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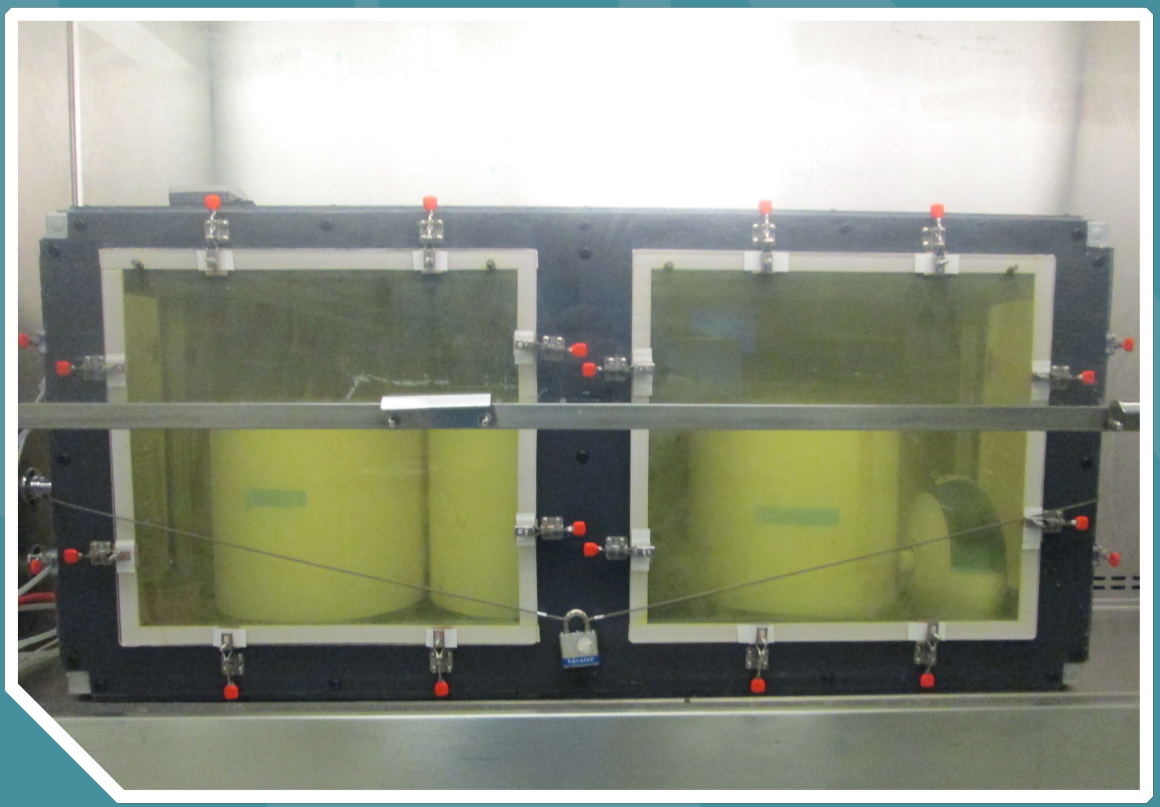
United States
Environmental Protection
Agency

# Assessment of the Decontamination of Soil Contaminated with *Bacillus anthracis* Spores Using Chlorine Dioxide Gas, Methyl Bromide, or Activated Sodium Persulfate



**Office of Research and Development**
Homeland Security Research Program

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## Disclaimer

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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 environmental contamination (such as 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 three chemical decontaminants for inactivating the causative agent for anthrax, *Bacillus anthracis* spores, in soil. The decontaminants that were evaluated included chlorine dioxide gas, a liquid biocide (sodium persulfate activated with aqueous hydrogen peroxide), and methyl bromide. The objective of this study was to provide an understanding of the performance (i.e., efficacy) of these decontamination technologies under a range of environmental conditions, thus guiding their use and implementation in HS applications for hard-to-decontaminate materials such as soil.

This investigation focused on decontaminating three types of soil materials - topsoil, sand, and a clayey soil - at soil depths of up to 5 inches. The three soil types were selected in an attempt to span the range of soil types (and associated properties, such as organic content, porosity, particle size). Decontamination efficacy tests were conducted using *B. anthracis* (Ames strain) spores. Decontamination efficacy was quantified in terms of log reduction (LR), based on the difference in the number of bacterial spores (as CFU) recovered from the positive controls (soil samples not exposed to decontaminant) and test samples. (A decontaminant is considered to be an effective sporicide if a 6 LR or greater is achieved based upon appropriate laboratory testing.) Tests were conducted with varying operational parameters (e.g., environmental conditions, contact time, decontaminant concentration) to assess the effect of these parameters on decontamination efficacy at each of the soil depths.

## Method Development

Prior to conducting the main decontamination studies just described, method development was required to determine techniques for preparing, placing, and quantitatively recovering *B. anthracis*-spiked soil samples from within a larger soil mass. In the end, a 1-gram soil mass was spiked with 0.1 mL of *B. anthracis* stock and was then sealed within custom-made Tyvek or polyvinylidene fluoride envelopes. This packet was referred to as a carrier soil packet, or CSP. To conduct an experiment, CSPs could then be placed at various depths within a larger soil column of the same soil type without contaminating the entire mass, were easily recovered as individual samples, and provided a quantitative amount of *B. anthracis* spores at each location.

Design of experiments using test columns was also evaluated prior to conducting the main decontamination studies. Small laboratory-sized test columns typically suffer from wall effects (e.g., channeling), making it difficult to simulate large outdoor soil characteristics accurately. To address this problem, we performed a series of method development tests using 4-, 6-, 8-, and 10-in diameter columns, commercially-available biological indicators (BIs) impregnated with *Bacillus atrophaeus* spores, and chlorine dioxide ($ClO_2$) gas or sodium persulfate liquid solution. This approach provided a cost-effective way to evaluate the expected column performance for treating *B. anthracis* with either gas or liquid decontaminants. Results showed that a 10-in diameter column was the preferred design, and that no wall effects were anticipated when BIs or CSPs were placed along the central axis of the column, down to five inches. Successful

conclusion of the method development phase allowed the project to then proceed to the main decontamination studies using the 10-inch diameter test columns with the three decontaminants.

## Summary of Decontamination Test Results

### Chlorine Dioxide

A total of seven ambient-temperature tests were performed using gaseous $ClO_2$ at target concentrations of 8.4 to 14 mg/L (3,000 to 5,000 ppm), exposure times of 3 to 27.5 hours, and relative humidity (RH) levels of ≥ 75%. In general, $ClO_2$ was ineffective at decontaminating topsoil at depths greater than 1". For depths up to 1", results ranged from ineffective to complete kill, depending on the exact test conditions. The most aggressive test was performed at an average $ClO_2$ concentration of 14.6 mg/L, 82.5% RH, and a 27.5-hr exposure. These conditions produced complete kill (≥ 7 LR) in topsoil at the 1" depth, but was ineffective at greater depths.

Sand was more easily treated by $ClO_2$ than the topsoil, and showed full decontamination down to 2" for most of the test conditions. Furthermore, two of the three 24-hr tests showed complete kill (≥7 LR) at all depths.

Clay was also more easily treated by $ClO_2$ than topsoil, and had full decontamination down to 3" for most of the test conditions. Two of the three 24-hr tests (~3,000 ppm) showed complete kill (≥7 LR) at all depths.

Data from one of the 24-hr tests showed no recovery from any of the clay test samples or positive controls, prompting additional tests that attempted to evaluate the decay of positive controls over a 1-week time period. Neither of these two additional tests showed complete decay in the clay material as had been observed in the previous test.

One test was performed using compacted soils for all three types. This condition showed that $ClO_2$ was unable to penetrate compacted topsoil to a 1" depth, but did not show a noticeable effect on either sand or clay.

### Sodium Persulfate

Three tests were performed using sodium persulfate (tradename Klozur™ SP) at concentrations of 0.5 to 1.0 M, activated immediately prior to use by mixing 50/50 (volume basis) with 8% aqueous hydrogen peroxide. The decontamination liquid was applied to the top of the test columns in up to six separate applications at either one or two day intervals. Total liquid volumes varied by experiment and soil type, with a maximum up to 0.54 mL per gram of soil based on a wetting depth of 6". Contact times were six to seven days, and environmental conditions were ambient temperature and RH.

The activated sodium persulfate decontamination solution proved to be extremely reactive with topsoil, and produced a vigorous foaming reaction upon application. Actual decontamination efficacy of topsoil, however, was poor, and was effective only down to 0.5" for the 0.5M solutions and to 1" for the 1.0 M solution. Sand showed less reactivity and formed more of a slurry with the liquid decontamination solution. Decontamination results were also poor for sand, and was effective to only a 1" depth. Clay, on the other hand, showed complete decontamination

down to 5” with only two applications of activated sodium persulfate, applied at a loading of 0.09 mL/g.

**Methyl Bromide**

A total of four tests were performed using gaseous methyl bromide (MeBr) at concentrations ranging from 224 to 325 mg/L (56,000 to 81,250 ppm), exposure times of 48 to 65.5 hrs, ambient temperature of 20 °C, and RH levels of ≥ 75%. Test 1, conducted at 236 mg/l MeBr for 48 hr, was not effective for any soil type, typically having a 1-2 LR. Maintaining similar test conditions except for increasing contact time (Test 2) or increasing concentration (Test 3) resulted in generally moderate improvement in efficacy. The pre-wetting of soils (Test 4) did not provide any improvement in MeBr decontamination efficacy. Efficacy was observed to be generally higher for topsoil and sand, and lowest for the clay soil; in fact, there were no test conditions in which any of the clay samples were decontaminated effectively, including the CSPs at the surface of the clay columns. Lastly, although there were a few exceptions, decontamination efficacy was generally similar across all depths for a particular soil and test condition, suggesting that penetration of the MeBr gas through the soil matrices was not a limiting factor.

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# Acronyms and Abbreviations

| | |
|---|---|
| BA | *Bacillus anthracis*, or *B. anthracis* |
| BI | Biological Indicator |
| BSC | Biological Safety Cabinet |
| BSL | Biological Safety Level |
| CC | Control Column |
| CFU | Colony Forming Units |
| COA | Certificate of Analysis |
| CSP | Carrier Soil Packet |
| EPA | U.S. Environmental Protection Agency |
| HS | Homeland Security |
| HSRP | Homeland Security Research Program |
| LED | Light Emitting Diode |
| LR | Log Reduction |
| MeBr | Methyl bromide |
| NCCC | Negative Control Control Column |
| NCTC | Negative Control Test Column |
| NHSRC | National Homeland Security Research Center |
| ORP | Oxidation Reduction Potential |
| PBS | Phosphate-Buffered Saline |
| PC | Polycarbonate or Positive Control |
| PCR | Polymerase Chain Reaction |
| PVC | Polyvinyl chloride |
| PVDF | Polyvinylidene Fluoride |
| QA | Quality Assurance |
| QAPP | Quality Assurance Project Plan |
| QC | Quality Control |
| QMP | Quality Management Plan |
| RH | Relative Humidity |
| SA | Select Agent |
| SBA | Sheep Blood Agar |
| TC | Test Column |

# Section 1.
# 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 study was to evaluate technologies for decontaminating soil contaminated with *Bacillus anthracis* (Ames) spores. (Note, in the rest of this report, we use the abbreviation "BA" to refer to spores of the *B. anthracis* Ames strain.) Decontamination of soil may be needed in the event of a wide area release of BA. Following such a contamination incident, spores may migrate below the soil surface by various transport mechanisms such as rainfall. However, there remains some uncertainty with regard to the depths in which spores may penetrate soil, and this may depend on a number of factors. For this reason, decontamination efficacy was evaluated as a function of soil depth in this study.

The decontaminants evaluated were chlorine dioxide ($ClO_2$) gas, activated sodium persulfate (SP), and methyl bromide (MeBr). This work built on previous laboratory studies involving soil decontamination [1-4], but was performed at a significantly larger scale. In contrast to prior studies, this study also investigated the effect of several operating parameters on decontamination efficacy, with the primary objective of finding the required conditions (e.g., concentration, contact time, mass quantity) needed for effective decontamination of soil as a function of parameters such as soil depth, soil type, moisture, and chemical composition (e.g., organic content, levels of selected cations).

Prior to embarking on the testing just described, however, an intensive series of method development tests was performed to address two primary concerns. First, a means of preparing, placing, and quantitatively recovering BA-spiked soil samples from within a larger soil mass was required, leading to development of the carrier soil packet (CSP). Second, a soil test column design was needed to simulate a large, outdoor soil mass while avoiding wall effects (e.g. channeling) that are typical with small laboratory-scale columns. The main body of this report, however, is focused on the actual decontamination test results. Details regarding the CSP development and wall-effects tests for the columns are found in the appendices.

The project was conducted at MRIGlobal's facilities in Kansas City (425 Volker, Kansas City, MO) and in Florida (1470 Treeland Blvd, S.E., Palm Bay, FL). Preliminary method development tests, such as the extraction and recovery of *Bacillus atrophaeus* (*B. atrophaeus*) biological indicators (BIs) from soil, were performed at the Florida location. Wall-effects tests using BIs, test chamber construction, and all work with Biosafety Level (BSL)-3 Select Agents (SAs), including spore preparation and testing with BA, were performed at the Kansas City location.

# Section 2.
# Technology Descriptions and Test Matrices

## 2.1 Technology Descriptions

Table 1 describes the three decontamination technologies evaluated in this study. Information is provided on the manufacturer, product name (where applicable), chemical components, and active ingredients. Further details on the chemical composition, preparation, and decontamination application procedures are provided in Section 4.

**Table 1. Decontamination Technology Description**

| Decontaminant | Product Name and Vendor | Active Ingredients | Components | EPA Pesticide Registration |
|---|---|---|---|---|
| $ClO_2$ | Minidox-M $ClO_2$ Generator; ClorDiSys Solutions, Inc., Lebanon, NJ | Chlorine dioxide gas | Chlorine dioxide gas ($ClO_2$); programmable from 0 to 30 mg/L. | 80802-1 |
| Sodium Persulfate | Klozur® SP PeroxyChem Philadelphia, PA | SP, activated with 8% hydrogen peroxide ($H_2O_2$) | SP ($Na_2S_2O_8$) >99% purity, used as either a 0.5 M or 1.0 M aqueous solution, activated with 8% hydrogen peroxide. | None |
| MeBr | Meth-O-Gas® 100 Commodity Fumigant; Cardinal Professional Products; Anaheim, CA | Methyl bromide | 100% methyl bromide | 5785-11 for pests associated with agricultural commodities |

Chlorine dioxide was selected for testing because an earlier screening study had shown good efficacy for inactivating BA in Arizona Test Dust [1]. This earlier study had also evaluated the performance with topsoil under limited laboratory conditions, so this was explored further across a wider range of test conditions. Sodium persulfate was included in this evaluation because it is used to remediate soil contaminated with organic chemicals. In addition, SP was shown to be effective in certain conditions against BA in soil in screening tests [2, 3] and on other outdoor materials [4], so more detailed testing was planned for this evaluation. Methyl bromide was selected for testing because it has been demonstrated to be effective against BA on building materials [5], and was shown to be effective against BA in small-scale soil tests [3]. Furthermore, although MeBr use is being phased out under the Montreal Protocol on Substances that Deplete the Ozone Layer, MeBr is still currently and widely used via critical use exemptions as a soil and commodity (quarantine) fumigant [6].

## 2.2 Test Matrix

A total of fourteen decontamination efficacy tests were performed, with an additional two tests performed to evaluate the decay of positive controls in clay. All of the tests were performed in a custom-built test chamber using BA at soil depths from zero (on top of surface) up to five inches within a 10-in diameter column. The exception to this procedure was for the clay positive controls decay tests, which did not require a soil column. Each soil column had six CSP samples of the appropriate soil type, with one set on the soil surface (0 inch depth) and the others buried at depths up to five inches. Five positive CSP controls of each soil type were set up in a separate Control Chamber and not exposed to the decontaminant. One negative control of each soil type was also set up in both the Test Chamber and the Control Chamber. The general test conditions are summarized in Table 2. Initial test conditions (e.g., concentration, relative humidity [RH], contact time) were selected based on previous test conditions shown to be effective for each decontaminant.

**Table 2. Overall Test Matrix for Decontamination of *B. anthracis* in Soil**

| Type of Test | No. of Tests | Soil Types | Decontaminant Concentration | Exposure Time (hrs) | Temperature/RH | Samples[a] |
|---|---|---|---|---|---|---|
| Chlorine Dioxide | 7 | Topsoil<br>Sand<br>Clay | 8.7 - 14.6 mg/L | 3 - 27.5 | 22.2 – 25.3°C<br>73.5 – 85.8 % RH | TC = 6<br>CC = 5<br>NCTC = 1<br>NCCC = 1 |
| Sodium Persulfate | 3 | Topsoil<br>Sand<br>Clay | 0.5 – 1.0 M<br>50/50 w/$H_2O_2$<br>0.09 – 0.18 mL/g<br>1 – 6 doses | 144 - 168<br>(6 - 7 days) | 21.5 – 22.6°C<br>91.3 – 92.9 % RH | TC = 6<br>CC = 5<br>NCTC = 1<br>NCCC = 1 |
| Methyl Bromide | 4 | Topsoil<br>Sand<br>Clay | 224 – 325 mg/L | 48 – 65.5 | 19.7 – 20.1°C<br>76.3 – 78.7 % RH | TC = 6<br>CC = 5<br>NCTC = 1<br>NCCC = 1 |
| Positive Controls Decay | 2 | Clay | NA | 0 - 168 hrs<br>(7 days) | 23.0 – 24.6°C<br>29.5 – 92.0 % RH | CC = 3[b] |

[a] Per soil type.
[b] Per time period.
TC = Test Column.
CC = Positive Control Column.
NCTC – Negative Control, Test Column.
NCCC – Negative Control, Control Column.

## 2.3 Test Matrices for Chlorine Dioxide Decontamination

The test matrix for $ClO_2$ is presented in Table 3, and shows the nominal target values for each of the test parameters. Actual measured values are presented later in the results section for $ClO_2$. Initial target test conditions for Test 1 were 8.4 mg/L (~3000 ppm, with 1 ppm = 2.81 mg/m$^3$, or 0.00281 mg/L for $ClO_2$) and ≥ 75% RH for three hrs. Conditions for each of the subsequent tests were determined based on results of previous tests. Fumigation conditions for each test were the same for each soil type.

**Table 3. Chlorine Dioxide Test Design**

| Test No. | Depth Increments Tested (inches) | Target $ClO_2$ Conc. (mg/L) | Target T (°C) | Target RH (%) | Target Contact Time (hrs) | Other Conditions |
|---|---|---|---|---|---|---|
| 1 | 1 | 8.4 | ≥ 21 | ≥75 | 3 | |
| 2 | 1 | 8.4 | ≥ 21 | ≥75 | 6 | |
| 3 | 0.5 | 8.4 | ≥ 21 | ≥75 | 3 | Saturated soil. |
| 4 | 1 | 8.4 | ≥ 21 | ≥75 | 24 | |
| 5 | 1 | 8.4 | ≥ 21 | ≥75 | 24 | Saturated soil. |
| 6 | 1 | 14.0 | ≥ 21 | ≥75 | 24 | |
| 7 | 1 | 8.4 | ≥ 21 | ≥80 | 6 | Compacted soil. |

## 2.4 Test Matrices for Sodium Persulfate Decontamination

The general approach for testing SP was similar to that of $ClO_2$, and again used three (3) soil types simultaneously in a 10" diameter column, inside the Test Chamber. Negative Controls and Positive Controls were identical to the $ClO_2$ testing.

The test matrix for SP is presented in Table 4, and shows the target values for each of the test parameters. Note that for Tests 2 and 3, decontaminant conditions varied by soil type.

**Table 4. Sodium Persulfate Test Design**

| Test No. | Depth(s) Tested | Target SP Conc. (mg/L) | Target T (°C) | Target RH (%) | Target Contact Time (days) | Target Application Conditions | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Soil | Quantity | No. of Doses | Interval |
| 1 | 1-in depths | 0.5 M, 50/50 with 8% $H_2O_2$ | Ambient T | Ambient RH | 7 | All types | 0.18 mL/g | 2 | 48-hr |
| 2 | 0.5-in depths (topsoil)<br>1-in depths (sand, clay) | 0.5 M, 50/50 with 8% $H_2O_2$ | Ambient T | Ambient RH | 7 | Topsoil<br>Sand<br>Clay | 0.09 mL/g<br>0.09 mL/g<br>0.09 mL/g | 6<br>6<br>2 | 24-hr<br>24-hr<br>24-hr |
| 3 | 0.5-in depths (topsoil)<br>1-in depths (sand, clay) | 1.0 M, 50/50 with 8% $H_2O_2$ | Ambient T | Ambient RH | 7 | Topsoil<br>Sand<br>Clay | 0.09 mL/g<br>0.09 mL/g<br>0.09 mL/g | 6<br>6<br>1 | 24-hr<br>24-hr<br>NA |

## 2.5 Test Matrices for Methyl Bromide Decontamination

The test design for MeBr is presented in Table 5, and shows the nominal target values for each of the test parameters. Actual measured values are presented later in the results section for MeBr. Initial target test conditions for Test 1 were 212 mg/L (~53,000 ppm, where 1 oz/1000 $ft^3 \approx 1$ mg/L $\approx$ 250 ppm for MeBr) and $\geq$ 75% RH for 48 hrs. Conditions for each of the subsequent tests were determined based on results of previous tests. MeBr fumigation conditions for each test were the same for each soil type.

**Table 5. Methyl Bromide Test Design**

| Test No. | Depth(s) Tested | Target MeBr Conc. (mg/L) | Target T (°C) | Target RH (%) | Target Contact Time (hrs) | Other Conditions |
|---|---|---|---|---|---|---|
| 1 | 1-in depths | 212 | ≥ 21 | ≥75 | 48 | |
| 2 | 1-in depths | 212 | ≥ 21 | ≥75 | 72 (48)[a] | |
| 3 | 1-in depths | 300 | ≥ 21 | ≥75 | 48 | |
| 4 | 1-in depths | 212 | ≥ 21 | ≥75 | 48 | Saturated soil. |

[a] One set of 0" depth samples removed at 48-hrs. All other samples to be removed at 72 hrs.

## 2.6 Test Matrices for Clay Positive Controls Decay Tests

When no viable spores were recovered from the clay positive controls during the first 24-hour $ClO_2$ test (Test 4, $ClO_2$), we thought this may have been due to interaction between clay and the spores. In addition, inadvertently, the temperature and RH for the positive controls was higher than typical (37 °C and 94% RH). In an effort to quantify this potential interaction over a 168-hr time period, a pair (ambient RH and high RH) of Clay Positive Controls Decay tests were conducted. These tests used three samples per time point, making a total of 18 spiked samples overall. One non-soil extraction control was also performed (to assess inoculation level). Refer to Table 6.

**Table 6. Clay Positive Controls Decay Test Design**

| Test No. | Depth(s) Tested | Target $ClO_2$ Conc. (mg/L) | Target T (°C) | Target RH (%) | Target Contact Time (hrs) | Other Conditions |
|---|---|---|---|---|---|---|
| 4a | 0 (Clay Positive Controls Decay) | None | Ambient T | Ambient RH | 0, 24, 48, 72, 96, 168 | Control Chamber only, for testing decay of clay positive controls. 3 samples each time period. |
| 7a | 0 (Clay Positive Controls Decay | None | Ambient T | High RH | 0, 24, 48, 72, 96, 168 | Control Chamber only, for testing decay of clay positive controls. 3 samples each time period. |

# Section 3.
# Summary of Test Procedures

Test procedures were performed according to the approved Quality Assurance Project Plan (QAPP) and are summarized below.

This project was designed to have two distinct phases. First, a set of preparations and soil column design tests was performed to determine the best technical approach for performing the actual decontamination tests. Once the preliminary tests were performed, the actual decontaminant tests were executed. The main experimental variables for the decontaminant tests included the decontaminant type (i.e., SP, $ClO_2$, or MeBr), soil type, and the specific physical parameters for each test (depth of soil, decontaminant concentration and/or quantity, contact time, and RH).

## 3.1 Biological Agent

*B. anthracis* (BA) Ames spores were prepared at the Kansas City location using the A0462 strain from the MRIGlobal Repository (originally from catalog number NR-411 from Biodefense and Emerging Infections Research Resources Repository, www.beiresources.org). The BA was prepared according to MRIGlobal procedures, including growth on liquid or solid sporulation media, microscopic examination to verify spore production, and a heat shock of the preparation (65°C for 30 minutes in a stationary water bath) to kill any remaining vegetative *B. anthracis* Ames cells. Spores were resuspended in 10% glycerol. The concentration of viable spores in the preparation was determined by viable plate counting methods using sheep blood agar (SBA).

An aliquot of the spore preparation was serially diluted with 100 µL aliquots plated onto each of three SBA plates. Plates were incubated for approximately 16-20 hours at 35°C. After incubation, colony forming units (CFU) were counted to determine spore concentration. Spore preparation volumes were adjusted to achieve at least $1\times10^9$ CFU/mL. BA spore preparations were mixed thoroughly and then aliquoted into 1-mL cryovials and stored at -80°C until use. All work with BA was conducted within a Biosafety Cabinet (BSC) under BSL-3 containment.

Project requirements stated that the efficacy of the three selected decontaminants would be evaluated against fully virulent BA. To verify that the Ames stock designated for use on this project contains pX01 and pX02 virulence plasmids, polymerase chain reaction (PCR) analysis was performed on the spore preparation using MRIGlobal in-house assays BA2 (targeting capB on pX02) and BA3 (targeting *LF* on pX01).

Appendix A contains a product information sheet for the Ames strain used in this study (A0462, BEI Resources catalog number NR-411). Appendix A also contains PCR reports for detecting the virulence plasmids pX01 and pX02 for the determination that was performed two times during the program. An initial plasmids analysis was performed in February 2016 prior to any BSL-3 work. A reanalysis was performed in June 2017 during the test series with MeBr. The presence of pX01 and pX02 plasmids was confirmed in both cases, indicating virulence of the *B. anthracis* strain. Assays BA2 and BA3 were used to evaluate serial dilutions of DNA prepared from the spore preparation. Quantification data indicate detection of both plasmids at the highest DNA dilution tested. Positive controls for both assays were positive; negative controls for both assays were negative.

## 3.2 Soils

Three soil types were used for this study: a topsoil, a sandy soil, and a clayey soil. These soils were purchased from commercial sources as outlined in Table 7. Analyses for moisture and density were performed at MRIGlobal. All other physical property tests were performed by Agvise Laboratories (North Dakota). A complete summary of physical properties of the soils is found in Appendix B.

Soils were purchased in 50-lb bags from a local supplier, and variations could not be avoided due to the large amounts of soil required for this project. To minimize changes in moisture content after purchase, soils were stored in a sealed plastic trash bag within a sealed plastic tub. However, as shown in Appendix B, soil moisture did diminish over time as the study proceeded.

**Table 7. Soil Suppliers and Primary Physical Properties**

| Soil Type | Lot, Batch, or Other Description | Manufacturer/ Supplier Name | Density Range (g/mL)[a] | Moisture Range (%)[b] | Organic Matter (%)[c] | Sand/Silt/Clay (%)[d] |
|---|---|---|---|---|---|---|
| Topsoil (loamy) | Timberline (Oldcastle) | Oldcastle Lawn & Garden (Home Depot, local supply) | 0.97 – 1.02 | 19.6 – 41.9 | 5.2 | 42/33/25 |
| Sand | Play Sand (Pavestone) | Pavestone (Home Depot, local supply) | 1.14 – 1.49 | 3.0 – 8.4 | 0.0 | 100/0/0 |
| Clay | Crimson Clay (Better Baseball) | Better Baseball (online supply) | 1.06 – 1.28 | 10.7 – 28.4 | 0.2 | 44/11/45 |

[a] Bulk density of undried, uncompacted soil as measured by weight in a beaker.
[b] Measured by ASTM D2974-87.
[c] By Walkley-Black method [7].
[d] Hydrometer method.

Soil density was measured prior to each test by weighing a 200-mL volume of each soil type in a beaker. Soil moisture was measured according to ASTM D 2974-87 prior to each test. Samples were weighed, dried in an oven for ≥16 hrs at 105 ± 5 °C, and weighed again, with the moisture calculated as:

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

A = mass of the as-received sample, g
B = mass of the oven-dried sample, g

Sand/Silt/Clay moisture content was measured by Agvise Laboratories using the hydrometer method. The soil textural triangle below in Figure 1 [8] shows results for the three types of soils tested. The topsoil sample fell into the “loam” category.

