Colony Forming Unit versus Enrichment-Polymerase Chain Reaction Assay for Sensitive Detection of *Bacillus atrophaeus* subsp. *globigii* Spores

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What's the Issue?

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- Remediation efforts could be extensive following a wide-area release of *Bacillus anthracis* (*B.a.*) spores into the environment.

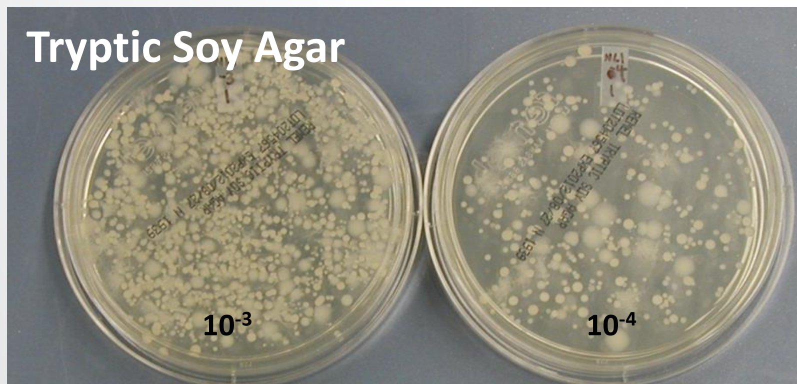
- In such a scenario, many types of materials and environments may need to be sampled, analyzed, and decontaminated, including soils.

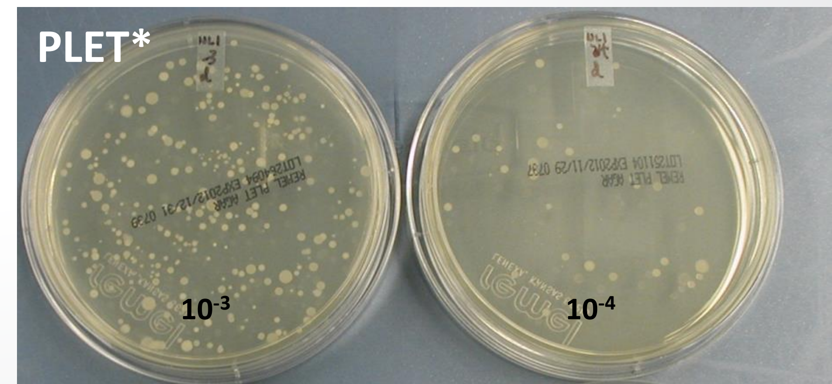
- Soil remains one of the most difficult materials to analyze and decontaminate for *B.a.*
  - Impurities, and other organisms in soil that impede detection.
  - Organic content of soil, as well as depth of soil, impede decon.

Issues Detecting Spores with Culture

- While culture is typically the “gold standard”, direct processing of soil via culture on a selective medium can be significantly hindered by the presence of other microorganisms in the soil

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





* *polymyxin-lysozyme-EDTA-thallous* acetate

Potential Inhibition

- *Bacillus* CFU recovered from soil spike = 200ul spread-plate on TSA
- Note carryover of soil particulate matter that could inhibit PCR

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Development of an Optimized Processing Protocol

