EPA
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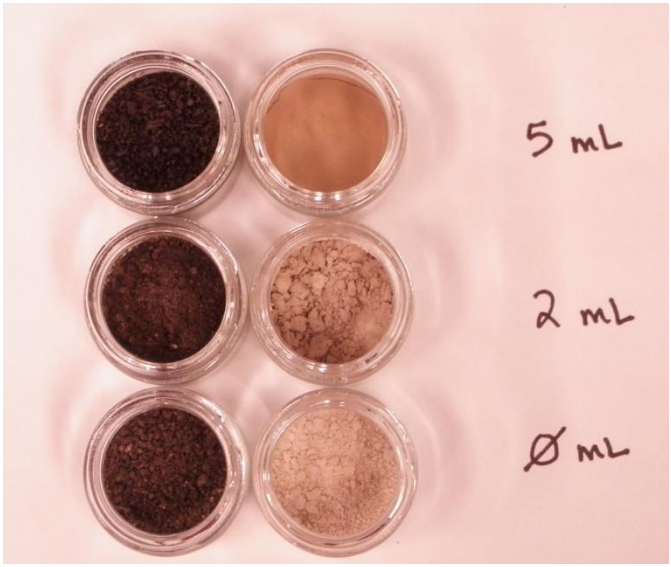
Environmental Protection
Agency

Remediation of Soil Contaminated with *Bacillus anthracis* spores

Erin Silvestri, Char Bowling, Joseph Wood



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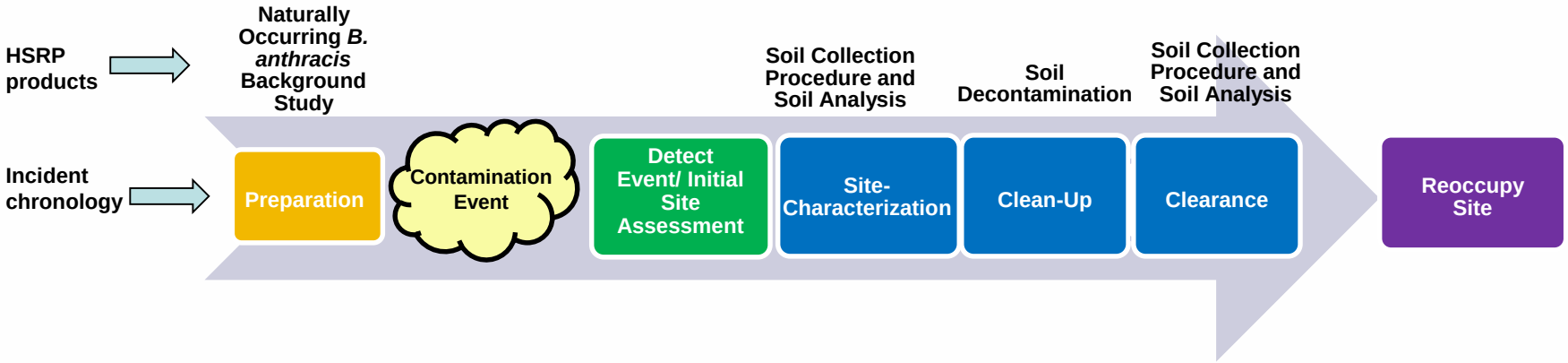
Acknowledgements and Disclaimer

- Acknowledgements
  - Leroy Mickelson, Dino Mattorano, Craig Ramsey
  - Dale Griffin, Timothy Boe, Frank Schaefer, Sanjiv Shah
  - Battelle, Pegasus

Outline

- Background of the problem
- Naturally occurring *Bacillus anthracis (B.a.)* (causative agent for anthrax)
- Methods to recover and analyze *B.a.* from soils
- Sample collection protocols
- Evaluation of soil decontamination technologies
- Lessons learned



Background of the problem

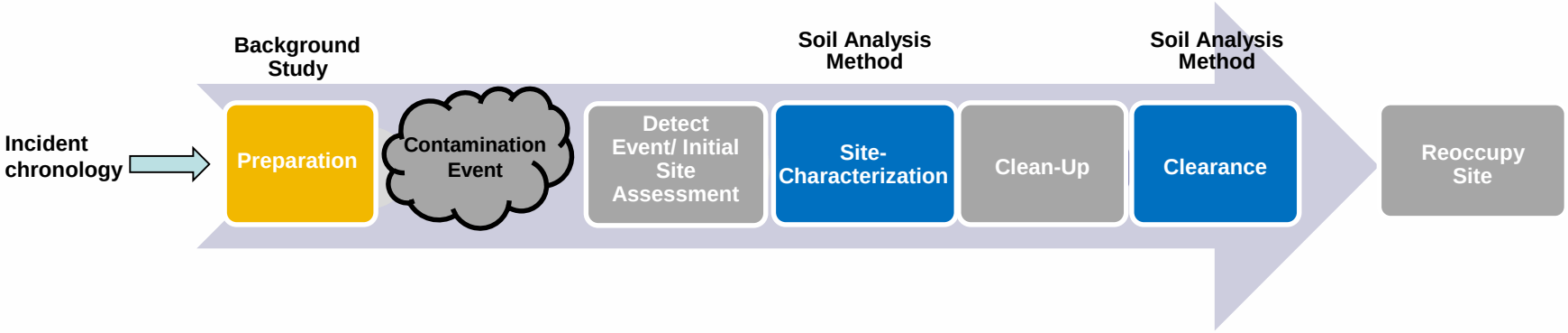
- Remediation efforts could be extensive following an aerosol release of *Bacillus anthracis* (*B.a.*) spores over a wide area

- In such a scenario, many types of materials and environments may need to be sampled, analyzed, and decontaminated, including soils

- Soils remains one of the most difficult materials to analyze and decontaminate for *B.a.*
  - Impurities, and other organisms in soil that impede detection
  - Organic content of soil, as well as depth of soil, impede decon

Naturally Occurring B. anthracis Background Study and Optimized Soil Analysis Method

Erin Silvestri

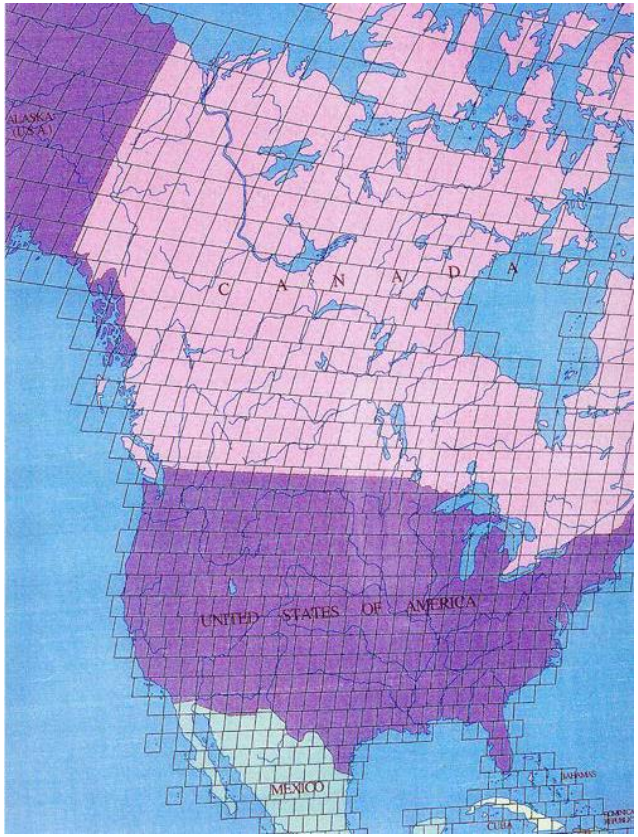


Naturally Occurring *B. Anthracis* and Incident Preparedness

- Knowledge of natural occurrence of *B. anthracis* in the environment helps decision makers better prepare for an incident and is a valuable tool for post-event investigations
  - Aids in communication of risk to the public
  - Aids in site characterization and decontamination decisions

U.S. Geological Survey (USGS) North American Soil Geochemical Landscapes Project (NASGLP)

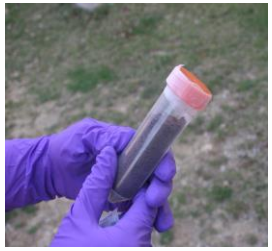
- Soil collected at a density of 1 site per 1600 km^2 (~13,500 sites) to expand baseline geochemical and microbiology data for the U.S., Canada, and Mexico.
- Generalized random tessellation stratified design for sample site selection
- Pilot studies began in 2004 and sample collection ran from 2007-2010



USGS and EPA Background Study Sample Analysis

USGS and EPA co-funded an effort to analyze 4800 samples from 48 conterminous states for presence of *Bacillus* species and *B. anthracis*