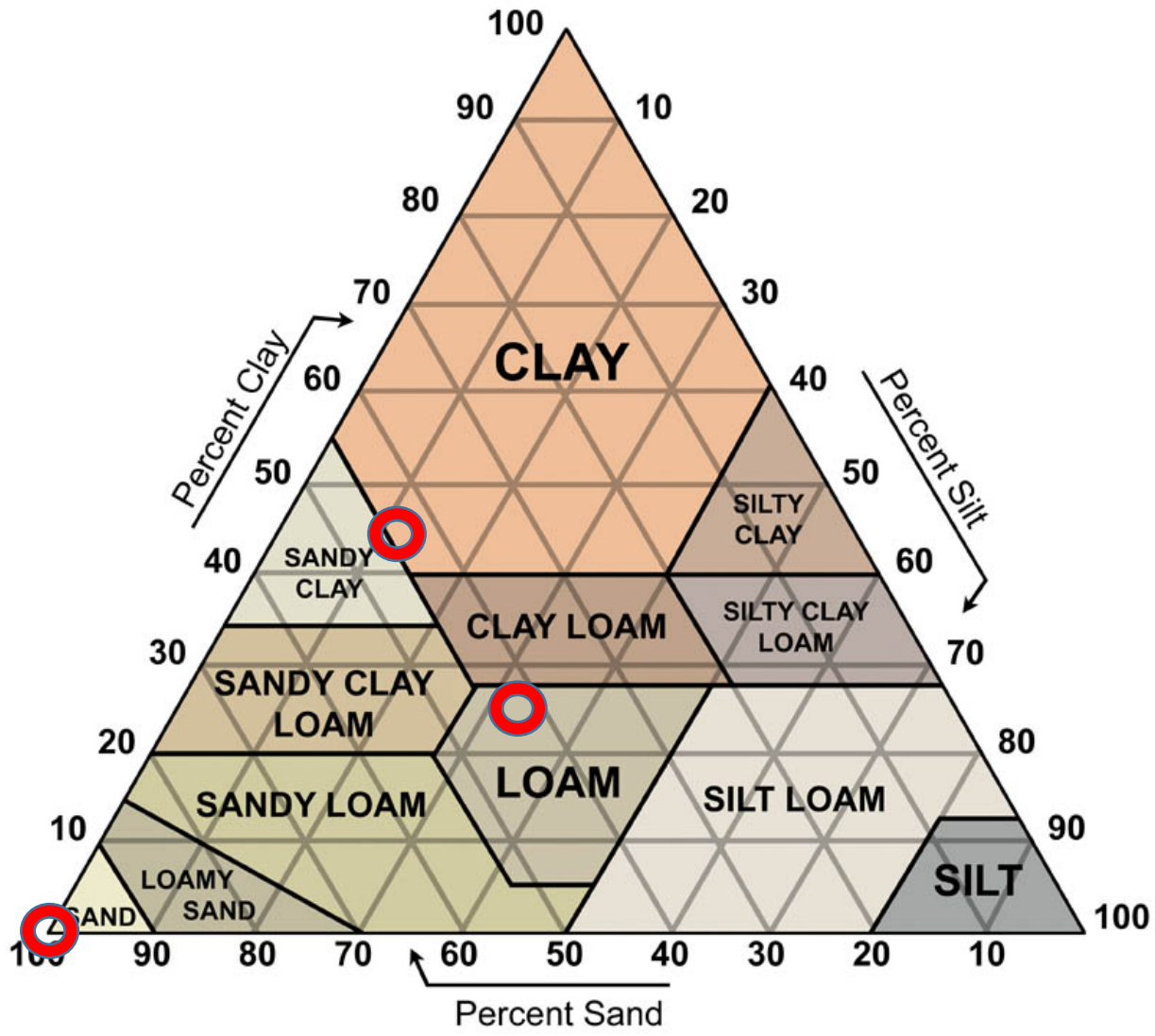


**Figure 1. Soil Textural Triangle [8]**

Detailed physical characteristics of the soils were obtained from a Series II analysis through Agvise Laboratories. Full results are contained in Appendix B. A small amount (~0.5 lb, or ~250 g) of each soil type was shipped to the analytical lab in a sealed plastic bag. Series II analysis consists of the following parameters, as per standard methods on file with Agvise Laboratories.

- pH (electrode)
- % organic matter (Walkley-Black procedure)
- Cation Exchange Capacity
- Ca, Mg, Na, K, and H (in conjunction with the above Cation Exchange Capacity)
- % moisture (gravimetric loss upon drying)
- % moisture at 1/3 bar (water holding capacity)

- % sand, silt, and clay (hydrometer method)
- USDA Textural Class (profiled according to USDA/NRCS System)
- Bulk Density (gm/cm3), (gravimetric weight)

Test soils for general column filling were not sterilized prior to use, and were used "as-is" from the suppliers. The smaller amounts of soil used for creating carrier soil packets spiked with BA Ames, however, were sterilized prior to use. Sterilization was performed by autoclaving at 121 °C for two hours.

As a final note, we acknowledge that clayey soils left undisturbed in the environment may become denser and less permeable to fluids due to their platelet/particle orientation. However, the procedures we used in the laboratory for the handling of soils and packing into test columns did not allow for us to use undisturbed clay soils. Thus most likely properties such as density and transmissivity of the clayey soils used in testing in this study may not be representative of actual field conditions. Additional tests are recommended to assess this issue.

## 3.3 Carrier Soil Packet (CSP) Development and Preparation

Carrier Soil Packets (CSPs) were created to provide primary containment of the BA Ames spores. CSPs also provided a convenient method for spiked soils to be placed at various depths within a soil column, to be easily recovered post-test, and allowed repeatable sample recovery and extraction procedures to be used.

The outer "pillow" material of each CSP was made from either Tyvek or polyvinylidine fluoride (PVDF), depending on the type of test. The initial plan was to use Tyvek (Mesa Labs, medical grade 1073B, 0.22-µm) throughout the study. Preliminary screening studies, however, showed that the liquid decontaminant (SP) was not able to penetrate Tyvek as quickly as expected. Further method development showed that PVDF filter media (EMD Millipore, Billerica, MA; 0.22-µm, hydrophilic) provided the necessary performance for use with SP. Consequently, CSPs for testing the gas-phase decontaminants ($ClO_2$, MeBr) were made from Tyvek, while CSPs for the liquid decontaminant (SP) were made from PVDF. Appendix C contains further details on the CSP method development.

Sterilized, 1-gram quantities of soil were placed into each CSP, and then spiked with ten, 10-µL droplets (100-µL total) of BA Ames spore prep, for a target inoculation level of $10^8$ CFU. Each CSP was then heat sealed using a lab heat sealer (Uline, Pleasant Prairie, WI). Dimensions of the CSPs were ~1.5" x 2" for the Tyvek and ~3" x 3" for the PVDF.

## 3.4 Test Column Preparation

Based on results from the preliminary wall effects testing (Appendix D), we fabricated 12" high test columns from 10" diameter polyvinyl chloride (PVC) pipe. This design allowed CSP placement at depths up to 5" without a wall effect (i.e. depth = radius).

Columns were fabricated with an open top to allow gas exposure or liquid application to the soil surface. The bottom of each column was closed with a PVC cap to prevent soil from falling out and to eliminate the possibility of decontaminant in-leakage from below.

Prior to each test, soil density was determined by weighing a 200-mL volume of soil in a glass beaker. The mass per unit depth (g/in) was then calculated for a 10-in diameter column. Depending on the test conditions, soil layers of either 1" or ½" depths were weighed using an A&D (Elk Grove, IL) FX-6000 electronic balance. Soil layers were stored in sealed ZipLoc® bags inside sealed plastic tubs to maintain moisture content at the original level prior to building a column for testing. The mass of soil for each 1-inch depth increment varied somewhat from test to test and for each type of soil; refer to Appendix B for soil density data.

To build each column, a 5" base of soil backfill was first placed on the bottom. Soil layers were then added one at a time, using the weighed ZipLoc bags and spiked CSPs as needed to meet the conditions for each test. This technique allowed five depths of CSPs to be tested simultaneously, along with a 0" depth sample placed on the top surface of the soil. CSPs were always placed in the center of each soil layer, thus maintaining a minimum clearance of 5" from the side walls of the column to eliminate any potential wall effects (gas leakage, channeling).

## 3.5 Sample Extraction and Biological Agent Quantification

Soil samples were extracted by removing soil from the CSP with a sterile spatula and placing it into 10 mL of sterile phosphate buffered saline (PBS) with 0.1% Triton-X 100. Samples were agitated at room temperature for 15 minutes at 200 rpm.

*Bacillus* spores were quantified using viable plate counting methodology with a dilution plating approach. 100-µL aliquots were removed from the extraction liquid after agitation, although if there was too much settling of soil particles, the extraction liquid was gently mixed again to restore turbidity. From that point, a series of 1:10 dilutions was performed and triplicate 100-µL aliquots of each dilution were plated onto sheep blood agar (SBA) culturing media. Plates were incubated for 16-20 hours at approximately 35 °C. Following incubation, samples were either immediately counted or were maintained in storage at 4-8 °C until enumeration. After counting was complete, laboratory staff destroyed all samples by autoclaving. Refer to Appendix C for further details about CSP development and CFU extraction and quantification method evaluations.

## 3.6 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:

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

where Mean $CFU_{pc}$ is the mean number of CFU counted from five replicate positive control samples from a single material, and $CFU_{target}$ is the number of CFU counted from the non-soil extraction control sample (i.e., 100-µL spiked into extraction buffer and recovered, not spiked onto soil). Mean % recovery was also calculated for each individual soil sample. Results are included in Sections 6 through 8.

Efficacy was defined as the comparison (as a $\log_{10}$ reduction) of viable counts between individual test samples after decontamination versus the average of the positive controls, as shown below:

$$\text{Efficacy (as log reduction, or "LR")} = (\text{Avg. } \log_{10} \text{CFU}_{pc}) - \log_{10} \text{CFU}_{sample} \quad \textbf{(2)}$$

At any given soil depth, a decontamination that achieved an efficacy of ≥ 6 LR was considered to be effective [9]. We note, however, that while a decontamination efficacy ≥ 6 LR may be considered "effective" when reporting test results, in an actual BA release event, the goal would be to minimize the number of recoverable viable spores, regardless of LR.

In cases where no viable spores were detected, a CFU value of 1 was assigned, producing a $\log_{10}$ value of zero for that sample. These samples were considered to be completely inactivated and therefore, achieved the maximum efficacy possible or quantifiable. That is, the final efficacy was reported as greater than or equal to (≥) the value calculated by Equation 2.

## 3.7 Biological Indicator (BI) Handling and Analysis

Prior to performing tests using BA within the custom-made CSPs, a series of preliminary method development and wall-effects tests was performed using commercially-available biological indicators, or BIs, from Mesa Laboratories, Inc. BIs consisted of a small stainless steel disc impregnated with *B. atrophaeus* spores to a level of $2.8 \times 10^6$ CFU per disc, and sealed within a Tyvek type 1073 (0.22-µm pore size) pouch to maintain sterility.

Method development tests were performed with the BIs as a cost-effective way to determine fundamental data on the expected performance of the CSPs and potential wall effects for the column designs. Details and results from these method development tests are included in Appendices C and D. The standard procedure for extraction and recovery of the BIs was as follows:

1. Cut Tyvek and aseptically dump BI disc into 15 mL tube containing 10 mL sterile water.
2. Sonicate tube for 15 min.
3. Heat shock (optional) by placing tube in water bath at 80-85 °C for 10 minutes.
4. Analyze by plate counting/serial dilution, using 0.1-mL aliquots.

# Section 4.
# Decontamination Procedures

## 4.1 Decontaminant Preparation

### 4.1.1 Chlorine Dioxide

A Minidox-M $ClO_2$ generator (ClorDiSys Solutions, Inc., Lebanon, NJ) was used to produce all of the $ClO_2$ required for the project. Prior to any laboratory use, the system was inspected and overhauled by a ClorDiSys field service engineer. The photometer was calibrated and fresh $ClO_2$ cartridges were installed. A complete checklist of the field maintenance performed is in the project file and is also found in the ClorDiSys Minidox-M Systems Operations Guide [10].

### 4.1.2 Sodium Persulfate

Klozur® SP (Peroxychem, Philadelphia, PA) was used as the source of SP, and is used for *in situ* and *ex situ* chemical oxidation of contaminants in environmental remediation applications (e.g., soil). Klozur® SP is a >99% pure SP ($Na_2S_2O_8$) in the form of white odorless crystals. In remediation applications, Klozur® SP is injected into contaminated soil or groundwater and is activated by mixing in appropriate proportions of 8% hydrogen peroxide ($H_2O_2$) (or other activators) by weight. Peroxychem recommends performing a bench-scale study to determine the optimum hydrogen peroxide to SP molar ratio for the remediation.

A full optimization was beyond the scope of this study. Guidance provided by Peroxychem, however, states that molar ratios of 1:1 to 10:1 (hydrogen peroxide to persulfate) are generally used, with a molar ratio of 5:1 typically being sufficient to treat most contaminants under a wide range of site conditions [11]. This information, along with previous EPA studies [2-4], led to an initial target condition of 0.5 M SP mixed in equal volumes with 8% hydrogen peroxide. This 50/50 mixture provides an actual molar ratio of 4.7 to 1. Later in the test series, the persulfate concentration was adjusted to 1.0 M, while still maintaining the 50/50 volume ratio with hydrogen peroxide. This mixture provides an actual molar ratio of 2.35 to 1.

Food-grade 8% hydrogen peroxide (Family Health, Miami FL) was purchased on-line, and fresh, unopened, 1-pint bottles were used as needed for each test. The hydrogen peroxide/persulfate solution was always mixed fresh immediately prior to application.

### 4.1.3 Methyl Bromide

Methyl bromide is a colorless and odorless volatile gas. Due to the toxicity of MeBr, previous EPA studies have used a commercial blend of 99.5% MeBr with 0.5% chloropicrin added as a warning irritant [12]. Laboratory staff were unable to locate a commercial supplier for MeBr containing 0.5% chloropicrin, and used 100% MeBr available through Cardinal Professional Products (Anaheim, CA) as Meth-O-Gas® 100 commodity fumigant. The Cardinal sales representative said that custom chloropicrin blends were no longer available due to market conditions (MeBr is now banned except for specific exempt industries).

## 4.2 Test and Control Chambers and Procedures

### 4.2.1 Test and Control Chambers

All testing was conducted using a custom-built Test Chamber to contain the soil columns exposed to decontaminant and a Control Chamber to contain positive control samples. The Test Chamber was a rigid structure with dimensions of 14” x 44” x 20” height, built from aluminum framing and chemical-resistant PVC walls. Temperature and RH were monitored with a HMD40/50 probe from Vaisala (Boston, MA). Other chamber conditions were modified slightly for each decontaminant type, and are described in the subsections below. A picture of the Test Chamber is shown below in Figure 2.

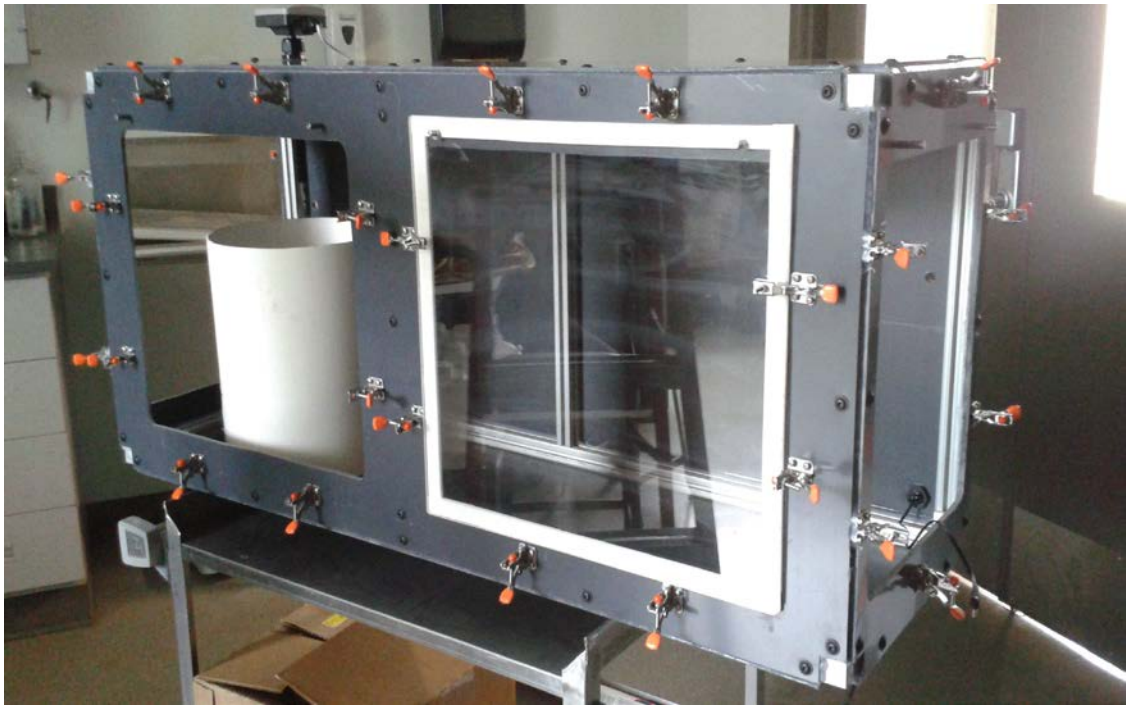

**Figure 2. Test Chamber**

The Control Chamber was a small plastic Life-Latch bucket for primary containment, placed into an incubator within the BSL-3 facility. The incubator temperature and RH were monitored and logged using an AmegaView centralized monitoring system (Mesa Monitoring, sensor model #3006-20; Lakewood, CO).

### 4.2.2 ChlorDiSys Minidox-M

The Test Chamber was connected to the Minidox-M using several ¼” Teflon tubes, as per the manufacturer’s guidance, for inlet/outlet $ClO_2$ to the photometer, feed $ClO_2$ to the Test Chamber, purge air to the Test Chamber, and a manual exhaust valve for purge air. Controls on the Minidox-M also allowed operation of a small humidifier (PureGuardian H1010; Euclid, OH) through a relay switch linked to a 120-VAC power supply. Dehumidification controls were not part of the design, thus allowing RH levels to be maintained at or above the set-point. Temperature controls were also not part of the design, although the Minidox-M did log

temperature throughout each test. Ambient operating conditions in the BSL-3 were targeted to be 70°F or greater.

Laboratory staff followed the manufacturer's procedures for operating the Minidox-M. First, the system performed a "conditioning" step where RH within the Test Chamber was raised to the target level. Chlorine dioxide gas "exposure" was then introduced to the target concentration and was held for the target time period. Note that the factory-installed program on the Minidox-M limited exposure time to a 10-hr time period, requiring lab staff re-entry and manually restarting the sequence for those tests that ran beyond 10 hours. After completing a test, the gas was removed and vented to external exhaust.

Chlorine dioxide concentration, temperature, and RH were logged at 1-min intervals throughout each test.

Vendor-supplied literature has the following information on the system controls:

- Humidifier auto on (RH is >2% below set point); auto off (RH is at the set point).
- $ClO_2$ auto on ($ClO_2$ is 0.3 mg/L below set point); auto off (0.3 mg/L above set point).
- Chlorine dioxide set point range 0 to 30 mg/L.
- Exposure time is 0 to 10 hours (600 minutes).
- Chamber volume is 1 to 28,000 cubic feet.
- Pressure set point, above atmospheric, 0 to 600 Pascals.

### 4.2.3 Sodium Persulfate Decontamination Procedure

Sodium persulfate was prepared at concentrations of either 0.5 M or 1.0 M and activated by mixing it 50/50 (volume basis) with 8% hydrogen peroxide. SP dry powder was weighed and mixed with deionized water to prepare the stock SP solution. (119.1 g dry SP into 1 L of water yields a 0.5 M solution.) Liquid hydrogen peroxide was mixed with the SP solution in 50/50 proportions immediately prior to soil application. Volumes and contact times were dependent on conditions determined for each individual test.

Liquid volume of each application was based on the approximate soil saturation point (in units of mL liquid per gram of soil) that was determined during preliminary testing. The liquid volume to be applied was then calculated for a wetted depth of 6 inches in a 10-in diameter column. Actual application of the liquid was performed by pouring the calculated volume through a hand-held garden sprinkler head onto the top of each column.

For the first test with SP, plastic end caps were in place on the bottom of each column as had been done with the previous $ClO_2$ tests. Although this design allowed liquid seepage (drainage) from the bottom, it was found to be more restrictive than desired. Consequently, several small ¼" holes were drilled into each end cap, improving the liquid drainage for the remaining tests.

Liquid drainage was collected in drip pans located beneath each column. Excess liquid was removed as needed to prevent the drip pans from overflowing. For the final test in this series, the oxidation reduction potential (ORP) was measured using a hand-held probe (Hach Pocket Pro™; Loveland, CO). ORP measurements were taken of the initial (fresh) activated SP and final

(partially spent) liquid emerging from the column bottom. Further details of the ORP measurements can be found in Appendix E.

Temperature and RH were not controlled for the SP testing, but were logged using a Hygroflex HF53W XMTR T/RH probe (Rotronic Instrument Corp., Hauppauge, NY).

### 4.2.4 Methyl Bromide Decontamination Procedure

The MeBr concentration in the test chamber was measured continuously using a Fumiscope™ Version 5.0 (Key Chemical and Equipment Company, Clearwater, FL). MeBr was added to the chamber as necessary to reach the specified concentration, and was maintained at or above the target set point by using an Omega Engineering CN7523 controller (Norwalk, CT).

The Fumiscope™ is calibrated annually by the manufacturer for MeBr, displaying the concentration on a digital LED display in ounces of MeBr per 1000 cubic feet. One oz per 1000 $ft^3$ is approximately 257 ppm at 25 °C, and is approximately 1 milligram (mg) per liter (independent of temperature). MeBr tests were expected to be run at ~212 to 300 mg/L (53,000 to 75,000 ppm). Calibration of the Fumiscope was conducted at the factory by the supplier. In addition, prior to being transferred to the BSL-3 facility, the Fumiscope™ calibration was verified with a 75,000 ppm calibration gas from Scott-Marrin, Inc (Riverside, CA).

The Fumiscope™ included an air pump that drew gas from the test chamber through the thermal conductivity detector at a controlled rate of ~1 LPM, then exhausted the gases back into the test chamber. Moisture was removed from the gas sample by using a small glass condenser trap just upstream of the Fumiscope™ At the end of a given trial, the test chamber was flushed with compressed air and then opened to flush with ambient laboratory air. Worker safety at the 5-ppm level was confirmed by testing the hood with a hand-held miniRAE 3000 PID sensor (RAE Systems, Inc., San Jose, CA). The miniRAE was used during Test 1 MeBr before, during, and after the test to spot check for safety/leaks (whenever staff were in the room taking care of something. There was not a set schedule.) After successfully demonstrating good engineering controls during Test 1, the safety office waived the need for using it, provided proper lab procedure was used, such as hood airflows and equipment setbacks.

Temperature and RH were monitored with the same Rotronic T/RH probe used for the previous SP tests. RH was controlled at or above the set point by using an Omega CN7523 controller with a small humidifier identical to the $ClO_2$ tests (i.e., the Pureguardian H1010 ultrasonic humidifier).

# Section 5.
# Quality Assurance and Quality Control

Quality assurance and quality control (QC) procedures were performed according to the program Quality Management Plan (QMP) and the QAPP. A summary of QA/QC procedures and results is below.

## 5.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, balances) and monitoring devices (e.g., thermometer, hygrometer, Minidox-M controls) used at the time of testing were verified as being within calibration or vendor certification.

## 5.2 Quality Control Results

Quality controls during the program included several types of sample performance controls, as summarized in Table 8. Specifically, there were two types of inoculation controls: a back titer (100-µL of the stock spore suspension on the test day was analyzed by direct plating) and the non-soil extraction controls (100-µL of stock spore suspension, spiked into buffer, recovered and plated identically to samples but without exposure to soil). Recovery of positive controls was then calculated versus the non-soil extraction control results for each test. Other QC samples included positive controls for each soil type (inoculated, not decontaminated), negative controls, and zero-depth controls (inoculated, unburied, placed on the top surface of each soil column, decontaminated).

**Table 8. Quality Control Sample Performance Criteria**

| Sample | Number of Samples | Performance Criteria | Corrective Action if Performance Criteria are not Attained |
|---|---|---|---|
| Inoculation Control – stock suspension directly analyzed by plating (back titer) | One per test at time-zero. | ±1 log from target value of 1 x $10^9$ CFU/mL | Identify and correct the cause of incorrect bacteria levels in the stock suspension. |
| Inoculation Control – stock suspension spiked into buffer w/o soil contact (non-soil extraction control) | One per test at time-zero. | ±1 log from target value of 1 x $10^9$ CFU/mL | Identify and correct the cause of incorrect bacteria levels in the result. |
| Positive Control – inoculated, not exposed to decontaminant, kept in separate Control Chamber | Five per soil type | Mean CFU ≥5% and ≤120% of inoculation control | Discuss results with team lead. |
| Negative Controls (laboratory blank) – not inoculated, not exposed to decontaminant | One per soil type | No observed CFU | Identify and remove source of contamination. |
| Negative Controls (procedural blank) – not inoculated, exposed to decontaminant | One per soil type. | No observed CFU | Identify and remove source of contamination. |
| 0” Control – inoculated, exposed to decontaminant, unburied, placed on top surface of soil | One per soil type | None; driven by the effectiveness of the decontaminant | Compare data with CSPs at depths; with highly-variable results attempt to identify source of the variability. |

Table 9 contains a summary of the quality control results for each of the tests. A "yes" indicates that the QC criteria from Table 8 were met for that test.

All back titers met the performance criteria of ±1 log from the target value of 1 x $10^9$ CFU/mL, except Test 7, $ClO_2$, which had a value higher than 1 x $10^{10}$ CFU/mL. In addition, all of the back titers showed a stock suspension value of at least 1 x $10^9$ CFU/mL, though there were no specific QC criteria for this. Non-soil extraction controls also met the QC criteria of ±1 log, with one exception, Test 3, $ClO_2$, that was low (6.6 x $10^7$ CFU). These few anomalies were not expected to impact results significantly.

For positive controls, a few samples fell beyond the 5% – 120% target criteria. Results shown in Table 9 show both yes/no and % recovery range for positive controls. (For each soil type, there were a total of 70 positive controls used in the study: 14 experiments X 5 replicates.) For topsoil, there were 6 of 70, or 8.6%, positive controls that fell outside this criterion. For sand: 7 of 70, or 10.0%; and for clay: 7 of 70, or 10.0%. Overall, 20 of 210, or 9.5%, of positive controls fell outside this recovery range.

As per Table 8, performance criteria that were not met were discussed with the principal investigator on a case-by-case basis, and corrective actions were taken where possible. Variations in % recovery are believed to be due to variations in the soil content. Since the testing required large volumes of soil, it was somewhat problematic to maintain consistency across the entire length of program. Poor recovery of clay positive controls from Test 4, $ClO_2$, led to an effort to characterize the decay of clay-based positive controls in Tests 4a and 7a.

For the $ClO_2$ and SP tests, all of the 0" controls (spiked CSP placed on the top surface of each soil column) showed complete kill (i.e., fully inactivated), with no observed CFU, with the exception of Test 3, SP. This sample did not show complete inactivation, presumably because the large amount of foaming partially blocked SP penetration into the PVDF packet.

For the MeBr tests, however, several of the CSPs on the soil surfaces (0" controls) unexpectedly did not show complete inactivation. Only one test (Test 2 MeBr, sand) of the fifteen total 0" samples showed complete kill. Three of the fifteen total 0" samples clearly showed *B. anthracis* colonies, although results fell below the quantitation range of 30 – 300 when undiluted. For project reporting purposes, these samples were counted as is, with a footnote of "below quantitation limit", i.e., > 0 but < 30 CFU, as per the project QAPP. Finally, the remaining eleven 0" samples for the MeBr testing showed a decontamination efficacy that was typically approximately the same as the buried samples, and frequently in the range of only 0 – 2 LR.