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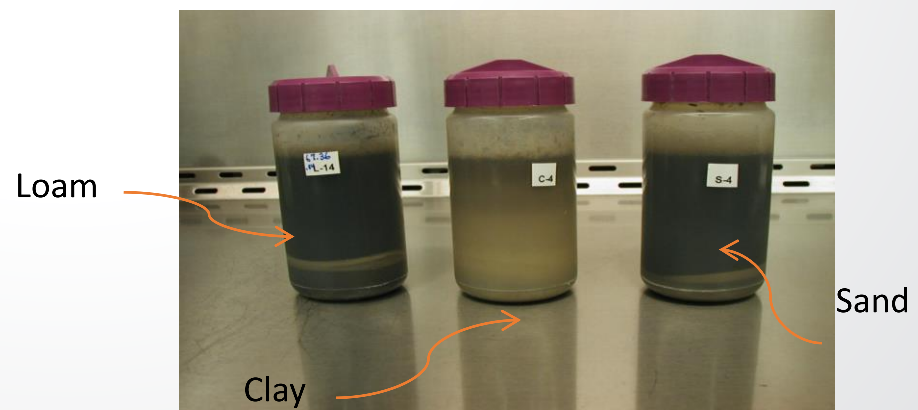
- A project team consisting of members from USGS, CDC, and EPA convened in May 2012 to work on optimizing extraction of *B. anthracis* spores from soil
- The method was developed using three soil types and two sample sizes (9 g and 45g)
- Method consists of a series of washes and centrifugation steps to concentrate the spores into a pellet
- The method will serve as a processing step prior to DNA extraction and further analysis



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Optimized Processing Protocol Details

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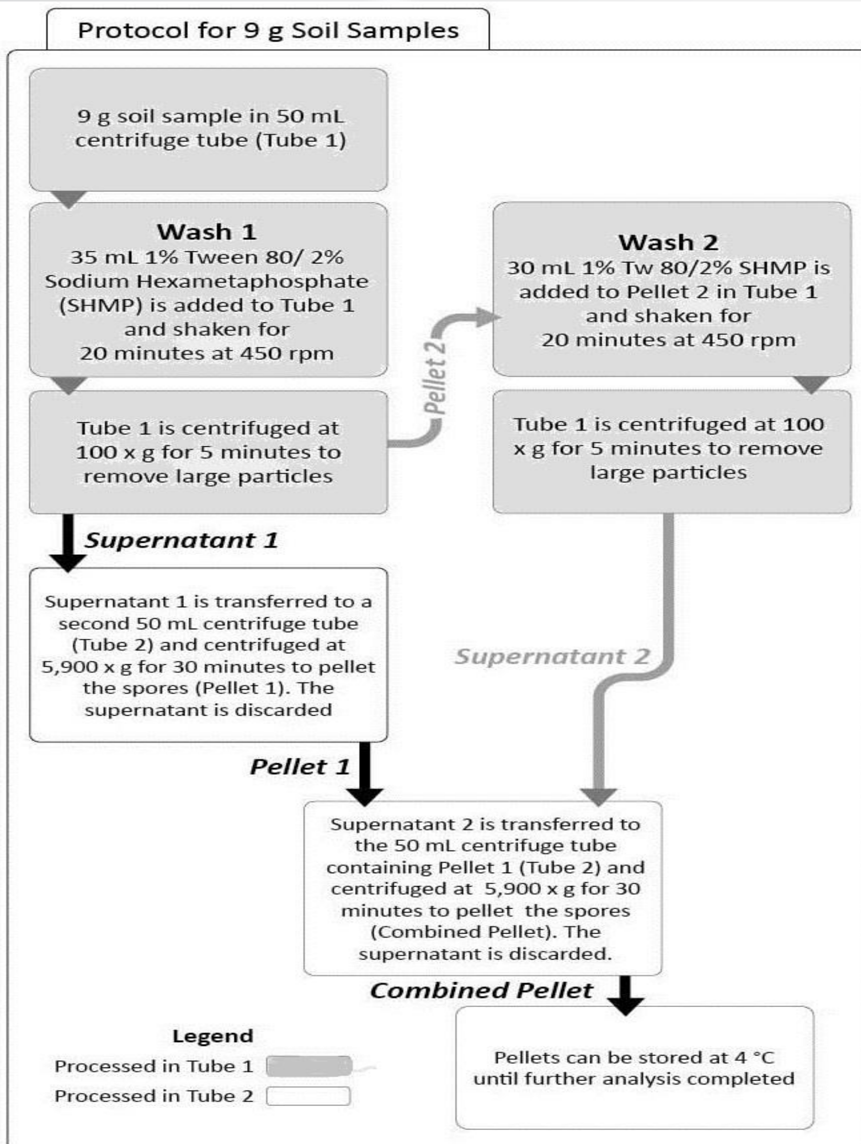