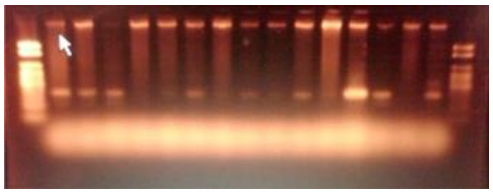
USGS/EPA Background Study Sample Analysis Procedure



Spore DNA extracted using 0.25 g sample aliquot and the MoBio PowerSoil DNA Isolation Kit

Amplification of DNA using Multiplex PCR

Limit of Detection = 10^6 spores/g soil

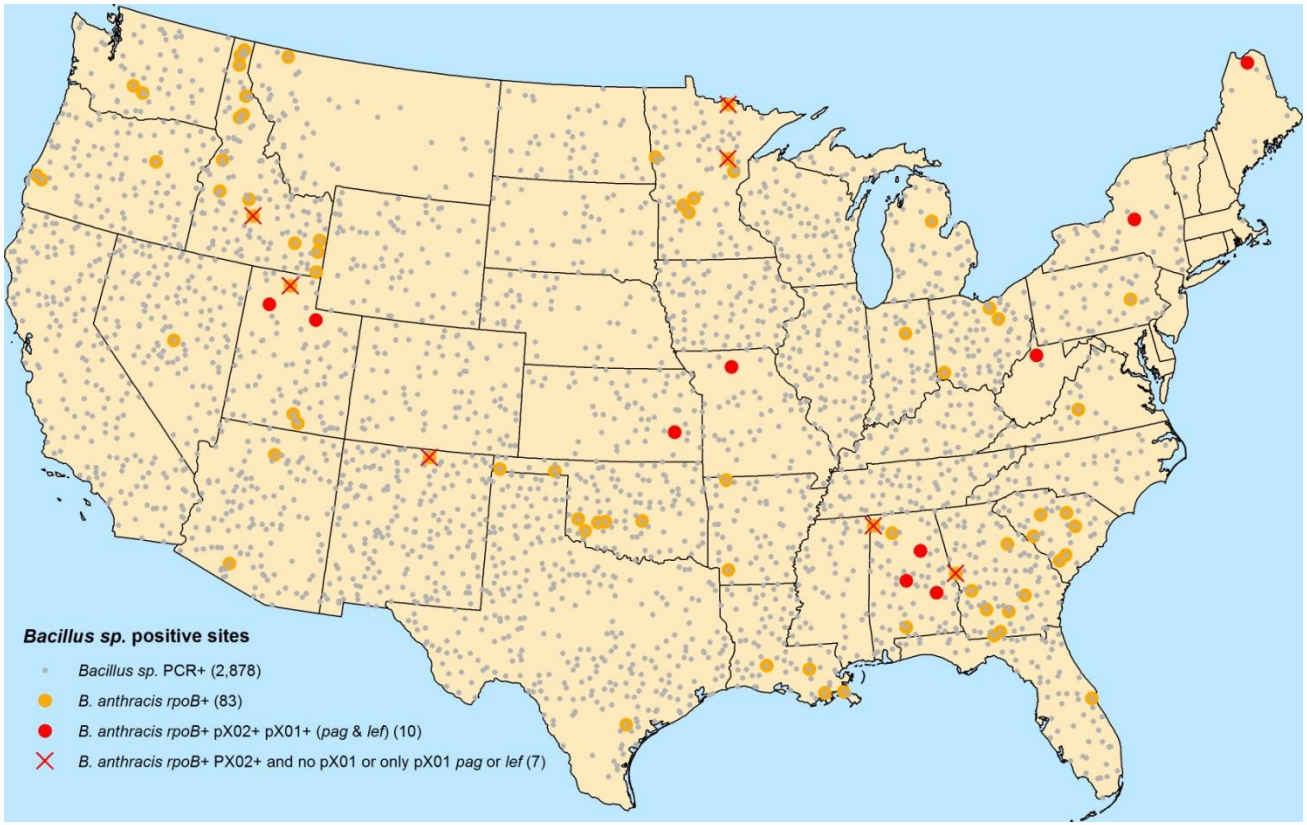


Amplified DNA visualized via SYBRGold stained gel electrophoresis

USGS and EPA Background Study
Results for *Bacillus* sp. and *B. anthracis*

Bacillus sp. were detected in 60.3% of the samples and 43 of 48 states

B. anthracis presumptively identified in 83 samples



Represents a "snapshot" in time

Variations to Background Study Analysis Method

Limit of Detection = 10^6 spores/g

0.25 g sample aliquot

45 g sample aliquot

Limit of Detection = 10^4 spores/g

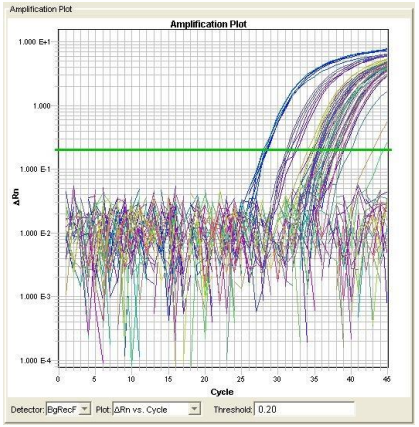
Entire aliquot is washed with phosphate buffered saline with TWEEN (PBST) and pelleted through centrifugation

Aliquot directly used

DNA extracted using MoBio PowerSoil DNA Isolation Kit

Pellet used

Quantitative PCR to amplify DNA

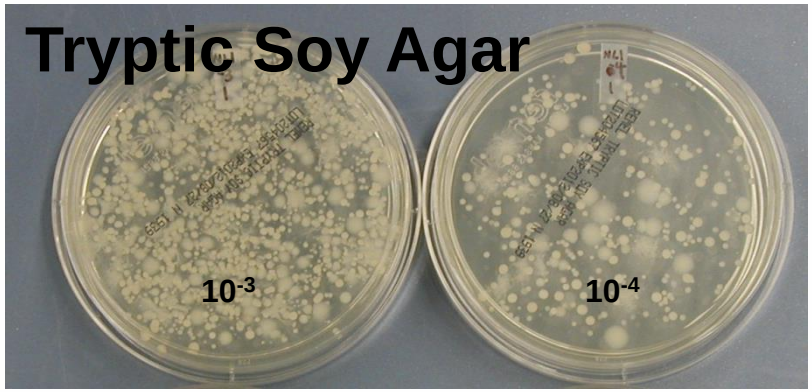


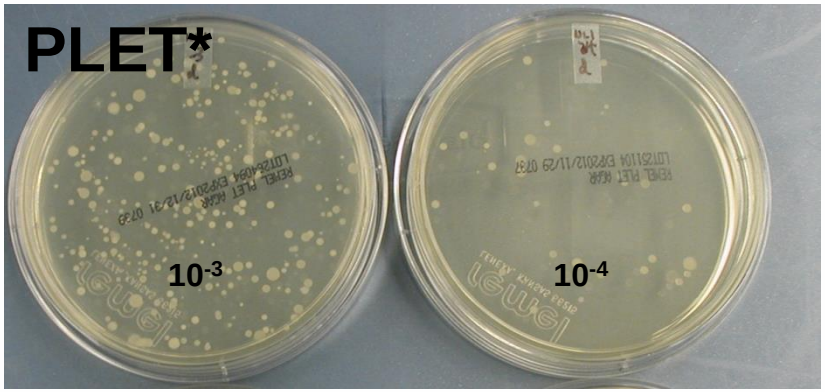
The lower the cycle threshold value, the more DNA is present

Limitations of Current Soil Methods

- Spores present in low numbers may not be detected due to high method LOD
- PCR does not allow for quantitative or viability analysis
- Interferences, inhibitors, impurities, and other organisms in the soil reduce recovery efficiency, especially when using culture based analysis methods

Native (nonsterile) Agvise Loam Soil with no *B. anthracis* spores added shows growth in selective media of background organisms