All negative controls (blanks) met the criteria of no observed CFU.

**Table 9. Quality Control Results Summary**

| Test ID | Back Titer | Non-Soil Extraction Control | Positive Controls % Recovery Range | | | Negative Controls | 0" Controls |
|---|---|---|---|---|---|---|---|
| | | | Topsoil | Sand | Clay | | |
| 1. $ClO_2$ | Yes | Yes | Yes (18 – 40) | No (4 – 51) | Yes (10 – 57) | Yes | Yes |
| 2. $ClO_2$ | Yes | Yes | Yes (25 – 96) | Yes (37 – 87) | Yes (8 – 69) | Yes | Yes |
| 3. $ClO_2$ | Yes | No | No (3 – 236) | No (1 – 114) | No (4 – 138) | Yes | Yes |
| 4. $ClO_2$ | Yes | Yes | No (17 – 315) | Yes (25 – 58) | No (0) | Yes | Yes |
| 5. $ClO_2$ | Yes | Yes | Yes (27 – 56) | No (116 – 165) | Yes (46 – 107) | Yes | Yes |
| 6. $ClO_2$ | Yes | Yes | Yes (47 – 81) | Yes (18 – 35) | Yes (10 – 23) | Yes | Yes |
| 7. $ClO_2$ | No[a] | Yes | Yes (19 – 47) | Yes (54 – 66) | Yes (7 – 16) | Yes | Yes |
| 4a. Decay | Yes | Yes | -- | -- | -- | -- | -- |
| 7a. Decay | Yes | Yes | -- | -- | -- | -- | -- |
| 1. SP | Yes | Yes | Yes (6 – 21) | Yes (18 – 36) | Yes (5 – 8) | Yes | Yes |
| 2. SP | Yes | Yes | Yes (27 – 37) | Yes (46 – 65) | Yes (7 – 22) | Yes | Yes |
| 3. SP | Yes | Yes | Yes (20 – 47) | Yes (21 – 37) | Yes (18 – 61) | Yes | No[b] |
| 1. MeBr | Yes | Yes | Yes (35 – 53) | Yes (87 – 113) | Yes (19 – 42) | Yes | No[c] |
| 2. MeBr | Yes | Yes | Yes (19 – 23) | Yes (84 – 97) | Yes (13 – 19) | Yes | No[d] |
| 3. MeBr | Yes | Yes | Yes (13 – 27) | Yes (70 – 83) | Yes (9 – 16) | Yes | No[e] |
| 4. MeBr | Yes | Yes | Yes (38 – 50) | Yes (74 – 94) | Yes (20 – 23) | Yes | No[c] |

[a] Results were high. No corrective action taken.
[b] Results did not show complete inactivation, believed due to excessive foaming during decontaminant application.
[c] Results did not show complete inactivation (48-hr exposure) for any of the three soil types.
[d] 48-hr 0" controls were not completely inactivated. At 65.5 hrs, sand 0" was completely inactivated, topsoil was >0 but <30 counts (below detection limit), while clay was not completely inactivated.
[e] 0" controls were >0 but <30 counts (below detection limit) for topsoil and sand, while clay was not completely inactivated.

## 5.3 Audits

### 5.3.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during the experiments. Table 10 summarizes the performance evaluation audits that were performed.

**Table 10. Performance Evaluation Audits**

| Measurement | Audit Procedure | Allowable Tolerance | Actual Measurement or Calibration Check |
|---|---|---|---|
| Volume of liquid from micropipettes | Annual calibration to meet manufacturers specifications, pass or replace pipette. | ±10% | 10 µL size: 1.0%<br>100 µL size: 0.8%<br>1000 µL size: 0.8% |
| Minidox-M $ClO_2$ concentration | Photometer calibrated by manufacturer prior to testing. | ±10% | 1% |
| Fumiscope thermal conductivity meter | Vendor certified and calibrated prior to test. Checked in laboratory using independent calibration gas. | ±10% | 1.4% |
| Balance | Compared to independent calibrated weight sets | ±0.5 g | 0.1 g |
| Temperature (lab incubator for controls) | Calibrated once annually by Amega, pass or replace sensor. | ±2°C | 0.6°C |
| RH (lab incubator for controls) | Calibrated once annually by Amega, pass or replace sensor. | ±5% | 1.7% |
| Temperature (refrigerator for storing extractions) | Calibrated once annually by Amega and continuously monitored. | ±2-8 °C | 1.2°C |
| Temperature (incubator used for plates) | Calibrated once annually by Amega, pass or replace sensor. | ±2°C | 0.5°C |
| Freezer (stock storage) | Calibrated once annually by Amega, pass or replace sensor. | ±2°C | 1.2°C |

### 5.3.2 Technical Systems Audit

As per the QAPP, a technical systems audit was not conducted as part of this program.

### 5.3.3 Data Quality Audit

As per the QAPP, 10% of the viable plate count data was audited by the QA representative assigned to this task. The data were traced from the initial acquisition through reduction and final reporting. Calculations and spreadsheets set up with the data were checked as part of the effort. The findings of the data quality audit showed that results are of known and acceptable quality. A copy of the data quality audit report is on file at MRIGlobal.

## 5.4 Test/Quality Assurance Plan Deviations

### 5.4.1 Countable Range

A countable range of 30 to 300 colonies for viable plating analysis was established in the QAPP for this project. Practical experience, however, required adjustment of this range on a case-by-case basis. The BA colonies proved to be quite large, making accurate counting above ~150

impossible. As a consequence, the analyst was sometimes forced to evaluate the next lowest dilution and use this value (e.g., 15 counts) even though it fell out of the target quantitation range. Any results outside the 30 to 300 range were flagged in the raw data given to the principal investigator for each individual test.

During the MeBr tests, several samples fell outside the quantitation range of 30 to 300 when undiluted sample aliquot was plated. For reporting purposes, these samples were counted as is, but footnoted "below quantitation limit, > 0 but < 30 counts".

### 5.4.2 Unburied versus Buried Positive Controls

When the program began, the initial plan was for positive control samples to be buried in a separate Control Column using the appropriate soil types. However, laboratory tests were conducted to demonstrate that a simpler method of using unburied positive controls would produce similar overall results. For Test 1, $ClO_2$, a set of five buried and five unburied positive controls were evaluated for each soil type. Table 11 compares the two sets of results, which had averages that agreed to within 15% for all three soil types. Consequently, at the direction of the principal investigator, all of the remaining tests used only unburied positive controls.

**Table 11. Comparison of Unburied and Buried Positive Controls**

| | Unburied (CFU) | Buried (CFU) | Difference (%) |
|---|---|---|---|
| Topsoil | $8.67 \times 10^7$ | $5.83 \times 10^7$ | |
| | $1.65 \times 10^8$ | $1.30 \times 10^8$ | |
| | $1.02 \times 10^8$ | $9.63 \times 10^7$ | |
| | $1.44 \times 10^8$ | $1.66 \times 10^8$ | |
| | $7.37 \times 10^7$ | $2.03 \times 10^8$ | |
| Topsoil Average | $1.14 \times 10^8$ | $1.31 \times 10^8$ | 13.3 |
| Clay | $4.20 \times 10^7$ | $1.62 \times 10^8$ | |
| | $1.44 \times 10^8$ | $1.15 \times 10^8$ | |
| | $9.30 \times 10^7$ | $9.83 \times 10^7$ | |
| | $5.73 \times 10^7$ | $1.82 \times 10^8$ | |
| | $2.36 \times 10^8$ | $9.27 \times 10^7$ | |
| Clay Average | $1.15 \times 10^8$ | $1.30 \times 10^8$ | 12.7 |
| Sand | $1.98 \times 10^8$ | $2.31 \times 10^8$ | |
| | $1.07 \times 10^8$ | $1.18 \times 10^8$ | |
| | $1.10 \times 10^8$ | $1.15 \times 10^8$ | |
| | NA[a] | $1.59 \times 10^8$ | |
| | $2.10 \times 10^8$ | $2.20 \times 10^8$ | |
| Sand Average | $1.56 \times 10^8$ | $1.69 \times 10^8$ | 7.6 |

[a] Sample did not meet criteria of 30-300 counts at any dilution and was not included in the average.

## 5.5 QA/QC Reporting

Each assessment and audit was documented in accordance with the QAPP and QMP. For this program, any findings were noted as not significant and no follow-up corrective actions were necessary.

## 5.6 Data Review

Records and data generated in the testing received a QC/technical review before they were used in calculations or determination of results, and prior to being incorporated into data reports to the principal investigator.

# Section 6.
# Results and Performance Summary for Chlorine Dioxide

## 6.1 Chlorine Dioxide Test Conditions

The actual fumigation conditions measured for the $ClO_2$ test series are presented in Table 12. Highlights of each test are given in the subsections below. Table 12 also includes summary information on decontamination efficacy, in terms of the maximum depth at which ≥ 6 LR was achieved for each soil type for each test.

**Table 12. Chlorine Dioxide Actual Test Conditions**

| Test No. | Depth(s) Tested | Avg. $ClO_2$ Conc. (mg/L) | Avg. T (°C) | Avg. RH (%) | Contact Time (hrs) | Other Conditions | Max depth (inches) achieving ≥ 6 LR topsoil | Max depth (inches) achieving ≥ 6 LR clayey | Max depth (inches) achieving ≥ 6 LR sandy |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0, 1, 2, 3, 4, 5” | 8.7 ± 0.30 | 25.3 ± 0.44 | 76.7 ± 3.65 | 3 | | 0 | 0 | 0 |
| | Positive Controls | NA | 26.5 ± 0.07 | 44.4 ± 0.33 | [a] | | | | |
| 2 | 0, 1, 2, 3, 4, 5” | 10.3 ± 2.98 | 24.4 ± 0.19 | 79.6 ± 6.49 | 6 | | 0 | 2 | 1 |
| | Positive Controls | NA | 25.7 ± 0.07 | 39.5 ± 0.19 | [a] | | | | |
| 3 | 0, 0.5, 1, 1.5, 2, 2.5” | 8.9 ± 0.85 | 24.3 ± 0.33 | 73.5 ± 3.62 | 3 | Saturated soil | 0 | 2.5 | 2 |
| | Positive Controls | NA | 25.2 ± 0.07 | 43.8 ± 0.59 | [a] | | | | |
| 4 | 0, 1, 2, 3, 4, 5 | 10.1 ± 2.16 | 23.3 ± 0.48 | 80.6 ± 3.02 | 24 | | 1 | 5* | 5 |
| | Positive Controls | NA | 36.7 ± 0.10 | 93.8 ± 1.34 | [a] | | | | |
| 5 | 0, 1, 2, 3, 4, 5” | 9.3 ± 1.34 | 23.2 ± 0.47 | 80.1 ± 2.76 | 24 | Saturated soil | 0 | 5 | 2 |
| | Positive Controls | NA | 24.6 ± 0.12 | 27.7 ± 4.37 | [a] | | | | |
| 6 | 0, 1, 2, 3, 4, 5” | 14.6 ± 2.13 | 22.2 ± 0.20 | 82.5 ± 1.24 | 27.5 | | 1 | 5 | 5 |
| | Positive Controls | NA | 23.5 ± 0.41 | 14.7 ± 3.98 | [a] | | | | |
| 7 | 0, 1, 2, 3, 4, 5” | 9.4 ± 0.89 | 22.3 ± 0.27 | 85.8 ± 0.43 | 7.75 | Compacted soil | 0 | 3 | 2 |
| | Positive Controls | NA | 23.4 ± 0.20 | 20.3 ± 4.23 | [a] | | | | |

[a] Positive controls had no decontaminant applied, but were collected and extracted at the end of the contact time for the test samples.

* No spores recovered from clay positive controls for Test 4, thus test results are questionable

## 6.2 Chlorine Dioxide Decontamination Results

### 6.2.1 Test 1 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥75% for a 3-hr contact time. This test used both buried and unburied positive control samples as described earlier in Section 5 (QC), while all subsequent tests used only unburied positive control CSPs. Results showed that the decontamination had virtually no effect for the topsoil and sand columns. The clay column showed limited effect (up to 2 LR) at depths up to 2".

### 6.2.2 Test 2 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥75% for a 6-hr contact time. This experiment was meant to determine if efficacy could be improved by increasing contact time, and in fact, efficacy generally did improve compared to Test 1. Results showed that the decontamination had a 3.5 LR at 1" and tapered off to no effect by 4" for the topsoil column. The sand column showed complete kill (8 LR) at 1" and tapered off to no effect by 4". The clay column showed complete kill (8 LR) at up to 2" and tapered off to no effect by 4".

### 6.2.3 Test 3 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥75% for a 3-hr contact time. The top 5" of soils were wetted pre-test to near the saturation point, which had been determined experimentally in preliminary tests to be 0.08 mL/g (topsoil), 0.10 mL/g (sand), and 0.04 mL/g (clay). CSPs were placed in 0.5-in increments, rather than the 1-in increments used previously, based on previous efficacy results.

Pre-wetting the soils did improve efficacy somewhat (compared to Test 1), although results were somewhat mixed. Results showed that for the topsoil, the $ClO_2$ provided a 1 LR at the 2" depth to the column, but was ineffective at shallower depths. The sand column showed complete kill at most depths. The clay column showed complete kill at most depths as well. For all three columns, it is believed that clumping or channeling due to the wetted soil was the cause of inconsistency across the range of depths.

### 6.2.4 Test 4 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥75% for an extended 24-hr contact time. Inadvertently, the actual mean temperature for the positive controls was 37 °C and the RH was elevated to 94%. Results showed that the extended contact time did continue to improve efficacy somewhat; decontamination had complete kill (8 LR) at 1" and tapered off to no effect by 3" for the topsoil column. The sand column showed complete kill (7 LR) at all depths. The clay column appeared to show complete kill at all depths, but positive controls for clay also showed no recovery, thus making the test results for clay questionable. Refer to Section 9 of this report for test results for the clay decay tests, conducted to explore reasons for no recovery of spores from clay positive control CSPs.

### 6.2.5 Test 5 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥75% for a 24-hr contact time. The top 5" of soils were wetted pre-test to near the saturation point, using the same experimentally-determined levels as in Test 3 $ClO_2$ described earlier. This experiment was conducted to elicit the effect of pre-wetting of soil, by comparing to Test 4. In this case, the effect of added soil moisture seems to have diminished efficacy somewhat. (The $ClO_2$ concentration for Test 5 was somewhat lower than for Test 4, and this may have also contributed to the somewhat lower efficacy.) Results showed that the decontamination had 1 LR at 1" for the topsoil column. The sand column showed complete kill (8 LR) at up to 2", dropping off to only a minor effect at 5". The clay column had complete kill (8 LR) at all depths.

### 6.2.6 Test 6 $ClO_2$

Target $ClO_2$ concentration was 14.0 mg/L and target RH was ≥75% for a target 24-hr contact time. This test was conducted to evaluate whether improved efficacy would occur for the topsoil with an increased $ClO_2$ concentration. Results showed that the decontamination had complete kill (8 LR) at 1" for the topsoil column, then tapered off to minor or no effect. This result is essentially the same for topsoil for Test 4. The sand column showed complete kill (8 LR) at all depths. The clay column had complete kill (8 LR) at all depths.

### 6.2.7 Test 7 $ClO_2$

Target $ClO_2$ concentration was 8.4 mg/L and target RH was ≥80% for a target 6-hr contact time. This test attempted to duplicate the conditions of Test 2 $ClO_2$, but with compressed soils. Once soil columns were built, soils were gently compacted by compression with a small hand tool. Results showed that the decontamination had no effect at any depth for the topsoil column. The sand column showed complete kill (8 LR) at up to 2". The clay column had complete kill (8 LR) at up to 3".

Tables 13 through Table 19 show the detailed results for the decontamination of BA spores in soil using $ClO_2$.

**Table 13. Test 1, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 1<br>8.7 mg/L, 3-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $4.13 \times 10^8$ | 0 | 0 | 8.04 |
| Test Sample, 1” | “ | 8.10 | 31 | -0.07 |
| Test Sample, 2” | “ | 8.16 | 35 | -0.12 |
| Test Sample, 3” | “ | 8.22 | 40 | -0.18 |
| Test Sample, 4” | “ | 8.26 | 44 | -0.22 |
| Test Sample, 5” | “ | 8.33 | 52 | -0.29 |
| Positive Controls (mean) | $4.13 \times 10^8$ | 8.04[a] | 18 – 40[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $4.13 \times 10^8$ | 0 | 0 | 7.98 |
| Test Sample, 1” | “ | 7.82 | 16 | 0.17 |
| Test Sample, 2” | “ | 8.00 | 24 | -0.02 |
| Test Sample, 3” | “ | 8.32 | 50 | -0.33 |
| Test Sample, 4” | “ | 8.39 | 59 | -0.40 |
| Test Sample, 5” | “ | 8.30 | 48 | -0.31 |
| Positive Controls (mean) | $4.13 \times 10^8$ | 7.98[a] | 4 – 51[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $4.13 \times 10^8$ | 0 | 0 | 7.98 |
| Test Sample, 1” | “ | 5.92 | <1 | 2.06 |
| Test Sample, 2” | “ | 6.06 | <1 | 1.92 |
| Test Sample, 3” | “ | 7.95 | 21 | 0.03 |
| Test Sample, 4” | “ | 7.96 | 22 | 0.02 |
| Test Sample, 5” | “ | 8.03 | 26 | -0.06 |
| Positive Controls (mean) | $4.13 \times 10^8$ | 7.98[a] | 10 – 57[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 14. Test 2, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 2<br>10.3 mg/L, 6-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $2.14 \times 10^8$ | 0 | 0 | 8.11 |
| Test Sample, 1” | “ | 4.60 | <1 | 3.51 |
| Test Sample, 2” | “ | 7.00 | 5 | 1.11 |
| Test Sample, 3” | “ | 7.32 | 10 | 0.79 |
| Test Sample, 4” | “ | 7.33 | 10 | 0.77 |
| Test Sample, 5” | “ | 7.85 | 33 | 0.26 |
| Positive Controls (mean) | $2.14 \times 10^8$ | 8.11[a] | 25 – 96[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $2.14 \times 10^8$ | 0 | 0 | 8.13 |
| Test Sample, 1” | “ | 0 | 0 | 8.13 |
| Test Sample, 2” | “ | 6.75 | 3 | 1.38 |
| Test Sample, 3” | “ | 7.03 | 5 | 1.10 |
| Test Sample, 4” | “ | 7.73 | 25 | 0.40 |
| Test Sample, 5” | “ | 7.75 | 26 | 0.38 |
| Positive Controls (mean) | $2.14 \times 10^8$ | 8.13[a] | 37 – 87[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $2.14 \times 10^8$ | 0 | 0 | 7.87 |
| Test Sample, 1” | “ | 0 | 0 | 7.87 |
| Test Sample, 2” | “ | 0 | 0 | 7.87 |
| Test Sample, 3” | “ | 5.82 | <1 | 2.05 |
| Test Sample, 4” | “ | 8.16 | 68 | -0.29 |
| Test Sample, 5” | “ | 8.27 | 88 | -0.40 |
| Positive Controls (mean) | $2.14 \times 10^8$ | 7.87[a] | 8 – 69[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 15. Test 3, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 3, saturated soils 8.9 mg/L, 3-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $6.67 \times 10^7$ | 0 | 0 | 7.55 |
| Test Sample, 0.5” | “ | 7.07 | 18 | 0.48 |
| Test Sample, 1.0” | “ | 7.24 | 26 | 0.30 |
| Test Sample, 1.5” | “ | 7.53 | 51 | 0.02 |
| Test Sample, 2.0” | “ | 6.20 | 2 | 1.35 |
| Test Sample, 2.5” | “ | 7.70 | 76 | -0.15 |
| Positive Controls (mean) | $6.67 \times 10^7$ | 7.55[a] | 3 – 236[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $6.67 \times 10^7$ | 0 | 0 | 6.59 |
| Test Sample, 0.5” | “ | 0 | 0 | 6.59 |
| Test Sample, 1.0” | “ | 3.79 | <1 | 2.79 |
| Test Sample, 1.5” | “ | 0 | 0 | 6.59 |
| Test Sample, 2.0” | “ | 0 | 0 | 6.59 |
| Test Sample, 2.5” | “ | 2.85 | <1 | 3.74 |
| Positive Controls (mean) | $6.67 \times 10^7$ | 6.59[a] | 1 – 114[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $6.67 \times 10^7$ | 0 | 0 | 7.18 |
| Test Sample, 0.5” | “ | 1.52 | <1 | 5.66 |
| Test Sample, 1.0” | “ | 0 | 0 | 7.18 |
| Test Sample, 1.5” | “ | 2.92 | <1 | 4.26 |
| Test Sample, 2.0” | “ | 0 | 0 | 7.18 |
| Test Sample, 2.5” | “ | 0 | 0 | 7.18 |
| Positive Controls (mean) | $6.67 \times 10^7$ | 7.18[a] | 4 – 138[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.

[b] Recovery range for five positive controls is reported. Low recovery possibly due to spore germination in freshly-autoclaved (moist) soil. Possible lab error also occurred during dilution of some positive controls, and some samples may be off by 1 log.

**Table 16. Test 4, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 4, saturated soils 10.1 mg/L, 24-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.20 \times 10^8$ | 0 | 0 | 8.14 |
| Test Sample, 1” | “ | 0 | 0 | 8.14 |
| Test Sample, 2” | “ | 6.62 | 3 | 1.52 |
| Test Sample, 3” | “ | 8.95 | 749 | -0.81 |
| Test Sample, 4” | “ | 8.89 | 652 | -0.75 |
| Test Sample, 5” | “ | 8.14 | 115 | 0.00 |
| Positive Controls (mean) | $1.20 \times 10^8$ | 8.14[a] | 17 – 315[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.20 \times 10^8$ | 0 | 0 | 7.62 |
| Test Sample, 1” | “ | 0 | 0 | 7.62 |
| Test Sample, 2” | “ | 0 | 0 | 7.62 |
| Test Sample, 3” | “ | 0 | 0 | 7.62 |
| Test Sample, 4” | “ | 0 | 0 | 7.62 |
| Test Sample, 5” | “ | 0 | 0 | 7.62 |
| Positive Controls (mean) | $1.20 \times 10^8$ | 7.62[a] | 25 – 58[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.20 \times 10^8$ | 0 | 0 | 0[c] |
| Test Sample, 1” | “ | 0 | 0 | 0[c] |
| Test Sample, 2” | “ | 0 | 0 | 0[c] |
| Test Sample, 3” | “ | 0 | 0 | 0[c] |
| Test Sample, 4” | “ | 0 | 0 | 0[c] |
| Test Sample, 5” | “ | 0 | 0 | 0[c] |
| Positive Controls (mean) | $1.20 \times 10^8$ | 0[a] | 0[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.
[c] Calculated as “0”, with 0% recovery for all positive controls and 0 seen for all test samples.

**Table 17. Test 5, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 5, saturated soils 9.3 mg/L, 24-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.90 \times 10^8$ | 0 | 0 | 7.87 |
| Test Sample, 1” | “ | 6.78 | 3 | 1.09 |
| Test Sample, 2” | “ | 7.99 | 52 | -0.12 |
| Test Sample, 3” | “ | 7.99 | 52 | -0.12 |
| Test Sample, 4” | “ | 8.04 | 58 | -0.17 |
| Test Sample, 5” | “ | 7.85 | 37 | 0.02 |
| Positive Controls (mean) | $1.90 \times 10^8$ | 7.87[a] | 27 – 56[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.90 \times 10^8$ | 0 | 0 | 8.41 |
| Test Sample, 1” | “ | 0 | 0 | 8.41 |
| Test Sample, 2” | “ | 0 | 0 | 8.41 |
| Test Sample, 3” | “ | 3.52 | <1 | 4.88 |
| Test Sample, 4” | “ | 4.22 | <1 | 4.19 |
| Test Sample, 5” | “ | 7.76 | 30 | 0.65 |
| Positive Controls (mean) | $1.90 \times 10^8$ | 8.41[a] | 116 – 165[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.90 \times 10^8$ | 0 | 0 | 8.17 |
| Test Sample, 1” | “ | 0 | 0 | 8.17 |
| Test Sample, 2” | “ | 0 | 0 | 8.17 |
| Test Sample, 3” | “ | 0 | 0 | 8.17 |
| Test Sample, 4” | “ | 0 | 0 | 8.17 |
| Test Sample, 5” | “ | 0 | 0 | 8.17 |
| Positive Controls (mean) | $1.90 \times 10^8$ | 8.17[a] | 46 – 107[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 18. Test 6, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extraction Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 6, saturated soils 14.6 mg/L, 27.5-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $4.40 \times 10^8$ | 0 | 0 | 8.42 |
| Test Sample, 1” | “ | 0 | 0 | 8.42 |
| Test Sample, 2” | “ | 7.77 | 13 | 0.65 |
| Test Sample, 3” | “ | 8.01 | 23 | 0.41 |
| Test Sample, 4” | “ | 8.02 | 24 | 0.40 |
| Test Sample, 5” | “ | 7.97 | 21 | 0.45 |
| Positive Controls (mean) | $4.40 \times 10^8$ | 8.42[a] | 47 – 81[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $4.40 \times 10^8$ | 0 | 0 | 8.05 |
| Test Sample, 1” | “ | 0 | 0 | 8.05 |
| Test Sample, 2” | “ | 0 | 0 | 8.05 |
| Test Sample, 3” | “ | 0 | 0 | 8.05 |
| Test Sample, 4” | “ | 0 | 0 | 8.05 |
| Test Sample, 5” | “ | 0 | 0 | 8.05 |
| Positive Controls (mean) | $4.40 \times 10^8$ | 8.05[a] | 18 – 35[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $4.40 \times 10^8$ | 0 | 0 | 7.82 |
| Test Sample, 1” | “ | 0 | 0 | 7.82 |
| Test Sample, 2” | “ | 0 | 0 | 7.82 |
| Test Sample, 3” | “ | 0 | 0 | 7.82 |
| Test Sample, 4” | “ | 0 | 0 | 7.82 |
| Test Sample, 5” | “ | 0 | 0 | 7.82 |
| Positive Controls (mean) | $4.40 \times 10^8$ | 7.82[a] | 10 – 23[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 19. Test 7, $ClO_2$ Decontamination Results**

| Test ID, summary | Non-Soil Extract. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 7, compacted soils 9.4 mg/L, 7.75-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.93 \times 10^8$ | 0 | 0 | 7.77 |
| Test Sample, 1” | “ | 8.09 | 63 | -0.32 |
| Test Sample, 2” | “ | 8.03 | 56 | -0.26 |
| Test Sample, 3” | “ | 8.02 | 54 | -0.25 |
| Test Sample, 4” | “ | 7.96 | 47 | -0.19 |
| Test Sample, 5” | “ | 7.93 | 44 | -0.16 |
| Positive Controls (mean) | $1.93 \times 10^8$ | 7.77[a] | 19 – 47[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.93 \times 10^8$ | 0 | 0 | 8.06 |
| Test Sample, 1” | “ | 0 | 0 | 8.06 |
| Test Sample, 2” | “ | 0 | 0 | 8.06 |
| Test Sample, 3” | “ | 3.82 | 0 | 4.24 |
| Test Sample, 4” | “ | 6.08 | 1 | 1.98 |
| Test Sample, 5” | “ | 7.67 | 24 | 0.40 |
| Positive Controls (mean) | $1.93 \times 10^8$ | 8.06[a] | 54 – 66[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.93 \times 10^8$ | 0 | 0 | 7.26 |
| Test Sample, 1” | “ | 0 | 0 | 7.26 |
| Test Sample, 2” | “ | 0 | 0 | 7.26 |
| Test Sample, 3” | “ | 0 | 0 | 7.26 |
| Test Sample, 4” | “ | 6.10 | 1 | 1.16 |
| Test Sample, 5” | “ | 7.64 | 23 | -0.39 |
| Positive Controls (mean) | $1.93 \times 10^8$ | 7.26[a] | 7 – 16[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

## 6.3 Chlorine Dioxide Log Reduction Charts

Figures 3 through Figure 9 show results for the $ClO_2$ tests in graphical form. These LR charts show the same "decontamination efficacy" data that were presented earlier in Section 6.2, but in a visual format.