- The protocol separates (using differential centrifugation) and concentrates the spores from bulk soil down to a pellet that can be used for further analysis.
  - Soil samples are washed twice with soil extraction solution to maximize recovery.
  - Low speed centrifugation (100 × *g*) sedimentates heavier and denser soil material.
- Limit of detection = 14 spores/g

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Enrichment of Soil Samples

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- Enrichment of samples might help improve detection of low number of viable spores in the presence of non-target organisms
- Enrichment PCR brings advantages from both culture and PCR techniques to bear on analytical challenge
- Viability is addressed by processing samples at Time= 0 and Time = 24 hours
    - No change in cycle numbers would be noted if spores didn’t germinate and grow

Enrichment-Polymerase Chain Reaction

- Add tryptic soy broth to spiked soil samples.
- Take time=0 200ul aliquot after vortexing and freeze until analysis.
- Incubate the samples at 36°C for 24 hours.
- Take time=24 200ul aliquot and freeze until analysis
- Use MoBio's PowerSoil Kit (now owned by Qiagen) to extract and purify DNA and elute DNA from the purification columns in Qiagen's AE buffer.
- Use 2ul of eluted DNA per reaction.
- Run a qPCR presence/absence reaction using a StepOne Tempcycler.

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Current Study Goals

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- The goal was to compare the optimized processing protocol (followed by use of culture) to an enrichment-PCR protocol to determine if lower numbers of viable spores could be detected

- The current experiments utilized:
  - 9g of soil (9g x 14 spores/g = 126 spore detection limit for the CFU protocol)

  - Spikes of 1350, 675, 225, 45 and 4.5 per 9g of soil

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Loam, Sand, and Clay Soils

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Sterile Agvise Loam Lot 11082014-3 DJF

Sterile Agvise Sandy Lot 12072014-3 DJF

Sterile Clay Lot 05292015-3 DJF

Soil Samples Weights

- ~9 g soil samples were weighed out and set-up in 50 mL tubes in duplicate (one set for CFU and one for enrichment-PCR)
- Samples autoclaved for 1 hour and cooled at room temperature prior to spiking.
- Samples are spiked and placed in containment chambers and rock at 165 rpm over the weekend.

Study Results

July 5, 2017- Spread Plate Counts

Dilution	Plate 1 Count	Plate 2 Count	Average
10^{-4}	TNTC	TNTC	
10^{-5}	TNTC	TNTC	
10^{-6}	80	77	78.5 (per 200 µL)
10^{-7}	4	9	6.5 (per 200 µL)
10^{-8}	0	0	0

TNTC= Too numerous to count; 10^{-6} average count used for seed spikes for 200 µL count and 39.3 per 100 µL

July 6, 2017- Seeded samples set-up using 9 g samples and done twice (one for standard plating protocol and one for enrichment). The July 5th 10^{-6} counts (average 39.3/100 µL) used to calculate spike volumes in the table to the right.

Spore Concentration/g	Spore Concentration in 9g	Volume of Spike in µL (Dilution)
0 (Assay- control)	0	0
150	1350	34.4 (10^{-4})
75	675	17.2 (10^{-4})
25	225	57.3 (10^{-5})
5	45	114.5 (10^{-6})
0.5	4.5	11.5 (10^{-6})

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Results, Continued

July 10, 2017:

- Enriched Samples
  - 25 mL cold tryptic soy broth was added to sample tubes, vortexed for ~5 seconds, and allowed to settle for 10 minutes.
  - The T = 0 sample consisted of a 1.0 mL subsample transferred to microfuge tubes and frozen at -20°C.
  - Samples were incubated at 36°C.
- Non-enrichment Samples
  - 9 g samples were processed and the final pellet was suspended in 25 mL diluent.
  - Ten 200 µL aliquots spread on spread-plates and incubated overnight at 36 °C.