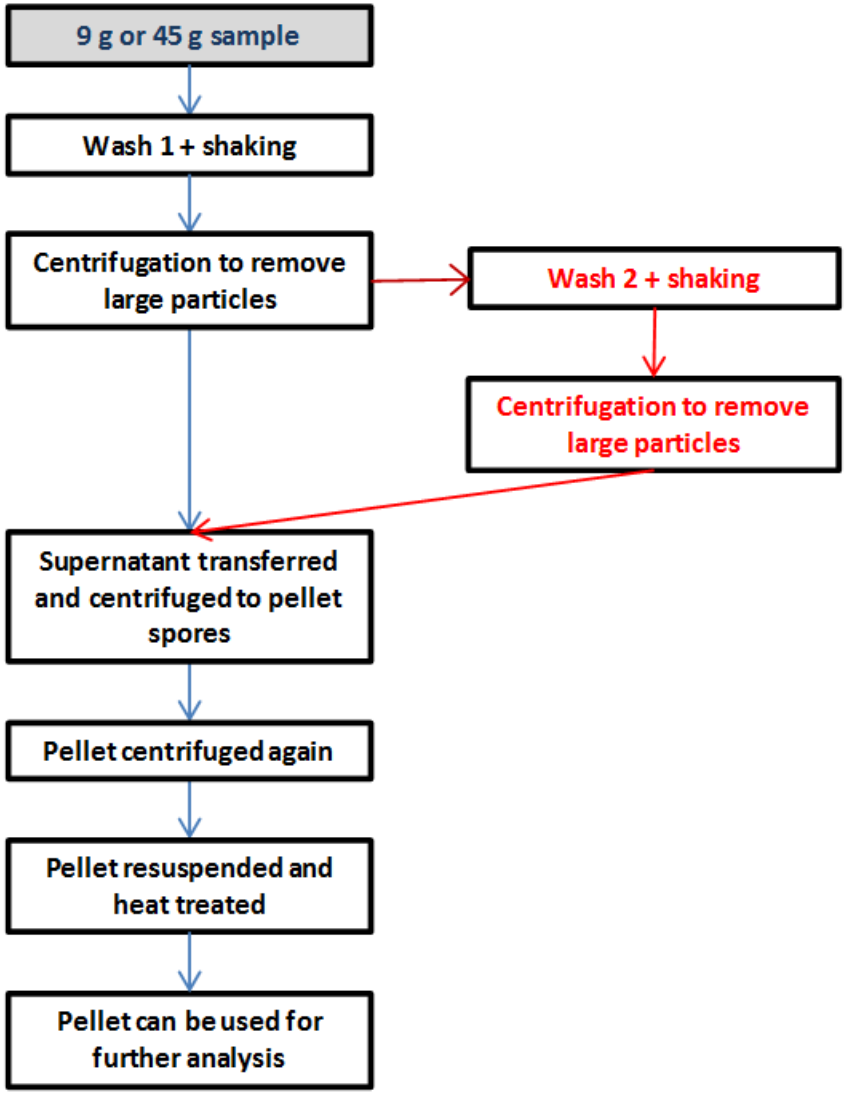
* *polymyxin-lysozyme-EDTA-thallous* acetate

Need for a Optimized Soil Method

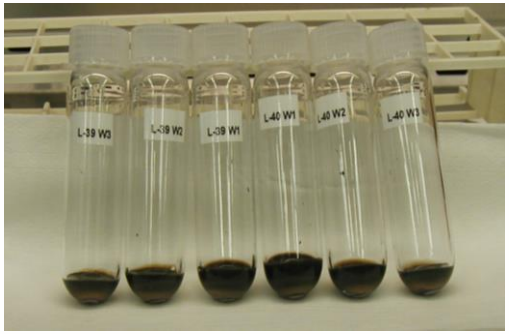
- Currently, no standard method for extraction and analysis of *B. anthracis* from soil available
  - Needed to fill gap in *Selected Analytical Methods for Environmental Remediation and Recovery* pathogens section
  - Needed to be able to detect spores in soil prior to decontamination efforts
- Ideal method would be amendable to use with culture
- Method with lower LOD capabilities needed

Development of an Optimized Extraction Method

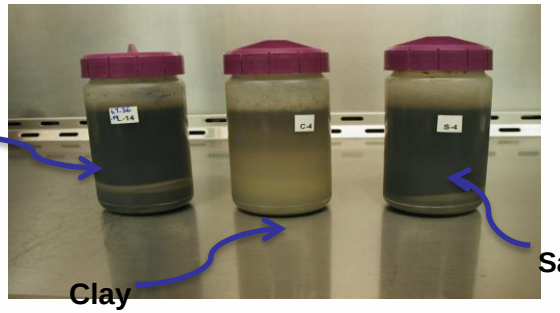
- EPA, USGS, and CDC project team
- Focus on optimizing extraction of spores from soil prior to DNA extraction or further analysis
- Uses a series of washes and centrifugation steps to concentrate the spores into a pellet
- Method being developed using three soil types (loam, clay, and sand) and two sample sizes



Method Evaluation Parameters



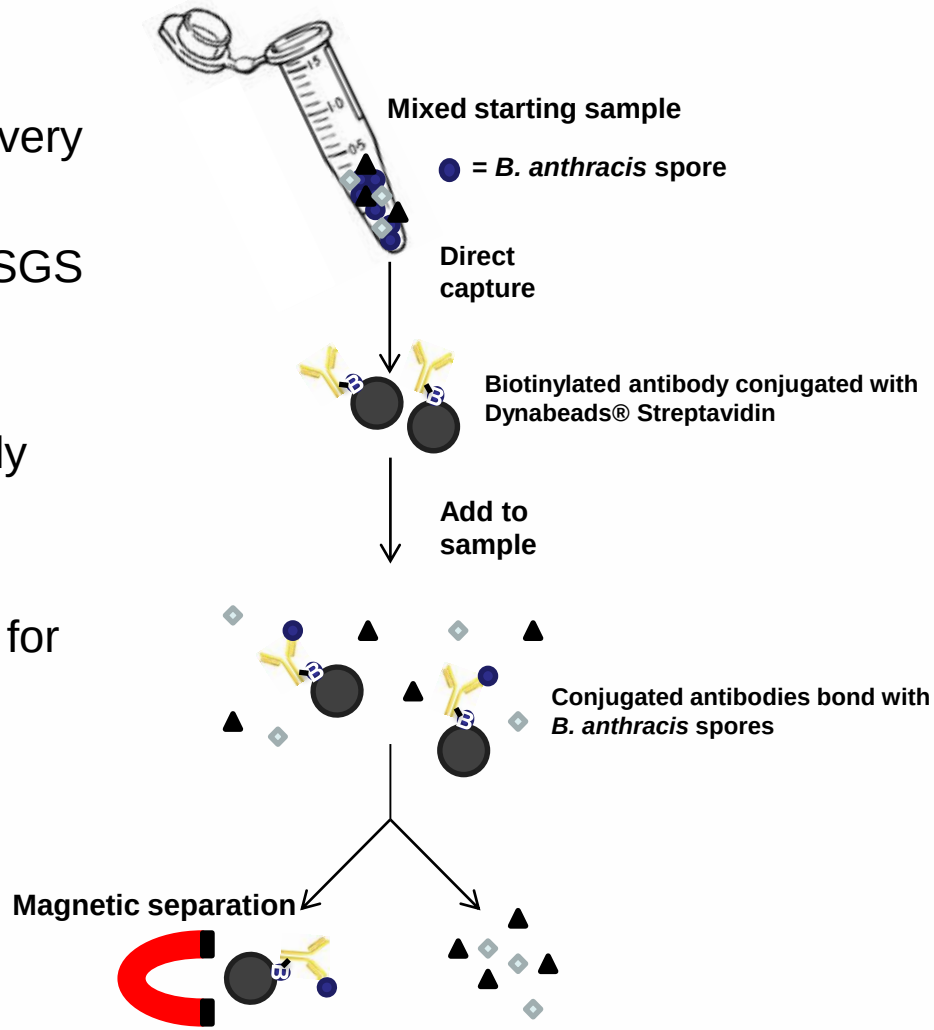
- Number of washes: diminishing return for more than two washes
- Recovery with the addition of sonication similar to recovery without sonication
- Three wash solutions tested: 1% Tween 80 with 2% Hexametaphosphate (HMP) showed slightly better recovery over PBST and phosphate buffered citrate solution
- Mechanical shaking preferred over hand shaking to reduce variability
- Centrifugation speed (using 100 x g and 250 x g): results similar, but 100 x g leaves slightly more spores



Future Efforts

- Evaluation of varied soil pH on spore recovery
- Verification of the extraction protocol at USGS to determine recovery efficiency and LOD
- Use of Fosfomycin (antibiotic) to selectively reduce background bacteria in the soil
- Use of immunomagnetic separation (IMS) for specific isolation of *B. anthracis* spores

IMS



Lessons Learned for Method to Recover and Analyze for *B.a.*

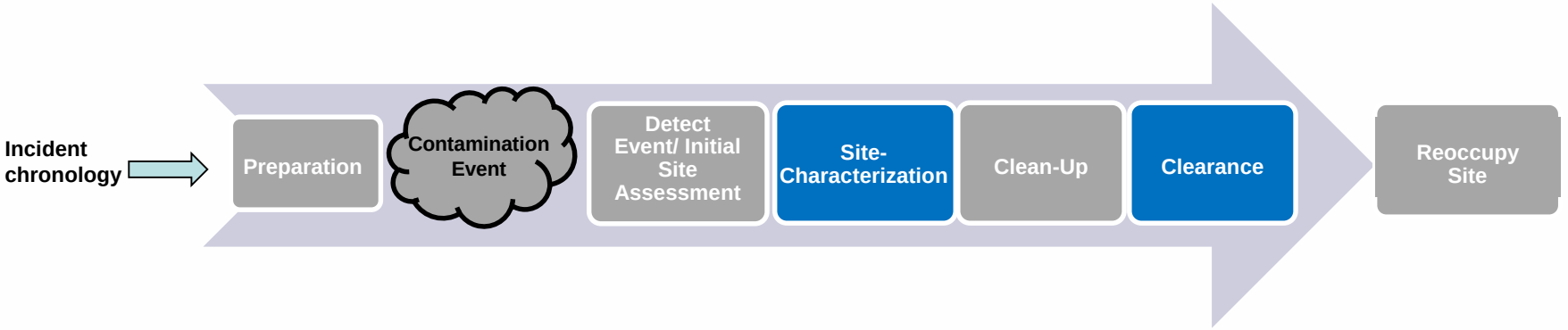
- Use of a larger sample size during the extraction procedure aids in recovery efficiency