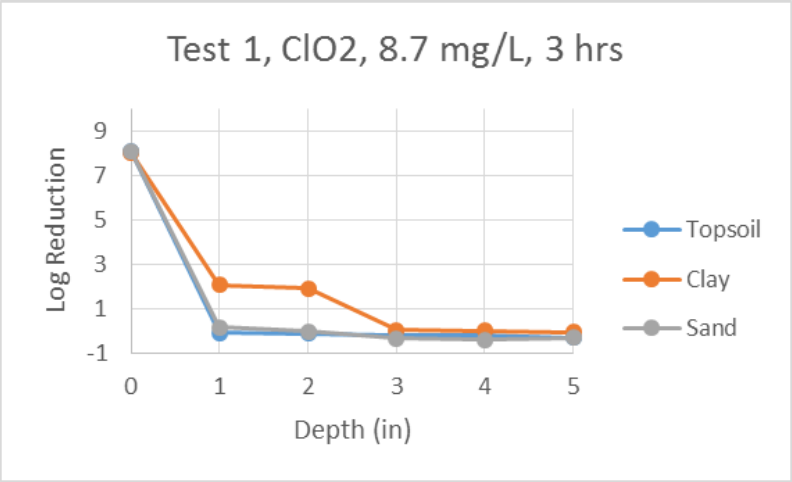


**Figure 3. Test 1, $ClO_2$: 8.7 mg/L, 3 hrs, 77% RH.**

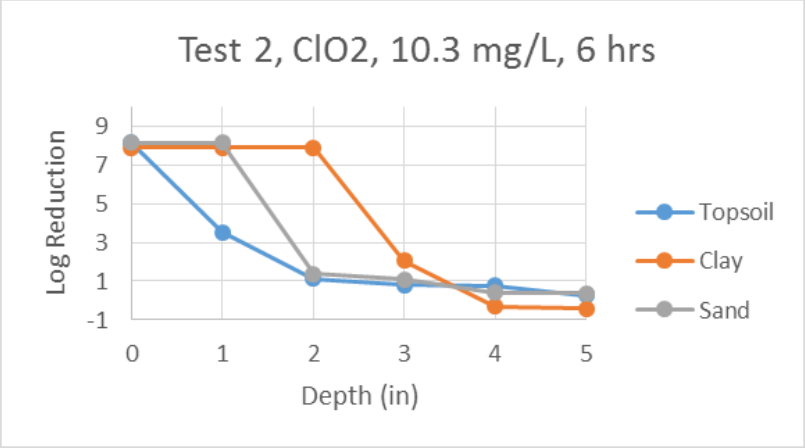


**Figure 4. Test 2, $ClO_2$: 10.3 mg/L, 6 hrs, 80% RH**

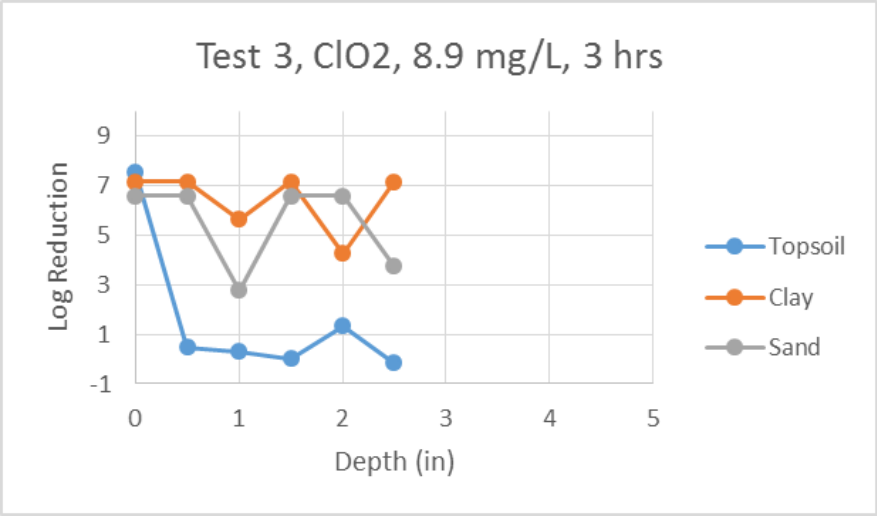


**Figure 5. Test 3, $ClO_2$: 8.9 mg/L, 3 hrs, 74% RH, [saturated soil]**

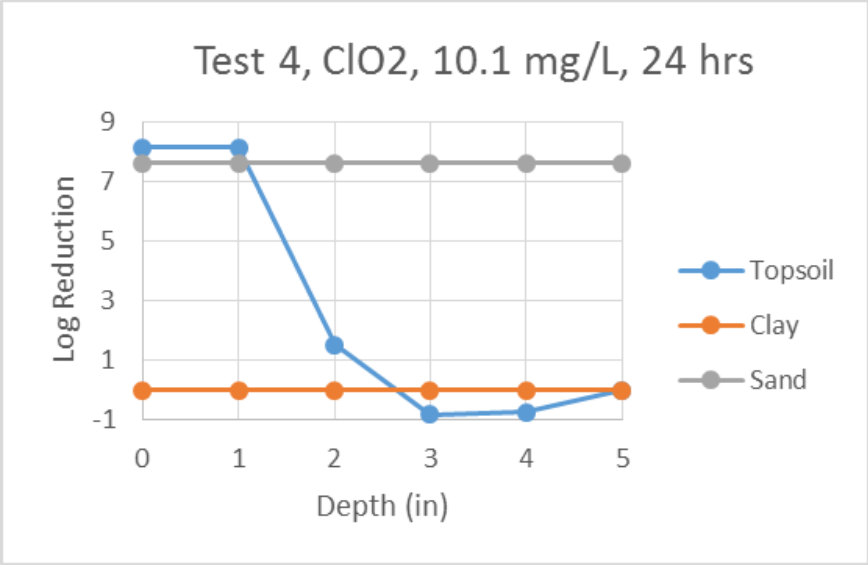


**Figure 6. Test 4, $ClO_2$: 10.1 mg/L, 24 hrs, 81% RH**

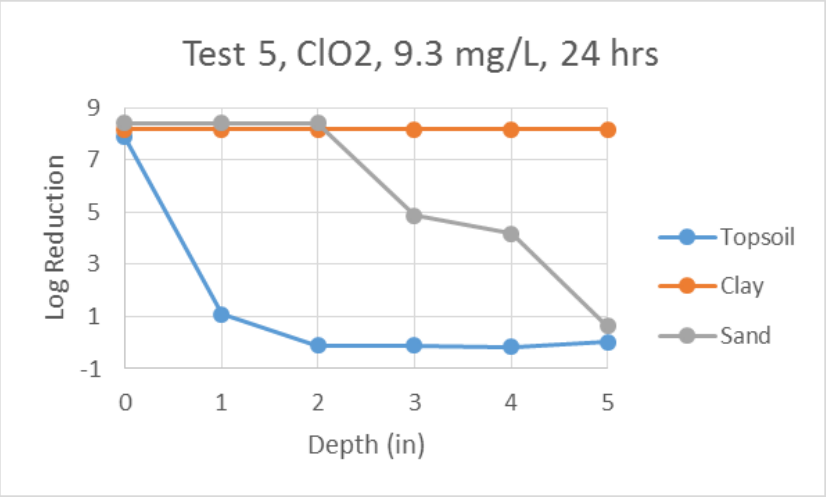


**Figure 7. Test 5, $ClO_2$: 9.3 mg/L, 24 hrs, 80% RH, [saturated soil]**

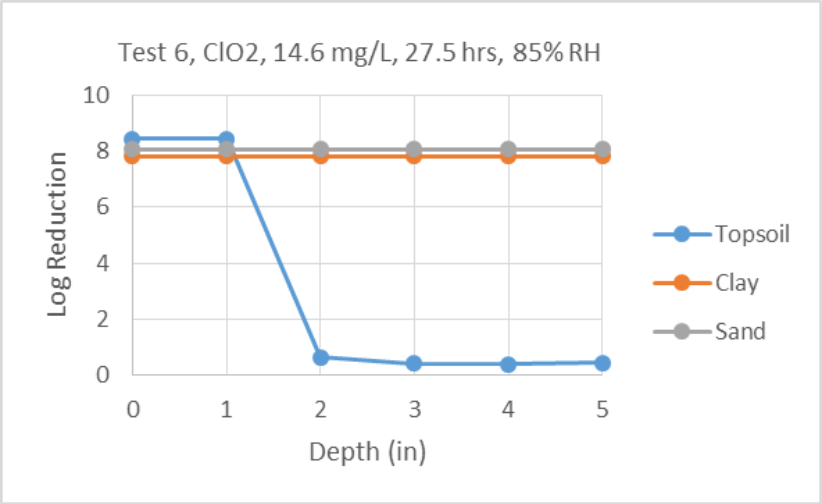


**Figure 8. Test 6, $ClO_2$: 14.6 mg/L, 24 hrs, 83% RH**

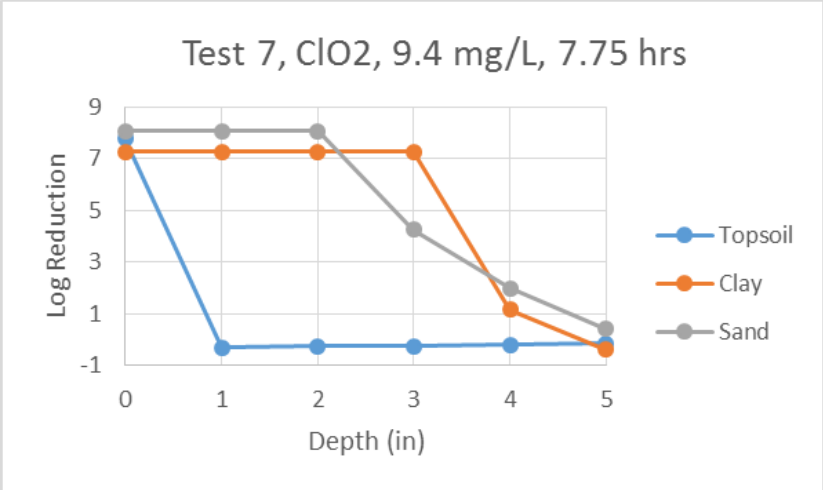


**Figure 9. Test 7, $ClO_2$: 9.4 mg/L, 7.75 hrs, 86% RH, [compacted soil]**

# Section 7.
# Results and Performance Summary for Sodium Persulfate

## 7.1 Sodium Persulfate Test Conditions

The actual conditions measured for the SP test series (three tests) are presented in Table 20. Highlights of each test are given in the subsections below. Table 20 also includes summary information on decontamination efficacy, in terms of the maximum depth at which ≥ 6 LR was achieved for each soil type for each test.

**Table 20. Sodium Persulfate Test Conditions**

| Test No. | Depth(s) Tested | SP Conc. (mg/L) | Avg. T (°C) | Avg. RH (%) | Contact Time (hrs) | Other Application Conditions | | | | Max depth (inches) achieving ≥ 6 LR |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Soil | Vol/Qty per dose | No. of Doses | Interval | |
| 1 | 0, 1, 2, 3, 4, 5” | 0.5 M, 50/50 with 8% $H_2O_2$ | 21.5 ± 0.70 | 92.9 ± 7.23 | 168 (7 days) | Topsoil | 1390 mL; 0.177 mL/g | 2 | 48-hr | 0 |
| | | | | | | Sand | 1390 mL; 0.155 mL/g | 2 | 48-hr | 1 |
| | | | | | | Clay | 1390 mL; 0.159 mL/g | 2 | 48-hr | 5 |
| | Positive Controls | NA | 23.7 ± 0.49 | 26.0 ± 13.68 | [a] | NA | NA | NA | NA | |
| 2 | 0.5, 1.0, 1.5, 2.0, 2.5” (topsoil) 0, 1, 2, 3, 4, 5” (sand, clay) | 0.5 M, 50/50 with 8% $H_2O_2$ | 22.0 ± 0.62 | 91.3 ± 9.04 | 144 (6 days) | Topsoil | 702 mL; 0.09 mL/g | 6 | 24-hr | 0.5 |
| | | | | | | Sand | 820 mL; 0.09 mL/g | 6 | 24-hr | 1 |
| | | | | | | Clay | 792 mL; 0.09 mL/g | 2 | 24-hr | 5 |
| | Positive Controls | NA | 23.8 ± 0.35 | 91.5 ± 2.22 | [a] | NA | NA | NA | NA | |
| 3 | 0.5, 1.0, 1.5, 2.0, 2.5” (topsoil) 0, 1, 2, 3, 4, 5” (sand, clay) | 1.0 M, 50/50 with 8% $H_2O_2$ | 22.6 ± 0.74 | 91.9 ± 8.70 | 168 (7 days) | Topsoil | 681 mL; 0.09 mL/g | 6 | 24-hr | 1 |
| | | | | | | Sand | 876 mL; 0.09 mL/g | 6 | 24-hr | 0 |
| | | | | | | Clay | 820 mL; 0.09 mL/g | 1 | NA | 4 |
| | Positive Controls | NA | 24.5 ± 0.47 | 94.3 ± 0.16 | [a] | NA | NA | NA | NA | |

[a] Positive controls had no decontaminant applied, but were collected and extracted at the end of the contact time for the test samples.

### 7.1.1 Test 1 SP

Target test conditions were two applications of 0.5 M SP (activated by 50/50 mixture with 8% hydrogen peroxide) separated by 48-hrs, with an overall contact time of 168 hrs (seven days). Each application was intended to provide wetting down to a 6" depth. A 1390 mL liquid volume was used for all soil types, making actual wetting levels of 0.177 mL/g (topsoil), 0.155 mL/g (sand), and 0.159 mL/g (clay) per application.

The SP solution reacted vigorously with the topsoil and created large volumes of foam. The foaming reaction with sand was minimal but still present. The sand also tended to become more of a slurry as the liquid was applied, causing buried CSPs to rise to nearer the surface, rather than remaining at their intended depths. Clay soils did not show any foaming reaction when the SP was applied.

Results showed that the decontamination had a 1 LR at 1" for the topsoil column, then tapered off to no effect. The sand column showed complete kill (7 LR) at 1". The clay column had complete kill (7 LR) at all depths.

### 7.1.2 Test 2 SP

Based on the results from Test 1 SP, this test was designed to increase the amount of activated SP for topsoil and sandy soil, but reduce the amount for clay. In addition, we hoped that smaller (but more and frequent) doses of SP for sand and topsoil would reduce the amount of foaming or reactivity. Target test conditions were increased to six applications of 0.5 M SP (activated by 50/50 mixture with 8% hydrogen peroxide) separated by 24-hrs, with an overall contact time of 168 hrs (seven days) for topsoil and sand. Given the success with clay in Test 1 SP, only two applications were performed. The dose or application volumes were reduced to 0.09 mL/g. For topsoil, CSPs were placed at 0.5-in depths.

The SP solution reactions with topsoil, sand, and clay were as described above for Test 1 SP. Due to the smaller application volumes, however, foaming was better controlled. The increased total liquid volumes for sand and topsoil for this test caused spent SP solution to begin emerging from the column bottoms during or after the third application.

Results showed that the decontamination had improved somewhat for the topsoil, which had complete kill (7 LR) at 0.5", then tapered off to no effect. The sand column showed complete kill (7 LR) at 1", then tapered off to no effect. Even with less SP applied, the clay column had complete kill (8 LR) at all depths.

### 7.1.3 Test 3 SP

This test was designed to improve efficacy by increasing the concentration of the SP. Target test conditions were six applications of 1.0 M SP (activated by 50/50 mixture with 8% hydrogen peroxide) separated by 24-hrs, with an overall contact time of 168 hrs (seven days) for topsoil and sand. Given the success with clay in Test 2 SP, only one application was performed. As with Test 2 SP, to reduce the problems with foaming, the application volumes were reduced to

0.09 mL/g, with liquid volumes being calculated from the soil density measured for the test. For topsoil, CSPs were placed at 0.5-in depths, rather than the 1-in depth used previously.

The SP solution reactions with topsoil, sand, and clay were as described above for Test 1 and 2 SP. Due to the smaller application volumes, foaming was again better controlled, but believed contributed to the recovery of spores from the 0” CSP for topsoil.

Spent SP solution that emerged from the column bottoms was evaluated in a more subjective manner during this test, with volumes being estimated and oxidation reduction potential (ORP) measurements being taken. See Appendix E for further details.

Results showed that the decontamination had complete kill (7 LR) down to 1” for the topsoil column, then dropped off to no effect. The sand column showed a 4 LR at 1”, then tapered off to no effect. The clay column had complete kill (7 LR) at all depths, except at 5”, which had a 1 LR.

## 7.2 Sodium Persulfate Decontamination Results

Table 21 through Table 23 show the detailed results for the decontamination of BA spores in soil using activated SP.

**Table 21. Test 1, SP Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 1 | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $2.23 \times 10^8$ | 0 | 0 | 7.29 |
| Test Sample, 1” | “ | 5.97 | < 1 | 1.32 |
| Test Sample, 2” | “ | 7.64 | 20 | -0.35 |
| Test Sample, 3” | “ | 7.53 | 15 | -0.24 |
| Test Sample, 4” | “ | 7.61 | 18 | -0.31 |
| Test Sample, 5” | “ | 7.33 | 24 | -0.44 |
| Positive Controls (mean) | $2.23 \times 10^8$ | 7.29[a] | 6 – 21[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $2.23 \times 10^8$ | 0 | 0 | 7.75 |
| Test Sample, 1” | “ | 0 | 0 | 7.75 |
| Test Sample, 2” | “ | 5.95 | < 1 | 1.80 |
| Test Sample, 3” | “ | 6.98 | 4 | 0.77 |
| Test Sample, 4” | “ | 7.05 | 5 | 0.70 |
| Test Sample, 5” | “ | 7.68 | 21 | 0.07 |
| Positive Controls (mean) | $2.23 \times 10^8$ | 7.75[a] | 18 – 36[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $2.23 \times 10^8$ | 0 | 0 | 7.14 |
| Test Sample, 1” | “ | 0 | 0 | 7.14 |
| Test Sample, 2” | “ | 0 | 0 | 7.14 |
| Test Sample, 3” | “ | 0 | 0 | 7.14 |
| Test Sample, 4” | “ | 0 | 0 | 7.14 |
| Test Sample, 5” | “ | 0 | 0 | 7.14 |
| Positive Controls (mean) | $2.23 \times 10^8$ | 7.14[a] | 5 – 8[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 22. Test 2, SP Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 2 | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $2.63 \times 10^8$ | 0 | 0 | 7.91 |
| Test Sample, 0.5” | “ | 0 | 0 | 7.91 |
| Test Sample, 1.0” | “ | 6.59 | 1 | 1.31 |
| Test Sample, 1.5” | “ | 7.50 | 12 | 0.40 |
| Test Sample, 2.0” | “ | 8.08 | 46 | -0.18 |
| Test Sample, 2.5” | “ | 8.27 | 70 | -0.36 |
| Positive Controls (mean) | $2.63 \times 10^8$ | 7.91[a] | 27 – 37[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $2.63 \times 10^8$ | 0 | 0 | 7.61 |
| Test Sample, 1” | “ | 0 | 0 | 7.61 |
| Test Sample, 2” | “ | 6.66 | 2 | 0.96 |
| Test Sample, 3” | “ | 4.00 | 0 | 3.61 |
| Test Sample, 4” | “ | 7.71 | 20 | -0.10 |
| Test Sample, 5” | “ | 5.98 | 0 | 1.63 |
| Positive Controls (mean) | $2.63 \times 10^8$ | 7.61[a] | 7 – 22[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $2.63 \times 10^8$ | 0 | 0 | 8.16 |
| Test Sample, 1” | “ | 0 | 0 | 8.16 |
| Test Sample, 2” | “ | 0 | 0 | 8.16 |
| Test Sample, 3” | “ | 0 | 0 | 8.16 |
| Test Sample, 4” | “ | 0 | 0 | 8.16 |
| Test Sample, 5” | “ | 0 | 0 | 8.16 |
| Positive Controls (mean) | $2.63 \times 10^8$ | 8.16[a] | 46 – 65[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 23. Test 3, SP Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 3 | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $3.87 \times 10^8$ | 4.29 | < 1 | 3.51 |
| Test Sample, 0.5” | “ | 0 | 0 | 7.80 |
| Test Sample, 1.0” | “ | 0 | 0 | 7.80 |
| Test Sample, 1.5” | “ | 7.91 | 39 | -0.11 |
| Test Sample, 2.0” | “ | 7.98 | 45 | -0.18 |
| Test Sample, 2.5” | “ | 8.15 | 67 | -0.35 |
| Positive Controls (mean) | $3.87 \times 10^8$ | 7.80[a] | 20 – 47[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $3.87 \times 10^8$ | 0 | 0 | 7.78 |
| Test Sample, 1” | “ | 2.82 | < 1 | 4.96 |
| Test Sample, 2” | “ | 3.98 | < 1 | 3.80 |
| Test Sample, 3” | “ | 4.82 | < 1 | 2.97 |
| Test Sample, 4” | “ | 7.10 | 6 | 0.69 |
| Test Sample, 5” | “ | 7.97 | 44 | -0.19 |
| Positive Controls (mean) | $3.87 \times 10^8$ | 7.78[a] | 21 – 37[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $3.87 \times 10^8$ | 0 | 0 | 7.88 |
| Test Sample, 1” | “ | 0 | 0 | 7.88 |
| Test Sample, 2” | “ | 0 | 0 | 7.88 |
| Test Sample, 3” | “ | 0 | 0 | 7.88 |
| Test Sample, 4” | “ | 0 | 0 | 7.88 |
| Test Sample, 5” | “ | 6.55 | 2 | 1.33 |
| Positive Controls (mean) | $3.87 \times 10^8$ | 7.88[a] | 18 – 61[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

## 7.3 Sodium Persulfate Log Reduction Charts

Figure 10 through Figure 12 show results for the SP tests in graphical form. This "decontamination efficacy" is the same (as log reduction, or LR) data that were presented earlier in Section 7.2, but in a visual format.

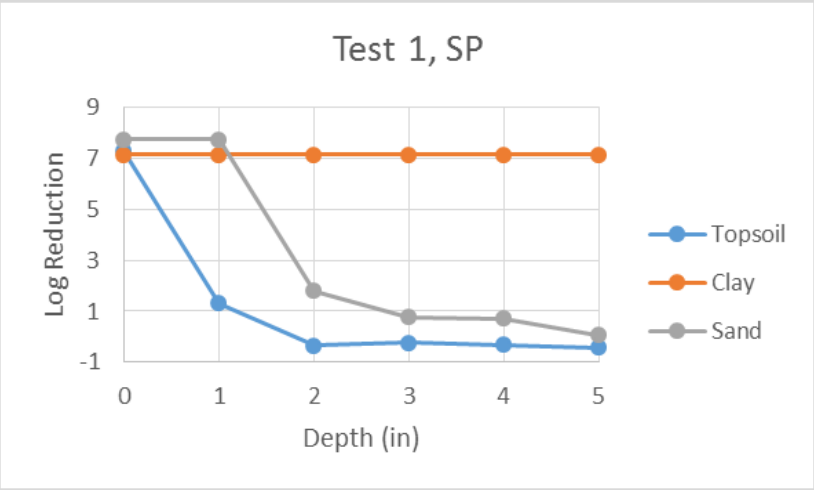


**Figure 10. Test 1, SP: 0.5 M, 0.16-0.18 mL/g, 2 applications**

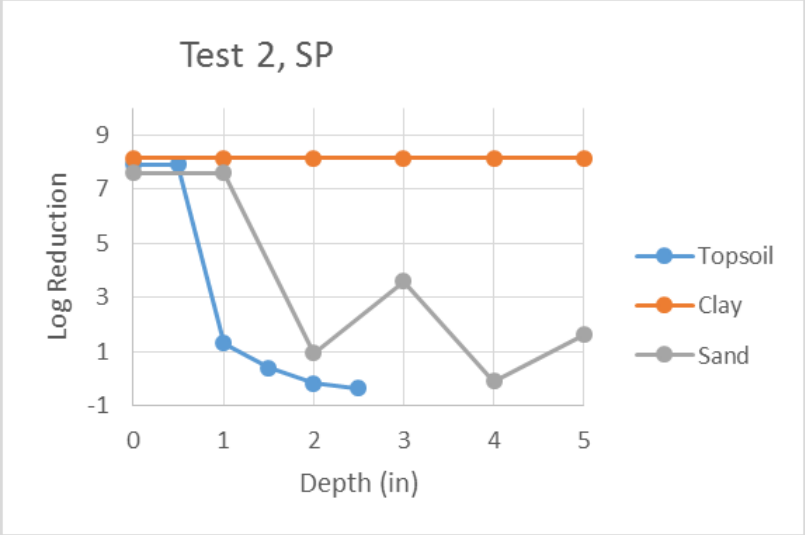


**Figure 11. Test 2, SP: 0.5 M, 0.09 mL/g, 6 applications (topsoil, sand), 2 applications (clay)**

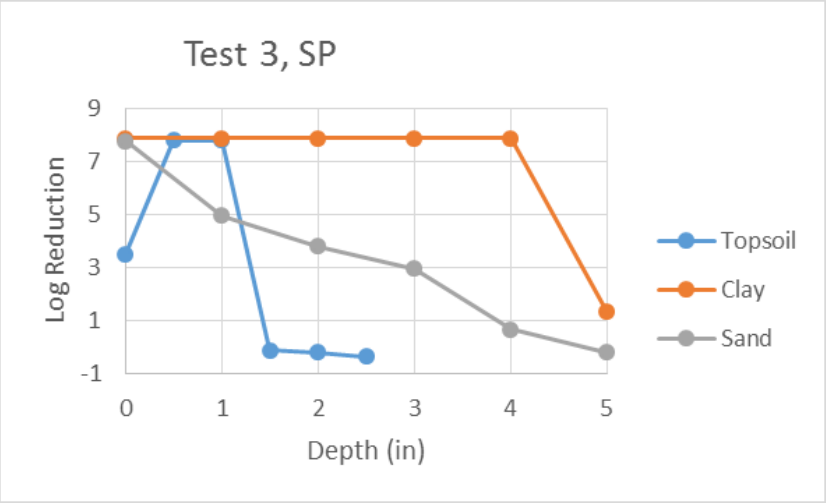


**Figure 12. Test 3, SP: 1.0 M, 0.09 mL/g, 6 applications (topsoil, sand), 1 application (clay)**

# Section 8.
# Results and Performance Summary for Methyl Bromide

## 8.1 Methyl Bromide Test Conditions

The actual conditions measured for the MeBr test series are presented in Table 24. Highlights of each test are given in the subsections below. Table 24 also includes summary information on decontamination efficacy, in terms of the maximum depth at which ≥ 6 LR was achieved for each soil type for each test.

**Table 24. Methyl Bromide Test Conditions**

| Test No. | Depth(s) Tested | Methyl Bromide Conc. (mg/L) | Avg. T (°C) | Avg. RH (%) | Contact Time (hrs) | No. of Samples[a] | Other Conditions | Max depth (inches) achieving ≥ 6 LR topsoil | Max depth (inches) achieving ≥ 6 LR clayey | Max depth (inches) achieving ≥ 6 LR sandy |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0, 1, 2, 3, 4, 5” | 236 ± 14.9 | 19.7 ± 0.11 | 78.0 ± 3.26 | 48 | TC=5<br>CC=5<br>NCTC=1<br>NCCC=1 | None | None | None | None |
| | Positive Controls | NA | 25.3 ± 0.17 | 93.3 ± 1.93 | [c] | | | | | |
| 2 | 0, 1, 2, 3, 4, 5” | 224 ± 11.5 | 20.0 ± 0.21 | 77.3 ± 2.68 | 65.5 (48)[b] | TC=5<br>CC=5<br>NCTC=1<br>NCCC=1 | None | None | None | 0 |
| | Positive Controls | NA | 24.8 ± 0.15 | 93.6 ± 1.21 | [c] | | | | | |
| 3 | 0, 1, 2, 3, 4, 5” | 325 ± 33.2 | 19.9 ± 0.08 | 76.3 ± 3.27 | 48 | TC=5<br>CC=5<br>NCTC=1<br>NCCC=1 | None | 0 | None | 0 |
| | Positive Controls | NA | 26.4 ± 1.80 | 94.3 ± 0.05 | [c] | | | | | |
| 4 | 0, 1, 2, 3, 4, 5” | 230 ± 16.8 | 20.1 ± 0.14 | 78.7 ± 3.43 | 48 | TC=5<br>CC=5<br>NCTC=1<br>NCCC=1 | Saturated soil | None | None | None |
| | Positive Controls | NA | 26.4 ± 0.58 | 94.3 ± 0.31 | [c] | | | | | |

[a] Per soil type.
[b] One 0” sample of each soil type was also removed at 48 hrs.
[c] Positive controls had no decontaminant applied, but were collected and extracted at the end of the contact time for the test samples.
TC = Test Column.
CC = Positive Control Column.
NCTC – Negative Control, Test Column.
NCCC – Negative Control, Control Column. “None” implies that no CSPs inactivated ≥ 6 LR, including CSPs on top of column (0 inch)

### 8.1.1 Test 1 MeBr

Target MeBr concentration was 212 mg/L and target RH was ≥75% for a 48-hr contact time.