July 11, 2017: Non-enrichment CFU Plate counts for June 5 samples.

TSA Plate #	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	0	9	6	2	0	0
2	0	7	6	1	1	0
3	0	6	3	1	0	0
4	0	8	1	1	0	0
5	0	5	7	3	1	0
6	0	9	2	1	0	0
7	0	6	2	0	0	0
8	0	9	7	2	0	0
9	0	9	4	0	0	0
10	0	12	9	2	0	0
Average	0	8.0	4.7	1.3	0.2	0

- 1 mL of enriched samples taken 24 hours after incubation were transferred to microfuge tubes and frozen at 70°C.

DNA Extraction for Enrichment Samples

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August 14, 2017

- Samples were thawed and 200 µL of Time 0 and Time 24 hour tubes were transferred to the Mobio PowerSoil bead beating tubes.
- DNA was extracted and eluted to 100 µL in Qiagen's AE buffer.
- DNA extract concentrations were checked using the Qubit HS Kit.
- 10 µL of each sample was assayed.

Time Point/Sample	ng/mL	Adjusted to µg/mL in Sample*
Time 0/1	<0.5	N/A
Time 0/2	1.22	0.025
Time 0/3	<0.5	N/A
Time 0/4	<0.5	N/A
Time 0/5	<0.5	N/A
Time 0/6	<0.5	N/A
Time 24/1	<0.5	N/A
Time 24/2	15.2	0.305
Time 24/3	15.9	0.318
Time 24/4	7.8	0.156
Time 24/5	9.83	0.197
Time 24/6	14.2	0.283