- Background organisms and other interferences makes analysis of soil samples using culture difficult
  - Currently using MoBio PowerSoil DNA extraction kit and qPCR for analysis
  - Extraction using fosfomycin and IMS may allow for culture based analysis

- Re-analysis of USGS soil samples using improved method may help identify additional locations where *B. anthracis* may be located

Sample Collection Protocol for Bacterial Pathogens in Surface Soil

Char Bowling



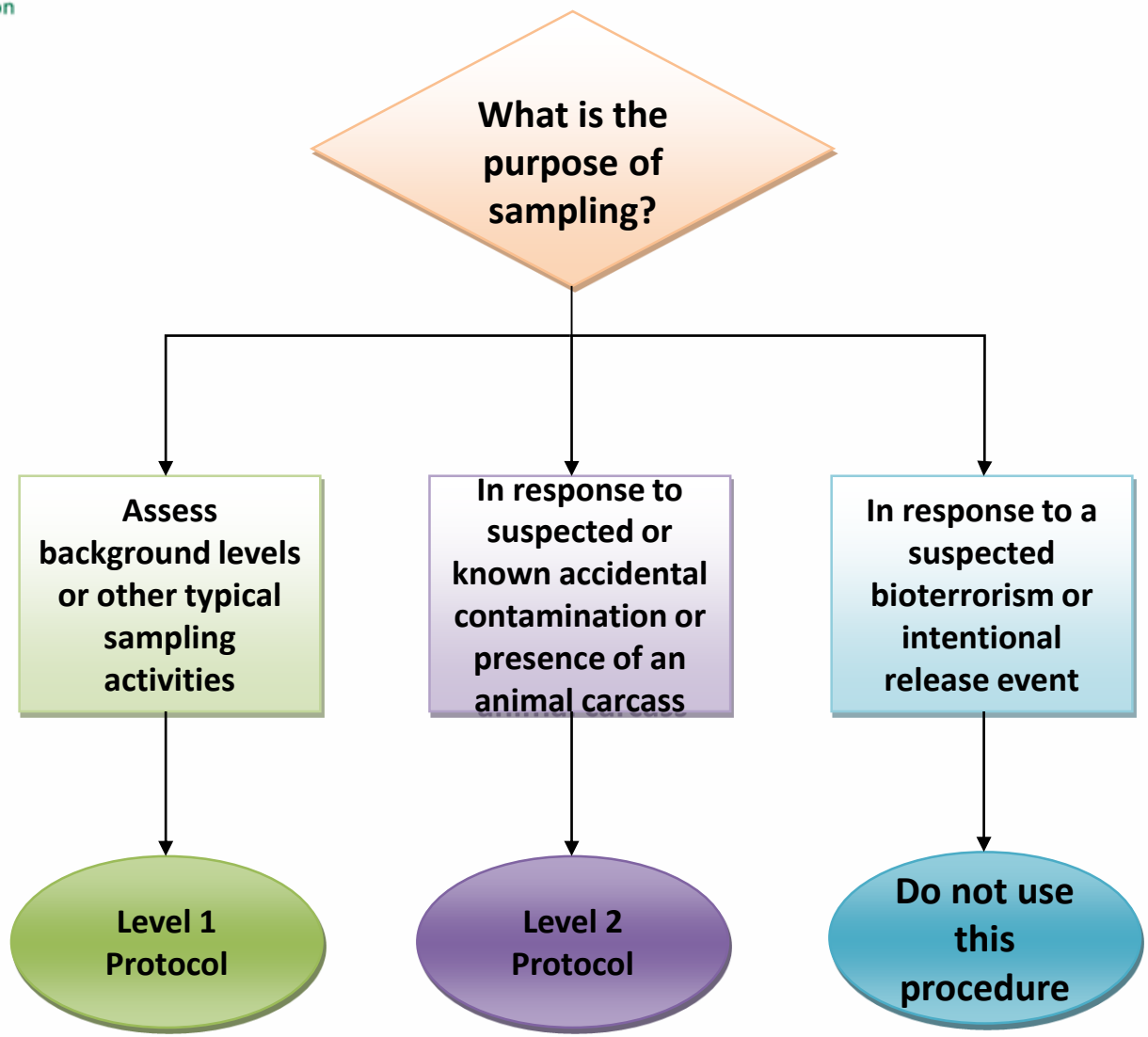
Sample Collection Protocol for Bacterial Pathogens in Surface Soil

- USGS and EPA effort
- Protocol for collecting, handling, shipping of soil samples for detection of naturally occurring pathogens of concern
- Based on the procedures used by U.S. Geological Survey (USGS) during its North American Geochemical Landscapes Pilot Studies
- Guide for developing sampling plans and other site-specific documentation

Use and Purpose

1. Surveillance to determine naturally occurring background levels in soil (i.e. no suspicion of contamination)
2. For suspected or known contamination (i.e. presence of animal carcass)

NOTE: Protocol does not cover sampling in response to a suspected bioterrorist or intentional release event


What is the purpose of sampling?
Assess background levels or other typical sampling activities
In response to suspected or known accidental contamination or presence of an animal carcass
In response to a suspected bioterrorism or intentional release event
Level 1 Protocol
Level 2 Protocol
Do not use this procedure

	Level 1 Protocol	Level 2 Protocol
Purpose	Background study Surveillance	Suspected or known accidental contamination
Sampling Team	1 person	2 people
Roles	All duties	Collector – collects samples
		Assistant – supplies and documentation
PPE	Gloves, dust mask, booties, safety glasses or goggles	Gloves, booties, full face respirator, and Tyvek suit

Protocol Overview

- Step-by-step instructions for Level 1 and Level 2 collection
- Applicable for most types of soil
- 50 mL sterilized tubes
- Top 0-5 cm of soil
  - Of interest due to:
    - Spore persistence
    - Humans and grazing animals have regular contact/surface disruption
- Field log and chain of custody forms

Protocol Summary

- Aseptic techniques
- Soil moisture, temperature, pH, and other landscape characteristics recorded
- Clean gloves for each sample
- Clean spatula/scoop and 50 mL tube
- Sealed with Parafilm and bagged
- Sample bags wiped with bleach wipe
- All samples from single location bagged for shipment
- Samples shipped to lab(s) in cooler(s)

Quality Assurance and Quality Control

- Field blanks
- Trip blanks
- Field replicates
- Background samples
- Custody seals
- Sample tracking & custody
- Meter calibration

NOTE: Type, number, and labeling of QA samples determined by site-specific sampling plan

Resources and Forms Included

- Health and Safety Plan development guidance
- Field Data Sheet
- Chain of Custody
- Laboratory Checklist
- Sample Kit Supply Checklist
- Land Cover Classification Descriptions
- Protocol for Sterilization of Soil by Autoclaving and Preparation of Blanks

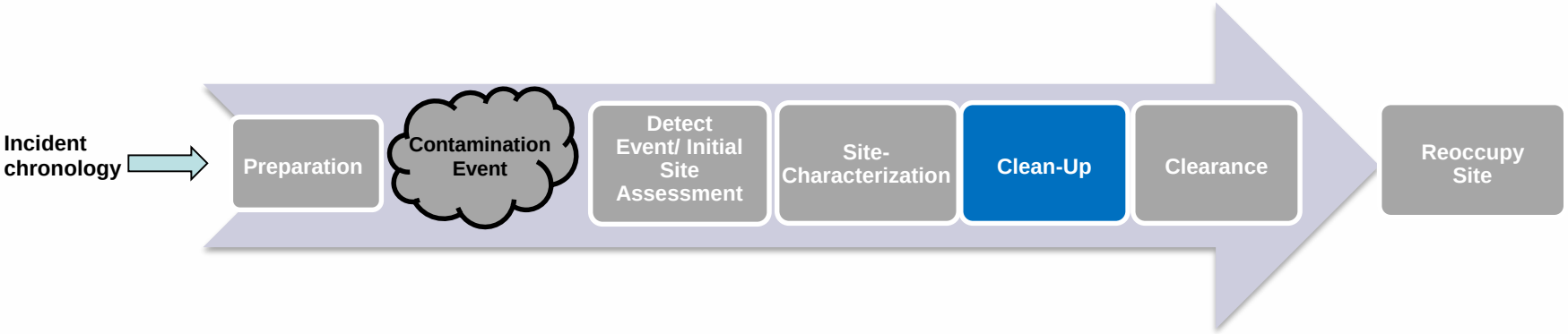
United States Environmental Protection Agency