Results from the first MeBr test showed that the decontamination had virtually no effect for the topsoil and clay columns, and the sand column had only a 1-2 LR. Furthermore, the 0" samples were not killed by direct exposure to the MeBr, but instead had decontamination levels nearly matching those of the buried samples. We were expecting that the samples on top of the soil surfaces would be effectively decontaminated, based on previous tests with MeBr [3, 5]. (Although we acknowledge that while the CSPs on the soil surfaces did not have soil depth to impact results, the CSPs were comprised of 1-gram of soil and this may have affected results.) Since we achieved only minimal inactivation of spores using MeBr at fumigation conditions which were effective in previous tests, this prompted us to investigate the cause of this discrepancy.

A visual examination of the agar plates showed that the morphology of the BA CFU was slightly different from previous tests, in that the colonies were clearly smaller in size. Figure 13 shows an example of the agar plates for this experiment. Because of this, initially we considered the possibility that our poor results with MeBr were due to inadvertently using an organism other than BA. The photo on the left is the MeBr exposed plate at the $1 \times 10^{-2}$ dilution, and the one on the right is the unexposed control sample plate at the $1 \times 10^{-7}$ dilution. Follow-up plating of a second generation cultured from the colonies on the agar plate pictured on the left side of Figure 13 revealed that the morphology returned to normal, indicating that the spores were being affected by the MeBr, but had not been actually killed at the time of the test ending (i.e. 48-hrs). Another explanation is that the plate on the left has ~50X as many CFU, thus competitive inhibition could explain the smaller colony size initially.

As an added check to provide further evidence to confirm we were in fact using our target organism, a second PCR analysis again showed the presence of the pX01 and pX02 plasmids in the affected morphology samples, identical to the results seen at the beginning of the study (Appendix A). The presence of both plasmids confirms a virulent strain of BA, although not necessarily the Ames strain. Refer to Section 3.1 and Appendix A for further documentation regarding the BA used in this study. The BA (Ames) used in this study originated from a stock obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository.

Additional discussions between the PI and members of the project team confirmed that the equipment used for taking measurements of temperature, RH, and MeBr concentration was functioning properly, and that there was no reason to suspect faulty data for these parameters. There are several other possibilities that may explain the difference in efficacy results between the present study and previous studies, but due to scheduling and budgetary constraints, we were unable to further investigate these. The differences in results may be due (but not limited to) to differences in microbiological methods, different methods to measure temperature, RH, and MeBr concentration; different laboratory personnel; or different BA Ames strain.


**Figure 13. BA Morphology Changes after Exposure to MeBr (left) vs Unexposed (right).**

### 8.1.2 Test 2 MeBr

Target MeBr concentration was 212 mg/L and target RH was ≥75%, with a target exposure time increased to 72 hours. The actual exposure time for this test was 65.5, due to miscommunication between lab staff. In an effort to better characterize the unexpected results from Test 1 MeBr, an additional set of 0" samples were generated for this test. One set of 0" samples was removed at the 48-hr contact time (as was done in Test 1), while the second set of 0" samples remained in place until the test end.

Results showed a measurable increase in efficacy between the 48-hr and 65.5-hr results. As had occurred in Test 1 MeBr, none of the 0" samples were completely inactivated at the 48-hr time. For the 65.5-hr time, sand 0" samples were completely inactivated, topsoil 0" depth samples still contained BA colonies, but were below quantitation limits (< 30 counts at the lowest dilution), and clay 0" samples were reduced by only a 3.5 LR. As with Test 1 MeBr results, efficacy seemed to be generally independent of depth of soil, suggesting that penetration of the MeBr gas through the soils was not a limiting factor.

While the 0" depth was the only set of paired samples allowing direct comparison for the 48-hr and 65.5-hr points, a general comparison can be made to the 48-hr results from Test 1 MeBr. The Test 1 MeBr results showed only a 1-2 LR for all soils types at all depths, while the Test 2 MeBr results were typically a 3-4 LR at all depths. Again note that several of the topsoil samples and one of the sand samples were below CFU quantitation limits (< 30 counts at the highest dilution), but were not completely inactivated. A visual examination of the agar plates showed that the morphology of the colonies for Test 2 MeBr essentially matched the morphology of Test 1 MeBr at both the 48-hr and 65.5-hr time periods.

### 8.1.3 Test 3 MeBr

In Test 3 MeBr, the target MeBr concentration was increased to 300 mg/L and target RH was ≥75% for a 48-hr contact time. The increased MeBr concentration improved efficacy (compared to Test 1 MeBr), but very few samples were effectively decontaminated.

The topsoil sample at a 1” depth was below quantitation limits. Other depths showed a 3-4 LR. Sand samples were reduced by 4 LR at all depths. Clay samples were reduced by 2-3 LR at all depths. Morphology of the colonies essentially matched the morphology of the previous MeBr tests.

### 8.1.4 Test 4 MeBr

Target MeBr concentration was 212 mg/L and target RH was ≥75% for a 48-hr contact time. After constructing the test columns, soils were wetted to their saturation point as had been done for Test 3 $ClO_2$ and Test 5 $ClO_2$ described earlier, to elicit this effect.

Similar to Test 1 MeBr, results showed that the 0” samples (topsoil, sand, and clay) still contained BA colonies, and were reduced by < 1 LR. Water-saturated soil did not increase the decontamination efficacy for the buried samples, as results were essentially the same as, or even slightly worse than, the 0” samples. Results for this test were similar to results for Test 1 MeBr, further suggesting that pre-wetting of soils does not improve efficacy.

## 8.2 Methyl Bromide Decontamination Results

Table 25 through Table 28 show detailed results for the decontamination of BA spores in soil using MeBr.

**Table 25. Test 1, MeBr Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 1 | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.16 \times 10^8$ | 6.49 | 3 | 1.22 |
| Test Sample, 1” | “ | 6.44 | 2 | 1.26 |
| Test Sample, 2” | “ | 6.81 | 6 | 0.90 |
| Test Sample, 3” | “ | 7.00 | 9 | 0.70 |
| Test Sample, 4” | “ | 6.82 | 6 | 0.88 |
| Test Sample, 5” | “ | 6.67 | 4 | 1.03 |
| Positive Controls (mean) | $1.16 \times 10^8$ | 7.70[a] | 35 – 53[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.16 \times 10^8$ | 6.72 | 4 | 1.35 |
| Test Sample, 1” | “ | 6.28 | 2 | 1.78 |
| Test Sample, 2” | “ | 5.66 | < 1 | 2.40 |
| Test Sample, 3” | “ | 6.86 | 6 | 1.21 |
| Test Sample, 4” | “ | 6.72 | 4 | 1.34 |
| Test Sample, 5” | “ | 6.66 | 4 | 1.40 |
| Positive Controls (mean) | $1.16 \times 10^8$ | 8.06[a] | 87 – 113[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.16 \times 10^8$ | 6.91 | 7 | 0.61 |
| Test Sample, 1” | “ | 6.90 | 7 | 0.62 |
| Test Sample, 2” | “ | 6.83 | 6 | 0.69 |
| Test Sample, 3” | “ | 6.92 | 7 | 0.60 |
| Test Sample, 4” | “ | 6.68 | 4 | 0.84 |
| Test Sample, 5” | “ | 6.87 | 6 | 0.65 |
| Positive Controls (mean) | $1.16 \times 10^8$ | 7.52[a] | 19 – 42[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

**Table 26. Test 2, MeBr Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 2: 224 mg/L, 65.5-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0" (48-hr) | $1.01 \times 10^8$ | 6.23 | 2 | 1.09 |
| Test Sample, 0" (65.5-hr) | " | 2.52[c] | <1 [c] | 4.80[c] |
| Test Sample, 1" | " | 3.52[c] | < 1[c] | 3.80[c] |
| Test Sample, 2" | " | 3.82 | < 1 | 3.51 |
| Test Sample, 3" | " | 2.82[c] | < 1[c] | 4.51[c] |
| Test Sample, 4" | " | 5.52[c] | < 1[c] | 1.80[c] |
| Test Sample, 5" | " | 4.08 | < 1 | 3.24 |
| Positive Controls (mean) | $1.01 \times 10^8$ | 7.32[a] | 19 – 23[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0" (48-hr) | $1.01 \times 10^8$ | 6.72 | 6 | 1.22 |
| Test Sample, 0" (65.5-hr) | " | 0 | 0 | 7.94 |
| Test Sample, 1" | " | 3.63 | < 1 | 4.30 |
| Test Sample, 2" | " | 3.66 | < 1 | 4.28 |
| Test Sample, 3" | " | 3.63 | < 1 | 4.31 |
| Test Sample, 4" | " | 3.82[c] | < 1[c] | 4.11[c] |
| Test Sample, 5" | " | 3.61 | < 1 | 4.33 |
| Positive Controls (mean) | $1.01 \times 10^8$ | 7.94[a] | 84 – 97[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0" (48-hr) | $1.01 \times 10^8$ | 6.87 | 7 | 0.36 |
| Test Sample, 0" (65.5-hr) | " | 3.71 | < 1 | 3.52 |
| Test Sample, 1" | " | 4.10 | < 1 | 3.13 |
| Test Sample, 2" | " | 4.10 | < 1 | 3.13 |
| Test Sample, 3" | " | 3.59 | < 1 | 3.64 |
| Test Sample, 4" | " | 4.20 | < 1 | 3.03 |
| Test Sample, 5" | " | 4.14 | < 1 | 3.09 |
| Positive Controls (mean) | $1.01 \times 10^8$ | 7.23[a] | 13 – 19[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.
[c] One or more plates of sample was below quantitation limit (< 30 CFU) at no dilution.

**Table 27. Test 3, MeBr Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 1: 325 mg/L, 48-hr | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.27 \times 10^8$ | 0 | 0 | 7.36 |
| Test Sample, 1” | “ | 3.52[c] | 0[c] | 3.84[c] |
| Test Sample, 2” | “ | 3.52 | < 1 | 3.84 |
| Test Sample, 3” | “ | 3.78 | < 1 | 3.58 |
| Test Sample, 4” | “ | 3.54 | < 1 | 3.82 |
| Test Sample, 5” | “ | 3.72 | < 1 | 3.64 |
| Positive Controls (mean) | $1.27 \times 10^8$ | 7.36[a] | 13 – 27[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.27 \times 10^8$ | 0 | 0 | 7.98 |
| Test Sample, 1” | “ | 3.64 | < 1 | 4.35 |
| Test Sample, 2” | “ | 3.83 | < 1 | 4.15 |
| Test Sample, 3” | “ | 3.97 | < 1 | 4.01 |
| Test Sample, 4” | “ | 3.98 | < 1 | 4.00 |
| Test Sample, 5” | “ | 3.94 | < 1 | 4.04 |
| Positive Controls (mean) | $1.27 \times 10^8$ | 7.98[a] | 70 – 83[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.27 \times 10^8$ | 4.54 | < 1 | 2.60 |
| Test Sample, 1” | “ | 4.39 | < 1 | 2.75 |
| Test Sample, 2” | “ | 4.72 | < 1 | 2.42 |
| Test Sample, 3” | “ | 4.62 | < 1 | 2.52 |
| Test Sample, 4” | “ | 4.18 | < 1 | 2.96 |
| Test Sample, 5” | “ | 3.91 | < 1 | 3.23 |
| Positive Controls (mean) | $1.27 \times 10^8$ | 7.14[a] | 9 – 16[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.
[c] One or more plates of sample was below quantitation limit (< 30 CFU) at no dilution.

**Table 28. Test 4, MeBr Decontamination Results**

| Test ID, summary | Non-Soil Ext. Control (CFU) | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Ext. Control) | Decontamination Efficacy |
|---|---|---|---|---|
| Test 4: 230 mg/L, 48-hr, saturated soil | | | | |
| Soil Type: Topsoil | | | | |
| Test Sample, 0” | $1.02 \times 10^8$ | 6.69 | 5 | 0.95 |
| Test Sample, 1” | “ | 6.82 | 6 | 0.82 |
| Test Sample, 2” | “ | 6.95 | 9 | 0.69 |
| Test Sample, 3” | “ | 7.12 | 13 | 0.52 |
| Test Sample, 4” | “ | 6.97 | 9 | 0.67 |
| Test Sample, 5” | “ | 7.08 | 12 | 0.55 |
| Positive Controls (mean) | $1.02 \times 10^8$ | 7.64[a] | 38 – 50[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Sand | | | | |
| Test Sample, 0” | $1.02 \times 10^8$ | 6.96 | 9 | 0.97 |
| Test Sample, 1” | “ | 7.02 | 10 | 0.91 |
| Test Sample, 2” | “ | 7.08 | 12 | 0.85 |
| Test Sample, 3” | “ | 7.08 | 12 | 0.85 |
| Test Sample, 4” | “ | 7.13 | 13 | 0.80 |
| Test Sample, 5” | “ | 7.26 | 18 | 0.67 |
| Positive Controls (mean) | $1.02 \times 10^8$ | 7.93[a] | 74 – 94[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |
| Soil Type: Clay | | | | |
| Test Sample, 0” | $1.02 \times 10^8$ | 6.74 | 5 | 0.60 |
| Test Sample, 1” | “ | 6.96 | 9 | 0.39 |
| Test Sample, 2” | “ | 6.92 | 8 | 0.42 |
| Test Sample, 3” | “ | 6.96 | 9 | 0.38 |
| Test Sample, 4” | “ | 7.12 | 13 | 0.22 |
| Test Sample, 5” | “ | 7.18 | 15 | 0.16 |
| Positive Controls (mean) | $1.02 \times 10^8$ | 7.34[a] | 20 – 23[b] | -- |
| Negative Controls (TC) | 0 | 0 | 0 | -- |
| Negative Controls (CC) | 0 | 0 | 0 | -- |

[a] Average for five positive controls is reported.
[b] Recovery range for five positive controls is reported.

## 8.3 Methyl Bromide Log Reduction Charts

Figure 13 through Figure 16 show results for the MeBr tests in graphical form. This "decontamination efficacy" (as log reduction, or LR) is the same data that were presented earlier in Section 8.2, but in a visual format.

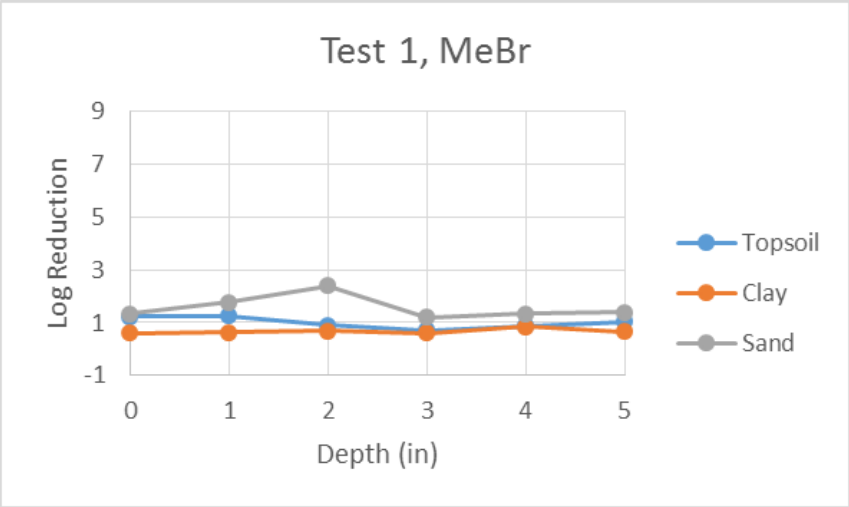


**Figure 14. Test 1, MeBr: 236 mg/L, 48 hrs, 78% RH**

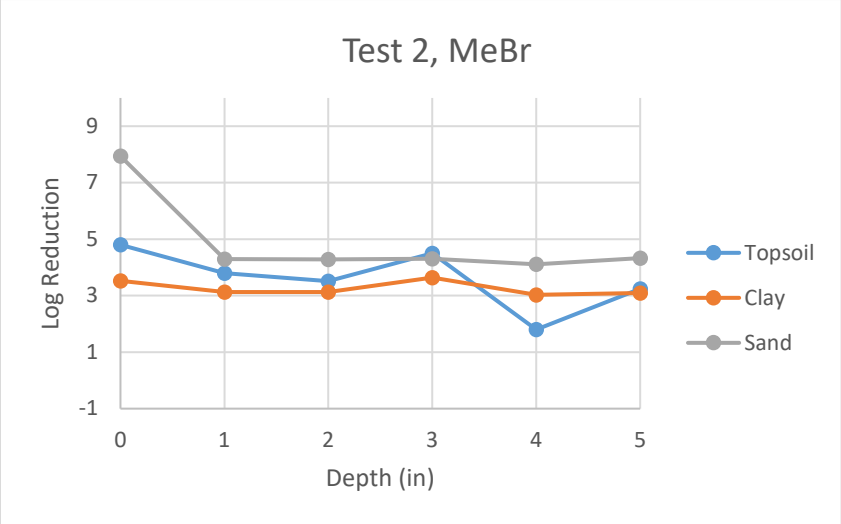


**Figure 15. Test 2, MeBr: 224 mg/L, 65.5 hrs, 77% RH**

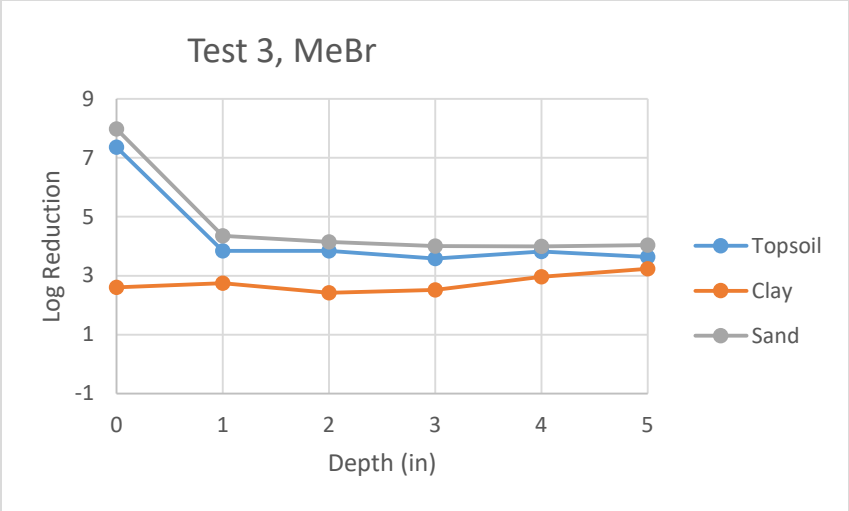


**Figure 16. Test 3, MeBr: 325 mg/L, 48 hrs, 76% RH**

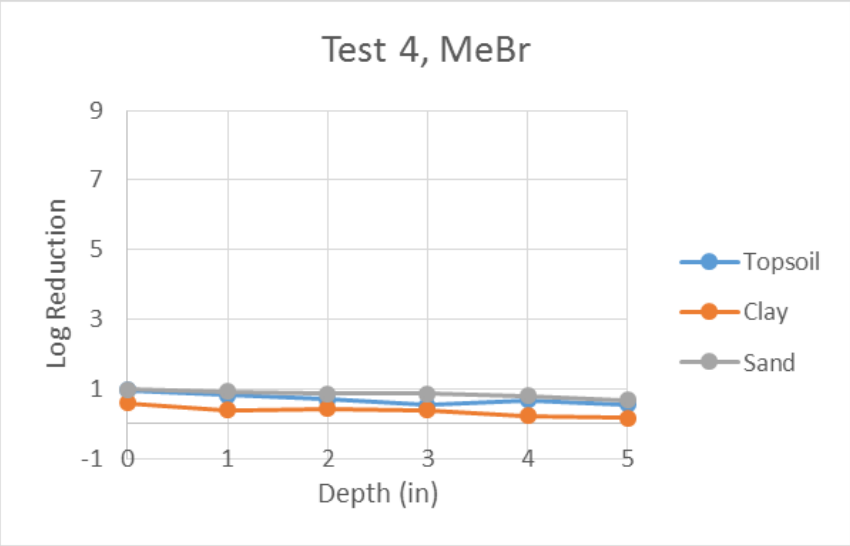


**Figure 17. Test 4, MeBr: 230 mg/L, 48 hrs, 79% RH, [saturated soil]**

# Section 9.
# Clay Positive Controls Decay Tests

## 9.1 Clay Positive Controls Decay Test Conditions

Two clay positive controls decay tests were performed during the same time period as the $ClO_2$ testing. Specifically, Test 4a was performed immediately following Test 4, $ClO_2$, and Test 7a was performed immediately following Test 7, $ClO_2$. The actual conditions measured for these two tests are presented in Table 29. Test 4a provided data for clay positive controls decay at low RH, while Test 7a was conducted at high RH.

**Table 29. Clay Positive Controls Decay Test Conditions**

| Test No. | Depth(s) Tested | Avg. $ClO_2$ Conc. (mg/L) | Avg. T (°C) | Avg. RH (%) | Time to Analysis (hrs) | Other Conditions |
|---|---|---|---|---|---|---|
| 4a | 0 (Clay Positive Controls Decay) | None | 24.6 ± 0.24 | 29.5 ± 5.8 | 0, 24, 48, 72, 96, 168 | Control Chamber only, for testing decay of clay positive controls. |
| 7a | 0 (Clay Positive Controls Decay | None | 23.0 ± 0.26 | 92.0 ± 1.68 | 0, 24, 48, 72, 96, 168 | Control Chamber only, for testing decay of clay positive controls. |

## 9.2 Clay Positive Controls Decay Test Results

Table 30 and Table 31 show results for the clay positive controls decay tests. Neither test showed complete decay (no spores recovered, or a LR ≥ 6) as had been observed during Test 4, $ClO_2$. With this in mind, it is clear that recovery of viable spores from clay is potentially inconsistent from test-to-test, and may be dependent on variables beyond the scope of this project to fully explore.

**Table 30. Test 4a, Clay Positive Controls Decay Results**

| Test ID, summary | Recovered CFU[a] | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extract. Control) | Log Reduction (vs. Non-Soil Extract. Control) |
|---|---|---|---|---|
| No $ClO_2$ decon, 0-hr | | | | |
| Control Sample 1 | $1.13 \times 10^8$ | 8.05 | 71 | 0.15 |
| Control Sample 2 | 1.07 " | 8.03 | 68 | 0.17 |
| Control Sample 3 | 1.12 " | 8.05 | 71 | 0.15 |
| Positive Control[b] | $5.67 \times 10^8$ | 8.75 | 358 | -0.55 |
| Non-Soil Extract. Control | $1.58 \times 10^8$ | 8.20 | -- | -- |
| No $ClO_2$ decon, 24-hr | | | | |
| Control Sample 1 | $7.83 \times 10^7$ | 7.89 | 50 | 0.30 |
| Control Sample 2 | 8.23 " | 7.92 | 53 | 0.27 |
| Control Sample 3 | 6.87 " | 7.84 | 44 | 0.35 |
| Positive Control[b] | $2.32 \times 10^8$ | 8.37 | 149 | -0.18 |
| Non-Soil Extract. Control | $1.56 \times 10^8$ | 8.19 | -- | -- |
| No $ClO_2$ decon, 48-hr | | | | |
| Control Sample 1 | $5.33 \times 10^7$ | 7.73 | 28 | 0.54 |
| Control Sample 2 | 5.90 " | 7.77 | 31 | 0.50 |
| Control Sample 3 | 6.07 " | 7.78 | 32 | 0.49 |
| Positive Control[b] | $1.79 \times 10^8$ | 8.25 | 95 | 0.02 |
| Non-Soil Extract. Control | $1.88 \times 10^8$ | 8.27 | -- | -- |
| No $ClO_2$ decon, 72-hr | | | | |
| Control Sample 1 | $4.07 \times 10^7$ | 7.61 | 23 | 0.63 |
| Control Sample 2 | 7.93 " | 7.90 | 46 | 0.34 |
| Control Sample 3 | 4.93 " | 7.69 | 28 | 0.55 |
| Positive Control[b] | $1.80 \times 10^8$ | 8.26 | 104 | -0.02 |
| Non-Soil Extract. Control | $1.74 \times 10^8$ | 8.24 | -- | -- |
| No $ClO_2$ decon, 96-hr | | | | |
| Control Sample 1 | $7.60 \times 10^7$ | 7.88 | 45 | 0.35 |
| Control Sample 2 | 9.10 " | 7.96 | 54 | 0.27 |
| Control Sample 3 | 8.50 " | 7.93 | 50 | 0.30 |
| Positive Control[b] | $1.49 \times 10^8$ | 8.17 | 88 | 0.06 |
| Non-Soil Extract. Control | $1.69 \times 10^8$ | 8.23 | -- | -- |
| No $ClO_2$ decon, 168-hr | | | | |
| Control Sample 1 | $4.63 \times 10^6$ | 6.67 | 2.7 | 1.57 |
| Control Sample 2 | 1.89 " | 6.28 | 1.1 | 1.96 |
| Control Sample 3 | 1.43 " | 6.16 | <1 | 2.08 |
| Positive Control[b] | $2.26 \times 10^8$ | 8.35 | 130 | -0.11 |
| Non-Soil Extract. Control | $1.74 \times 10^8$ | 8.24 | -- | -- |

[a] All samples were inoculated with $1.58 \times 10^8$ CFU (100-µL of $1.58 \times 10^9$ CFU/mL).
[b] No extraction, no soil.

**Table 31. Test 7a, Clay Positive Controls Decay Results**

| Test ID, summary | Recovered CFU[a] | Log of Recovered CFU from the CSP | Mean % Recovery (vs. Non-Soil Extract. Control) | Log Reduction (vs. Non-Soil Extract. Control) |
|---|---|---|---|---|
| No $ClO_2$ decon, 0-hr | | | | |
| Control Sample 1 | $1.15 \times 10^8$ | 8.06 | 71 | 0.15 |
| Control Sample 2 | $9.93 \times 10^7$ | 8.00 | 61 | 0.21 |
| Control Sample 3 | $8.70 \times 10^7$ | 7.94 | 54 | 0.27 |
| Positive Control[b] | $1.72 \times 10^8$ | 8.24 | 106 | -0.03 |
| Non-Soil Extract. Control | $1.62 \times 10^8$ | 8.21 | -- | -- |
| No ClO2 decon, 24-hr | | | | |
| Control Sample 1 | $7.60 \times 10^7$ | 7.88 | 26 | 0.59 |
| Control Sample 2 | $1.11 \times 10^8$ | 8.05 | 38 | 0.42 |
| Control Sample 3 | $9.17 \times 10^7$ | 7.96 | 31 | 0.51 |
| Positive Control[b] | $3.77 \times 10^8$ | 8.58 | 128 | -0.11 |
| Non-Soil Extract. Control | $2.93 \times 10^8$ | 8.47 | -- | -- |
| No $ClO_2$ decon, 48-hr | | | | |
| Control Sample 1 | $5.07 \times 10^7$ | 7.70 | 32 | 0.50 |
| Control Sample 2 | 6.50 " | 7.81 | 41 | 0.39 |
| Control Sample 3 | 4.43 " | 7.65 | 28 | 0.55 |
| Positive Control[b] | $1.49 \times 10^8$ | 8.17 | 94 | 0.03 |
| Non-Soil Extract. Control | $1.58 \times 10^8$ | 8.20 | -- | -- |
| No $ClO_2$ decon, 72-hr | | | | |
| Control Sample 1 | $1.07 \times 10^8$ | 8.03 | 62 | 0.21 |
| Control Sample 2 | $7.03 \times 10^7$ | 7.85 | 41 | 0.39 |
| Control Sample 3 | $9.40 \times 10^7$ | 7.97 | 55 | 0.27 |
| Positive Control[b] | $1.31 \times 10^8$ | 8.12 | 76 | 0.12 |
| Non-Soil Extract. Control | $1.72 \times 10^8$ | 8.24 | -- | -- |
| No $ClO_2$ decon, 96-hr | | | | |
| Control Sample 1 | $2.93 \times 10^7$ | 7.47 | 19 | 0.72 |
| Control Sample 2 | 2.73 " | 7.44 | 18 | 0.75 |
| Control Sample 3 | 3.83 " | 7.58 | 25 | 0.61 |
| Positive Control[b] | $1.19 \times 10^8$ | 8.07 | 76 | 0.12 |
| Non-Soil Extract. Control | $1.56 \times 10^8$ | 8.19 | -- | -- |
| No $ClO_2$ decon, 168-hr | | | | |
| Control Sample 1 | $5.83 \times 10^7$ | 7.77 | 42 | 0.38 |
| Control Sample 2 | 8.20 " | 7.91 | 59 | 0.24 |
| Control Sample 3 | 6.87 " | 7.84 | 49 | 0.31 |
| Positive Control[b] | $4.00 \times 10^8$ | 8.60 | 286 | -0.45 |
| Non-Soil Extract. Control | $1.40 \times 10^8$ | 8.15 | -- | |

[a] All samples were inoculated with $1.60 \times 10^8$ CFU (0.1 mL of $1.60 \times 10^9$ CFU/mL).
[b] No extraction, no soil.

