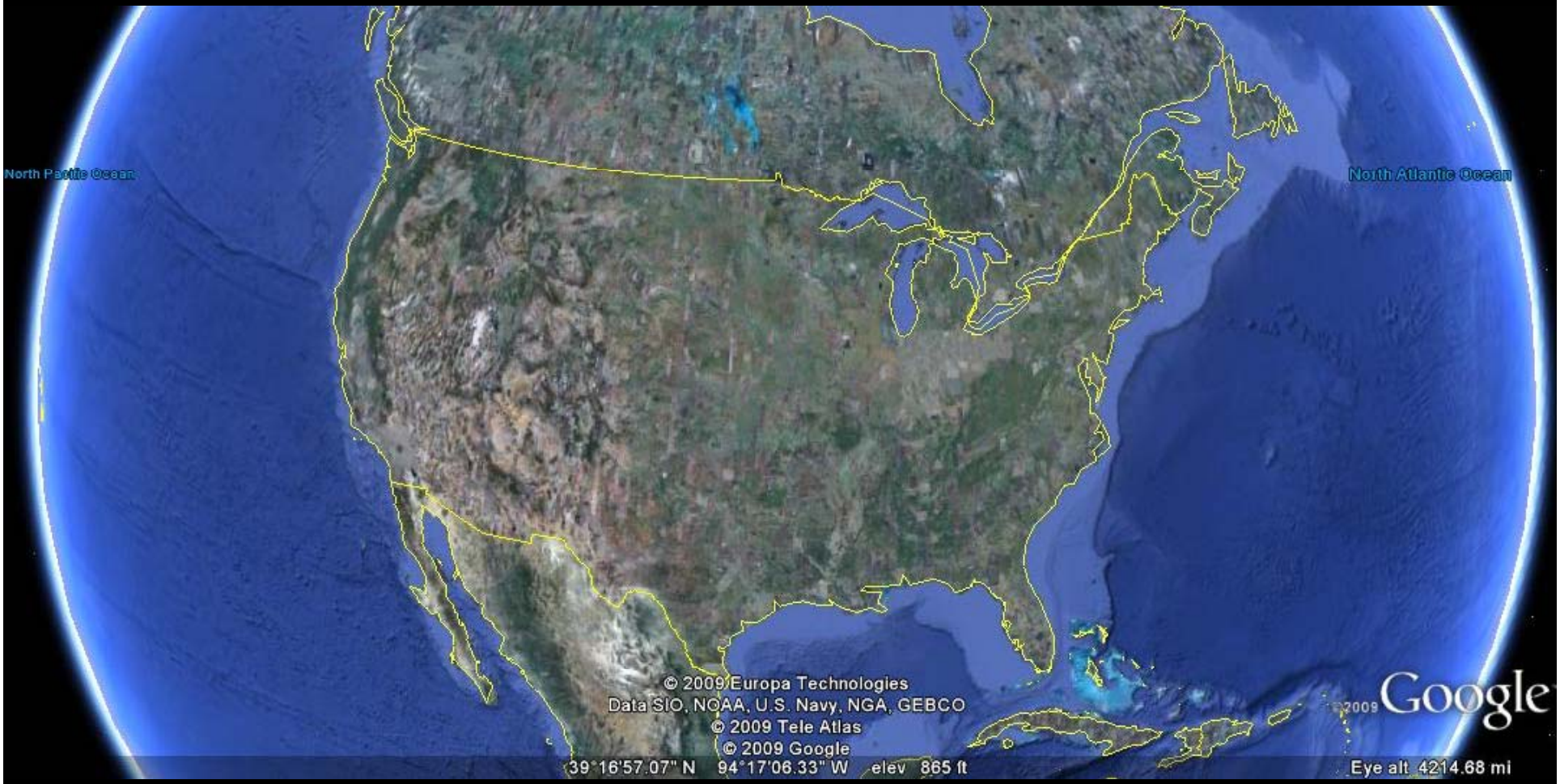
PATHOGENIC BACTERIA IN NORTH AMERICAN SOILS: A JOINT USGS-USEPA SURVEY



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Richard Allen, Ph.D. - USEPA

Study Objectives

- Study scale - Continental United States, 1 site per 1600 km^2, 4,851 samples.
  1. For pilot study data see *Smith et al. 2009. Geochemical Studies of North American Soils: Results from the Pilot Study Phase of the North American Soil Geochemical Landscapes Project. Applied Geochemistry, 24(8):1355-1356.*
- Determine the most sensitive and specific polymerase chain reaction (PCR)-based detection of pathogens from a wide range of soil types.
  1. Determine the presence of specific pathogens in the soil samples.
  2. *Bacillus anthracis* (4,851 samples, standard PCR, presence/absence, with verification by the University of South Florida's Center for Biological Defense, Tampa, Florida).
  3. *Yersinia pestis* (2,133 samples, quantitative-PCR).
  4. *Fransicella tularensis* (2,133 samples, quantitative-PCR).
- Relate pathogen data to geochemistry and climate to aid in –
  1. Outbreak investigations (natural and human induced)
     1. Wildlife
     2. Livestock
     3. Human
  2. Model development.

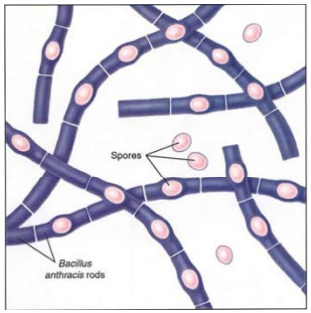
Soil Microbiology

1. Bacteria populations in soils typically range from 10^6 to 10^9 cells/gram as determined via direct count assay.
2. Culturable bacteria numbers may range from 0 to 10^7 colony forming units/gram of soil.
3. The current estimate of culturable bacteria and any sample type is 0.1 to 10% of the total population.
4. Current estimates put the typical number of bacteria genotypes per gram of soil at 10,000.
5. The dominant bacteria genera typically found is *Bacillus*.
6. Virus populations are typically1 to 2 logs less than the bacteria populations (opposite of aquatic environments).
7. ~ 10^6 fungi per gram of soil.
8. ~ 10^4 protozoa per gram of soil

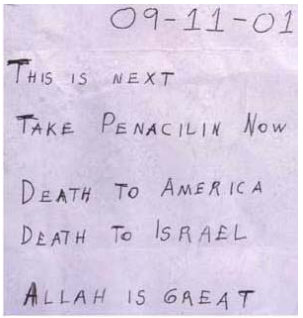
Bacillus anthracis

1. **Gram positive**
2. **Non-motile**
3. **Rod-shaped with square-shaped ends forming chains**
4. **1 to 1.2µm in width**
5. **3 to 5µm in length**
6. **Endospore former (spores 0.1 to 0.5µm)**
7. **Genome – 5,227,293 bp**
8. **Virulence plasmids – pX01 (189,000 bp, toxin), pX02 (96,000 bp, capsule)**
9. **Can be grown under aerobic or anaerobic conditions**
10. **Growth rate – in vitro, typically less than 30 minutes**
11. **Prevalence in soil survey studies – typically less than 5%**
12. **First identified as the causative agent of anthrax by Robert Koch**

(Koch, R., 1876. Die aetiologie der milzgrand-krankheit, begrundet auf die entwicklunsgeschichte de *Bacillus Anthracis. Beitr. Biol. Pflanz. 2, 277-311*)

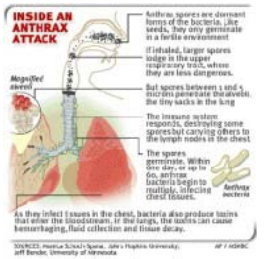


Anthrax



• Cutaneous – primary cause = occupational exposure (cuts/abrasions) to contaminated hides, symptoms/illness = ~2 week incubation, sore development with swelling, possible black crusted pustule with a broad zone of edema, may also develop painful lymph nodes, fever and headache. Fatality rate for untreated cases ~20%

• Gastrointestinal – primary cause = consumption of contaminated meat, symptoms/illness = fever, abdominal pain, vomiting, bloody diarrhea which may progress to toxemia, shock and death. Fatality rates for untreated cases range from 25 – 60%.



•Pulmonary – primary cause = occupational exposure to contaminated dust, hair, hides, symptoms/illness = flu-like, fever, fatigue, headache, muscle aches, shortness of breath that progress to bronchitis, shock and death. Fatality rate for untreated cases, 100%. Can be fatal when treatment started after symptoms appear.

Outbreaks associated with heavy precipitation and flooding

Topographical lows in grazing areas that may allow ponding of water can present higher risk

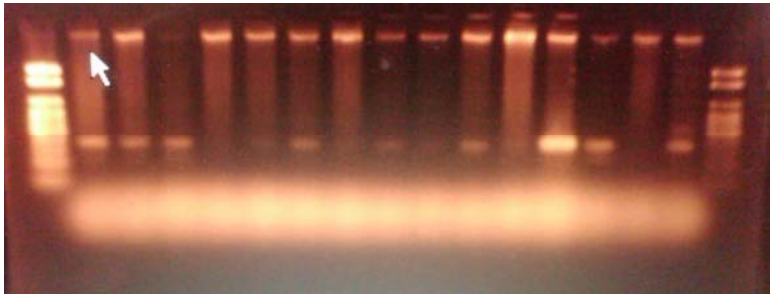
Bacillus sp./anthracis Detection Protocol