*10 µL used for the reading

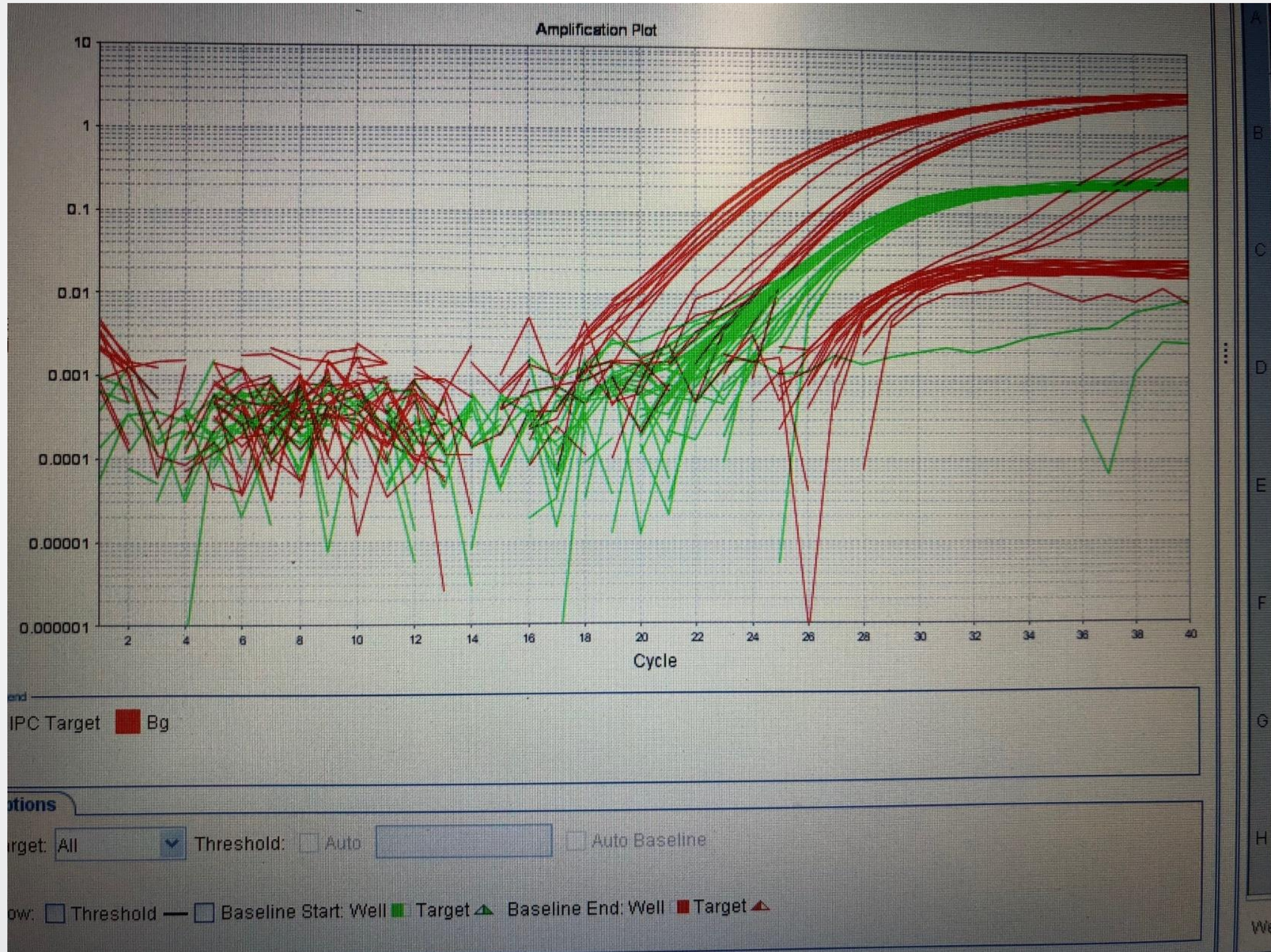
PCR Run Set-Up

Set-Up PCR Run Using 2 µL of 10^{-1} Template in Duplicate

Sample	Volume Assayed in µL	IPC Cross Rn of 0.1*	PCR Signal Cross Rn of 0.1**
Time 0, 1	2	30	0
Time 0, 2	2	30	0
Time 0, 3	2	30	0
Time 0, 4	2	30	0
Time 0, 5	2	30	0
Time 0, 6	2	30	0
Time 24, 1	2	30	0
Time 24, 2	2	33	25
Time 24, 3	2	32	25
Time 24, 4	2	32	26
Time 24, 5	2	40	25
Time 24, 6	2	38	24
Negative Control	2	29	0
IPC Block	2	Blocked	N/A

* Internal positive control cycle number that crossed an Rn threshold of 0.1
**PCR cycle number that crossed an Rn threshold of 0.1
Rn is the normalized reporter: the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye

PCR Results



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CFU Data

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Combined CFU averages from 10 spread plates for loam, clay, and sand samples

Sample	Loam	Sand	Clay
Sample 1 = 0 spores	0	0	0
Sample 2 = 1350 spores	7.2	7.2	16.9
Sample 3- 675 spores	2.3	4.8	7.5
Sample 4= 225 spores	1.1	0.8	2.5
Sample 5 = 45 spores	0.1	0.1	0.6
Sample 6 = 4.5 spores	0.2	0	0.1

Enrichment-PCR Data

- Enrichment Assays with 10^{-1} dilution of 100 µL extract of 200 µL TSB from overnight incubation at 36°C.
- Number values for PCR data are the cycle number that crossed an Rn threshold of 0.1
- No Amp.= no amplification/ negative reaction.
- N/A= Not applicable for that experiment

Sample Spikes
Sample 1 = 0 Spores
Sample 2 = 1350 Spores
Sample 3 = 675 Spores
Sample 4 = 225 Spores
Sample 5 = 45 Spores
Sample 6= 4.5 Spores