## 9.3 Clay Positive Controls Decay Test Log Reduction Charts

Figure 17 and Figure 18 show results for the clay positive controls decay tests in graphical form. While these figures show some decay over the course of the 168-hr time period, a sharp decay to at least LR of ≥ 6 did not occur. The figures also show a rise and fall over the test period, illustrating the variability encountered.

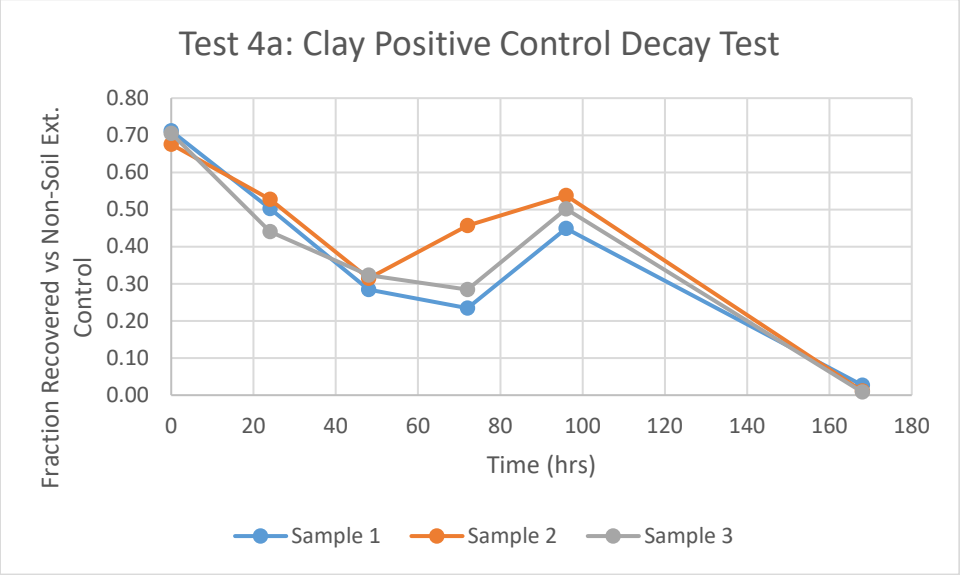


**Figure 18. Test 4a: Clay Positive Control Decay Test, 30% RH**

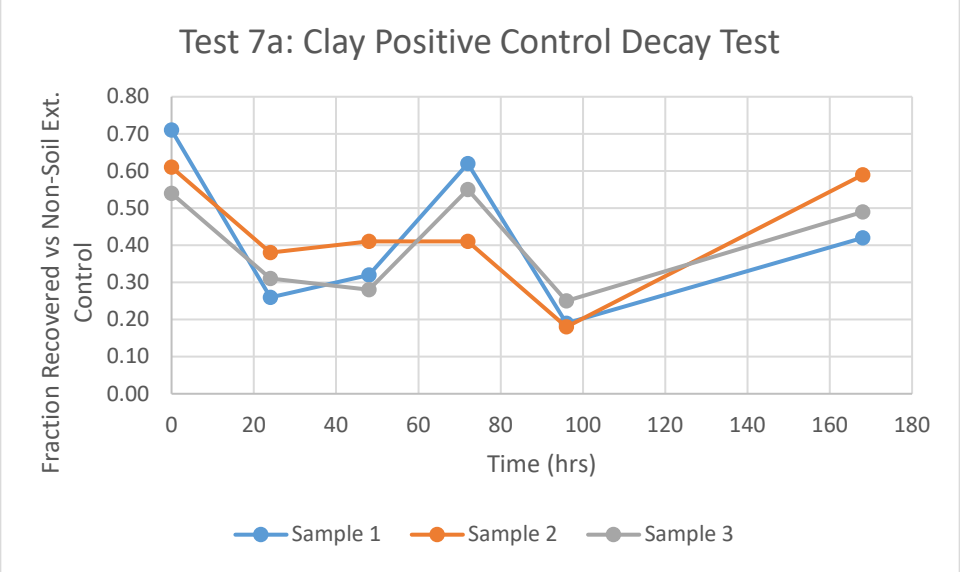


**Figure 19. Test 7a: Clay Positive Control Decay Test, 92% RH**

# Section 10. Summary of Results and Conclusions

The decontaminants evaluated in this study ($ClO_2$ gas, activated SP, and MeBr) were selected based on their efficacious results from previous bench-scale soil decontamination tests in which only 1-2 cm of topsoil were used in Petri dishes. In this study the scale of testing was enlarged, vis-à-vis using 10" diameter columns filled with soil to a depth of six inches, and using three types of soil materials: a topsoil, a sandy soil, and a clayey soil.

Due to the enlarged scale of testing, a method development program was needed to establish experimental procedures related to preparing, placing, and quantitatively recovering BA spores from within the large soil mass in the test columns. We settled on an approach in which BA spores would be contained in CSPs and placed in the center of the soil columns at various depths, ranging from 0 inches (on soil surface) down to five inches.

$ClO_2$ gas

With $ClO_2$ gas, topsoil was the most difficult of the soil materials to decontaminate. Of the seven tests conducted, the maximum depth in which topsoil was effectively decontaminated was just one inch, and this occurred in only two tests (with either extended contact time and/or increased concentration). For the clay and sandy soils, effective decontamination was achieved in a few of the tests down to a depth of 5 inches. Sand showed full decontamination down to 2 inches for most of the test conditions, while clay had full decontamination down to 3 inches for most of the tests. The depth of the soil in which effective decontamination was achieved generally increased with an increase in contact time or $ClO_2$ concentration.

Sodium Persulfate

Three tests were performed using activated SP, with variations in the concentration, application rate, the number of applications of this liquid decontaminant, or the contact time. The SP solution proved to be highly reactive with topsoil, and produced a vigorous foaming reaction upon application. Decontamination efficacy of topsoil was effective to 0.5 inch for the 0.5 M solutions and to 1 inch for the 1.0 M solution. Sand showed less reactivity with the SP, but the activated SP was effective to only a maximum of a 1-inch depth. Clay, on the other hand, showed complete decontamination down to either 4 or 5 inches in all three tests.

MeBr

Four tests were conducted using MeBr at concentrations ranging from 224 to 325 mg/L, contact times of 48 to 65.5 hrs, ambient temperature of 20 °C, and RH levels of ≥ 75%. The decontamination efficacy of MeBr was less than expected based on previous studies using this fumigant. In the majority of the experiments, BA was not effectively inactivated even at the surface of the soil columns. This prompted us to confirm again via PCR that the microorganism we were working with was in fact BA. We are uncertain why the MeBr results in this study are inconsistent with previous EPA decontamination studies using MeBr, although there are several possibilities. Further research may be needed to clarify this discrepancy.

In the first test, conducted at 236 mg/l MeBr for 48 hr, MeBr was not effective for any soil type, and resulted only in 1-2 LR, including the CSPs on the surface. Maintaining similar test conditions except for increasing contact time or increasing concentration resulted in only

moderate improvement in decontamination efficacy, primarily in the range of 3-4 LR. The pre-wetting of soils did not provide any improvement in MeBr decontamination efficacy either, producing efficacy results similar to Test 1.

Overall, efficacy was observed to be generally higher for topsoil and sand, and lowest for the clay soil. Lastly, although there were a few exceptions, decontamination efficacy was generally similar across all depths for a particular soil and test condition, suggesting that penetration of the MeBr gas through the soil matrices was not a limiting factor.

Implications

This study demonstrated that $ClO_2$ gas and activated SP may be suitable candidates for decontamination of soil contaminated with BA, depending on a number of factors, but primarily the soil type and depth of spores. Inactivation of spores beyond 1-inch depth may require more aggressive decontamination conditions as demonstrated in this study, depending on soil type.

For the activated SP in particular, relatively large amounts of this decontaminant were needed to be effective for the topsoil and sandy soils. This suggests this decontamination technique may be more suitable if implemented ex-situ (e.g., soil removed and placed in a tank without drainage), to allow for longer and improved contact between the BA spores and the decontaminant.

Further research is needed to understand the poor performance of MeBr in the present study, in light of its demonstrated effectiveness in previous research.

Lastly, future field-scale soil decontamination studies are recommended, in particular to assess issues related to encountering soils (such as those with clay) that may be denser and less permeable to decontaminants (as compared to this lab study).

# Section 11. References


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6. The Phase-out of Methyl Bromide. https://www.epa.gov/ods-phaseout/methyl-bromide Accessed 8/24/17.
7. Walkley, A. and I.A. Black. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. 37:29-38.)
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12. U.S. EPA. *Methyl Bromide Decontamination of Indoor and Outdoor Materials Contaminated with Bacillus anthracis spores*. United States Environmental Protection Agency, EPA/600/R-14/170, August 2014.

# Appendix A.
# *Bacillus Anthracis* Source Information and Plasmids Analysis

The source sheet (catalog number NR-411) from Biodefense and Emerging Infections (BEI) Research Resources Repository from which all BA (Ames strain) for this study originated is below. Laboratory staff confirmed the presence of the capB gene (testing for the presence of plasmid pXO2) and the LF gene (testing for the presence of plasmid pXO1) by PCR assay. Presence of both plasmids, which were found, indicates a virulent strain of the *B. anthracis* stock was received from BEI, as expected.

Plasmid confirmation of pXO1 and pXO2 in the BA employed for this program was done at the start of the program (February 16, 2016) and again near the end of the program (June 21, 2017). The instrument employed for these assays was a real-time PCR instrument (Bio-Rad CFX 96 Hercules, CA) using a custom-developed assay that has been validated and confirmed using numerous strains of BA.

The initial plasmids analysis instrument print-out for pXO1 and pXO2, February 16, 2016 is below. The second analysis (June, 2017) instrument print-out is listed second.

beiresources
BIODEFENSE & EMERGING INFECTIONS RESEARCH RESOURCES REPOSITORY

# Product Information Sheet for NR-411

## *Bacillus anthracis*, Strain Ames (A0462)

## Catalog No. NR-411

## For research use only. Not for human use.

### Contributor:
Professor Paul Keim, Keim Genetics Laboratory, Director of Department of Biological Sciences, College of Arts and Sciences, Northern Arizona University, Flagstaff, Arizona

### Product Description:
Bacteria Classification: *Bacillaceae*, *Bacillus*, *Bacillus cereus* group
Species: *Bacillus anthracis*
Strain: Ames (A0462, Asc 159)
Original Source: *Bacillus anthracis* (*B. anthracis*), strain Ames (A0462, Asc 159) was isolated from a dead cow in Jim Hogg County, Texas in 1981.[1,2] The organism was sent to USAMRIID where it was passaged in a guinea pig and mistakenly given the name "Ames". In the mid-1980s *B. anthracis*, strain Ames was provided to the Chemical and Biological Defence Sector and the Centre for Applied Microbiology and Research (CAMR) at Porton Down, Salisbury, UK. Professor Paul Keim received the bacteria from CAMR.

*B. anthracis* is an aerobic, Gram-positive, spore-forming, rod-shaped bacillus that causes the acute infectious disease anthrax. Herbivores are the natural hosts and become infected by consuming soil. Humans are incidentally infected by coming into contact with infected animals or their products. *B. anthracis* virulence is dependent on the expression of a polysaccharide capsule and an extracellular toxin that is composed of three proteins: lethal factor, edema factor, and protective antigen.[3]

The presence of pXO1 and pXO2 in NR-411 has been confirmed by PCR amplification of plasmid-specific sequences from extracted DNA.

### Material Provided:
Each vial contains approximately 0.5 mL of bacterial culture in 0.5X Tryptic Soy Broth supplemented with 10% glycerol.

Note: If homogeneity is required for your intended use, please purify prior to initiating work.

### Packaging/Storage:
NR-411 was packaged aseptically in screw-capped plastic cryovials. The product is provided frozen and should be stored at -60°C or colder immediately upon arrival. For long-term storage, the vapor phase of a liquid nitrogen freezer is recommended. Freeze-thaw cycles should be avoided.

### Growth Conditions:
Media:
Tryptic Soy Broth
Tryptic Soy Agar with 5% defibrinated sheep blood
Incubation:
Temperature: 37°C
Atmosphere: Aerobic
Propagation:
1. Keep vial frozen until ready for use; thaw slowly.
2. Transfer the entire thawed aliquot into a single tube of broth.
3. Use several drops of the suspension to inoculate an agar slant and/or plate.
4. Incubate the tubes and plate at 37°C for 24 hours.

### Citation:
Acknowledgment for publications should read "The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *Bacillus anthracis*, Strain Ames (A0462), NR-411."

### Biosafety Level: 3
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th ed. Washington, DC: U.S. Government Printing Office, 2007; see www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm. This publication recommends that all persons working in or entering laboratory or animal care areas where frequent activities with clinical specimens or diagnostic cultures of *Bacillus anthracis* are being conducted should have documented evidence of satisfactory vaccination.

### Disclaimers:
You are authorized to use this product for research use only. It is not intended for human use.

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**References:**

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5. Keim, P., et al. "Multiple-Locus Variable-Number Tandem Repeat Analysis Reveals Genetic Relationships within *Bacillus anthracis*." J. Bacteriol. 182 (2000): 2928-2936. PubMed: 10781564.



Support Provided by NIAID

**Biodefense and Emerging Infections Research Resources Repository**
www.beiresources.org

E-mail: contact@beiresources.org
Tel: 800-359-7370
Fax: 703-365-2898

Initial plasmids analysis for pXO1 and pXO2, February 16, 2016.

BIO-RAD

# XC0620160216_1_BA2_BA3_Quant_DK.pcrd

## 2/16/2016 2:37 PM

### Report Information

**User:** BioRad/lgreene
**Data File Name:** XC0620160216_1_BA2_BA3_Quant_DK.pcrd
**Data File Path:** C:\Users\Public\Documents\Bio-Rad\CFX\Users\data
**Well Group Name:** All Wells
**Report Differs from Last Save:** Yes

### Run Setup

#### Run Information

**Run Date:** 2/16/2016 12:20 PM
**Run User:** lgreene
**Run Type:** User-defined
**Plate File:** Florida Ba Test.pltd
**ID:**
**Notes:**
**Sample Volume:** 25
**Temperature Control Mode:** Calculated
**Lid Temperature:** 105
**Base Serial Number:** CT013497
**Optical Head Serial Number:** 785BR11154

#### Protocol

**1:** 50.0°C for 2:00
**2:** 95.0°C for 11:00
**3:** 95.0°C for 0:15
Plate Read
**4:** 56.0°C for 1:00
**5:** GOTO 3, 39 more times

#### Plate Display

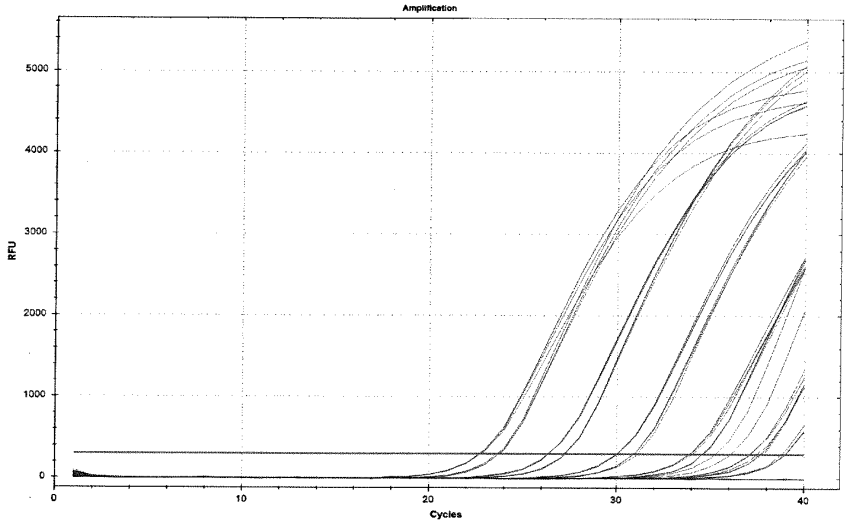
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Unk BA2 BA2_1:10_ 01 | Unk BA2 BA2_1:10_ 02 | Unk BA2 BA2_1:10_ 03 | Unk BA2 BA2_1:100 _01 | Unk BA2 BA2_1:100 _02 | Unk BA2 BA2_1:100 _03 | Unk BA2 BA2_1:100 0_01 | Unk BA2 BA2_1:100 0_02 | Unk BA2 BA2_1:100 0_03 | Unk BA2 BA2_1:100 00_001 | Unk BA2 BA2_1:100 00_002 | Unk BA2 BA2_1:100 00_003 |
| B | Unk BA2 BA2_1:100 000_001 | Unk BA2 BA2_1:100 000_002 | Unk BA2 BA2_1:100 000_003 | Unk BA2 BA2_PC_0 1 | Unk BA2 BA2_PC_0 2 | Unk BA2 BA2_NTC_ 01 | Unk BA2 BA2_NTC_ 02 | | | | | |
| C | Unk BA3 BA3_1:10_ 01 | Unk BA3 BA3_1:10_ 02 | Unk BA3 BA3_1:10_ 03 | Unk BA3 BA3_1:100 _01 | Unk BA3 BA3_1:100 _02 | Unk BA3 BA3_1:100 _03 | Unk BA3 BA3_1:100 0_01 | Unk BA3 BA3_1:100 0_02 | Unk BA3 BA3_1:100 0_03 | Unk BA3 BA3_1:100 00_001 | Unk BA3 BA3_1:100 00_002 | Unk BA3 BA3_1:100 00_003 |
| D | Unk BA3 BA3_1:100 000_001 | Unk BA3 BA3_1:100 000_002 | Unk BA3 BA3_1:100 000_003 | Unk BA3 BA3_PC_0 1 | Unk BA3 BA3_PC_0 2 | Unk BA3 BA3_NTC_ 01 | Unk BA3 BA3_NTC_ 02 | | | | | |
| E | | | | | | | | | | | | |

Plate Display

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

## Quantification

**Step #:** 3
**Analysis Mode:** Target
**Cq Determination:** Single Threshold
**Baseline Method:**
BA2: Auto Calculated
BA3: Auto Calculated
**Threshold Setting:**
BA2: 294.70, Auto Calculated
BA3: 306.43, Auto Calculated




Quantification Data

| Well | Fluor | Target | Content | Sample | Cq | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|
| A01 | FAM | BA2 | Unkn | BA2_1:10_01 | 23.70 | 23.70 | 0.000 |
| A02 | FAM | BA2 | Unkn | BA2_1:10_02 | 23.67 | 23.67 | 0.000 |
| A03 | FAM | BA2 | Unkn | BA2_1:10_03 | 23.57 | 23.57 | 0.000 |
| A04 | FAM | BA2 | Unkn | BA2_1:100_01 | 27.20 | 27.20 | 0.000 |
| A05 | FAM | BA2 | Unkn | BA2_1:100_02 | 27.17 | 27.17 | 0.000 |
| A06 | FAM | BA2 | Unkn | BA2_1:100_03 | 27.17 | 27.17 | 0.000 |
| A07 | FAM | BA2 | Unkn | BA2_1:1000_01 | 30.85 | 30.85 | 0.000 |
| A08 | FAM | BA2 | Unkn | BA2_1:1000_02 | 31.06 | 31.06 | 0.000 |
| A09 | FAM | BA2 | Unkn | BA2_1:1000_03 | 30.86 | 30.86 | 0.000 |
| A10 | FAM | BA2 | Unkn | BA2_1:10000_001 | 34.53 | 34.53 | 0.000 |
| A11 | FAM | BA2 | Unkn | BA2_1:10000_002 | 34.53 | 34.53 | 0.000 |
| A12 | FAM | BA2 | Unkn | BA2_1:10000_003 | 34.53 | 34.53 | 0.000 |

## Quantification Data

| Well | Fluor | Target | Content | Sample | Cq | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|
| B01 | FAM | BA2 | Unkn | BA2_1:100000_001 | 37.58 | 37.58 | 0.000 |
| B02 | FAM | BA2 | Unkn | BA2_1:100000_002 | 38.99 | 38.99 | 0.000 |
| B03 | FAM | BA2 | Unkn | BA2_1:100000_003 | 37.67 | 37.67 | 0.000 |
| B04 | FAM | BA2 | Unkn | BA2_PC_01 | 38.93 | 38.93 | 0.000 |
| B05 | FAM | BA2 | Unkn | BA2_PC_02 | 38.72 | 38.72 | 0.000 |
| B06 | FAM | BA2 | Unkn | BA2_NTC_01 | N/A | 0.00 | 0.000 |
| B07 | FAM | BA2 | Unkn | BA2_NTC_02 | N/A | 0.00 | 0.000 |
| C01 | FAM | BA3 | Unkn | BA3_1:10_01 | 22.78 | 22.78 | 0.000 |
| C02 | FAM | BA3 | Unkn | BA3_1:10_02 | 22.75 | 22.75 | 0.000 |
| C03 | FAM | BA3 | Unkn | BA3_1:10_03 | 22.70 | 22.70 | 0.000 |
| C04 | FAM | BA3 | Unkn | BA3_1:100_01 | 26.33 | 26.33 | 0.000 |
| C05 | FAM | BA3 | Unkn | BA3_1:100_02 | 26.37 | 26.37 | 0.000 |
| C06 | FAM | BA3 | Unkn | BA3_1:100_03 | 26.39 | 26.39 | 0.000 |
| C07 | FAM | BA3 | Unkn | BA3_1:1000_01 | 30.03 | 30.03 | 0.000 |
| C08 | FAM | BA3 | Unkn | BA3_1:1000_02 | 30.03 | 30.03 | 0.000 |
| C09 | FAM | BA3 | Unkn | BA3_1:1000_03 | 30.07 | 30.07 | 0.000 |
| C10 | FAM | BA3 | Unkn | BA3_1:10000_001 | 33.92 | 33.92 | 0.000 |
| C11 | FAM | BA3 | Unkn | BA3_1:10000_002 | 33.89 | 33.89 | 0.000 |
| C12 | FAM | BA3 | Unkn | BA3_1:10000_003 | 34.05 | 34.05 | 0.000 |
| D01 | FAM | BA3 | Unkn | BA3_1:100000_001 | 37.14 | 37.14 | 0.000 |
| D02 | FAM | BA3 | Unkn | BA3_1:100000_002 | 37.48 | 37.48 | 0.000 |
| D03 | FAM | BA3 | Unkn | BA3_1:100000_003 | 37.11 | 37.11 | 0.000 |
| D04 | FAM | BA3 | Unkn | BA3_PC_01 | 36.08 | 36.08 | 0.000 |
| D05 | FAM | BA3 | Unkn | BA3_PC_02 | 35.24 | 35.24 | 0.000 |
| D06 | FAM | BA3 | Unkn | BA3_NTC_01 | N/A | 0.00 | 0.000 |
| D07 | FAM | BA3 | Unkn | BA3_NTC_02 | N/A | 0.00 | 0.000 |

# QC Parameters

## Data

| Description | Value | Use | Results | Exclude Wells | All excluded wells |
|---|---|---|---|---|---|
| Negative control with a Cq less than | 38 | True | | False | |
| NTC with a Cq less than | 38 | True | | False | |
| NRT with a Cq less than | 38 | True | | False | |
| Positive control with a Cq greater than | 30 | True | | False | |

## Data

| Description | Value | Use | Results | Exclude Wells | All excluded wells |
|---|---|---|---|---|---|
| Unknown without a Cq | N/A | True | BA2:B6, B7. BA3:D6, D7. | False | |
| Standard without a Cq | N/A | True | | False | |
| Efficiency greater than | 110.0 | True | | | |
| Efficiency less than | 90.0 | True | | | |
| Std Curve R^2 less than | 0.980 | True | | | |
| Replicate group Cq Std Dev greater than | 0.20 | True | | False | |

Plasmids confirmation reanalysis of pXO1 and pXO2, June 21, 2017.