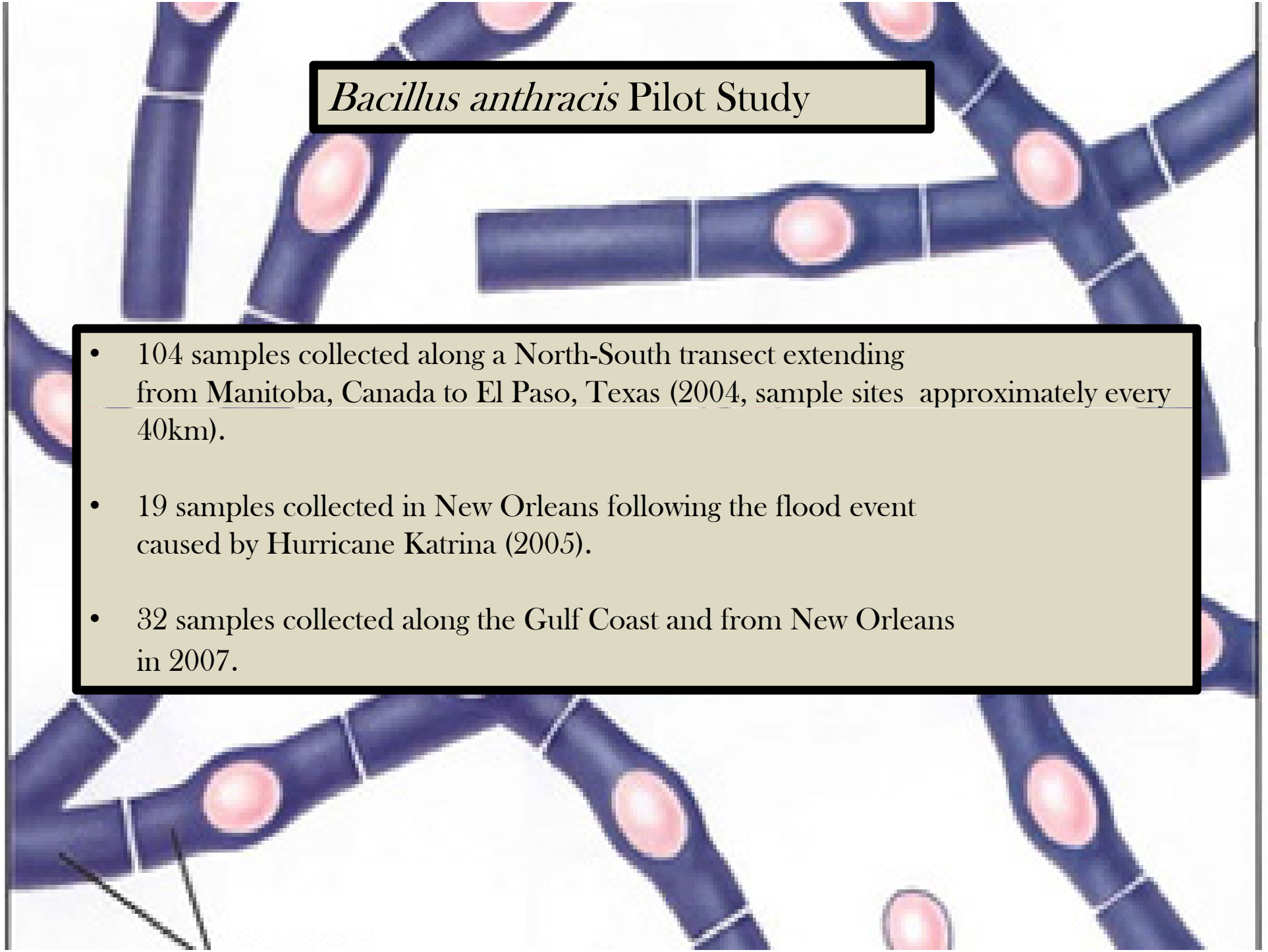
~0.25g of soil

BA-RF = GACGATCATYTWGGAAACCG
BA-RR = GGNGTYTCRATYGGACACAT
= 359-base pair region of rpoB gene (encodes the RNA polymerase b-subunit) that is specific for *Bacillus* species at the genus level

Ba-SF = TTCGTCCTGTTATTGCAG
= 208-base pair region of the same gene that is specific for *B. anthracis*

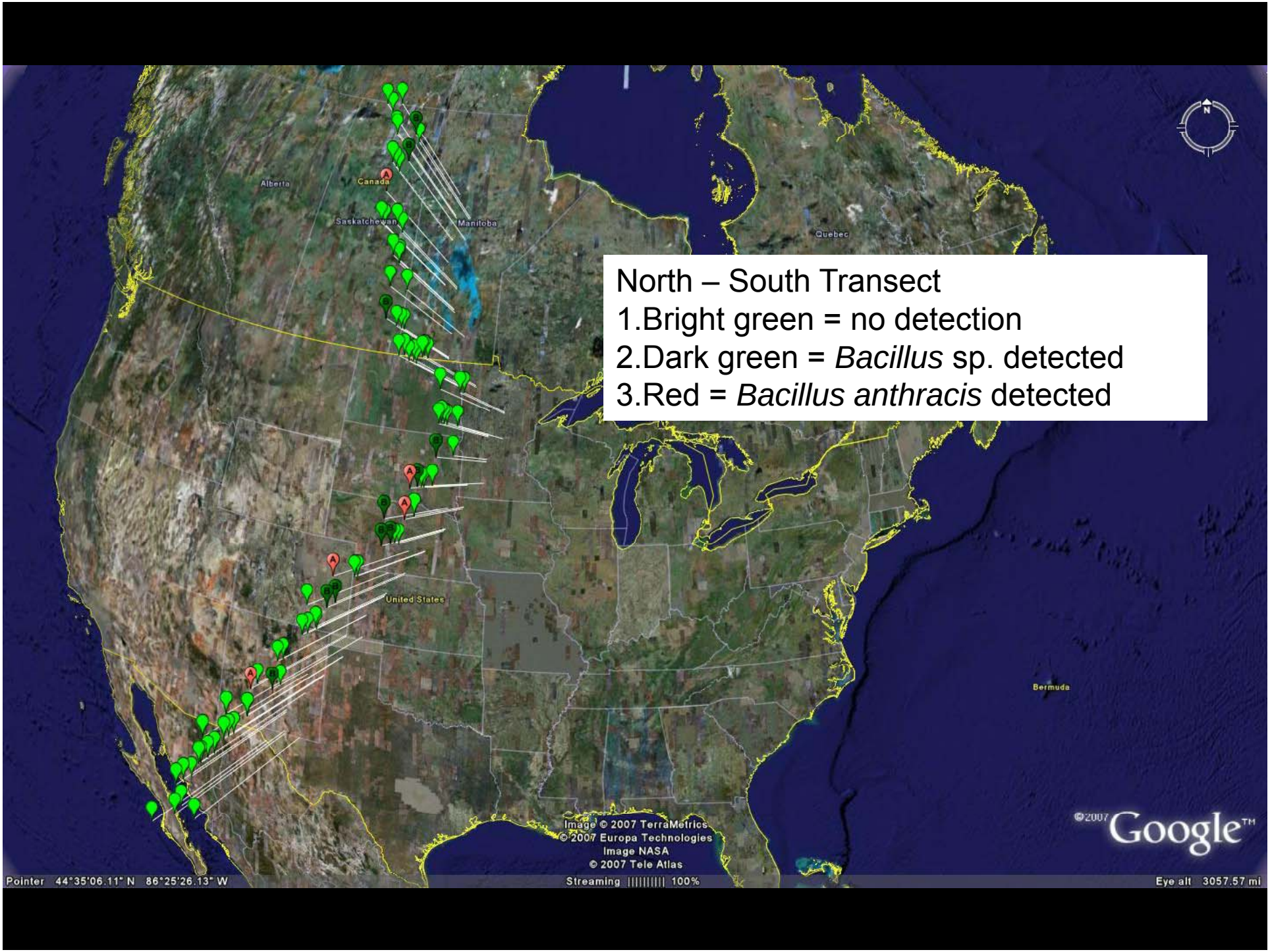
(PCR amplification profile and primer sequences from - Ko et al., 2003. Identification of *Bacillus anthracis* by *rpoB* Sequence Analysis and Multiplex PCR. Journal of Clinical Microbiology. 41(7):2908-2914)





Bacillus anthracis Pilot Study

- 104 samples collected along a North-South transect extending from Manitoba, Canada to El Paso, Texas (2004, sample sites approximately every 40km).
- 19 samples collected in New Orleans following the flood event caused by Hurricane Katrina (2005).
- 32 samples collected along the Gulf Coast and from New Orleans in 2007.


North – South Transect
1.Bright green = no detection
2.Dark green = *Bacillus* sp. detected
3.Red = *Bacillus anthracis* detected
Alberta
Canada
Saskatchewan
Manitoba
Quebec
United States
Bermuda
Image © 2007 TerraMetrics
© 2007 Europa Technologies
Image NASA
© 2007 Tele Atlas
©2007 Google™
Pointer 44°35'06.11" N 86°25'26.13" W
Streaming 100%
Eye alt 3057.57 mi