Lessons Learned and Next Steps

- Final version of SCP still under management review
- Currently no collection procedure for intentional or bioterrorism events
- May be issues with rocky or very hard/compacted soils
- Verification of SCP needed
- Training video planned

Soil Decontamination Technologies

Joe Wood



Soil Decontamination Technologies Evaluated

- Chlorine dioxide (ClO_2) gas
- Aqueous ClO_2 solution
- pH-amended (acidified) bleach
- Sodium persulfate activated with hydrogen peroxide
- Methyl bromide
- Metam sodium
- Natural attenuation of vegetative *B.a.*

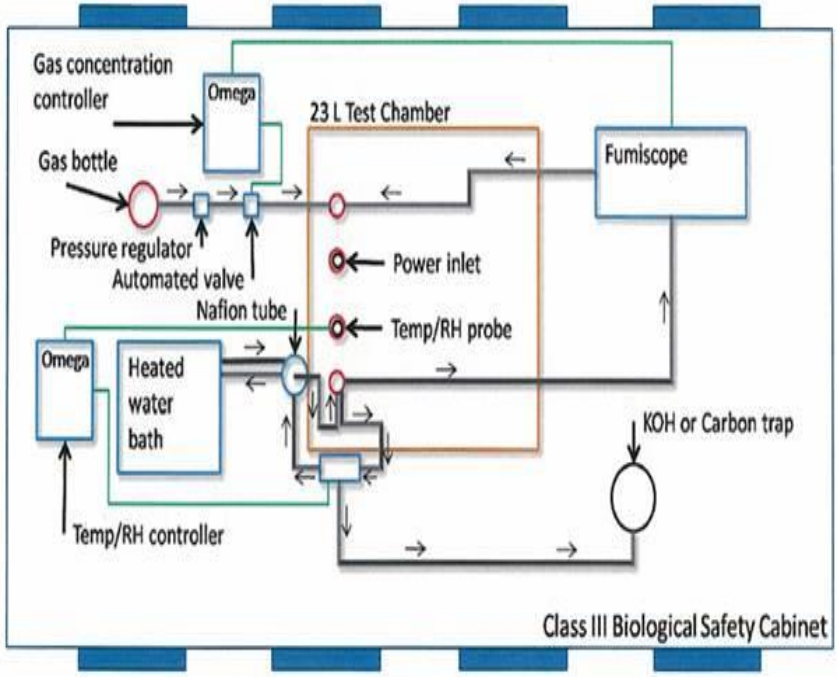


Diagram of MeBr decon chamber

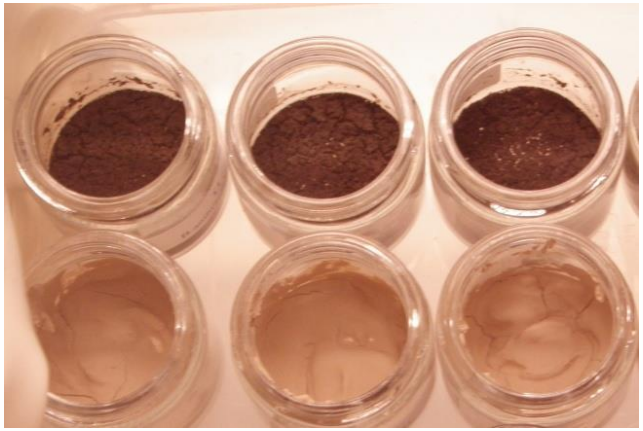
Testing Parameters

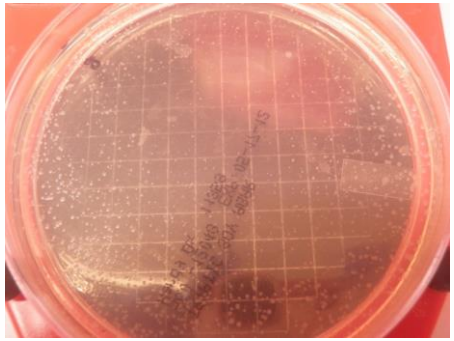
- Tests were conducted with varying operational parameters to assess and improve their effect on decontamination efficacy
- Variables tested depended on decon tech., but included:
  - contact time
  - number of applications (liquids)
  - decontaminant concentration
  - temperature, relative humidity (RH)
  - soil depth
  - soil moisture

ClO_2 generator

Bacillus Strains and Soil Types Tested

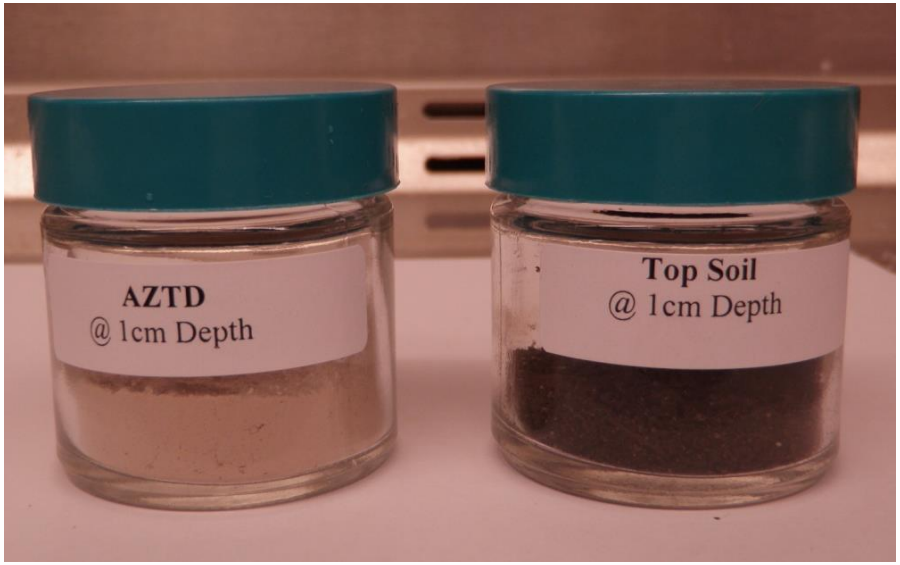
- *B.anthracis* (Ames strain)
- *Bacillus subtilis* (*B.s.*; ATCC 19659)
- Topsoil
- Arizona Test Dust (AZTD)





Soil Inoculation Methods

- 1.5 in glass jars filled with sterile soil to a depth of 1 cm
- Samples inoculated with ~1x10^8 CFU of viable *B. a. or B. s.* spores using 100 µL liquid suspension
- Samples allowed to dry in Class III Bio Safety Cabinet overnight at ambient temperature and %RH
- Positive controls recovered; test samples exposed to decontaminant



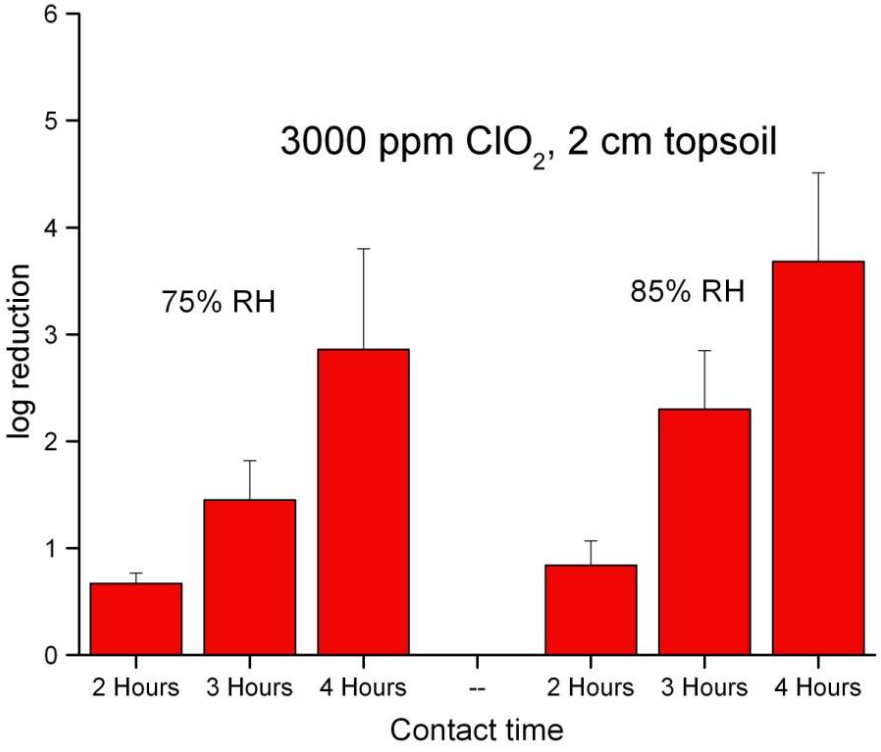
Microbiological Assays and Methods

- Extraction
- Serial dilution and plating
- Incubation
- Colonies enumerated and the CFU/mL determined
- Decontamination efficacy quantified in terms of log reduction (LR)

