
pH-amended bleach + PBS + Triton® X-100 + 1.5% STS + spores	1.24×10^8	1.03×10^8	97.5
pH-amended bleach + PBS + Triton® X-100 + 2.0% STS + spores	1.24×10^8	1.04×10^8	98.2

Table B-4 Neutralization Testing with *Bacillus subtilis* Spores with pH-Amended Bleach, 60 Minute Contact Time, Two Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
AZTD			
pH-amended bleach + spores	1.07×10^8	0	0
pH-amended bleach + PBS + Triton® X-100 + spores	1.07×10^8	1.44×10^5	0.268
PBS + Triton® X-100 + spores (Control)	1.07×10^8	5.38×10^7	-
pH-amended bleach + PBS + Triton® X-100 + 1.0% STS + spores	1.07×10^8	6.39×10^7	119
pH-amended bleach + PBS + Triton® X-100 + 1.5% STS + spores	1.07×10^8	6.88×10^7	128
pH-amended bleach + PBS + Triton® X-100 + 2.0% STS + spores	1.07×10^8	7.94×10^7	148

Table B-5 Neutralization Testing with *Bacillus subtilis* Spores with Klozur™, Seven-Day Contact Time, Six Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
Klozur™ + spores	1.11×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.11×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.11×10^8	1.20×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.11×10^8	1.22×10^8	102
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.11×10^8	1.17×10^8	97.9
Klozur™ + PBS + Triton® X-100 + 5.0% STS + spores	1.11×10^8	1.13×10^8	94.6
AZTD			
Klozur™ + spores	1.11×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.11×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.11×10^8	1.34×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.11×10^8	1.43×10^8	107
Klozur™ + PBS + Triton® X-100 + 1.5% STS + spores	1.11×10^8	1.39×10^8	104
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.11×10^8	1.06×10^8	79.3

Table B-6 Neutralization Testing with *Bacillus subtilis* Spores with Klozur™, Seven-Day Contact Time, Three Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
Klozur™ + spores	1.24×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.24×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.24×10^8	1.15×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.24×10^8	1.05×10^8	90.9
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.24×10^8	1.06×10^8	91.9
Klozur™ + PBS + Triton® X-100 + 5.0% STS + spores	1.24×10^8	9.88×10^7	85.7
AZTD			
Klozur™ + spores	1.24×10^8	0	0
Klozur™ + PBS + Triton® X-100 + Spores	1.24×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.24×10^8	1.06×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.24×10^8	1.12×10^8	106
Klozur™ + PBS + Triton® X-100 + 1.5% STS + spores	1.24×10^8	1.13×10^8	106
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.24×10^8	1.01×10^8	95.5

Table B-7 Neutralization Testing with *Bacillus subtilis* Spores with Klozur™, Seven-Day Contact Time, Three Total Applications (Days 0, 2 and 4)

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
Klozur™ + spores	1.16×10^8	0	0
Klozur™ + PBS + Triton® X-100 + Spores	1.16×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.16×10^8	1.01×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.16×10^8	1.04×10^8	103
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.16×10^8	1.02×10^8	101
Klozur™ + PBS + Triton® X-100 + 5.0% STS + spores	1.16×10^8	9.84×10^7	97.2
AZTD			
Klozur™ + spores	1.16×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.16×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.16×10^8	9.36×10^7	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.16×10^8	1.21×10^8	130
Klozur™ + PBS + Triton® X-100 + 1.5% STS + spores	1.16×10^8	1.06×10^8	113
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.16×10^8	9.15×10^7	97.8

Table B-8 Neutralization Testing with *Bacillus subtilis* Spores with Klozur™, Seven-Day Contact Time, One Total Application

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
Klozur™ + spores	1.34×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.34×10^8	0	0
PBS + Triton X-100 + spores (Control)	1.34×10^8	1.25×10^8	-
Klozur™+ PBS + Triton® X-100 + 1.0% STS + spores	1.34×10^8	1.18×10^8	94.4
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.34×10^8	1.21×10^8	96.8
Klozur™ + PBS + Triton® X-100 + 5.0% STS + spores	1.34×10^8	1.14×10^8	90.7
AZTD			
Klozur™ + spores	1.34×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.34×10^8	0	0
PBS + Triton X-100 + spores (Control)	1.34×10^8	1.10×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.34×10^8	1.05×10^8	96.2
Klozur™+ PBS + Triton® X-100 + 1.5% STS + spores	1.34×10^8	9.00×10^7	82.2
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.34×10^8	7.81×10^7	71.3

Table B-9 Neutralization Testing with *Bacillus subtilis* spores with Klozur™, 7 Day Contact Time, 2 Total Applications

Treatment	Inoculum (CFU)	Total Observed (CFU)	% of Control
Topsoil			
Klozur™ + spores	1.17×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.17×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.17×10^8	1.27×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.17×10^8	1.06×10^8	83.3
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.17×10^8	1.02×10^8	80.5
Klozur™ + PBS + Triton® X-100 + 5.0% STS + spores	1.17×10^8	9.89×10^7	77.7
AZTD			
Klozur™ + spores	1.17×10^8	0	0
Klozur™ + PBS + Triton® X-100 + spores	1.17×10^8	0	0
PBS + Triton® X-100 + spores (Control)	1.17×10^8	1.15×10^8	-
Klozur™ + PBS + Triton® X-100 + 1.0% STS + spores	1.17×10^8	1.15×10^8	99.9
Klozur™ + PBS + Triton® X-100 + 1.5% STS + spores	1.17×10^8	1.07×10^8	93.1
Klozur™ + PBS + Triton® X-100 + 2.0% STS + spores	1.17×10^8	1.01×10^8	87.6

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