New Orleans post Katrina sites and results, 2005

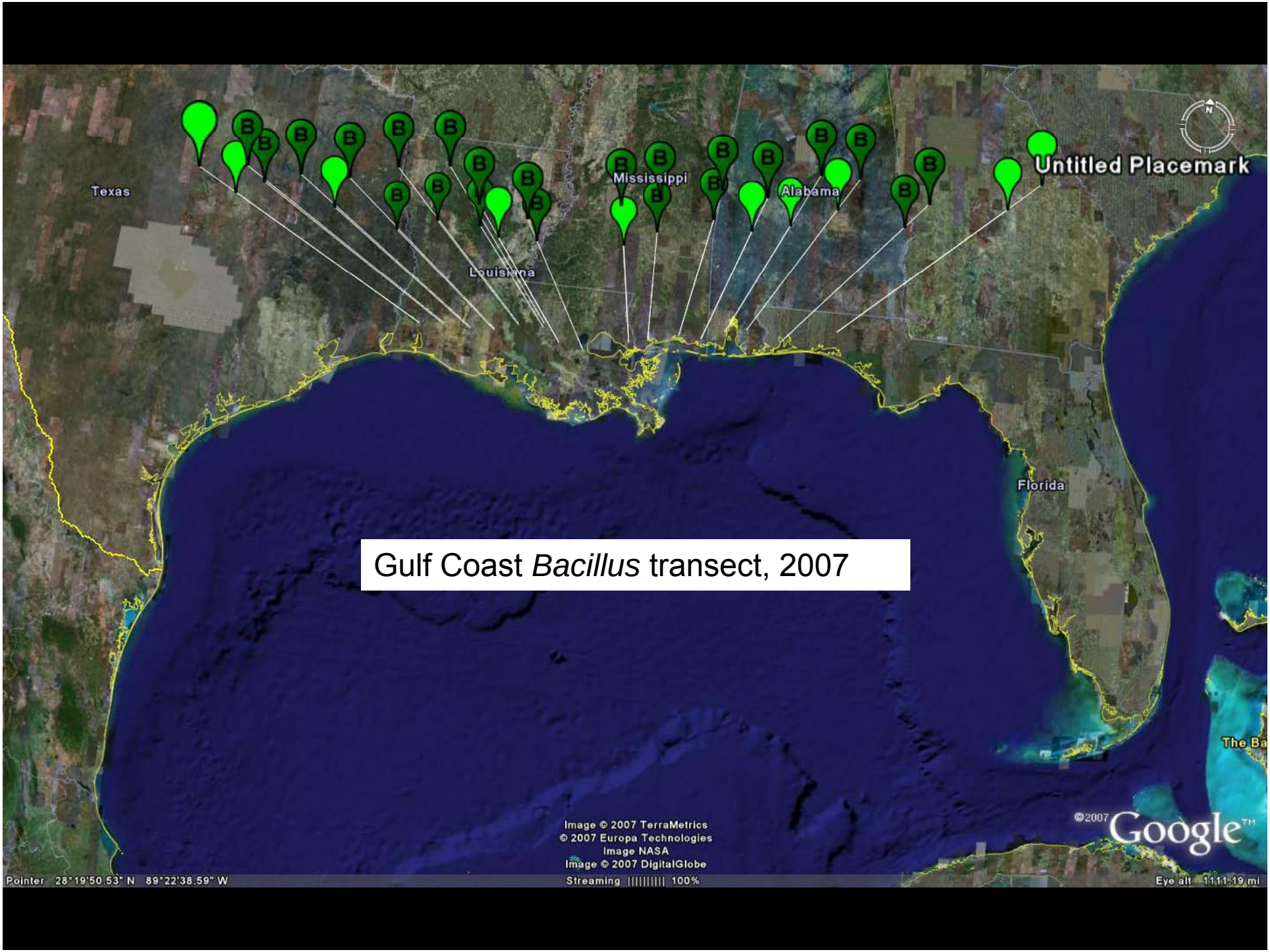
Fig. 2. 2005 Post-Katrina New Orleans and Chalmette, LA, sample sites.

Post-Katrina New Orleans

Katrina passed east of New Orleans 8/29/05
Samples collected 9/15/05 and assayed on 10/4/05

- 5 of 19 samples were PCR positive for *Bacillus sp.* and *Bacillus anthracis* (six sites were positive for human enteroviruses. One site positive for both *B. anthracis* and enteroviruses).
- All positive for pX01 plasmid and one positive for pX01/pX02 per USF's Center for Biological Defense.
- PCR positive sites were sampled and screened again on 8/10/07. No *B. anthracis* positive samples. Two positive for *Bacillus sp.*
- 32 Gulf Coast sites extending from Sulfur, Louisiana, to DeFuniakSprings, Florida – No *B. anthracis* detected, 22 were positive for *Bacillus sp.*

Additionally, motility, Gram stain, hemolysis, and γ–phage sensitivity were determined on isolates obtained from samples


Texas
Louisiana
Mississippi
Alabama
Florida
Untitled Placemark
Gulf Coast *Bacillus* transect, 2007
Image © 2007 TerraMetrics
© 2007 Europa Technologies
Image NASA
Image © 2007 DigitalGlobe
©2007 Google™
Pointer 28°19'50.53" N 89°22'38.59" W
Streaming 100%
Eye alt 1111.19 mi

USGS geochemistry data obtained at each sample site

Table 2. Major and trace element data for soils collected at a depth of 0-5 cm.

Lab No.	Field No.		Latitude	Longitude	COUL TITR CRBNT_C	Biolog normalized	CVAA Hg	ICPAES_MS Al	ICPAES_MS Ca	ICPAES_MS Fe	ICPAES_MS K	ICPAES_MS Mg	ICPAES_MS Na	ICPAES_MS S	ICPAES_MS Ti	ICPAES_MS Ag	ICPAES_MS As
					%	median	ppm	%	%	%	%	%	%	%	%	ppm	ppm
C-241462	48-4d1-PH	KY	38.0	-85.0	0.02	105	0.05	6.62	0.66	3.66	2.66	0.59	0.42	0.05	0.36	<1	7
C-241563	48-4d2-PH	KY	38.0	-85.0	0.02	105	0.05	6.5	0.42	3.56	2.55	0.59	0.48	0.04	0.36	<1	7
C-241436	48-3-PH	KY	38.0	-84.8	0.16	103	0.05	3.84	1.09	2.52	1.25	0.37	0.28	0.06	0.25	<1	8
C-241461	46-2d1-PH	WV	38.0	-80.5	0.01	106	0.04	3.27	0.06	1.48	0.57	0.14	0.11	0.03	0.26	<1	5

Table 2. Major and trace element data for soils collected at a depth of 0-5 cm.

Lab No.	Field No.	ICPAES_MS Ba	ICPAES_MS Be	ICPAES_MS Bi	ICPAES_MS Cd	ICPAES_MS Ce	ICPAES_MS Co	ICPAES_MS Cr	ICPAES_MS Cs	ICPAES_MS Cu	ICPAES_MS Ga	ICPAES_MS In	ICPAES_MS La	ICPAES_MS Li	ICPAES_MS Mn	ICPAES_MS Mo	ICPAES_MS Nb
		ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
C-241462	48-4d1-PH	384	2.3	0.22	0.2	85.9	16.9	60	4.78	21.3	16.3	0.06	42.8	38	1280	0.67	10.4
C-241563	48-4d2-PH	472	1.8	0.23	0.2	84	18.2	48	4.47	14.5	14.2	0.06	40.1	32	1180	0.64	8
C-241436	48-3-PH	412	1.6	0.18	0.2	82.6	14.9	40	2.58	22.4	9.6	0.04	39.5	22	2780	0.89	7.2
C-241461	46-2d1-PH	175	0.7	0.16	<0.1	40.8	3.8	33	2.75	6.8	7.63	0.03	19.5	26	88	1.02	7.9

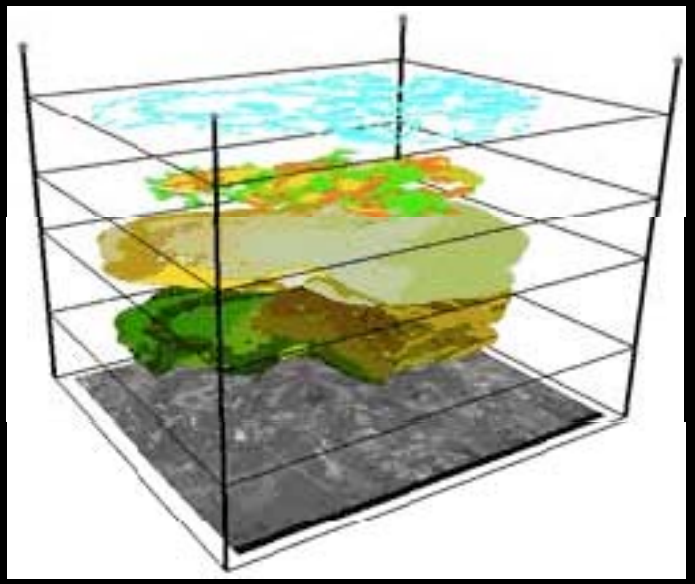
Table 2. Major and trace element data for soils collected at a depth of 0-5 cm.

Lab No.	Field No.	ICPAES_MS Ni	ICPAES_MS P	ICPAES_MS Pb	ICPAES_MS Rb	ICPAES_MS Sb	ICPAES_MS Sc	ICPAES_MS Sr	ICPAES_MS Sr	ICPAES_MS Te	ICPAES_MS Th	ICPAES_MS Tl	ICPAES_MS U	ICPAES_MS V	ICPAES_MS W	ICPAES_MS Y	ICPAES_MS Zn
		ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
C-241462	48-4d1-FH	27	1190	24.5	93.5	0.52	13.2	2	69.3	<0.1	12	0.6	3.2	81	0.8	27.8	83
C-241563	48-4d2-FH	30.8	1140	24.1	93.1	0.42	12.2	1.9	63	<0.1	12.3	0.6	3.3	80	0.6	26.6	91
C-241436	48-3-PH	17	3580	34.8	59.8	0.42	7.9	1.3	89.9	<0.1	10.5	0.5	3.7	53	0.6	28.5	63
C-241461	46-2d1-FH	10.3	404	18.5	37.3	0.47	4.4	1.2	39.5	<0.1	6.7	0.3	2.2	46	0.7	8.1	30

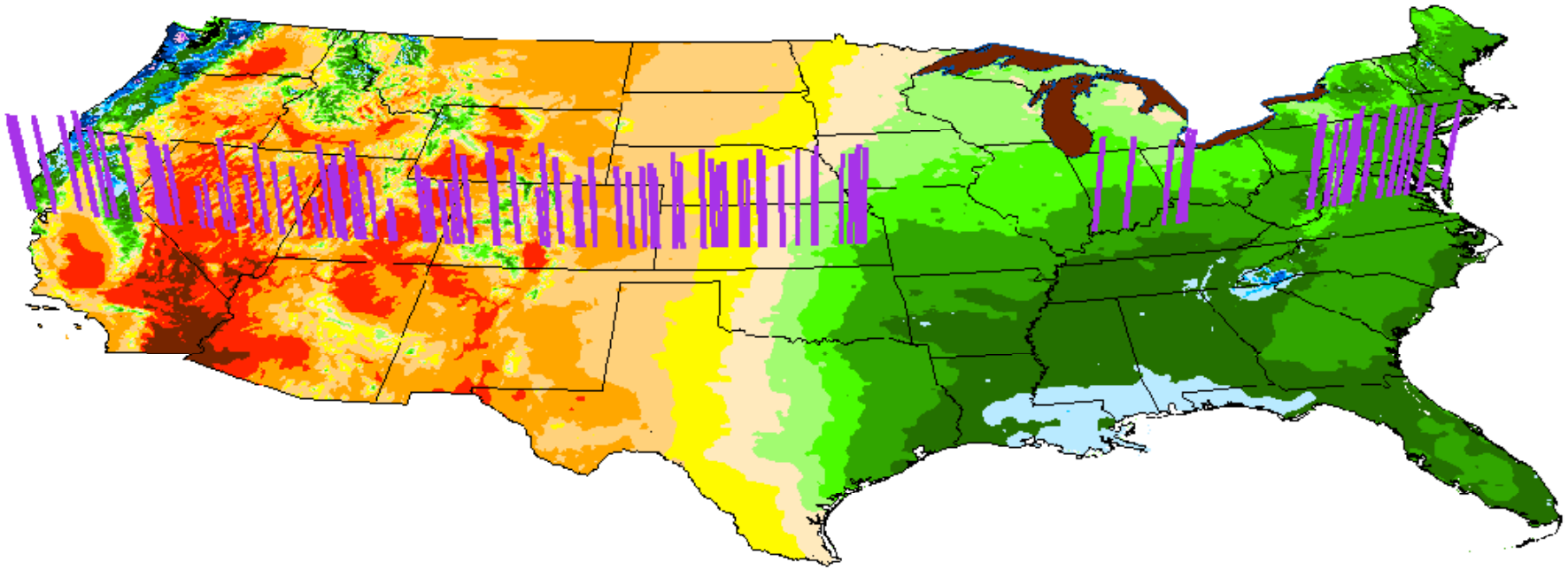
Table 2. Major and trace element data for soils collected at a depth of 0-5 cm.