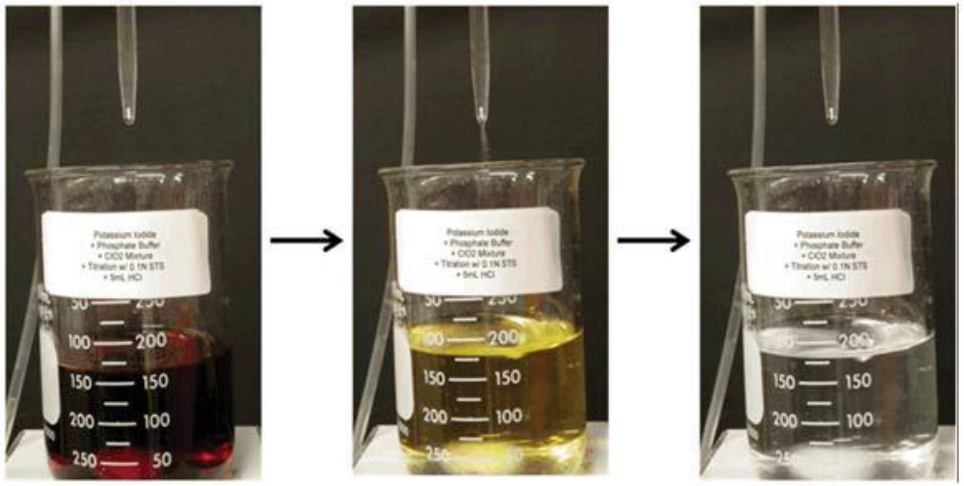
Chlorine Dioxide Gas

- Nearly all AZTD samples completely decontaminated
- Greater than 6 LR for topsoil at 1 cm depth, 2 hour, both RH levels
- 2 cm topsoil much

more difficult to decon

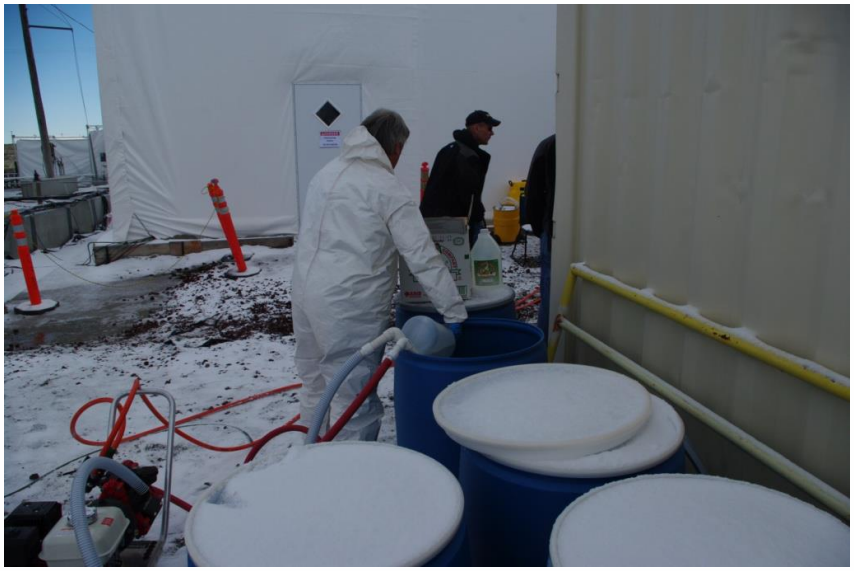


Results - Aqueous ClO_2



Aqueous ClO_2 completely ineffective (< 0.5 LR) at most robust test conditions (4000 ppm, 2 hr contact Time, 4 spray applications)

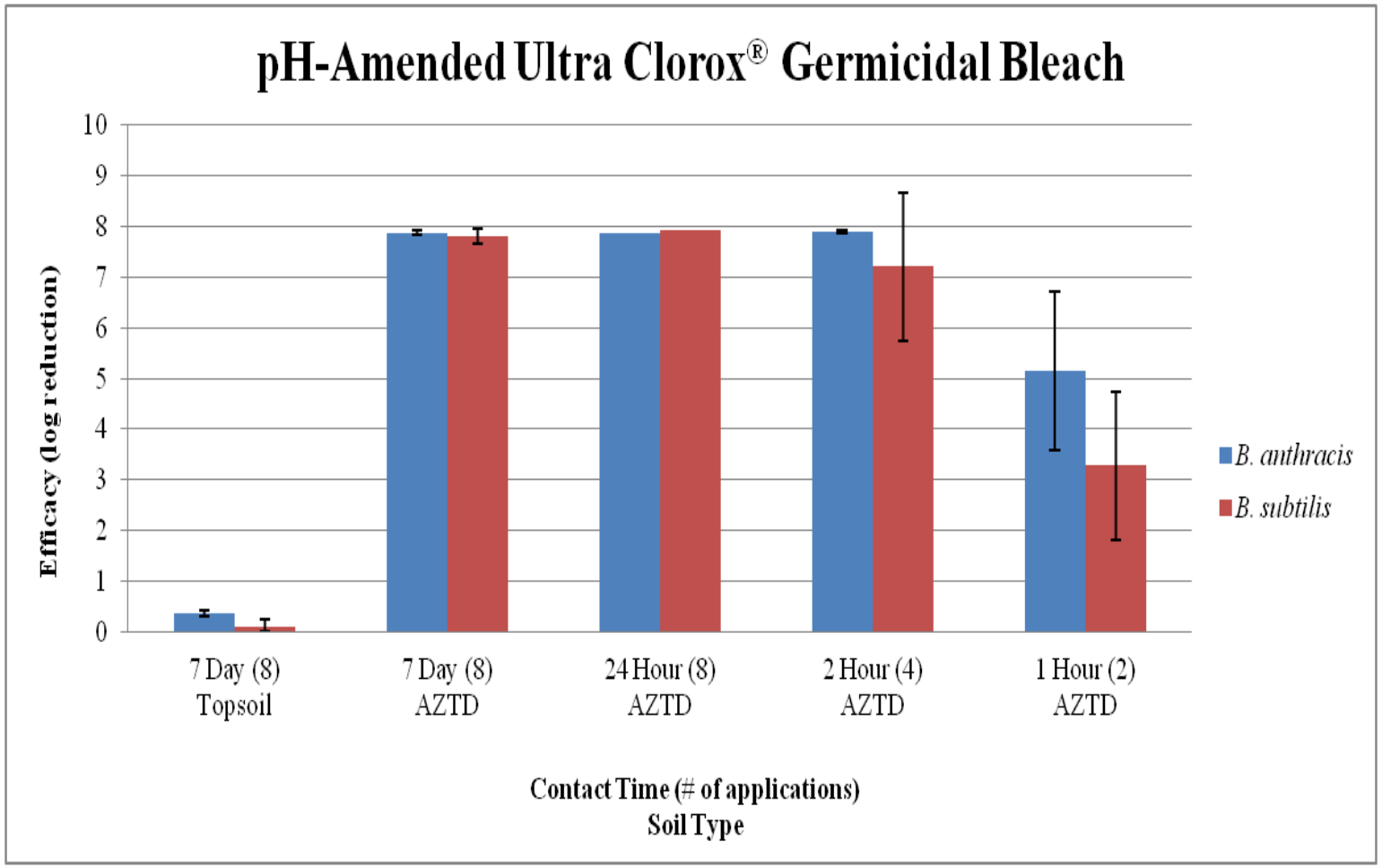
pH-Amended (acidified) Bleach



They told us not to mix them, but we did anyway

Mixing of pH-adjusted bleach solution in 55-gal drums at the Bio-Response Operational Testing and Evaluation Project