Time point (hrs)/ Sample	Loam 1	Loam 2	Loam 3	Sand 1	Sand 2	Sand 3	Clay 1	Clay 2	Clay 3
T=0/1	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=0/2	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=0/3	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=0/4	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=0/5	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=0/6	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=24/1	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.	No Amp.
T=24/2	25.5	27	25	27	25	24	24	25	24.5
T=24/3	25.5	27	23.5	26	25	24	23.5	27	24.5
T=24/4	25.5	27	25	27	26	24	24	27.5	24.5
T=24/5	25.5	27	27	27	25	24	24.5	29	24.5
T=24/6	No Amp.	27	25	35	24	25	24	31	25

- T=6 samples 1-6 were also run using Loam 1: no amplification was noted
- T= 48 samples 1-6 were also run using Loam 2 and Sand 1.
  - No amplification was noted for sample 1 in either set.
  - Loam 2 samples 2-6 amplified between 22-24 cycle number that crossed an Rn threshold of 0.1
  - Sand 1 samples 2-6 all amplified at 23 cycle number that crossed an Rn threshold of 0.1

PCR Amplification

Loam soil with a 4.5 spore spike showing good early amplification at 24hr incubation (red) versus the internal positive control (green) and the 0 hr (where it appears that late amplification is occurring in one of the duplicate reactions.

CFU vs. Enrichment-PCR Data

Spike Level	Loam 2 Average CFU/PCR Signal	Loam 3 Average CFU/PCR Signal	Sand 2 Average CFU/PCR Signal	Sand 3 Average CFU/PCR Signal	Clay 1 Average CFU/PCR Signal	Clay 3 Average CFU/PCR Signal
Sample 1 = 0 Spores	0/0	0/0	0/0	0/0	0/0	0/0
Sample 2 = 1350 Spores	6.1/27	8.2/25	8.0/25	6.4/24	17.8/24	15.9/24.5
Sample 3 = 675 Spores	1.4/27	3.2/23.5	4.7/25	4.9/24	9.1/23.5	5.9/24.5
Sample 4 = 225 Spores	0.9/27	1.2/25	1.3/26	0.3/24	2.5/24	2.4/24.5
Sample 5 = 45 Spores	0.1/27	0/27	0.2/25	0/24	0.6/24.5	0.5/24.5
Sample 6 = 4.5 Spores	0.2/27	0.1/25	0/24	0/25	0/24	0.1/25

CFU numbers are averages from 10 spread-plates.
Number values for PCR data are the cycle number that crossed an Rn threshold of 0.1

Sand/Clay Experiment

Sand/Clay 1 CFU	Sand/Clay 2 CFU	Sand/Clay Average CFU	Soil Mixture (Sand/Clay)
11.4	11	11.2	100% Sand
11	13.8	12.4	80%/20%
12.6	12.1	12.4	60%/40%
13.5	14	13.8	40%/60%
14.7	13	13.9	20%/80%
14.4	13.4	13.9	100% Clay