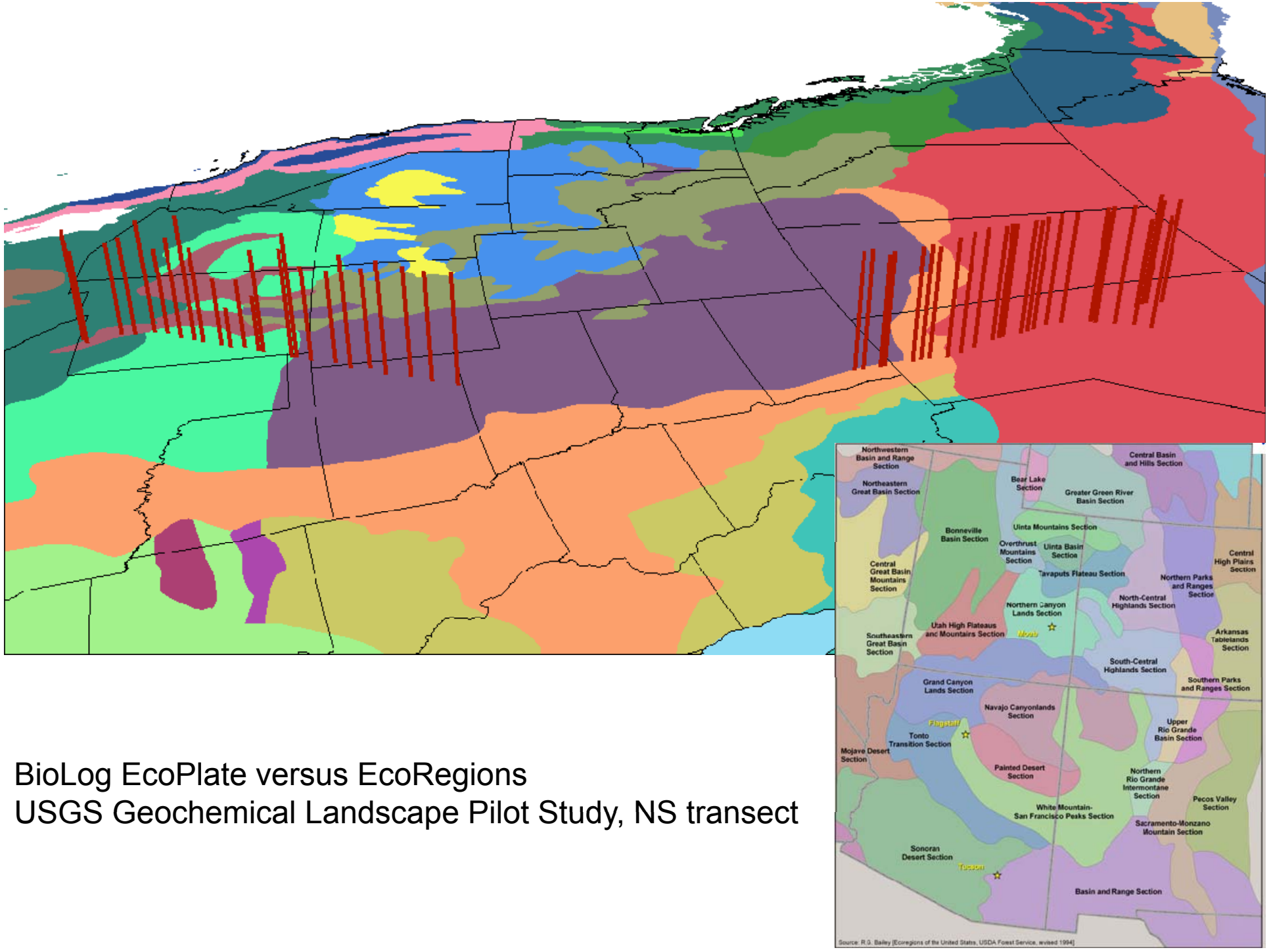
Lab No.	Field No.	Hyd Sb	Hyd Se	Combust. Total C	Combust. Total S
		ppm	ppm	%	%
C-241462	48-4d1-PH	<0.6	0.5	4.78	<0.05
C-241563	48-4d2-PH	<0.6	0.4	2.96	<0.05
C-241436	48-3-PH	<0.6	0.3	4.03	<0.05
C-241461	46-2d1-PH	<0.6	0.6	3.33	<0.05

Top soil map, USGS



BioLog EcoPlate versus Precipitation
USGS Geochemical Landscape Project Pilot Study, EW transect





BioLog EcoPlate versus EcoRegions
USGS Geochemical Landscape Pilot Study, NS transect

Pilot Study Summary

- The highest *B. anthracis* prevalence was noted following flooding of New Orleans.
- In non-flooded soils *B. anthracis* prevalence was less than 5% for the North-South transect and 0% for the Gulf Coast transect.

- There was a statistically significant relationship between soils with elevated moisture content (≥15.0% weight) and the presence of *Bacillus sp.*/*B. anthracis* in both the North-South and Gulf Coast transects (*p*-value *Bacillus sp.*/*B. anthracis* = 0.003/0.001).
- In the North-South transect statistically significant relationships were noted between the occurrence of *Bacillus sp.* and the elements Co, Cu, Pb. Sn, Tl, and Zn. These relationships were not observed in the New Orleans samples or along the Gulf Coast.

Elements such as Cu, and Zn are utilized by *Bacillus sp.* to enhance virulence and impart resistance to antibiotics, H_2O_2, and UV stress.

Survey – Continental U.S.

1. 4,851 sites uniformly distributed.

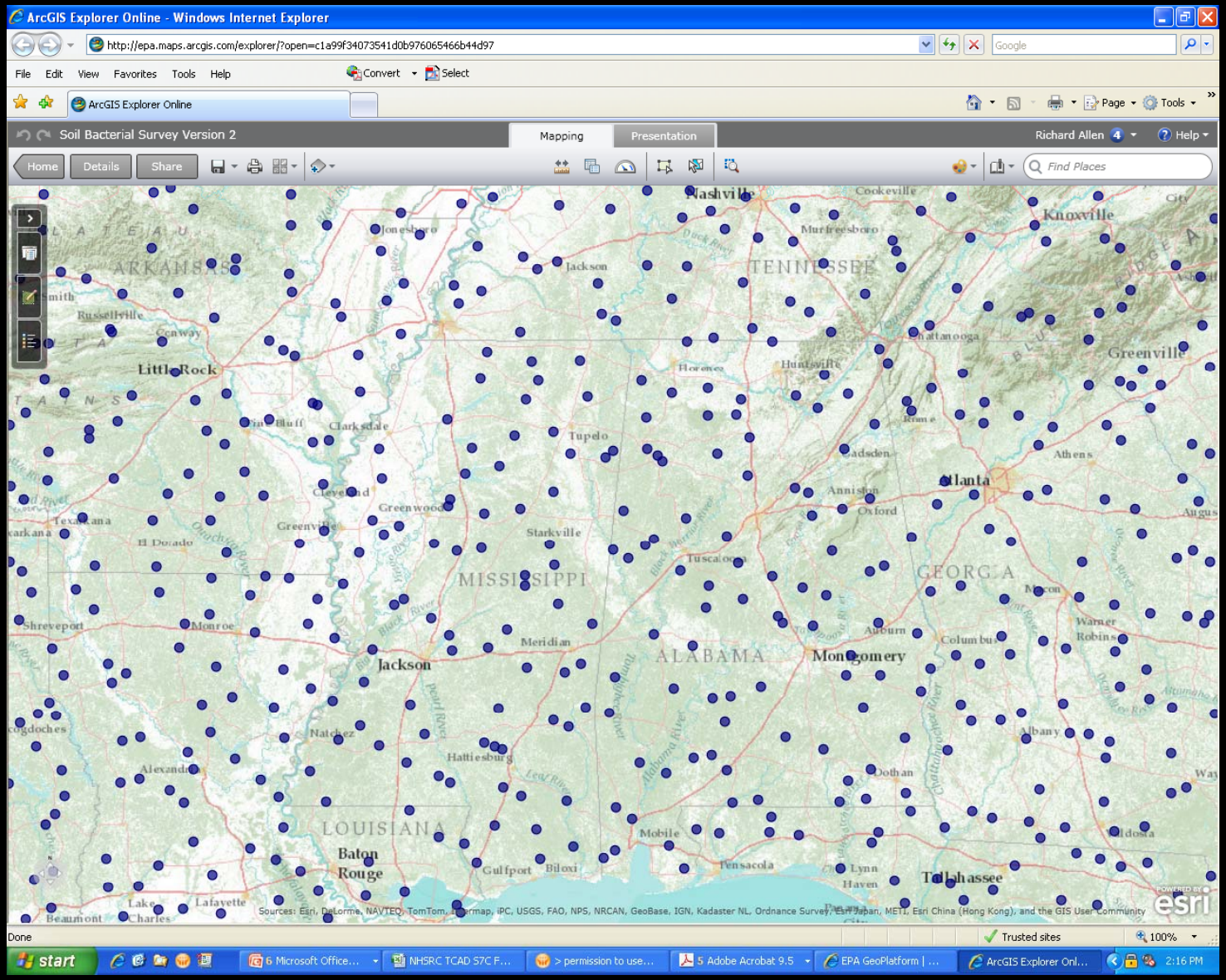
2. MoBio's PowerSoil Kit which utilizes 0.25g for DNA extractionand is more sensitive than their UltraClean Soils Kit which screens 1.0g of soil (4CFU vs 170CFU/g of soil) will be utilized for DNA extraction.