BIO-RAD

# XC0520170621_2_BA2_BA3_DK.pcrd

7/7/2017 11:06 AM

## Report Information

**User:** BioRad/dkiekel
**Data File Name:** XC0520170621_2_BA2_BA3_DK.pcrd
**Data File Path:** C:\Users\mducote\AppData\Local\Microsoft\Windows\Temporary Internet Files\Content.Outlook\GKJT4YFC
**Well Group Name:** All Wells
**Report Differs from Last Save:** No

## Notes

## Run Setup

### Run Information

**Run Date:** 6/21/2017 10:46 AM
**Run User:** dkiekel
**Run Type:** User-defined
**ID:**
**Notes:**
**Sample Volume:** 25
**Temperature Control Mode:** Calculated
**Lid Temperature:** 105
**Base Serial Number:** CT013504
**Optical Head Serial Number:** 785BR11128

### Protocol

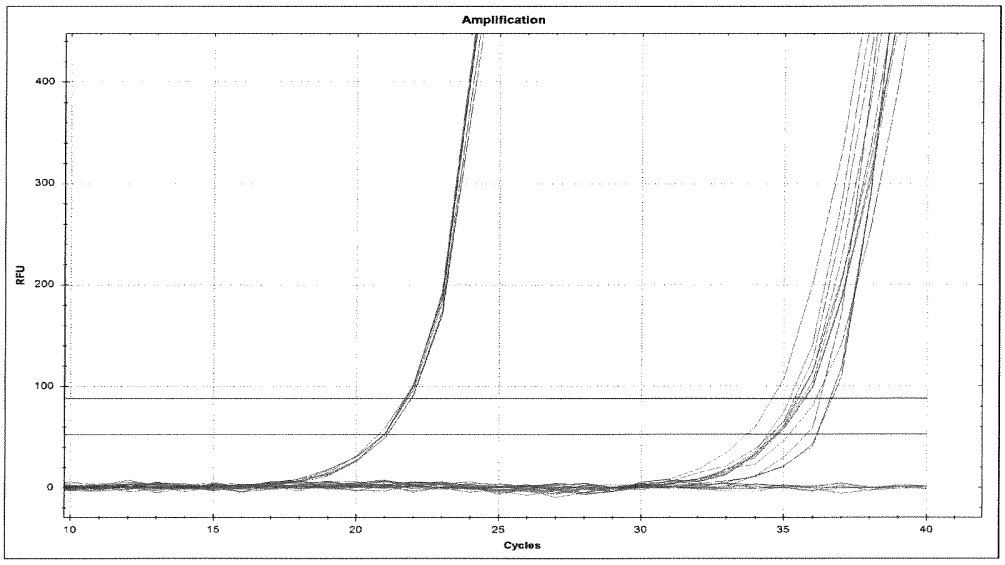
**1:** 50.0°C for 15:00
**2:** 95.0°C for 2:00
**3:** 95.0°C for 0:15
**4:** 60.0°C for 1:00
Plate Read
**5:** GOTO 3, 39 more times

### Plate Display

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Unk BA2 BA3 S1-1 | Unk BA2 BA3 S1-2 | Unk BA2 BA3 S1-3 | Unk BA2 BA3 S2-1 | Unk BA2 BA3 S2-2 | Unk BA2 BA3 S2-3 | Unk BA2 BA3 PC-1 | Unk BA2 BA3 PC-2 | Unk BA2 BA3 PC-3 | Unk BA2 BA3 NTC-1 | Unk BA2 BA3 NTC-2 | Unk BA2 BA3 NTC-3 |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

## Quantification

**Step #:** 4
**Analysis Mode:** Fluorophore
**Cq Determination:** Single Threshold
**Baseline Method:**
Cy5: Auto Calculated
Tex 615: Auto Calculated
**Threshold Setting:**
Cy5: 88.16, Auto Calculated
Tex 615: 52.57, Auto Calculated




## Quantification Data

| Well | Fluor | Target | Content | Sample | Cq | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|
| A01 | Cy5 | BA3 | Unkn | S1-1 | 21.93 | 21.93 | 0.000 |
| A02 | Cy5 | BA3 | Unkn | S1-2 | 21.93 | 21.93 | 0.000 |
| A03 | Cy5 | BA3 | Unkn | S1-3 | 21.72 | 21.72 | 0.000 |
| A04 | Cy5 | BA3 | Unkn | S2-1 | 21.70 | 21.70 | 0.000 |
| A05 | Cy5 | BA3 | Unkn | S2-2 | 21.81 | 21.81 | 0.000 |
| A06 | Cy5 | BA3 | Unkn | S2-3 | 21.76 | 21.76 | 0.000 |
| A07 | Cy5 | BA3 | Unkn | PC-1 | 36.69 | 36.69 | 0.000 |
| A08 | Cy5 | BA3 | Unkn | PC-2 | 36.26 | 36.26 | 0.000 |
| A09 | Cy5 | BA3 | Unkn | PC-3 | 36.61 | 36.61 | 0.000 |
| A10 | Cy5 | BA3 | Unkn | NTC-1 | N/A | 0.00 | 0.000 |
| A11 | Cy5 | BA3 | Unkn | NTC-2 | N/A | 0.00 | 0.000 |
| A12 | Cy5 | BA3 | Unkn | NTC-3 | N/A | 0.00 | 0.000 |
| A01 | Tex 615 | BA2 | Unkn | S1-1 | 34.54 | 34.54 | 0.000 |
| A02 | Tex 615 | BA2 | Unkn | S1-2 | 34.81 | 34.81 | 0.000 |
| A03 | Tex 615 | BA2 | Unkn | S1-3 | 35.18 | 35.18 | 0.000 |

## Quantification Data

| Well | Fluor | Target | Content | Sample | Cq | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|
| A04 | Tex 615 | BA2 | Unkn | S2-1 | 34.70 | 34.70 | 0.000 |
| A05 | Tex 615 | BA2 | Unkn | S2-2 | 33.72 | 33.72 | 0.000 |
| A06 | Tex 615 | BA2 | Unkn | S2-3 | 34.76 | 34.76 | 0.000 |
| A07 | Tex 615 | BA2 | Unkn | PC-1 | 34.72 | 34.72 | 0.000 |
| A08 | Tex 615 | BA2 | Unkn | PC-2 | 34.43 | 34.43 | 0.000 |
| A09 | Tex 615 | BA2 | Unkn | PC-3 | 34.59 | 34.59 | 0.000 |
| A10 | Tex 615 | BA2 | Unkn | NTC-1 | N/A | 0.00 | 0.000 |
| A11 | Tex 615 | BA2 | Unkn | NTC-2 | N/A | 0.00 | 0.000 |
| A12 | Tex 615 | BA2 | Unkn | NTC-3 | N/A | 0.00 | 0.000 |

# Appendix B.
# Soil Properties

Soil density was measured by weighing a 200-mL volume of each soil type in a beaker. Soil moisture was measured according to ASTM D 2974-87 prior to each test. Samples were weighed, dried in an oven for ≥16 hrs at 105 ± 5°C, and weighed again, with the moisture calculated as:

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

A = mass of the as-received sample, g
B = mass of the oven-dried sample, g

**Table B-1. Ongoing Soil Properties Checks (density, moisture)**

| | Density (g/mL) | | | Moisture (%) | | | |
|---|---|---|---|---|---|---|---|
| **Test/Date** | **Topsoil** | **Sand** | **Clay** | **Topsoil** | **Sand** | **Clay** | **Notes** |
| Initial (preliminary tests) | 1.00 | 1.39 | 1.06 | -- | -- | -- | |
| Test 1, ClO2 (08/05/16) | -- | -- | -- | 41.5 | 5.5 | 22.3 | Density not measured; assumed to be unchanging from Test 1 |
| Test 2, ClO2 (08/24/16) | -- | -- | -- | 41.9 | 3.0 | 22.9 | Density not measured; assumed to be unchanging from Test 1 |
| Test 3, ClO2 (09/06/16) | 1.01 | 1.49 | 1.11 | 27.5 | 8.4 | 26.3 | Values are initial, unwetted. For testing, soils were wetted to near saturation by adding 0.08 mL/g (topsoil), 0.04 mL/g (clay), 0.10 mL/g (sand). |
| Test 4, ClO2 (09/26/16) | 0.97 | 1.25 | 1.27 | 21.5 | 5.2 | 28.4 | |
| Test 5, ClO2 (11/28/16) | 1.02 | 1.14 | 1.28 | 21.6 | 4.7 | 15.1 | Values are initial, unwetted. For testing, soils were wetted to near saturation by adding 0.08 mL/g (topsoil), 0.04 mL/g (clay), 0.10 mL/g (sand). |
| Test 6, ClO2 (12/06/16) | 0.93 | 1.14 | 1.01 | 19.9 | 4.4 | 14.9 | |
| Test 7, ClO2 (12/21/16) | 1.03 | 1.39 | 1.16 | 20.4 | 3.5 | 15.6 | Uncompressed. |
| Test 7, ClO2 (12/21/16) | 1.37 | 1.59 | 1.55 | 20.4 | 3.5 | 15.6 | Compressed. Same soil as uncompressed, measured after compression. |
| Test 1, SP (02/06/17) | 1.02 | 1.16 | 1.13 | 20.0 | 4.2 | 10.8 | |
| Test 2, SP (03/02/17) | 1.01 | 1.18 | 1.14 | 20.3 | 4.2 | 10.7 | |
| Test 3, SP (04/11/17) | 0.98 | 1.26 | 1.18 | 19.6 | 5.1 | 10.7 | |
| Test 1, MeBr (06/06/2017) | 0.97 | 1.18 | 1.13 | 21.6 | 3.4 | 10.8 | |
| Test 2, MeBr (06/19/2017) | 0.96 | 1.19 | 1.04 | 24.1 | 2.1 | 8.0 | |
| Test 3, MeBr (07/05/2017) | 1.00 | 1.18 | 1.06 | 21.9 | 2.7 | 8.4 | |
| Test 4, MeBr (07/19/2017) | 1.02 | 1.22 | 1.13 | 25.7 | 4.5 | 8.0 | Values are initial, unwetted. For testing, soils were wetted to near saturation by adding 0.08 mL/g (topsoil), 0.04 mL/g (clay), 0.10 mL/g (sand). |

08/18/2016 Agvise Laboratories Series II Soil Characterization Test:

| Type | Density[a] (g/mL) | Moisture[b] (%) | % OM[c] | % Sand/Silt/Clay | USDA Textural Class | Cation Exchange Capacity (meq/ 100 g) | pH (Water) | Buffer pH (Adams-Evans) |
|---|---|---|---|---|---|---|---|---|
| Topsoil (Oldcastle) | 0.81 | 54.1 | 33.9 | 64/18/18 | Sandy Loam | 57.0 | 8.1 | 7.9 |
| Sand (Play Sand) | 1.64 | 2.7 | 0.1 | 100/0/0 | Sand | 4.3 | 8.4 | 8.0 |
| Clay (Crimson Clay) | 1.11 | 31.9 | 0.2 | 40/16/44[d] | Clay | 8.3 | 4.7 | 7.2 |

[a] Disturbed Bulk Density.
[b] Field capacity at 1/3 bar.
[c] Organic matter by Walkley-Black method.
[d] Supplier (Better Baseball) claims mix is 25/40/35 but cites no source or method.

08/18/2016 Agvise Laboratories Series II Soil Characterization Test, Base Saturation Data:

| Type | K (%, ppm) | Ca (%, ppm) | Mg (%, ppm) | Na (%, ppm) | H (%, ppm) |
|---|---|---|---|---|---|
| Topsoil (Oldcastle) | 27.8<br>6,178 | 42.5<br>4,839 | 14.5<br>989 | 14.6<br>1,909 | 0.7<br>4 |
| Sand (Play Sand) | 2.5<br>42 | 80.7<br>688 | 12.7<br>65 | 2.1<br>21 | 1.9<br>1 |
| Clay (Crimson Clay) | 0.9<br>29 | 16.5<br>274 | 4.5<br>45 | 0.9<br>17 | 77.2<br>64 |

# Appendix C.
# CSP Development

Carrier soil packet (CSP) development was performed through a multi-step process that evaluated the types of materials and techniques that would give good recovery, yet allow permeation of the decontaminant. Some of the method development steps used a commercially-available product containing a small stainless steel disc impregnated with *B. atrophaeus* spores and sealed within a Tyvek packet. This commercial product, produced by Mesa Labs, is known as the Apex biological indicator, or BI, while the custom-made packets containing soil spiked with BA were referred to as CSPs. Using the commercial BIs as the basis for developing BA-spiked CSPs, six steps were performed for CSP development:

1. Develop a method for removing the Tyvek BI envelopes from the soil columns.
2. Perform extractions of *B. atrophaeus* spores from the stainless steel carriers within the BIs (after sitting in soil).
3. Develop a method for aseptic removal of discs from BI Tyvek envelopes.
4. Determine penetration of SP liquid into Tyvek and PVDF envelopes.
5. Develop Tyvek and PVDF envelopes for carrier soil packets.
6. Perform extractions of *B. atrophaeus* spores from spiked autoclaved soils placed within the soil contained in a column.

A summary of all the CSP development tests is presented in Table C-1. The resulting data from these tests are described in the sections below.

**Table C-1. Summary of CSP Development Tests**

| Date | Soil Type | Columns | Description | Location of Additional Details |
|---|---|---|---|---|
| **Extraction of *B. atrophaeus* from the BI Stainless Steel Carriers** | | | | |
| 01/06/2016 | Topsoil | NA | BI recovery from Tyvek in soil with and w/o heat shock. | Table C-2 |
| 02/12/2016 | NA | NA | BI recovery test. | Table C-3 |
| **SP Soak (no soil)** | | | | |
| 02/24/2016 | NA | Glass Jars | BIs in Tyvek in SP for 7 days. | Table C-4 |
| 05/11/2016 | NA | Glass Jars | BIs in Tyvek in SP for 5, 10, 30, 60 min. | Table C-5 |
| 06/04/2016 | NA | Glass Jars | BIs in PVDF in SP for 5, 10, 30, 60 min. | On file. |
| 06/22/2016 | NA | Glass Jars | BIs in PVDF in SP for 5, 10, 30, 60 min. | Table C-6 |
| 06/27/2016 | NA | Glass Jars | BIs in PVDF in SP for 4 to 48 hrs. | Table C-7 |
| **PVDF Development Tests** | | | | |
| 05/18/2016 | NA | NA | PVDF and PC filters immersed in SP. | Visual exam |
| 05/20/2016 | NA | NA | PVDF, PC, and Tyvek materials wetted with a drop of SP. | Visual exam |
| **Extraction of *B. atrophaeus* from Spiked Autoclaved Soil** | | | | |
| 05/16/2016 | All types | NA | *B. atrophaeus*-spiked soil recovery test in Tyvek. | Table C-8 |
| 06/04/2016 | All types | NA | *B. atrophaeus* -spiked soil recovery test in PVDF. | Table C-9 |

## Tyvek Envelope Removal Method Development

Tests were conducted to assess procedures to remove the Tyvek envelopes from soil columns. Preliminary tests included attaching a wire or dental floss to the envelope and pulling it up through the soil column. After using this method for the first wall effects test, however, it was abandoned in favor of simply tipping the soil column on its side, carefully dumping the soil into a second bucket, and removing each BI envelope by hand as it reached the top surface.

## Extraction of *B. atrophaeus* from the BI carriers

Using the Mesa Labs protocol, the extraction efficiency of *B. atrophaeus* from the BI stainless steel carriers within the Tyvek envelopes was evaluated. One experiment was performed on 01/06/2016 using four BIs that sat in soil for 5 days, two of which were heat-shocked for 10 min at 80-85°C, while two were not (Table C-2).

**Table C-2. BI Extraction Efficacy (5 days in soil, with and without heat shock)**

| Sample | Type | Method | Observed (CFU) | Recovery (%)[a] |
|---|---|---|---|---|
| 1 | Apex BI in Tyvek | Standard | $2.67 \times 10^6$ | 95.4 |
| 2 | Apex BI in Tyvek | Standard | $1.64 \times 10^6$ | 58.6 |
| 3 | Apex BI in Tyvek | Standard + Heat Shock | $1.86 \times 10^6$ | 66.4 |
| 4 | Apex BI in Tyvek | Standard + Heat Shock | $1.02 \times 10^6$ | 36.4 |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis (COA).

The standard extraction procedure using the Mesa Labs protocol is as follows:

1. Cut Tyvek, and aseptically dump BI disc into 50 mL tube containing 10 mL sterile water.
2. Sonicate tube for 15 min.
3. Heat shock (optional) by placing tube in water bath at 80-85°C for 10 minutes.
4. Analyze by plate counting/serial dilution.

A second experiment was performed on 02/12/2016 using four BIs. Two BIs were removed from the Tyvek envelopes and directly recovered using the procedure above. One BI envelope was stirred in sterile water for 10 minutes prior to recovery. One BI was soaked in bleach for 10 minutes prior to recovery. Table C-3 presents the results.

**Table C-3. BI Extraction Efficacy**

| Sample | Type | Treatment | Observed (CFU) | Recovery (%)[a] |
|---|---|---|---|---|
| 1 | Apex BI in Tyvek | Simple recovery | $1.50 \times 10^6$ | 53.6 |
| 2 | Apex BI in Tyvek | Simple recovery | $1.53 \times 10^6$ | 54.6 |
| 3 | Apex BI in Tyvek | Stir in sterile water 10 min, then recover. | $1.42 \times 10^6$ | 50.8 |
| 4 | Apex BI in Tyvek | Soak in bleach for 10 min, then recover. | 0 | 0 |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from COA.

## Aseptic Removal of BI Discs from Tyvek Envelopes

Procedures were developed for aseptic removal of the stainless steel carriers from the BI Tyvek and (later) PVDF envelopes. These procedures were then adapted for use with BA inoculated soil later in the project.

The envelopes were opened by peeling apart the two papers near the top of the envelope or by cutting with sterile scissors. The stainless steel BI was removed by tipping the opened Tyvek envelope and allowing the BI disc to fall out, or by grasping the disc by sterile forceps. The BI discs were placed in 1 mL of sterile phosphate buffered saline extraction buffer containing 0.1% Triton X-100 surfactant and agitated at ~200 rpm at room temperature for 15 minutes. Samples were diluted and plated as described in the main body of the report and incubated at ~35°C for 16-20 hours. *B. atrophaeus* was present as orange colonies and enabled discrimination from contaminants.

## Determining Penetration of Sodium Persulfate into Tyvek and PVDF Envelopes

It was anticipated that SP decontamination would require extended exposure times (1 to 7 days). Hence, a 5-day test was performed on 02/24/2016 to determine minimum exposure time in which persulfate permeated the commercially-available Tyvek envelope BIs. Glass jars were set up and filled with ~1" depth of activated SP (0.5 M, 1:1 with 8% hydrogen peroxide) to allow for complete immersion of the BI. One BI was placed in each jar and was briefly swirled. Due to the hydrophobic nature of Tyvek, the BIs tended to float but eventually were permeated. For comparison, a set of BIs soaked in sterile water was also included. The permeation data from these experiments are summarized below.

**Table C-4. BIs Soaked in Activated Sodium Persulfate (Tyvek Envelope)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] | Decon. Efficacy (LR)[a] |
|---|---|---|---|---|---|
| 15-sec exposure | Tyvek | Activated SP | $5.93 \times 10^5$ | 21 | 0.68 |
| 1-day exposure | Tyvek | Activated SP | 0 | 0 | 6.45 |
| 4-day exposure | Tyvek | Activated SP | 0 | 0 | 6.45 |
| 5-day exposure | Tyvek | Activated SP | 0 | 0 | 6.45 |
| 15-sec exposure | Tyvek | Sterile water | $1.72 \times 10^6$ | 62 | 0.21 |
| 1-day exposure | Tyvek | Sterile water | $5.43 \times 10^5$ | 19 | 0.72 |
| 4-day exposure | Tyvek | Sterile water | $5.23 \times 10^5$ | 19 | 0.73 |
| 5-day exposure | Tyvek | Sterile water | $4.53 \times 10^5$ | 16 | 0.79 |

[a] Based on BI standard of $2.80 \times 10^6$ CFU (from supplier's COA).

Results showed complete kill (6.45 LR) at the 1-day mark, while the 15-sec sample only showed partial kill of 0.68 LR. The data from these tests does not unambiguously determine if slow permeation or slow decontamination rates are the cause of complete kill on or before 24 hours of exposure. In an attempt to determine the source of the time to kill, a test was performed on 05/11/2016 using BIs contained within the Tyvek and also removed from the Tyvek, in which 100 µL of SP was directly applied onto the spore-laden concave side of the disc. This application allowed the surface area of the disc to be entirely covered with liquid, but not overflow. Discs kept in Tyvek were gently swirled in liquid as had been done during the previous soak test.

Results show that the persulfate is, in fact, fast-acting upon direct contact with the *B. atrophaeus* contained on the BI disc. This test demonstrated that the rate of persulfate permeation through the Tyvek envelope is significant and affects the actual decontamination rate, and thus an alternative material was investigated.

**Table C-5. BIs in Activated Sodium Persulfate (Direct to Disk vs. Tyvek Envelope)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] | Decon. Efficacy (LR)[a] |
|---|---|---|---|---|---|
| 5-min exposure | none | Activated SP | 0 | 0 | 6.45 |
| 10-min exposure | none | Activated SP | 0 | 0 | 6.45 |
| 30-min exposure | none | Activated SP | 0 | 0 | 6.45 |
| 60-min exposure | none | Activated SP | 0 | 0 | 6.45 |
| 30-min exposure | Tyvek | Activated SP | $6.00 \times 10^5$ | 21 | 0.67 |
| 60-min exposure | Tyvek | Activated SP | $6.47 \times 10^4$ | 2 | 1.64 |
| 15-sec exposure | Tyvek | Sterile water | $1.10 \times 10^6$ | 39 | 0.41 |
| 60-min exposure | Tyvek | Sterile water | $1.23 \times 10^5$ | 4 | 1.36 |

[a] Based on BI standard of $2.80 \times 10^6$ CFU (from supplier's COA).

Porous polyvinylidene fluoride (PVDF) was chosen as a potential candidate based on its known aqueous permeation rate identified by the manufacturer. Envelopes were custom made by using 90-mm, 0.22-µm, hydrophilic, filters from Millipore (GVWP 00010). Envelopes were made by folding the filter in half, heat sealing two sides, aseptically transferring a BI disk by pouring it from a cut Tyvek envelope, and heat-sealing the third side.

A decontamination efficacy test was performed on 06/22/2016 using BI disks in PVDF envelopes prepared as described. Results showed increasing decontamination efficacy with time for the PVDF envelopes over the time period of 5 minutes to 60 minutes (Table C-6). Since a complete kill (LR ~6.5) was not seen within the hour, however, a follow-up test was performed on 06/27/2016 where time periods of 4 to 48 hours were tested. These results are shown in Table C-7.

**Table C-6. BIs in Activated Sodium Persulfate, 5 to 60 min (PVDF vs. Tyvek Envelope)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 5-min exposure | PVDF | Activated SP | $3.73 \times 10^6$ | 133 | 0.17 |
| 10-min exposure | PVDF | Activated SP | $1.97 \times 10^6$ | 70 | 0.45 |
| 30-min exposure | PVDF | Activated SP | $3.70 \times 10^5$ | 13 | 1.17 |
| 60-min exposure | PVDF | Activated SP | $7.33 \times 10^3$ | < 1 | 2.88 |
| 30-min exposure | Tyvek | Activated SP | $2.63 \times 10^6$ | 94 | 0.32 |
| 60-min exposure | Tyvek | Activated SP | $3.07 \times 10^6$ | 110 | 0.25 |
| 60-min exposure | PVDF | Sterile water | $5.90 \times 10^6$ | 211 | -0.03 |
| Control sample | none | Direct to sterile water | $5.50 \times 10^6$ | 196 | NA |

[a] Based on BI standard of $2.80 \times 10^6$ CFU (from supplier's COA).
[b] Based on recovery of control sample and not the COA from the BI supplier.

**Table C-7. BIs in Activated Sodium Persulfate, 4 to 48 hr (PVDF only)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 4-hr exposure | PVDF | Activated SP | 0 | 0 | 6.47 |
| 18-hr exposure | PVDF | Activated SP | 0 | 0 | 6.47 |
| 24-hr exposure | PVDF | Activated SP | 0 | 0 | 6.47 |
| 48-hr exposure | PVDF | Activated SP | 0 | 0 | 6.47 |
| Control sample | none | Direct to sterile water | $2.93 \times 10^6$ | 105 | NA |

[a] Based on BI standard of $2.80 \times 10^6$ CFU (from supplier's COA).
[b] Based on recovery of control sample and not the COA from the BI supplier.

## Developing Tyvek and PVDF Envelopes for Carrier Soil Packets

Physical evaluations (size, shape, material strength) were performed to characterize the Tyvek envelopes used for making CSPs for the spiked soils. A 1-gram quantity of soil was found to be appropriate. Heat-sealing the envelopes with a Uline lab heat sealer was found to be sufficient.

Upon determining that Tyvek was not the best choice for SP testing, an alternative material was sought. A brief review of suppliers showed that polyvinylidene fluoride (PVDF) and polycarbonate (PC) were available as 0.2-µm, hydrophilic filter media from Millipore. Samples of both PVDF and PC were obtained and tested for physical strength and ability to heat seal. Both materials were immersed in activated SP and showed no deterioration after 12 hours. Finally, both materials were tested for penetration of activated SP by placing a single drop of liquid on each, and observing the rate of liquid flow through the filter. While both materials were hydrophilic and allowed penetration, PVDF was clearly superior. Figure C-1 is a picture of the PC (left), PVDF (upper and middle right), and Tyvek (lower right) five minutes after the drop was placed. Note that complete disbursement of the water drop occurs on the PVDF, while for the other materials, a portion of the drop was still intact.

Because of the data in this and the previous section, all decontamination tests described in this report using SP employed PVDF envelopes. The commercially-available Tyvek BIs were simply cut open and the disc was poured into a PVDF envelope, then heat sealed in place.

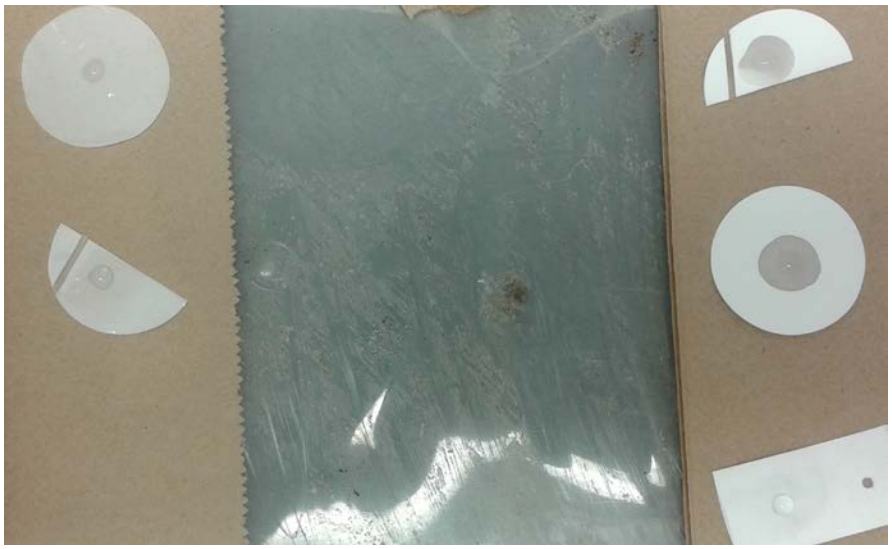


**Figure C-1. Persulfate Penetration into PC, PVDF, and Tyvek**

## Extracting *B. atrophaeus* from Spiked Autoclaved Soil

Two sets of tests were done to evaluate recovery of *B. atrophaeus*-spiked soil. One set of tests was performed using 1-gram quantities of autoclaved soil in Tyvek envelopes. After preliminary wall-effects tests showed problems with using Tyvek for SP testing, another identical test was performed using PVDF envelopes.

For the Tyvek envelope tests, soils were spiked ten times with 10-µL aliquots of *B. atrophaeus* (100 µL total from a master cell bank containing 6.7e8 CFU/mL, resulting in a 6.7e7 CFU spike) per 1-gram sample. Three samples of each soil type were prepared, along with one negative control (unspiked) for each soil type. Tyvek and PVDF envelopes were then heat sealed and placed in a biosafety cabinet (BSC) for 30 minutes. Samples were recovered by cutting envelopes open with sterile scissors, placing soil into 50-mL conical tubes, adding 10 mL of extraction buffer, agitating at 200 rpm for 15 minutes, removing 100 µL of extract, and plating serial dilutions. Table C-8 shows the results.

**Table C-8. *B. atrophaeus*-Spiked Soil Extraction Efficacy (Tyvek Envelopes)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] |
|---|---|---|---|---|
| Topsoil | Tyvek | Sit 30 min | $1.77 \times 10^7$ | 40 |
| Topsoil | Tyvek | Sit 30 min | $2.03 \times 10^7$ | 46 |
| Topsoil | Tyvek | Sit 30 min | $1.97 \times 10^7$ | 45 |
| Neg. Control (Topsoil) | Tyvek | Sit 30 min | 0 | 0 |
| Sand | Tyvek | Sit 30 min | $2.84 \times 10^7$ | 64 |
| Sand | Tyvek | Sit 30 min | $3.03 \times 10^7$ | 69 |
| Sand | Tyvek | Sit 30 min | $3.03 \times 10^7$ | 69 |
| Neg. Control (Sand) | Tyvek | Sit 30 min | 0 | 0 |
| Clay | Tyvek | Sit 30 min | $2.01 \times 10^7$ | 46 |
| Clay | Tyvek | Sit 30 min | $2.22 \times 10^7$ | 51 |
| Clay | Tyvek | Sit 30 min | $2.28 \times 10^7$ | 52 |
| Neg. Control (Clay) | Tyvek | Sit 30 min | 0 | 0 |
| No Soil Spike (control) | none | Direct to buffer | $4.40 \times 10^7$ | NA |

[a] Based on control sample (direct to buffer) recovery of $4.4 \times 10^7$ CFU.

For the PVDF envelope tests, soils were spiked as described above, but ten 10-µL aliquots of *B. atrophaeus* diluted 1:1 with sterile water were used (100 µL total from a master cell bank of 3.35e8 CFU/mL, resulting in a 3.35e7 CFU spike) per 1-gram sample. One sample of each soil type was prepared, and one control sample was used. Table C-9 shows the results.

**Table C-9. *B. atrophaeus*-Spiked Soil Extraction Efficacy (PVDF Envelopes)**

| Sample | Envelope | Treatment | Observed (CFU) | Recovery (%)[a] |
|---|---|---|---|---|
| Topsoil | PVDF | Sit 30 min | $2.00 \times 10^7$ | 716 |
| Sand | PVDF | Sit 30 min | $7.47 \times 10^6$ | 268 |
| Clay | PVDF | Sit 30 min | $9.50 \times 10^5$ | 34 |
| No Soil Spike (control) | none | Direct to buffer | $2.79 \times 10^6$ | NA |

[a] Based on control sample (direct to buffer) recovery of $2.79 \times 10^6$ CFU.

# Appendix D.
# Preliminary Tests

Several preliminary tests were conducted prior to executing the main study. These tests included preliminary wall-effects and soil saturation tests, and are summarized in the sections that follow and also in Table D-1.