Geochemistry of Soils

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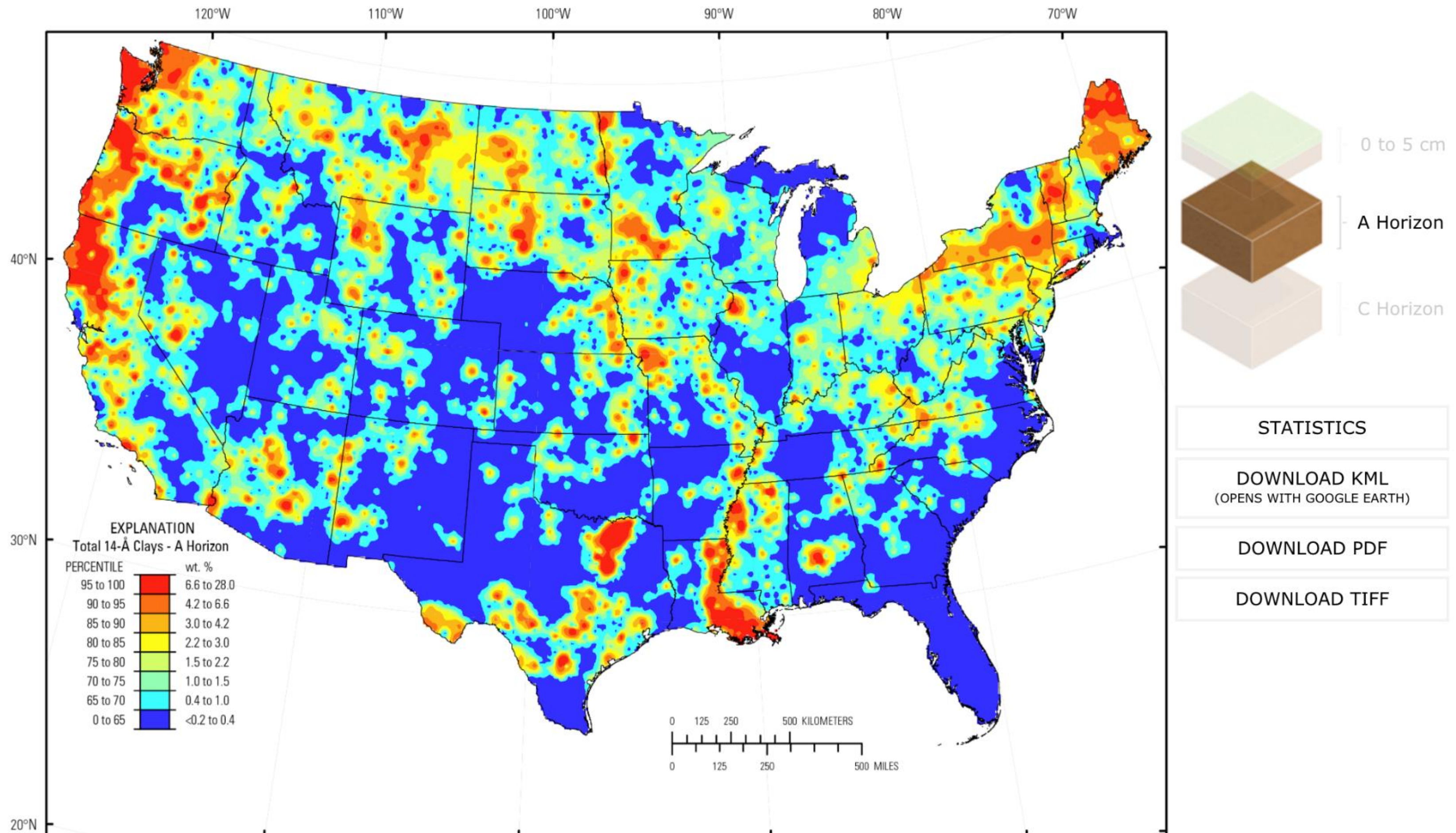
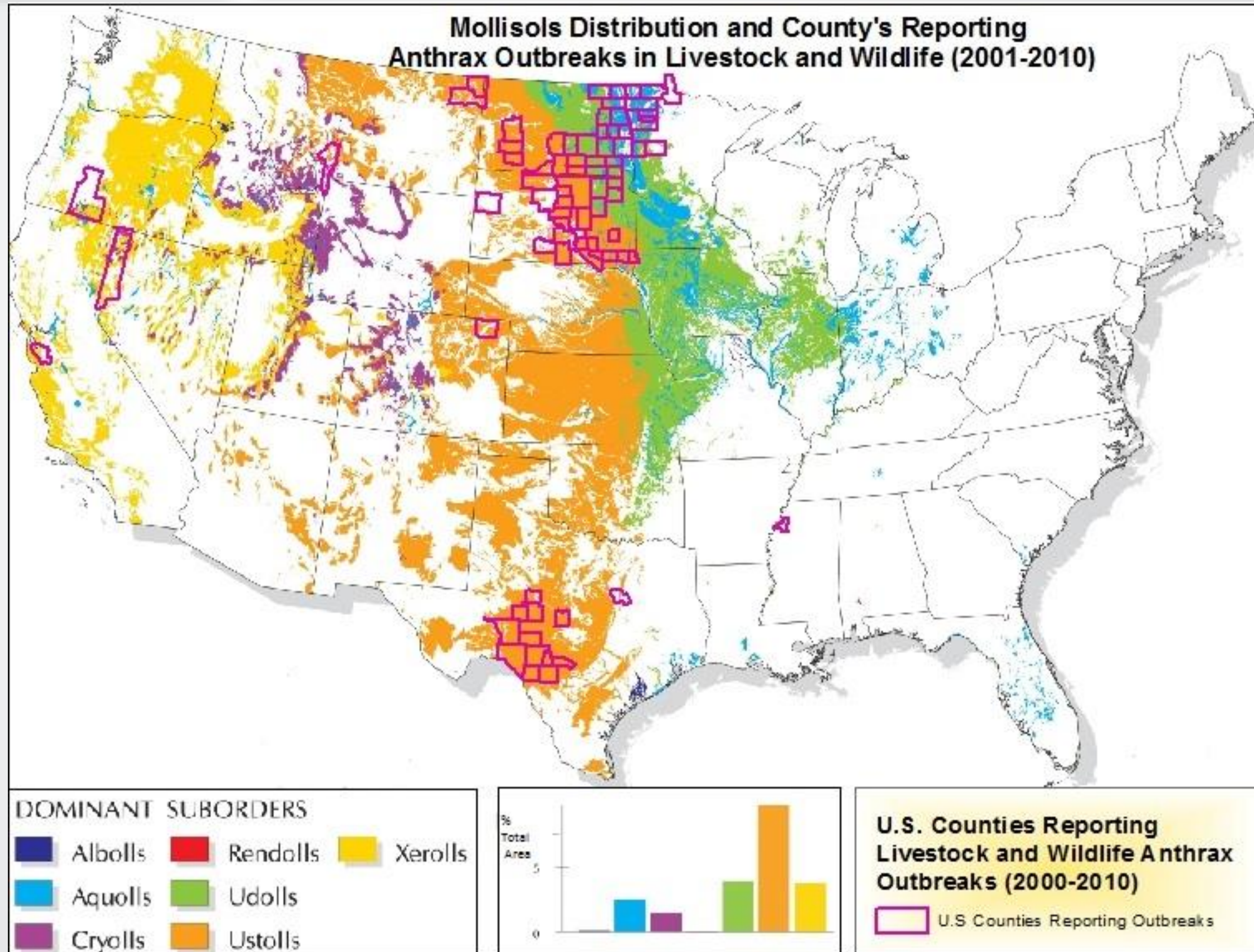


Soil Distribution and Outbreaks



Conclusions

- The CFU assay consistently detected spores at spike ranges of 225, 225 and 45 in loam, sand and clay soils respectively.

- Volume adjusted percent recoveries of spores from these same soil types averaged 56%, 63% and 146%, respectively.

- The enrichment-PCR assay was potentially able to detect spores at all spike concentrations and at lower levels compared to the optimized processing protocol.

- Clay soil CFU percent recoveries were greater than 100% of the spike, indicating a potential influence of clay in spore germination.

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Conclusions, Continued

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- To assess the influence of clays on spore germination pure sand and clay and sand:clay soil mixtures (%) of 80:20, 60:40, 40:60, 20:80 were spiked with concentrations of spores as previously described.
  - The CFU assay detected an average of 11 CFU/9 grams of soil for 100% sand through a stepped increase of 14 CFU/9 grams of soil for 100% clay.
- Collectively, these results show the enrichment-PCR assay has the potential to be more sensitive method for detecting spores in soil samples than the optimized processing protocol and that clay may contribute to spore germination.
- Future research? Addressing enrichment quantification using MPN-PCR

Disclaimer and Acknowledgements

Disclaimer

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