3. Primers for detection of *Bacillus sp.* (*rpoB rif* region 359 bp) and *B. anthracis* (*rpoB* 208bp).

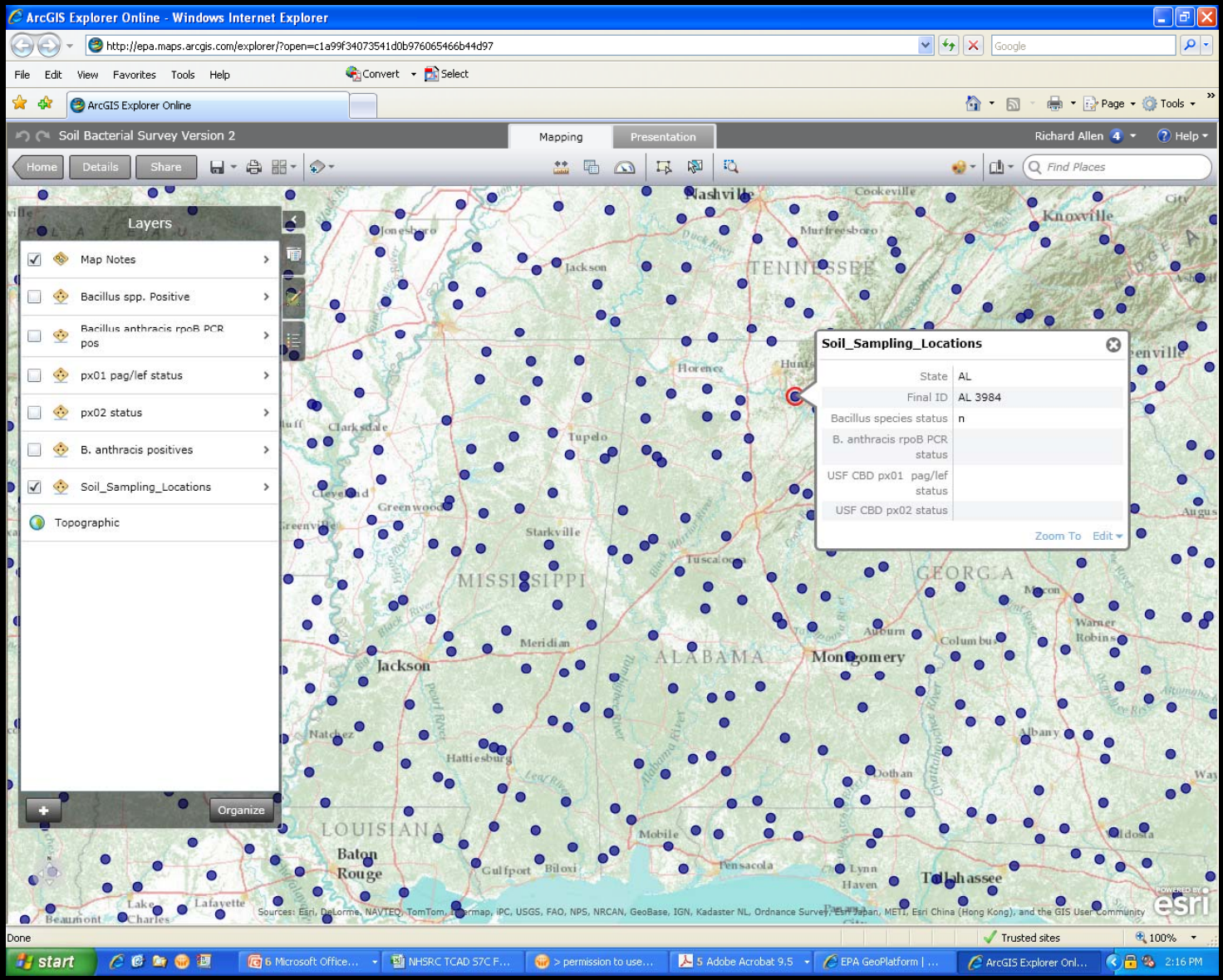
4. Qiagen's HotStart Master Mix Plus Kit and gel/amplicon visualization with SYBRGold will be utilized to determine presence/absence.