**Table D-1. Summary of Wall-Effects Tests**

| Date | Soil Type | Columns | Description | Data Location |
|---|---|---|---|---|
| **$ClO_2$ Wall-Effects Tests** | | | | |
| 02/18/2016 | Topsoil | 10”, 8”, 6” buckets | 8.4 mg/L, 3 hr, 75% RH | Table D-2 |
| 03/04/2016 | Sand | 10”, 8”, 6” columns | 8.4 mg/L, 3 hr, 75% RH | Table D-3 |
| 03/16/2016 | Topsoil | 8”, 6” columns | 8.4 mg/L, 6 hr, 75% RH | Table D-4 |
| 03/30/2016 | Clay | 8”, 6” columns | 8.4 mg/L, 6 hr, 75% RH | Table D-5 |
| **Sodium Persulfate Wall-Effects Tests** | | | | |
| 03/28/2016 | Topsoil | 10”, 8”, 6” columns | ~0.1 mL/g, 1 day | Table D-6 |
| 04/20/2016 | Topsoil | 10”, 8”, 6” columns | 0.14 mL/g total, 7 day | Table D-7 |
| 06/06/2016 | Topsoil | 6” column | 0.14 mL/g total, 1 day | Table D-8 |
| **Sodium Persulfate Wettability Tests** | | | | |
| 04/11/2016 | Topsoil | 10” bucket | Multiple applications to match previous water test and observe similarities. | Visual exam |
| 04/13/2016 | Topsoil | 8” column | Fresh applications every 20 min until saturation point is reached at 0.14 mL/g. | Visual exam |

## Preliminary Wall Effects Tests

Four wall effects tests were performed using $ClO_2$, and three were performed using SP, as described below.

### Chlorine Dioxide with Topsoil, 8.4 mg/L, 3 hr

A wall effects test was performed on 02/18/2016 using $ClO_2$, BIs, commercial topsoil and a small containment box. Chlorine dioxide was generated by the Minidox-M with a concentration set point of 8.4 mg/L and an exposure time of 3 hrs. A containment box was made from a 35-gallon plastic tub (Sterilite™; Townsend, MA) with the top sealed by duct tape. Connections to the Minidox-M were made by using tubing and standard fittings obtained from ClorDiSys. Three test columns were built as follows: 10” diameter x 10” depth (5-gallon plastic bucket), 8” diameter x 10” depth (PVC pipe with an end cap on the bottom); 6” diameter x 10” depth (PVC pipe with an end cap on the bottom).

Timberline™ topsoil (Oldcastle Lawn & Garden, Inc., available at Home Depot) was used for the test soil. Soil density was established to be 1.0 $g/cm^3$ based on previous EPA work [4]. For test purposes, this density was obtained by weighing the appropriate mass of soil per unit depth for the three column diameters tested (6”, 8”, and 10”).

BIs were placed in duplicate in each column at depths of 2” and 5” and were located side by side in the center of the column (i.e., at least three inches from the wall). A single BI was placed on the top surface of the soil in each column. Two additional BIs were not placed in the containment, but were analyzed as controls, making a total of 17 BIs for testing the three configurations.

Results showed that the $ClO_2$ gas did not penetrate soil at ≥2" depth in any of the columns, while the BIs on the top surface had complete kill. Control samples showed normal recovery in the range of approximately 50%. Average air temperature was 20.2°C (68.4°F) and average RH was 84.6% during the test. Table D-2 presents the results.

**Table D-2. Wall Effects Test ($ClO_2$ and Topsoil, 02/18/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | % Recovery[a] | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|---|
| 10 | 0" (top surface) | A | 0 | 0 | -- | 6.22 |
| 10 | 2" | A | $1.51 \times 10^6$ | 54 | 6.18 | 0.04 |
| 10 | 2" | B | $1.51 \times 10^6$ | 54 | 6.18 | 0.04 |
| 10 | 5" | A | $1.55 \times 10^6$ | 55 | 6.19 | 0.03 |
| 10 | 5" | B | $1.58 \times 10^6$ | 56 | 6.20 | 0.02 |
| 8 | 0" (top surface) | A | 0 | 0 | -- | 6.22 |
| 8 | 2" | A | $1.36 \times 10^6$ | 49 | 6.13 | 0.09 |
| 8 | 2" | B | $1.43 \times 10^6$ | 51 | 6.15 | 0.07 |
| 8 | 5" | A | $1.53 \times 10^6$ | 55 | 6.18 | 0.04 |
| 8 | 5" | B | $1.53 \times 10^6$ | 55 | 6.18 | 0.04 |
| 6 | 0" (top surface) | A | 0 | 0 | -- | 6.22 |
| 6 | 2" | A | $1.43 \times 10^6$ | 51 | 6.16 | 0.07 |
| 6 | 2" | B | $1.38 \times 10^6$ | 49 | 6.14 | 0.08 |
| 6 | 5" | A | $1.39 \times 10^6$ | 50 | 6.14 | 0.08 |
| 6 | 5" | B | $1.41 \times 10^6$ | 50 | 6.14 | 0.07 |
| Control | Not exposed | A | $1.77 \times 10^6$ | 63 | 6.25 | NA |
| Control | Not exposed | B | $1.57 \times 10^6$ | 56 | 6.19 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Chlorine Dioxide with Sand, 8.4 mg/L, 3 hr

A wall effects test was performed on 03/04/2016 using $ClO_2$, BIs, and commercial sand (Pavestone™ play sand from Home Depot). The containment box, columns, test depths, $ClO_2$ concentration, and exposure time were identical to the topsoil test described above. Sand density was determined to be 1.4 g/cm$^3$ by weighing a measured volume of sand in a beaker.

Results showed that the $ClO_2$ gas penetrated sand at the 2" depth, producing a log reduction of approximately 2, but did not penetrate at the 5" depth. BIs on the top surface had complete kill. Control samples showed normal recovery in the range of approximately 50%. Average air temperature was 19.8°C (67.6°F) and ranged from 19.0 to 20.6°C during the test. Average RH was 81.4% and ranged from 76.8 to 84.1% during the test. Table D-3 presents the results.

**Table D-3. Wall Effects Test ($ClO_2$ and Sand, 03/04/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 10 | 0” (top surface) | A | 0 | -- | 6.12 |
| 10 | 2” | A | 0 | -- | 6.12 |
| 10 | 2” | B | 0 | -- | 6.12 |
| 10 | 5” | A | $1.42 \times 10^6$ | 6.15 | -0.03 |
| 10 | 5” | B | $1.46 \times 10^6$ | 6.16 | -0.04 |
| 8 | 0” (top surface) | A | 0 | -- | 6.12 |
| 8 | 2” | A | 0 | -- | 6.12 |
| 8 | 2” | B | 0 | -- | 6.12 |
| 8 | 5” | A | $1.39 \times 10^6$ | 6.14 | -0.03 |
| 8 | 5” | B | $1.43 \times 10^6$ | 6.16 | -0.04 |
| 6 | 0” (top surface) | A | 0 | -- | 6.12 |
| 6 | 2” | A | $6.00 \times 10^3$ | -- | 6.12 |
| 6 | 2” | B | $1.00 \times 10^4$ | -- | 6.12 |
| 6 | 5” | A | $1.25 \times 10^6$ | 6.10 | 0.03 |
| 6 | 5” | B | $1.32 \times 10^6$ | 6.12 | 0.00 |
| Control | Not exposed | A | $1.33 \times 10^6$ | 6.12 | NA |
| Control | Not exposed | B | $1.33 \times 10^6$ | 6.12 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Chlorine Dioxide with Topsoil, 8.4 mg/L, 6 hr

A wall effects test was performed on 03/16/2016 using $ClO_2$, BIs, and commercial topsoil as described earlier. The containment box was identical to previous tests. Columns were 6” and 8” PVC pipe. Test depths were from 0 to 6” in 1-in intervals. Chlorine dioxide concentration was 8.4 mg/L, with a total exposure time of 6 hrs.

Table D-4 presents the results for these 6-hr topsoil exposure tests. Results showed that the $ClO_2$ gas did not penetrate the topsoil. All samples except one had a log reduction of <1, indicating a possible wall effect for that sample. BIs on the top surface had complete kill. Control samples showed normal recovery in the range of approximately 50%. Average air temperature was 23.2°C (73.8°F) ranging from 21.8°C to 24.5°C during the test. Average RH was 78.8% and ranged from 73.0 to 86.3% during the test.

**Table D-4. Wall Effects Test ($ClO_2$ and Topsoil, 03/16/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 8 | 0” (top surface) | A | 0 | -- | 6.06 |
| 8 | 1” | A | $4.27 \times 10^6$ | 6.63 | -0.58 |
| 8 | 2” | A | $1.24 \times 10^6$ | 6.09 | -0.04 |
| 8 | 3” | A | $5.60 \times 10^4$ | 4.75 | 1.31 |
| 8 | 4” | A | $5.93 \times 10^5$ | 5.77 | 0.28 |
| 8 | 5” | A | $4.83 \times 10^5$ | 5.68 | 0.37 |
| 8 | 6” | A | $6.00 \times 10^5$ | 5.78 | 0.28 |
| 6 | 0” (top surface) | A | 0 | -- | 6.06 |
| 6 | 1” | A | $1.35 \times 10^6$ | 6.13 | -0.08 |
| 6 | 2” | A | $1.35 \times 10^6$ | 6.13 | -0.07 |
| 6 | 3” | A | $1.32 \times 10^6$ | 6.12 | -0.07 |
| 6 | 4” | A | $1.64 \times 10^6$ | 6.22 | -0.16 |
| 6 | 5” | A | $1.10 \times 10^6$ | 6.04 | 0.01 |
| 6 | 6” | A | $9.30 \times 10^5$ | 5.97 | 0.09 |
| Control | Not exposed | A | $8.27 \times 10^5$ | 5.92 | NA |
| Control | Not exposed | B | $1.56 \times 10^6$ | 6.19 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of controls samples (last two entries in the table).

## Chlorine Dioxide with Clay, 8.4 mg/L, 6 hr

A wall effects test was performed on 03/30/2016 using $ClO_2$, BIs, and commercial clay soil as described earlier. The containment box was identical to previous tests. Columns were 6” and 8” PVC pipe. Test depths were from 0 to 6” in 1-in intervals. Chlorine dioxide concentration was 8.4 mg/L for an exposure time of 6 hrs. Manual homogenization of the clay was performed wherein clumps of > ~1/4” were broken up by hand during column filling. The clay was not mechanically sieved.

Table D-5 presents the results for these 6-hr clay tests. Results showed that the $ClO_2$ gas penetrated up to 3 to 4” with complete kill, then dropped off to <1 log reduction at greater depths. BIs on the top surface had complete kill. Control samples showed normal recovery in the range of approximately 50%. Average air temperature was 23.2°C (73.8°F) and ranged from 21.8 to 24.5°C during the test. Average RH was 78.8% and ranged from 73.0 to 86.3% during the test.

**Table D-5. Wall Effects Test ($ClO_2$ and Clay, 03/30/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 8 | 0" (top surface) | A | 0 | -- | 6.31 |
| 8 | 1" | A | 0 | -- | 6.31 |
| 8 | 2" | A | 0 | -- | 6.31 |
| 8 | 3" | A | 0 | -- | 6.31 |
| 8 | 4" | A | 0 | -- | 6.31 |
| 8 | 5" | A | $4.70 \times 10^5$ | 5.67 | 0.64 |
| 8 | 6" | A | $9.00 \times 10^4$ | 4.95 | 1.36 |
| 6 | 0" (top surface) | A | 0 | -- | 6.31 |
| 6 | 1" | A | 0 | -- | 6.31 |
| 6 | 2" | A | 0 | -- | 6.31 |
| 6 | 3" | A | 0 | -- | 6.31 |
| 6 | 4" | A | $1.30 \times 10^5$ | 5.14 | 1.17 |
| 6 | 5" | A | $5.70 \times 10^5$ | 5.76 | 0.55 |
| 6 | 6" | A | $6.63 \times 10^5$ | 5.82 | 0.49 |
| Control | Not exposed | A | $2.04 \times 10^6$ | 6.31 | NA |
| Control | Not exposed | B | $2.05 \times 10^6$ | 6.31 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Sodium Persulfate with Topsoil, 0.5 M, 0.10 mL/g, 1 day

A wall effects test was performed on 03/28/2016 using activated SP, BIs, and commercial topsoil as described earlier. No containment box was used for these tests. Three column configurations were tested with 6", 8", and 10" diameter PVC pipe placed in an open-topped plastic tub (to contain any drips) in a fume hood. Test depths were at 0, 2", and 5". Sodium persulfate was mixed at 0.5 M concentration, and was activated by mixing with fresh 8% hydrogen peroxide immediately prior to application. The liquid was applied at a total volume of 0.09 to 0.10 mL/g of topsoil calculated for a depth of 5" of soil. Soak time after application was 1 day. One additional 10" column was also dosed with deionized water as a control. Liquid was applied in ~100-mL increments every 2 minutes as follows:

- 10" column: 6x 97-mL applications, 582 mL total
- 8" column: 4x 100-mL applications, 400 mL total
- 6" column: 2x 100-mL applications, 200 mL total

Table D-6 presents the results of the persulfate/topsoil exposures. Results showed little, if any, kill versus the controls. BIs on the top surface did not all show complete kill. During later tests, as previously described, problems were traced to hydrophobic qualities of Tyvek, which essentially slows down or even repels the persulfate liquid.

Average air temperature was 24.1°C (75.4°F) and ranged from 22.5 to 25.5°C during the test. Average RH was 19.8% and ranged from 17.0 to 25.0% during the test.

**Table D-6. Wall Effects Test (Sodium Persulfate and Topsoil, 03/28/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 10 control | 0" (top surface) | A | $2.65 \times 10^5$ | 5.42 | 0.32 |
| 10 control | 2" | A | $3.30 \times 10^5$ | 5.52 | 0.22 |
| 10 control | 2" | B | $3.33 \times 10^5$ | 5.52 | 0.22 |
| 10 control | 5" | A | $3.23 \times 10^5$ | 5.51 | 0.23 |
| 10 control | 5" | B | contaminated | -- | -- |
| 10 | 0" (top surface) | A | $3.33 \times 10^2$ | 2.52 | 3.22 |
| 10 | 2" | A | $3.27 \times 10^5$ | 5.51 | 0.23 |
| 10 | 2" | B | $3.53 \times 10^5$ | 5.55 | 0.19 |
| 10 | 5" | A | $2.02 \times 10^5$ | 5.30 | 0.44 |
| 10 | 5" | B | $3.83 \times 10^5$ | 5.58 | 0.16 |
| 8 | 0" (top surface) | A | 0 | -- | 5.75 |
| 8 | 2" | A | $1.79 \times 10^5$ | 5.25 | 0.49 |
| 8 | 2" | B | $1.73 \times 10^5$ | 5.24 | 0.50 |
| 8 | 5" | A | $2.59 \times 10^5$ | 4.41 | 1.33 |
| 8 | 5" | B | $2.88 \times 10^4$ | 4.46 | 1.28 |
| 6 | 0" (top surface) | A | $1.03 \times 10^3$ | 3.01 | 2.73 |
| 6 | 2" | A | $2.02 \times 10^5$ | 5.31 | 0.44 |
| 6 | 2" | B | $1.88 \times 10^5$ | 5.27 | 0.47 |
| 6 | 5" | A | $1.82 \times 10^5$ | 5.26 | 0.48 |
| 6 | 5" | B | $1.78 \times 10^5$ | 5.25 | 0.49 |
| Control | Not exposed | A | $1.04 \times 10^6$ | 6.02 | NA |
| Control | Not exposed | B | $2.94 \times 10^5$ | 5.47 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Sodium Persulfate with Topsoil, 0.5 M, 0.14 mL/g, 7 day

A wall effects test was performed on 04/20/2016 using activated SP, BIs, and commercial topsoil as described earlier. No containment box was used for these tests. Three columns configurations were tested with 6", 8", and 10" diameter PVC pipe placed in an open-topped plastic tub (to contain any drips) in a fume hood. Test depths were from 0 to 6" in 1-inch increments. SP was mixed at 0.5 M concentration, and was activated by mixing with fresh 8% hydrogen peroxide immediately prior to application. The liquid was applied at a total volume of 0.14 mL/g of topsoil calculated for a depth of 6" of soil, the amount determined to be the saturation point of soil in earlier lab tests. Soak time after application was 7 days. One additional sample was dosed with deionized water in topsoil as a wet control. Liquid was applied in 2 equal increments, 30 minutes apart, as follows:

- 10" column: two (2) 550-mL applications; 1,100 mL total
- 8" column: two (2) 350-mL applications; 700 mL total
- 6" column: two (2) 195-mL applications; 390 mL total

Table D-7 presents the results of these 7-day persulfate exposures. Results showed little, if any, kill versus the controls. Unlike the previous test, all BIs on the top surface did show complete kill, indicating that the 7-day exposure time is more than adequate for the persulfate to permeate the Tyvek CSP (as was shown earlier in this report).

Average air temperature was 22.7°C (72.9°F) and ranged from 22.5 to 24.0°C during the test. Average RH was 48.7% and ranged from 40.0 to 58.0% during the test. Table D-7 presents the results.

**Table D-7. Wall Effects Test (Sodium Persulfate and Topsoil, 04/20/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 10 | 0" (top surface) | A | 0 | -- | 5.53 |
| 10 | 1" | A | $5.67 \times 10^5$ | 5.75 | -0.22 |
| 10 | 2" | A | $5.97 \times 10^5$ | 5.78 | -0.25 |
| 10 | 3" | A | $7.57 \times 10^5$ | 5.88 | -0.35 |
| 10 | 4" | A | $3.93 \times 10^5$ | 5.59 | -0.07 |
| 10 | 5" | A | $2.56 \times 10^5$ | 5.41 | 0.12 |
| 10 | 6" | A | $5.27 \times 10^5$ | 5.72 | -0.19 |
| 8 | 0" (top surface) | A | 0 | -- | 5.53 |
| 8 | 1" | A | $3.13 \times 10^5$ | 5.50 | 0.03 |
| 8 | 2" | A | $4.93 \times 10^5$ | 5.69 | -0.16 |
| 8 | 3" | A | $4.97 \times 10^5$ | 5.70 | -0.17 |
| 8 | 4" | A | $3.87 \times 10^5$ | 5.59 | -0.06 |
| 8 | 5" | A | $4.20 \times 10^5$ | 5.62 | -0.09 |
| 8 | 6" | A | $4.97 \times 10^5$ | 5.70 | -0.17 |
| 6 | 0" (top surface) | A | 0 | -- | 5.53 |
| 6 | 1" | A | $4.30 \times 10^5$ | 5.63 | -0.10 |
| 6 | 2" | A | $5.70 \times 10^5$ | 5.76 | -0.23 |
| 6 | 3" | A | $3.20 \times 10^5$ | 5.51 | 0.02 |
| 6 | 4" | A | $5.77 \times 10^5$ | 5.76 | -0.23 |
| 6 | 5" | A | $5.13 \times 10^5$ | 5.71 | -0.18 |
| 6 | 6" | A | $3.07 \times 10^5$ | 5.49 | 0.04 |
| Wet Control | Not exposed | A | $4.63 \times 10^5$ | 5.67 | -0.14 |
| Control | Not exposed | A | $3.33 \times 10^5$ | 5.52 | NA |
| Control | Not exposed | B | $3.43 \times 10^5$ | 5.54 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Sodium Persulfate with Topsoil, 0.5 M, 0.14 mL/g, 1 day, PVDF packets

A wall effects test was performed on 06/06/2016 using activated SP, BIs repackaged in PVDF, and commercial topsoil as described earlier. PVDF BIs were prepared by cutting open a standard Tyvek BI packet, pouring the actual BI disc into a PVDF packet, then heat sealing the PVDF.

A single column configuration was used for this test. The column was a 6" PVC pipe placed in an open-topped plastic tub (to contain any drips) in a fume hood. Test depths were at 0, 1, and 2". SP was mixed at 0.5 M concentration, and was activated by mixing with fresh 8% hydrogen peroxide immediately prior to application. The liquid was applied at a total loading of 0.14 mL/g of topsoil calculated for a depth of 6" of soil, the amount determined to be the saturation point of soil in earlier lab tests. Soak time after application was 1 day. Liquid was applied in 2 equal increments, 30 minutes apart, as follows:

- 6" column: two (2) 195-mL applications; 390 mL total

Table D-8 presents the results, which showed ~0.4 log reduction versus the controls.

Average air temperature was 22.5°C (72.5°F) and ranged from 22.5 to 23.0°C during the test. Average RH was 48.4% and ranged from 41.0 to 52.0% during the test.

**Table D-8. Wall Effects Test (Sodium Persulfate and Topsoil, 06/06/2016)**

| Column Diameter (in) | BI Depth (in) | Sample ID | Observed (CFU) | Log of Recovered CFU | Decon. Efficacy (LR)[b] |
|---|---|---|---|---|---|
| 6 | 0" (top surface) | A | 0 | -- | 5.05 |
| 6 | 1" | A | $4.37 \times 10^4$ | 4.64 | 0.41 |
| 6 | 2" | A | $4.53 \times 10^4$ | 4.66 | 0.39 |
| | | | | | |
| Control | Not exposed | A | $9.50 \times 10^4$ | 4.98 | NA |
| Control | Not exposed | B | $1.30 \times 10^5$ | 5.11 | NA |

[a] BI standard is $2.8 \times 10^6$ CFU, obtained from certificate of analysis.
[b] Compared against the average of control samples (last two entries in the table).

## Sodium Persulfate Soil Saturation Tests

Due to the large masses of soil being tested in this project, we were not able to rely on liquid loading volumes used in previous EPA studies. Previous work (EPA, 2015), for example, used a liquid volume of 3 applications of 0.18 mL activated SP per gram of soil (three (3) 0.18-mL applications, or 0.54 mL/g total, which worked well on a petri-dish scale. For the current project, large test columns containing >25 lbs of soil were required, so it was necessary to experimentally determine a more appropriate soil saturation point.

An initial test using a 6" depth of topsoil in a 5-gal bucket was performed on 02/11/2016. Topsoil (density ~1.0 g/mL) was weighed and measured. For the 5-gal bucket, this amounts to 3.4 lb/inch of soil depth, or 20.4 lbs for a 6" depth. The bucket was assembled so that the original bottom was replaced with a metal screen, and elevated onto a platform for observation.

100 mL of deionized water was evenly distributed across the surface of the soil. This was repeated every 2 minutes, and visual observations were made for leakage of water through the metal screen at the bottom of the bucket. After 800 mL had been poured onto the soil (~16 minutes after beginning the test), water began dripping through the metal screen. A beaker was placed under the dripping water, and approximately 1 mL was collected before the dripping stopped. Based on these data, a saturation point of 0.09 mL/g was estimated.

The first SP wall-effects test was performed on 03/28/2016 (see previous section) using a volume of 0.09 mL/g, and a target penetration depth of 5". When no decontamination was observed at this depth, it was decided to determine the soil saturation point using activated SP, rather than water. When large volumes of soil are wetted by large volumes of SP, a vigorous oxidizing reaction occurs that immediately liberates extensive volumes of gas, causing foaming. This, in turn, causes the liquid to puddle up on the soil surface in a foamy layer that ultimately penetrates over the next 30 minutes or so. This is a phenomenon not seen during previous EPA studies because of the relatively small volumes of soil and decontaminant volumes used during petri-dish sized experiments.

A SP saturation test was performed on 04/13/2016 that used the same general approach as the previous water tests. An 8” column was filled to a 6” depth with topsoil (4,952 g), and 100-mL volumes of activated SP were applied. Applications were initially in 20-min intervals. By the 3rd application, the reaction had created enough foam to slow penetration of the persulfate, so that there was standing liquid on the top surface that becomes a sticky, porous area as it seeps in (Figure D-1). By the 5th application, a penetration rate was achieved such that fresh applications were slowed to once per hour. Shortly after the 7th application, liquid began dripping from the bottom surface, indicating that the saturation point had been reached (Figure D-2). Dripping liquid was captured in a glass beaker and was poured over fresh topsoil in a nearby bucket. No reaction was observed with the fresh topsoil, suggesting that the captured persulfate was now inert, or at least its reactive components reduced.

**Figure D-1. Topsoil Persulfate Saturation Test (just prior to 5th application of sodium persulfate)**

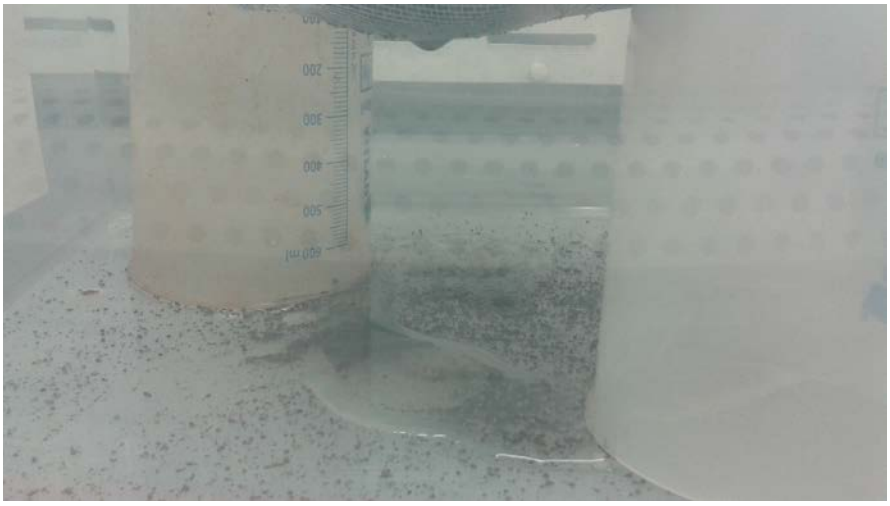


**Figure D-2. Sodium Persulfate Dripping From Bottom of Topsoil Column**

The persulfate saturation level for the topsoil was calculated to be 0.14 mL/g. Again, this level is less than the previous 0.54 mL/g used in earlier EPA work. It is also important to note that the applications must be spaced well apart, or else surface reactions and gas release will inhibit the timely flow of sodium persulfate into the soil.

# Appendix E.
# ORP Measurements

During the SP test series, any liquid that permeated through the column was collected in drip pans located beneath each column. Excess liquid was removed as needed to prevent the drip pans from overflowing. For the final test in this series (Test 3 SP), the oxidation reduction potential (ORP) was measured using a hand-held probe (Hach Pocket Pro™). ORP measurements were taken of the initial (fresh) activated SP and final (partially spent) liquid emerging from the column bottom. Liquid volumes were also estimated by the laboratory staff.

As shown in Table E-1, liquid emerging from the sand column was nearly the same as the application volume (876 mL) after the 3rd application, and also showed a clearly different ORP value from the freshly-mixed solution. For topsoil, ORP from the emerging liquid was only slightly different from the freshly-mixed solution, and only limited drainage occurred. Clay was not included in the ORP evaluation because only one liquid application was used, and no liquid was found to emerge from the bottom of the clay column.

Results show that the ORP changes during permeation through the clay and topsoil. Interestingly, the ORP, in general, for the topsoil permeate is very close to the activity (i.e., ORP reading) as freshly-mixed persulfate. In the clay experiments, the ORP increased significantly and in several cases by almost a factor of two. The ORP device was checked repeatedly between readings using a certified standard and was always shown to be within specifications. The source of this increase was not investigated further, but could be due to extraction from the clay and soil, increasing the ORP reading.

**Table E-1. ORP Results for Test 3 SP**

| | | ORP Reading of Permeated Liquid[a] | | Volume of Collected Permeate | |
|---|---|---|---|---|---|
| Day | Application | Topsoil (mV) | Sand (mV) | Topsoil (mL) | Sand (mL) |
| 0 | 1 | -- | -- | -- | -- |
| 1 | 2 | -- | -- | -- | -- |
| 2 | 3 | -- | 638 | -- | 339 |
| 3 | 4 | -- | 661 | -- | 794 |
| 4 | 5 | 432 | 653 | 180 | 825 |
| 5 | 6 | 512 | 559 | 261 | 860 |

[a] Freshly-mixed SP, activated with 8% hydrogen peroxide, had an average ORP response of 437 mV. The response to a certified calibration solution was stable at 225 mV (Zobell's solution from Hach).

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