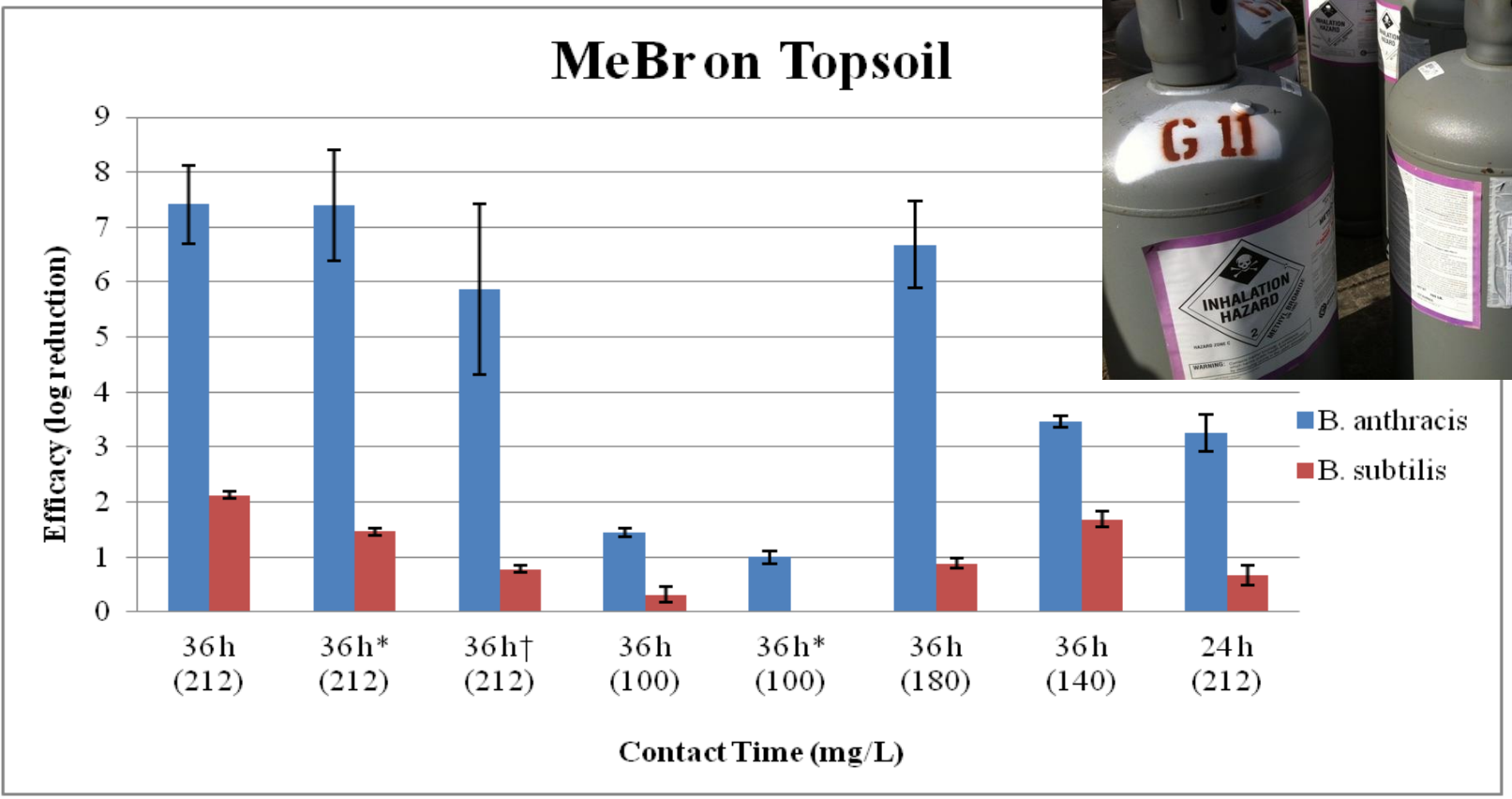
Results – Amended Bleach



Results - Sodium Persulfate

† = The decontaminant was applied every 60 minutes until the total number of applications were reached.
‡ =The decontaminant was applied on days 0, 2 and 4.

Results - Methyl Bromide



* = 2 mL sterile filtered water added to each sample prior to inoculation.
† = samples dried in oven prior to inoculation.

Metam Sodium

RESTRICTED USE PESTICIDE

Due to acute inhalation toxicity to humans.
For retail sale to and use by certified applicators or persons under their direct supervision and only for those uses covered by the certified applicator's certification.

VAPAM® HL

SOIL FUMIGANT

A SOIL FUMIGANT SOLUTION FOR SPECIFIC CROPS AS LISTED IN THIS LABEL

MAY BE APPLIED BY WATER-RUN APPLICATIONS (*e.g.*, CHEMIGATION), SOIL INJECTION OR SOIL BEDDING EQUIPMENT TO SUPPRESS AND/OR CONTROL SOIL-BORNE PESTS IN LISTED ORNAMENTALS, FOOD AND FIBER CROPS

For the control or suppression of Weeds, Diseases and Nematodes. Controls or suppresses weeds such as Bermudagrass, Chickweed, Dandelion, Ragweed, Henbit, Lambsquarter, Pigweed, Watercress, Amaranths species: Watergrass, Johnsongrass, Nightshade, Nutsedge, Wild Morning-Glory and Purslane, Nematodes and Symphylids. Soil-borne diseases such as Rhizoctonia, Pythium, Phytophthora, Verticillium, Sclerotinia, Oak Root Fungus and Club Root of Crucifers. Refer to specific cropping and application methods to determine control or suppression of the target.

ACTIVE INGREDIENT:

Sodium methyldithiocarbamate (anhydrous)*	42.0%
INERT INGREDIENTS:	58.0%
TOTAL:	100.0%

*Contains 4.26 lbs. Metam Sodium per gallon

KEEP OUT OF REACH OF CHILDREN

DANGER - PELIGRO

Si usted no entiende la etiqueta, busque a alquien para que se la explique a usted en detalle.
(If you do not understand the label, find someone to explain it to you in detail.)

PERSONAL PROTECTIVE EQUIPMENT (PPE)

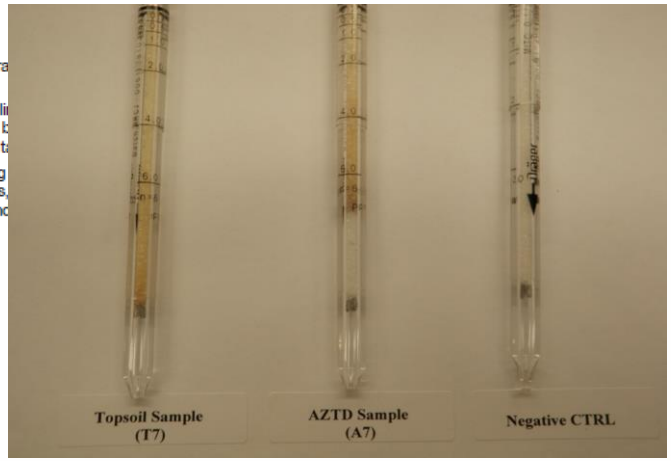
Some materials that are chemical-resistant to this product are barrier laminate or viton ≥ 14 mils. For more options, follow the instructions for category H on an EPA chemical-resistance category selection chart.

Handlers applying via weed sprayer while irrigation sprinklers are running or handlers who may be exposed to liquid spray while repairing a malfunctioning chemigation system or shutting off equipment must wear:

- Chemical-resistant coveralls over long-sleeve shirt and long pants,
- Chemical-resistant gloves,
- Chemical-resistant footwear plus socks,
- Chemical-resistant headgear,
- Protective eyewear, and
- Respirator of the type specified in the respira
  on this label.

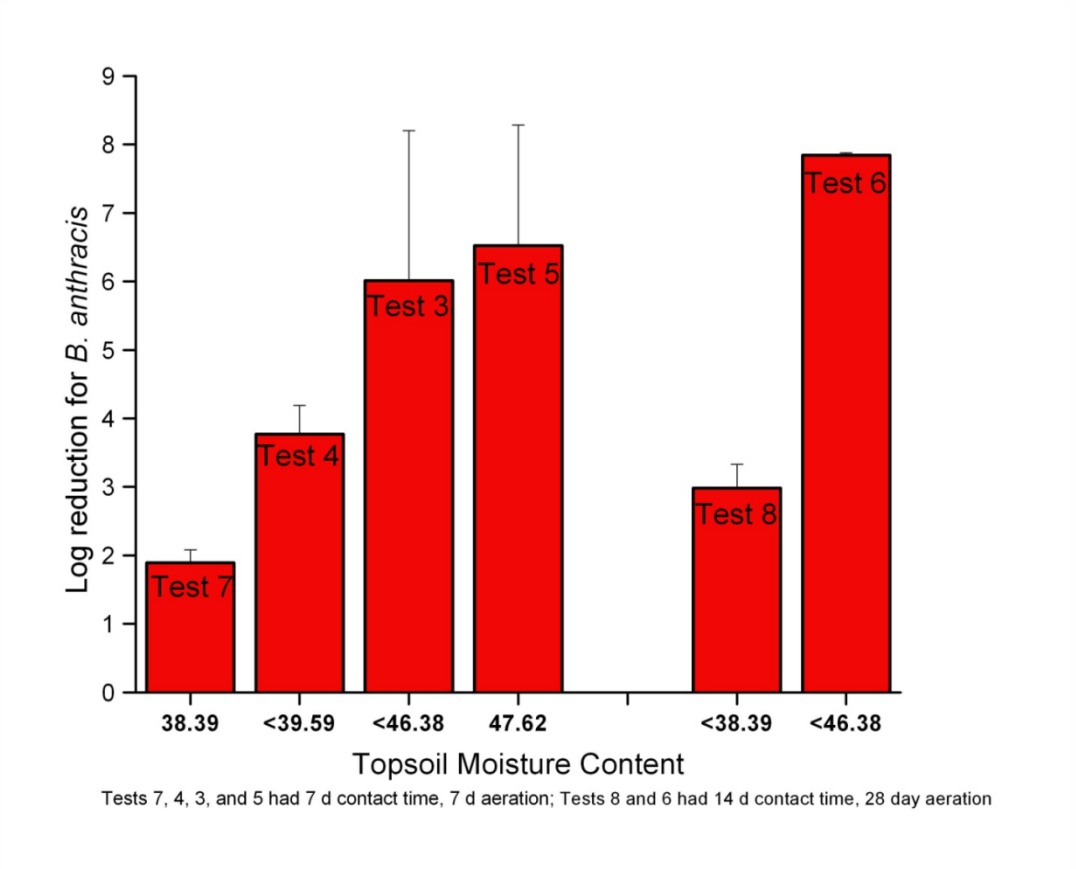
Handlers wearing chemical-resistant attire are li
period to prevent heat illness, and, as required b
Pesticides, employers of these handlers must t