5. Confirmation of PCR positives by the USF's Center for Biological Defense and Northern Arizona University.

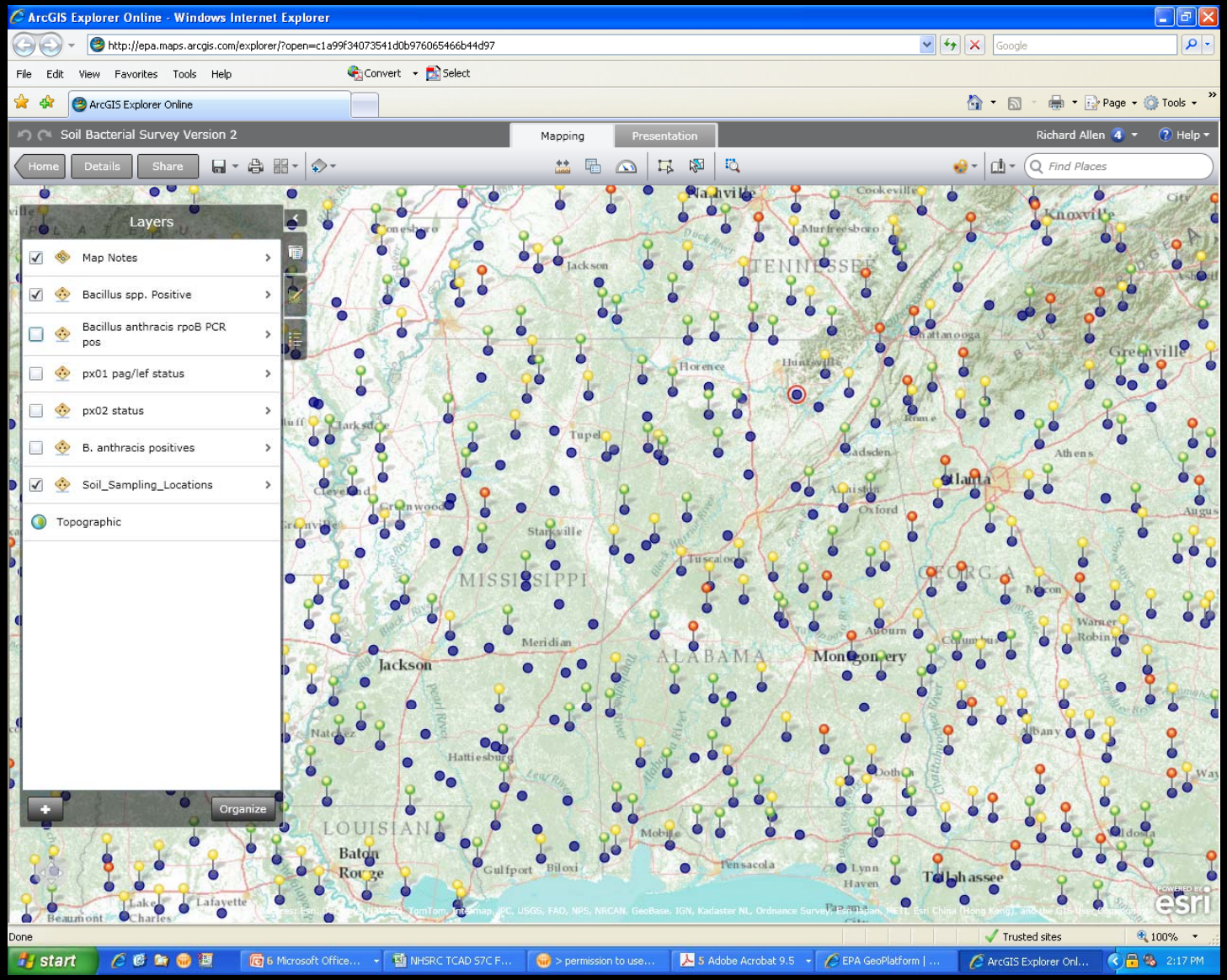
USGS-USEPA sample site layer



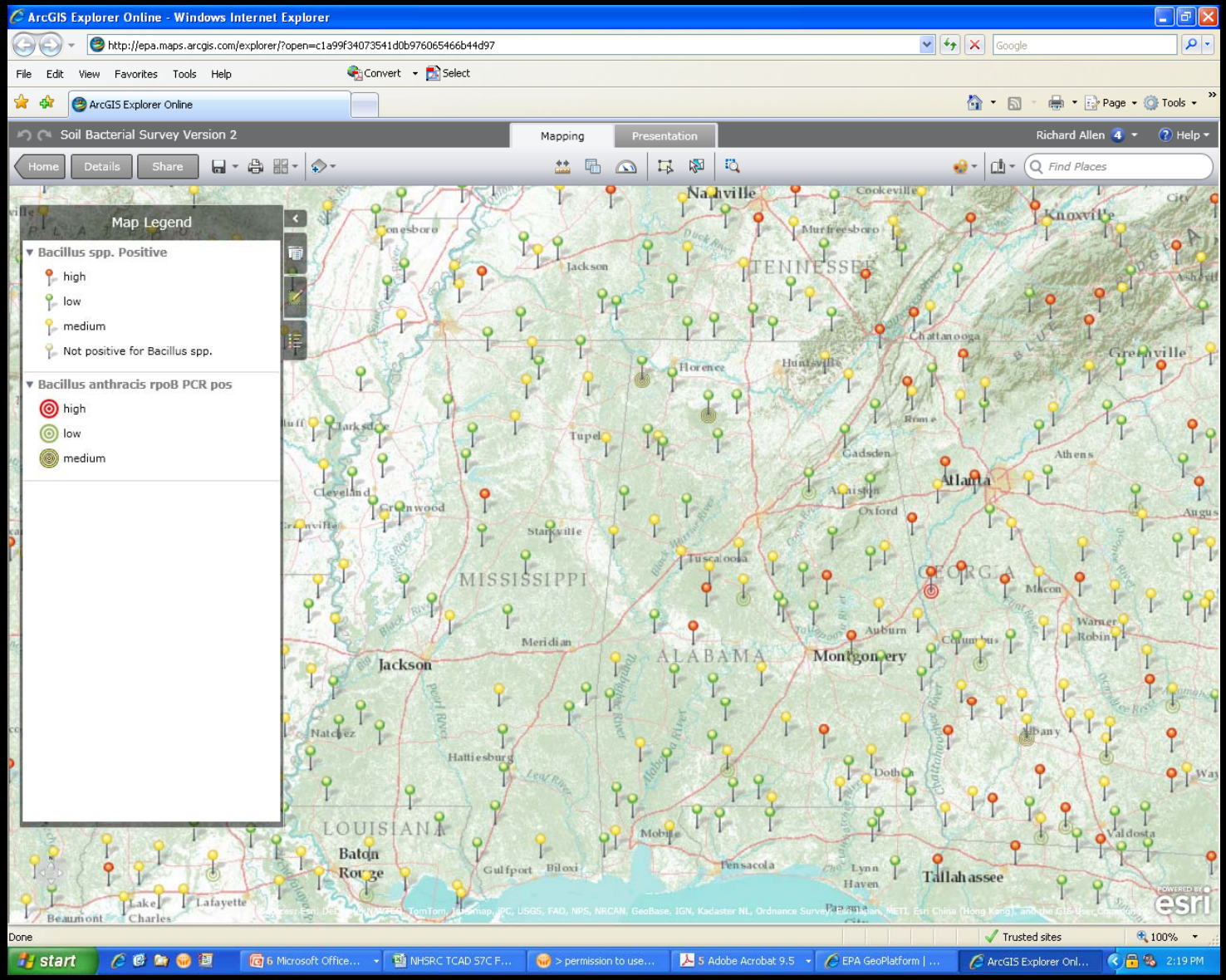
USGS-USEPA sample site layer – Site specific information



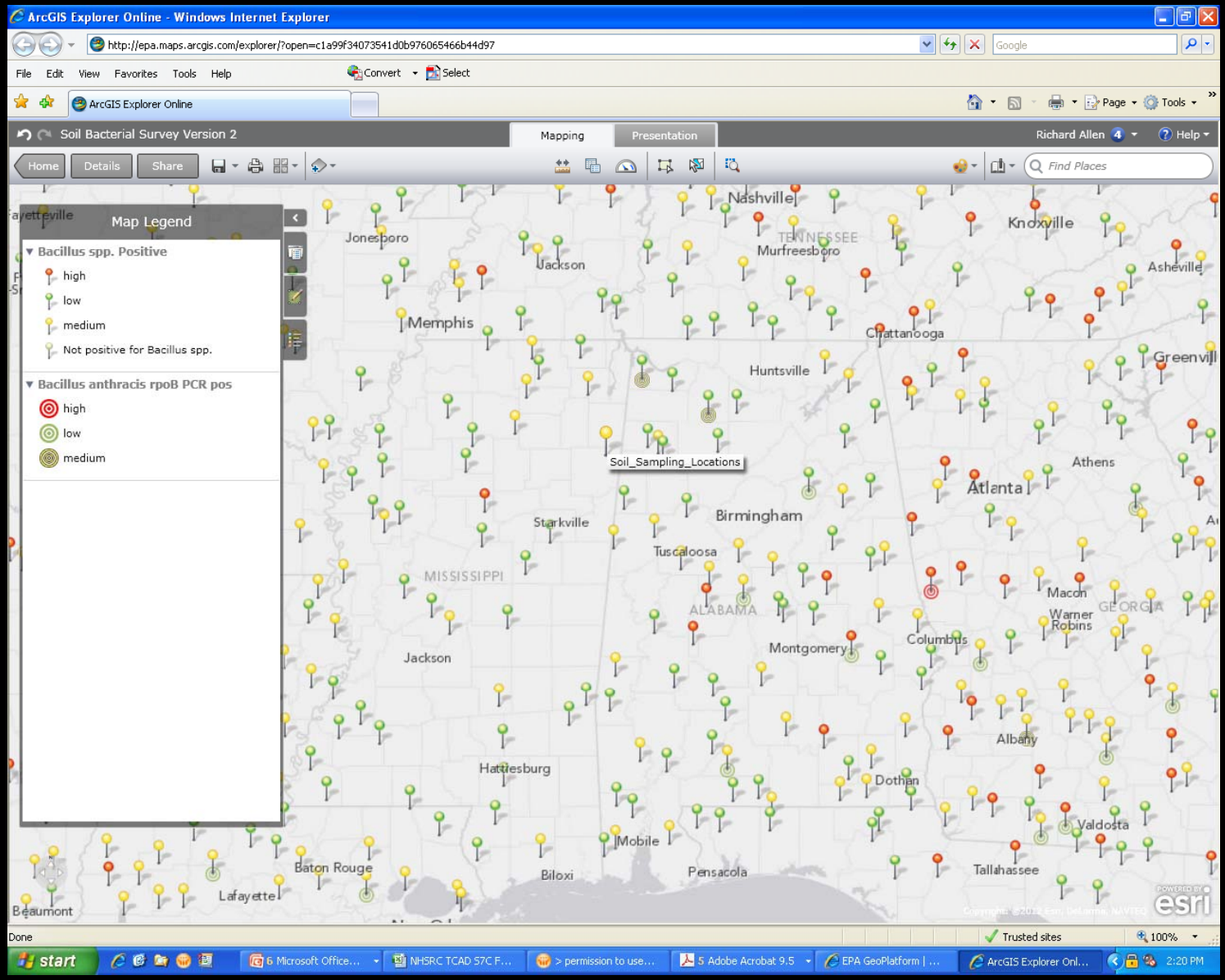
USGS-USEPA *Bacillus* sp. positive layer – Color coded for strength of PCR signal



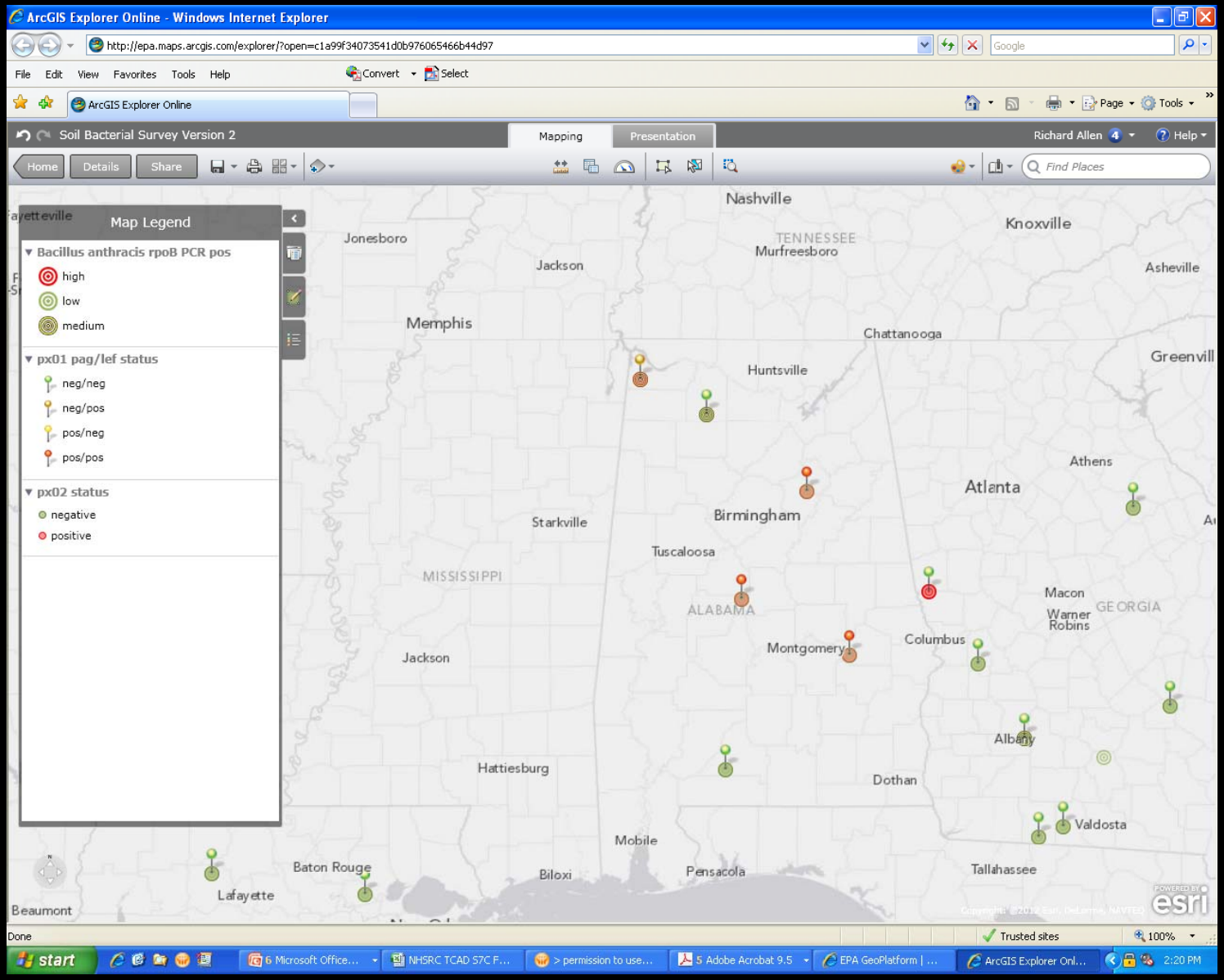
USGS-USEPA *Bacillus* sp. and *B. anthracis* positive layer – Color coded for strength of PCR signal, w/topo



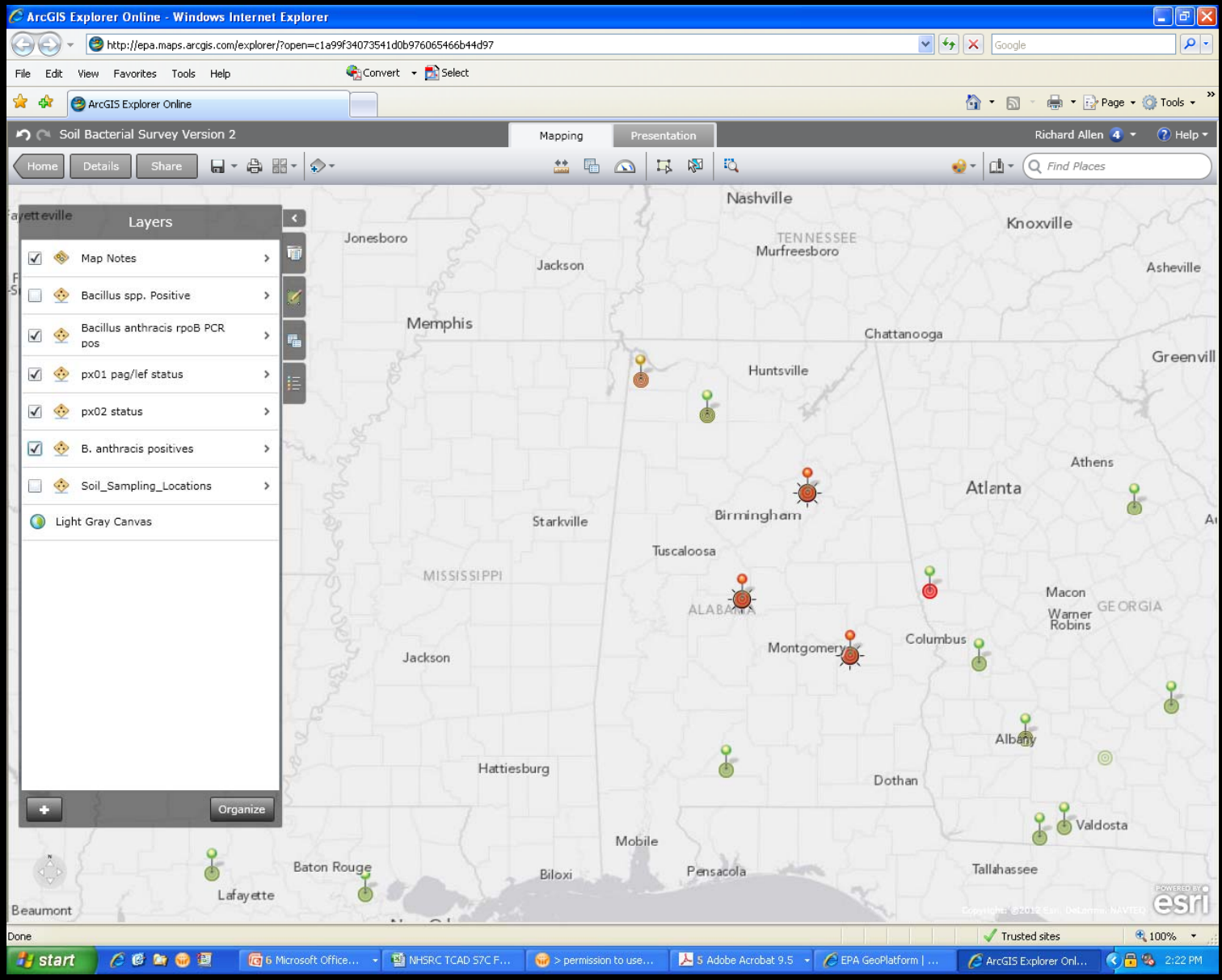
USGS-USEPA *Bacillus* sp. and *B. anthracis* positive layer – Color coded for strength of PCR signal, w/o topo



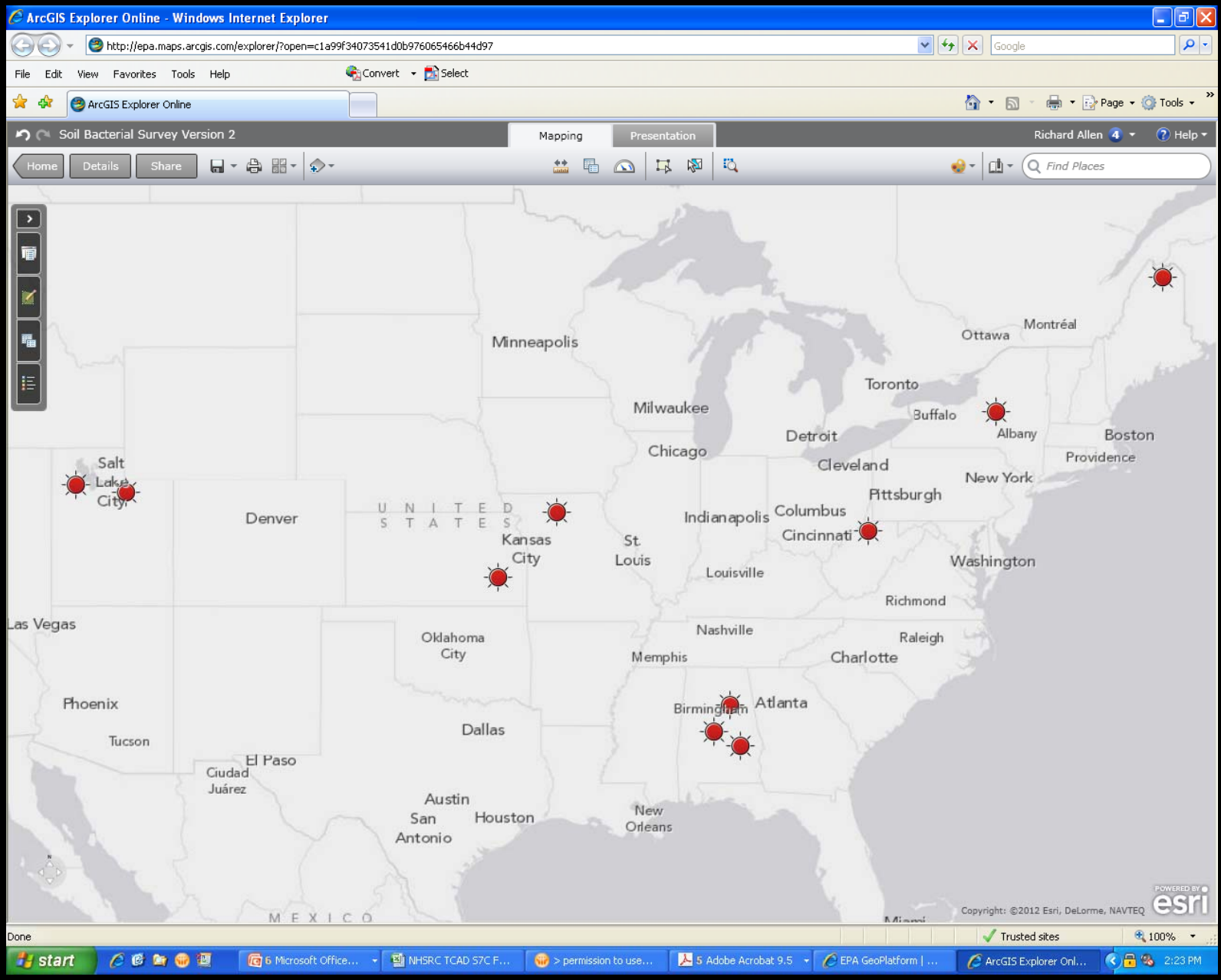
USGS-USEPA *B. anthracis/plasmid* positive layer – Color coded for strength of PCR signal and presence or absence of plasmid genes



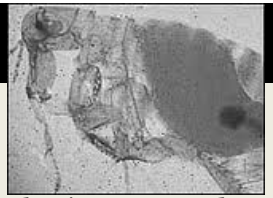
USGS-USEPA *B. anthracis* positive layer –Color coded for strength of PCR signal, presence or absence of plasmid genes and highlighted red star for all positive



USGS-USEPA *B. anthracis* positive layer – All genetic markers screened present

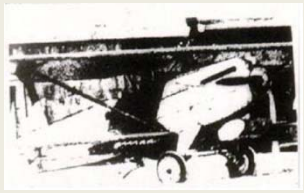


Yersinia pestis

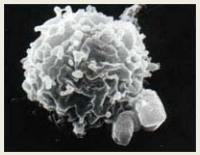


Gram negative, rod-shaped, facultative anaerobe. A flea-borne category A pathogen. Causative agent of the death of ~1/3 of European population in the 14th Century, ~75 million worldwide 19th Century outbreak killed ~12 million in China and India
Annually, about 12 cases in the US, 2,000 worldwide.

"I was fifteen years old at the time, and I remember everything clearly. The Japanese plane spread something that looked like smoke. A few days later we found dead rats all over the village. At the same time, people came down with high fevers and aches in the lymph nodes. Every day, people died....."



Fransicella tularensis



Gram negative, facultative intracellular (infects macrophages).
A highly virulent category A pathogen. Inhalation can lead to lethal pneumonic rabbit fever.
Previously developed as a biological weapon (easily spread via aerosols and low infectious dose 10-50 CFU).
Widespread disease (USA) in animals, ~200 US human cases per year.