Except as required above, handlers transferring
motorized ground equipment with open cabs,
chemigation equipment during application, and
wear:



Measurement of methylisothiocyanate

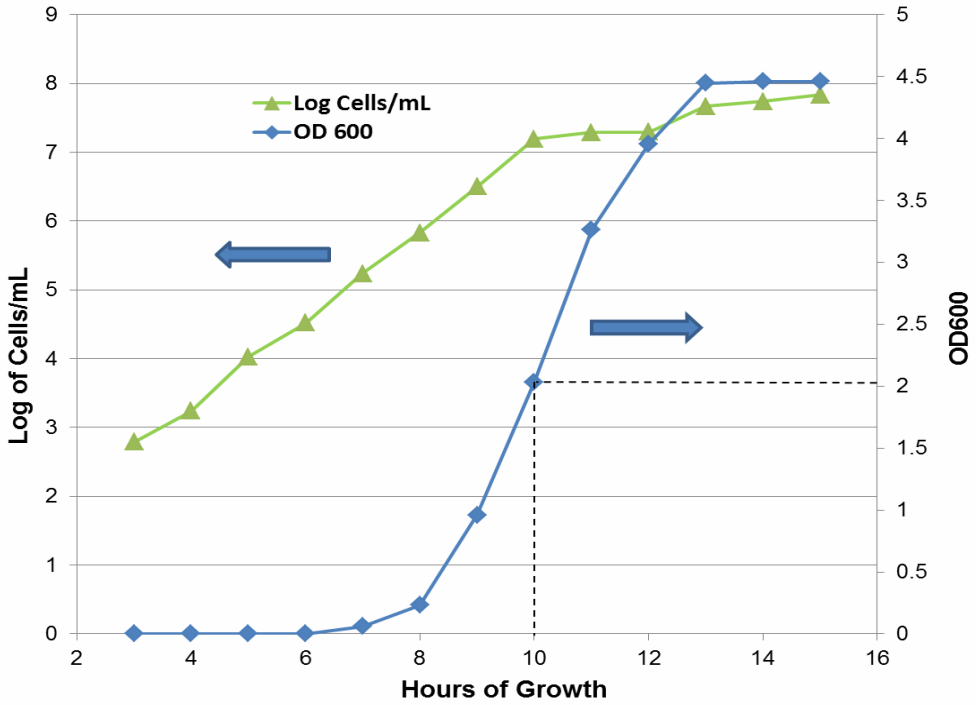
Results - Metam Sodium



Effect of soil moisture content

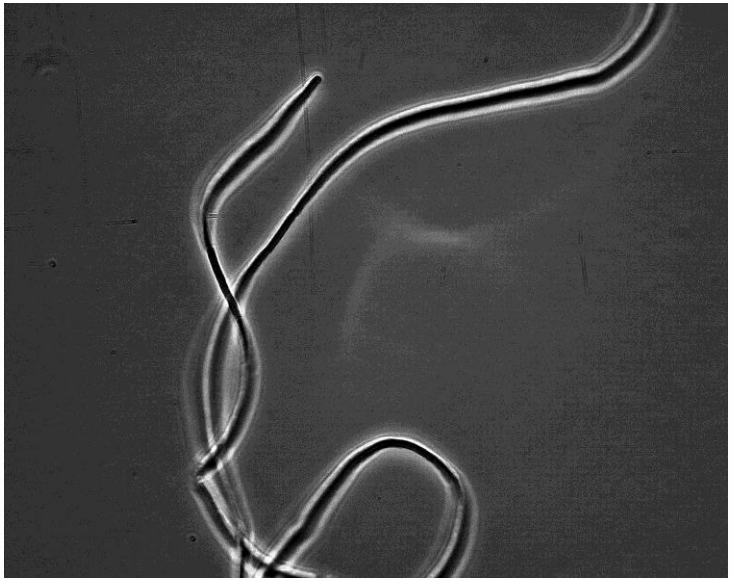
Natural Attenuation of Vegetative *B.a.*

- *B.a.* spores can persist in soil for decades
- However, germinants (under development) could be employed to convert spores into vegetative cells, which are much less resistant to environmental stressors
- Tests were conducted to assess persistence of vegetative *B.a.* in soil



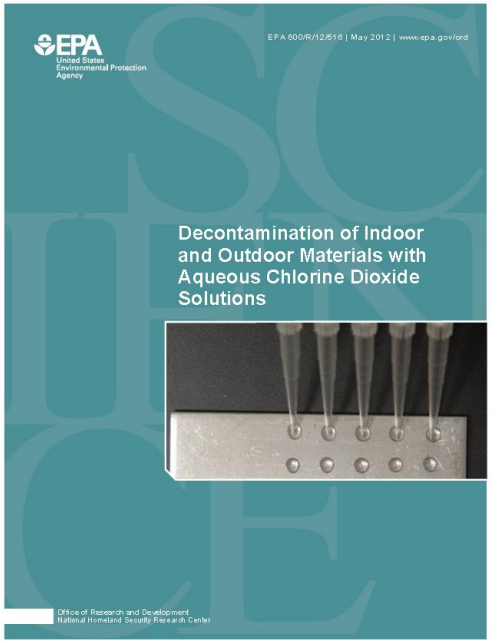
Natural Attenuation of Vegetative *B.a.*

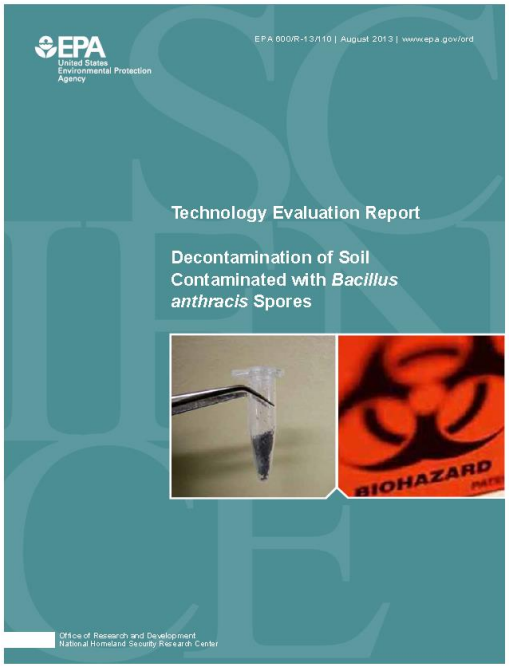
- Persistence of *B.a* cells in topsoil, at ambient lab temperature and RH, was 5 days
- Minimal impact of simulated sunlight (UV-A/B) on persistence
- Higher RH (e.g., 75%) enhances persistence
- However, in moist soil, high humidity (95%), Within 1 week, cell population had grown 10-fold & completely sporulated
  - Once sporulated, can persist for decades



Some References

- Poster presented at American Society of Microbiology 2013 meeting
- EPA reports





Next Steps Soil Decon

- Assess additional commercial, off the shelf pesticidal and chemical oxidation in-situ technologies
- Assess additional soils, effect of soil depth
- Larger scale tests

Lessons Learned for Soil Decon

- Decon efficacy: > 6 LR for *B.a.* obtained with all decontaminants studied, on both soil types, except pH-amended bleach and aqueous ClO_2
- Soil Type: AZTD generally easier to decon, but depends on decontaminant (e.g. sodium persulfate efficacy for *B.a.* similar for both soil types)
- Soil depth: In tests with ClO_2 gas, increasing soil depth significantly impacted efficacy. Further research needed to asses impact of soil depth