"People who inhale an infectious aerosol would generally experience severe respiratory illness, Including life-threatening pneumonia and systemic infection, if they are not treated. The bacteria that causes tularemia occur widely in nature and could be isolated and grown in quantity in a laboratory, although manufacturing an effective aerosol weapon would require considerable sophistication"

WWW.BT.CDC.GOV

Yersinia pestis and *Fransicella tularensis* Detection Protocols

~0.25g of soil

Yersinia pestis

67bp amplicon, located between two common genomic *Yersinia* insertion elements (1541 and 285)...ID'd via suppression subtractive hybridization

3aF - GGACGGCATCACGATTCTCT, 3aR - CCTGAAAACTTGGCAGCAGTT

3a Probe - [6~FAM]AAACGCCCTCGAATCGCTGGC[BHQ1a~6FAM]

(Primers – Radnedge et al., 2001. AEM. 67:3759-3762. Probe - Qu et al., 2010. PLOS Neglected Tropical Diseases. 4(3)e629...modified amplification profile...AB TaqMan start then Qu et al. cycles)

Fransicella tularensis

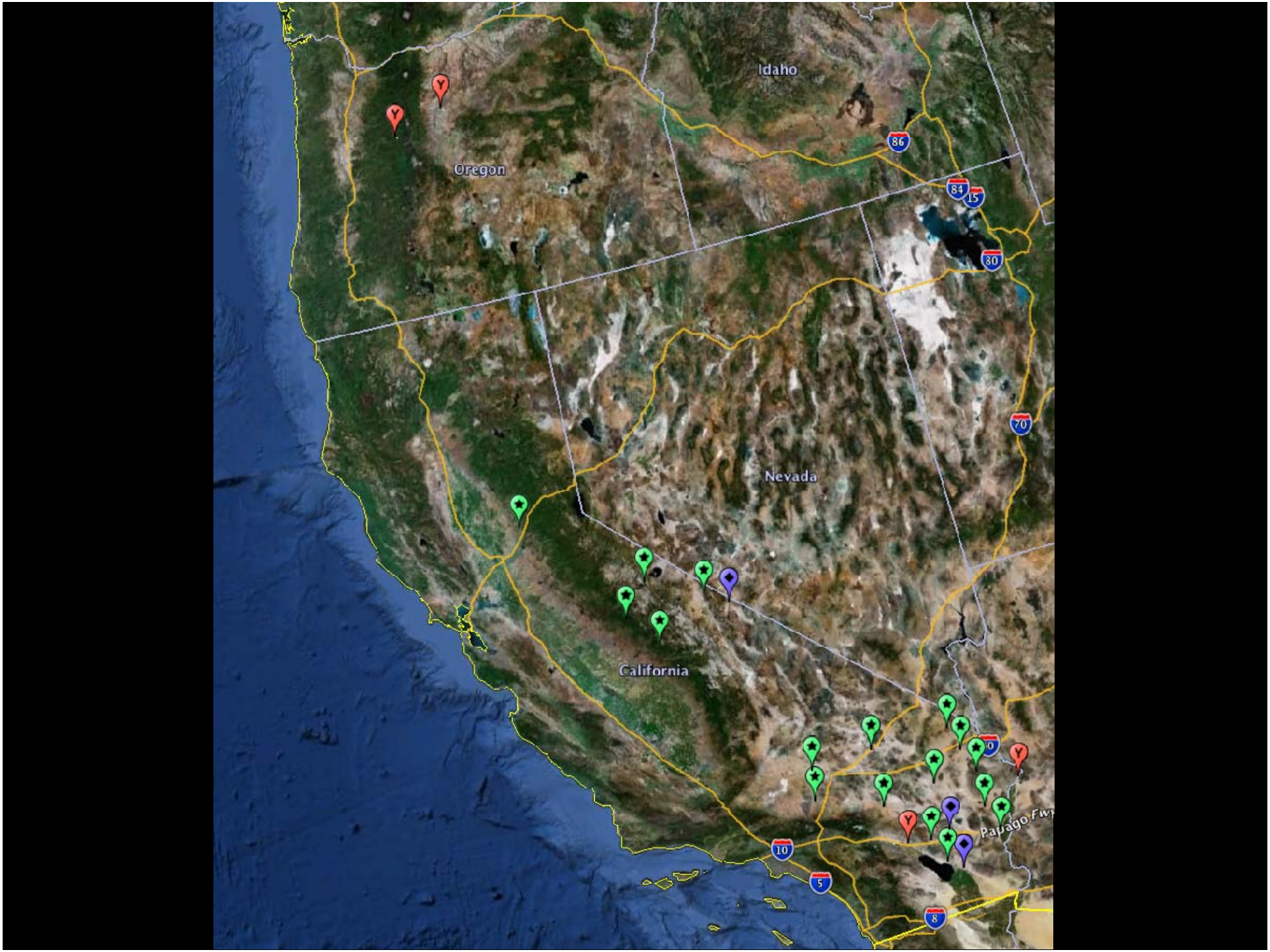
86bp amplicon of the fopA gene (encodes an outer membrane protein).

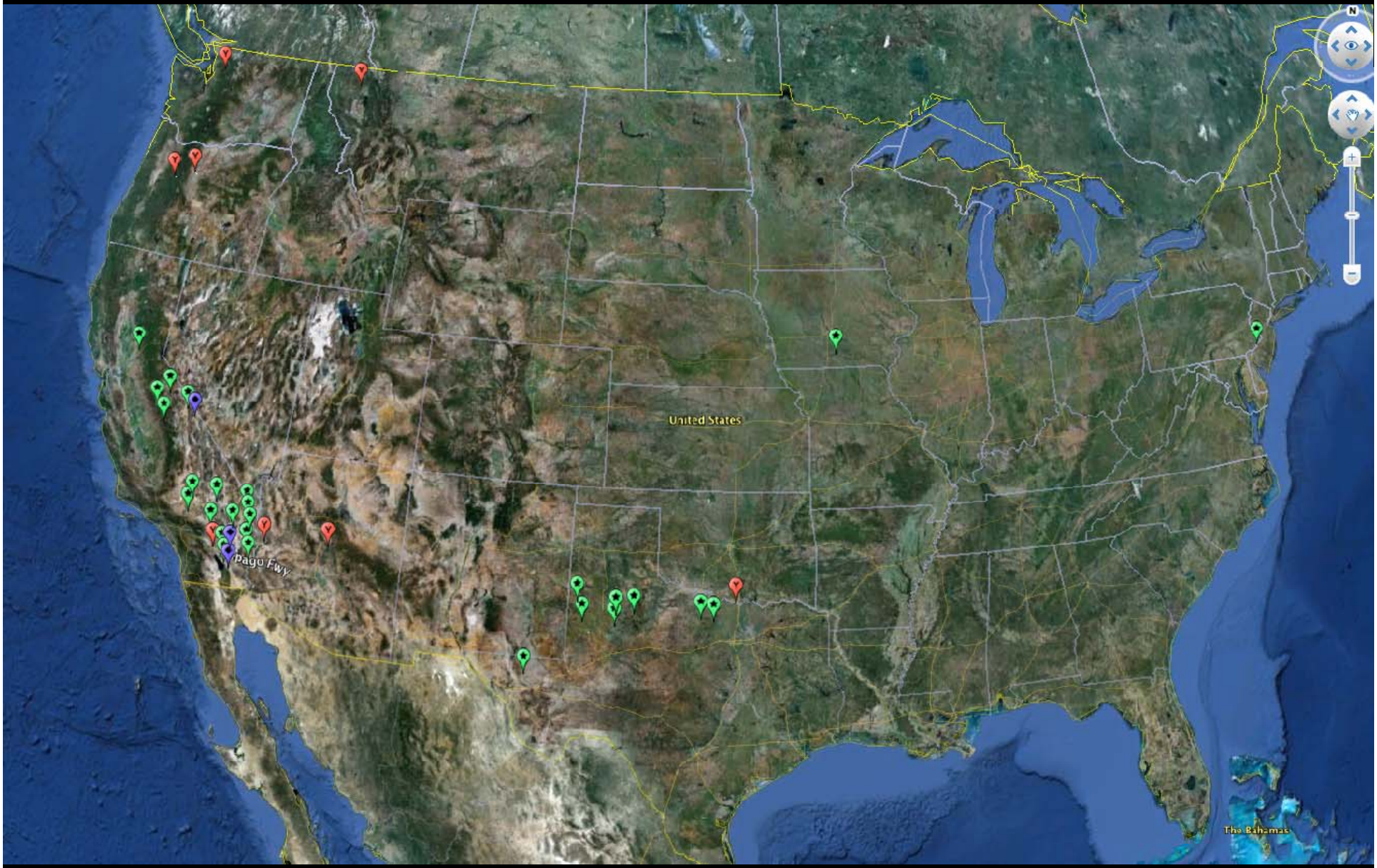
Up – AACAATGGCACCTAGTAATATTTCTGG,

Dn - CCACCAAAGAACCATGTTAAACC,

Probe - [6~FAM]TGGCAGAGCGGGTACTAACATGATTGGT[BHQ1a~6FAM]

(PCR amplification profile and primer/probe sequences from – Emanuel et al., 2003. Journal of Clinical Microbiology. 41(2):689-693)


Idaho
Oregon
Nevada
California
86
84
15
80
70
10
5
8


United States
The Bahamas

Summary/Conclusions

- 4,851 sites analyzed for *Bacillus* species.
- ~50% positive for the genus *Bacillus.*
- 79 PCR positive for *B. anthracis.*

  10 PCR positives verified by USF.
  62 PCR positives pending verification and reassessment
  Presumptive prevalence rate at ~1.0% of sites at this point in the study
- *Yersinia pestis* was detected in 9 of 2133 samples (0.4%).
- *Fransicella tularensis* was detected in 30 of 2133 samples (1.4%).

- As the New Orleans post-Katrina data demonstrates, *B. anthracis* is more readily detected following flood events. Surprisingly, post flood isolates could not be detected several years later.
- *B. anthracis* post flood surveys are needed as well as survival experiments in surface and subsurface environments.
- This study will provide 'natural occurrence data' that will be valuable in site specific risk assessments conducted during future events.

Acknowledgements

U.S. Environmental Protection Agency collaborators:
Tonya Nichols and Sarah Perkins

Disclaimer:
This presentation has been reviewed by the Agencies but does not necessarily reflect the Agencies' views. No official endorsement should be inferred.

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United States
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